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Usefulness of Flow Cytometry in Diagnosis of IgG4-Related Ophthalmic Disease and Extranodal Marginal Zone B-Cell Lymphoma of the Ocular Adnexa

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Abstract. *Background/Aim: Although flow cytometry (FCM) is used to evaluate cell surface markers of various leucocyte populations quantitatively, little is known about the usefulness of FCM in lymphoproliferative disorders of the ocular adnexa. The aim of this study was to disclose results of FCM, which were compared among IgG4-related ophthalmic disease (IgG4-ROD), idiopathic orbital inflammation (IOI), and extranodal marginal zone B-cell lymphoma (EMZL). Materials and Methods: This is a retrospective observational study. Sixty-nine tumors comprising of 16 IgG4-ROD, 24 IOI, and 29 EMZL were enrolled in the study. All tumors, surgically excised, were diagnosed based on histopathology, immunoglobulin (Ig) heavy chain gene rearrangement, and FCM. In FCM, the percentage of T-cell markers (CD2, CD3, CD4, CD5, CD7, CD8), B-cell markers (CD10, CD19, CD20, CD23), NK cell marker (CD56) and cell surface kappa/lambda was searched based on medical records. Ig light chain restriction was evaluated from results in kappa/lambda deviation by FCM. Results: The percentage of CD2, CD3, CD4, CD7, and CD10 was significantly higher in IgG4-ROD/IOI than EMZL ($p<0.05$ in every factor). In contrast, CD19 and CD20 percentages were significantly greater in EMZL than IgG4-ROD/IOI ($p<0.01$). There was no significant difference in any marker between IgG4-ROD and IOI. Kappa-positive cells were significantly greater in EMZL than IgG4-ROD/IOI ($p<0.05$). In kappa/lambda deviation, false-positive was noted in 3 (7.5%) benign IgG4-ROD/IOI and false-negative was observed in 10 (34.5%) EMZL cases. Sensitivity and specificity of Ig light chain restriction were 65.5 and 92.5%, respectively. Conclusion: Analyses of cell surface markers using FCM were useful in differentiating EMZL from IgG4-ROD/IOI. Sensitivity of Ig light chain restriction was relatively low in diagnosis of EMZL using FCM.*

IgG4-related ophthalmic disease (IgG4-ROD), recently defined as reactive lymphoplasmacytic infiltration, is characterized by tissue enlargements, increased serum IgG4 levels and histology-proven IgG4-positive plasma cell infiltration. The Japanese multicenter analyses disclosed that the frequency of diagnosis with IgG4-ROD would increase among orbital lymphoproliferative disorders (1). IgG4-ROD can manifest systemic involvements such as autoimmune pancreatitis, lung and renal diseases, and pituitary inflammation, all of which can affect patients' life. Therefore, since the clinical course of IgG4-ROD is suggested to be different from idiopathic orbital inflammation (IOI), it is essential to diagnose IgG4-ROD correctly although both IgG4-ROD and IOI reveal benign reactive lymphoid hyperplasia based on histopathology.

IgG4-ROD simulates B-cell malignant lymphomas such as extra nodal marginal zone B-cell lymphoma (EMZL) (2, 3), and the differentiation is sometimes difficult based on histopathological examination only. In addition, we have shown that CD20 and vascular endothelial growth factor-immunopositive B lymphoid cells were detected in tumor tissues of both ocular adnexal EMZL and benign reactive lymphoid hyperplasia (4). Therefore, further information should be collected to differentiate benign lymphoproliferative disorders from EMZL for ophthalmologists. Flow cytometry (FCM) is a method to evaluate cell surface markers of various leucocyte populations quantitatively. Recently, it is likely that FCM is useful in diagnosis of orbital lymphoma (5, 6);

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however, little is known about usefulness of FCM in making differential diagnoses of IgG4-ROD, IOI and ocular adnexal lymphoma.

The aim of this study was to disclose results of FCM, that were compared among IgG4-ROD, IOI and EMZL.

Materials and Methods

Patients. This is a retrospective observational study. Institutional review board in Hokkaido University Hospital and Teine Keijinkai Hospital approved this study (IRB number: 016-0297). Patients who were diagnosed between January 2007 and April 2016 were eligible. All the patients having ocular adnexal tumors underwent surgical resection of the tumors. The excised tumor tissues were immediately submitted for FCM without fixation. Histopathology and immunoglobulin heavy chain (IgH) gene arrangements were also examined using the remaining tumor tissues. This study enrolled sixty-nine tumors consisting of 16 IgG4-ROD, 24 IOI, and 29 EMZL. Diagnosis of EMZL was made based on histopathology, IgH, and FCM. In this study, patients with IgG4-producing EMZL and other types of lymphoma arising from the ocular adnexa such as diffuse large B-cell lymphoma, follicular lymphoma, mantle cell lymphoma, and NK/T cell lymphoma were excluded.

Patients with IgG4-ROD were diagnosed based on the recent diagnostic criteria (7). Briefly, there were 3 characteristic findings including tissue enlargements of the ocular adnexa, elevated serum IgG4 levels (>135 mg/dl) and marked IgG4-positive plasma cell infiltration in the tissues. If all the 3 factors were satisfied, then the patients were diagnosed with definitive IgG4-ROD. This study enrolled patients with definitive IgG4-ROD whereas patients with probable/possible IgG4-ROD were excluded. Further, patients with IOI who underwent tumor tissue resection were also examined in this study. Diagnosis of IOI was made by histology showing non-specific lympho-plasmacytic inflammation which was not filled with histological criteria of IgG4-ROD (7). In addition, blood tests revealed no elevation of serum IgG4 levels if tested, or no detection of specific autoantibodies. Wegener granulomatous inflammation, bacterial/fungal infection, and orbital granulomatous inflammation caused by sarcoidosis were excluded.

FCM. Cells were stained using FITC or PE-conjugated monoclonal antibodies against T-cell markers (CD2, CD3, CD4, CD5, CD7, CD8), B-cell markers (CD10, CD19, CD20, CD23), and NK cell marker (CD56), and polyclonal antibodies directed against kappa and lambda. Cells were resuspended in phosphate-buffered saline supplemented with 1% bovine serum albumin and antibody reactions at room temperature for 30 min. After a washing step, the cells were resuspended, and then five-color flow cytometric analysis was performed using the flow cytometer (Beckman Coulter Cytomics FC 500) within the next 2 h. Multi-parameter analyses of the different cell populations were performed based on the morphologic properties and FITC/PE intensity of the pan-leukocyte antigen CD45. Relative percentages of various cell subsets from each case were subjected to statistical analysis.

Kappa/lambda deviation. After obtaining populations of cell surface kappa/lambda by FCM, kappa/lambda ratios were then calculated. Ratios less than 0.7 or greater than 5.5 are evaluated as positive for immunoglobulin light chain restriction according to the previous report (8).

Statistical analysis. Patients' age, and percentage of cell populations in T-cell markers, B-cell markers, NK cell marker, and cell surface kappa/lambda were compared among IgG4-ROD, IOI and EMZL using Mann-Whitney *U*-test. Frequency of gender was evaluated using Chi-square test among groups. A value of less than 0.05 was considered significant.

Results

T-cell, B-cell and NK cell linages. The patients' age was 60.3±8.6, 63.4±12.7, and 69.2±10.7 years in IgG4-ROD, IOI and EMZL, respectively. The age of disease onset was significantly higher in EMZL than IgG4-ROD/IOI ($p<0.05$). Eight males and 8 females were involved in IgG4-ROD. In contrast, 29 EMZL patients included 12 males and 17 females, in which the number of female patients was significantly greater in EMZL than IgG4-ROD ($p<0.05$). All the patients with IgG4-ROD showed high serum IgG4 levels (136-1,550 mg/dl: mean, 487.8). The percentage of CD2, CD3, CD4, CD7 (Table I), and CD10 (Table II) was significantly lower in EMZL than IgG4-ROD and IOI ($p<0.05$ in every factor). CD23 population was significantly higher in IgG4-ROD than EMZL whilst there was no significant difference in CD23 between IOI and EMZL (Table II). CD5, CD8 (Table I) and CD56 (Table II) percentages were not significantly different between EMZL and IgG4-ROD, whereas CD19 and CD20 percentages were significantly greater in EMZL than IgG4-ROD/IOI ($p<0.05$, Table II). In contrast, there were no significant differences in the cell surface markers between IgG4-ROD and IOI.

Immunoglobulin light chain analyses. In cell surface immunoglobulin light chain, kappa was predominant in 15 out of 16 IgG4-ROD (93.7%), 23 of 24 IOI (96.0%), and 21 of 29 EMZL cases (72.4%). Kappa -positive cell population was 31.2±13.6, 31.5±10.8 and 48.3±26.6, and lambda-positive cell population was 18.9±9.3, 20.6±9.4 and 22.0±23.6 in IgG4-ROD, IOI and EMZL, respectively. Kappa-positive cells were significantly greater in EMZL than IgG4-ROD/IOI ($p<0.05$; Table II), while lambda-positive cells were not significantly different. Kappa/lambda deviation was present in 1 (6.3%) IgG4-ROD, 2 (8.3%) IOI and 19 (65.5%) EMZL cases. False positive was noted in 3 (7.5%) benign IgG4-ROD/IOI and false negative was observed in 10 (34.5%) EMZL cases. Therefore, the sensitivity and specificity of kappa/lambda deviation were 65.5 and 92.5 %, respectively.

Discussion

It is important to know that IgG4-ROD can simulate lymphoid malignancies. Indeed, malignant lymphoma in the ocular adnexa possibly arose from IgG4-ROD (2). We have reported a coincidence of IgG4-ROD and EMZL proved by

Table I. *T cell linage population (%) in lymphoproliferative disorders of the ocular adnexa.*

	IgG4-ROD (N=16)	IOI (N=24)	EMZL (N=29)	*p*-Values		
				IgG4 *vs*. EMZL	IOI *vs*. EMZL	IgG4 *vs*. IOI
CD2	39.1±17.2	41.4±16.4	23.9±12.7	0.0121	0.0002	ns
CD3	35.6±15.6	40.6±17.1	23±11.4	0.0266	0.0004	ns
CD4	34.4±7.3	33.4±11.4	17.4±9.4	<0.0001	<0.0001	ns
CD5	42.2±21.7	45.5±17.8	32.4±17.3	ns	0.0237	ns
CD7	31.6±14.7	33.4±13.2	18.9±11.8	0.0293	0.0005	ns
CD8	8.7±5.6	11.4±8.1	6.8±5.1	ns	0.0062	ns

IgG4-ROD, IgG4-related ophthalmic disease; IOI, idiopathic orbital inflammation; EMZL, extranodal marginal zone B-cell lymphoma; ns, not significant.

Table II. *B cell and other cell linages population in lymphoproliferative disorders of the ocular adnexa.*

	IgG4-ROD (N=16)	IOI (N=24)	EMZL (N=29)	*p*-Values		
				IgG4 *vs*. EMZL	IOI *vs*. EMZL	IgG4 *vs*. IOI
CD19	59.1±18.9	56.7±19	74.3±14.8	0.013	0.0003	ns
CD20	50.9±13.7	52.9±13.3	72.8±16.7	0.0002	<0.0001	ns
CD23	27.2±16.8	19.9±13.2	15.4±14.7	0.0441	ns	ns
Kappa	31.2±13.6	31.5±10.8	48.3±26.6	0.0467	0.003	ns
Lambda	18.9±9.3	20.6±9.4	22±23.6	ns	ns	ns
CD10	7.8±9.3	9.7±11.4	3.2±4.4	0.0356	0.0006	ns
CD56	2.8±3.2	2.6±2.3	1.8±3.1	0.057	0.0212	ns

IgG4-ROD, IgG4-related ophthalmic disease; IOI, idiopathic orbital inflammation; EMZL, extranodal marginal zone B-cell lymphoma; ns, not significant.

histological as well as FCM analyses (3). Therefore, to compare FCM findings in IgG4-ROD and EMZL is critical for differentiation of these disorders. In this study, the B-cell linages, CD19, 20 and CD23-positive cell population were significantly greater in EMZL than IgG4-ROD, whereas CD2, 3, 4 and CD7-positive T cells were significantly lower in the former than the latter. Taken together, FCM could show significant differences regarding B-cell and T-cell linages quantitatively in EMZL and IgG4-ROD. Therefore, FCM is a useful method to differentiate IgG4-ROD from EMZL.

Recently, Ueda *et al*. demonstrated that T-cell markers (CD3, 4, 8, 10, 25) and B-cell markers (CD19, 20, 23) tested by FCM were useful in differentiating benign lymphoproliferative disorders from malignant lymphoma (6). This study was consistent with the previous report. Moreover, this study further showed other T-cell markers including CD2 and CD7, both of which were more characteristic in benign IgG4-ROD/IOI than in EMZL. Rabinowich *et al*. reported that CD2 and CD7 were expressed not only in T cells, but also in NK cells (9). In addition, CD7, but not CD2, -positive NK cells play a critical role in the various immune responses through cell adhesion molecules, integrins (9). Further studies are needed to elucidate localization of CD2 and CD7 and their functions in the immune cell surfaces in IgG4-ROD.

In this study, according to the FCM findings, benign IgG4-ROD/IOI demonstrated that cells having both T-cell and B-cell linages were moderately detected in the tumor tissues. These results clearly show that benign lymphoproliferative disorders in the ocular adnexa are made up of polyclonal populations in lymphoid cells. However, there was no statistical difference between definitive IgG4-ROD and IOI. These results suggest that FCM findings might not contribute to the differentiation of IgG4-ROD from IOI. It has been reported that Th2 cells play a pivotal role in germinal center formation and IgG4 production in IgG4-related disease (10). Moreover, CD19(+) CD27(+) CD20(–) CD38(hi) plasmablasts take part in the active inflammation in IgG4-related disease (11). Although this study examined

representative T-cell and B-cell linages, further cell surface markers such as CD38 and CD27 in the tumor tissues would contribute to differentiation in both disorders.

The presence of B-cell monoclonality is an important proof of B-cell malignancy. Ways to prove B-cell monoclonality comprise IgH gene rearrangement by PCR method/Southern blot analysis, and/or immunoglobulin light chain restriction by FCM/immunohistochemistry. Indeed, examination of IgH gene rearrangement is useful to confirm the B-cell monoclonality; however, it may be challenging to gain reliable results if an insufficient number of cells was collected from the isolated tissues. Sometimes local resected tumor tissues may not have enough cells to be examined in ophthalmology practice. Therefore, further data should be collected to validate true diagnosis of ocular adnexal lymphoproliferative disorders. This study further examined cell surface immunoglobulin light chains in ocular adnexal lymphoproliferative disorders. Indeed, kappa-positive cell number was significantly greater in EMZL than IgG4-ROD/IOI. And then kappa/lambda deviation was determined based on FCM analysis. False positive was noted in 3 (7.5%) benign IgG4-ROD/IOI and false negative was observed in 10 (34.5%) EMZL cases. Samoszuk *et al*. reported that a false-positive rate was less than 10%, whereas a false-negative rate was approximately one fourth (8). The current data showed that a false positive rate was similar to the previous report; however, false negative was slightly higher. In fact, this study demonstrated that the sensitivity and specificity of kappa/lambda deviation were 65.5 and 92.5%, respectively, indicating that analyses of B-cell clonality using FCM revealed relatively low sensitivity and high specificity in ocular adnexal lymphoproliferative disorders.

Interestingly, the number of B cell markers CD19/20 was lower as shown above, whereas CD23 was significantly greater in IgG4-ROD than in EMZL, the latter of which was not observed between IOI and EMZL. It has recently been reported that CD23-immunopositive cells were clearly detected in the tissues of IgG4-ROD (6). CD23 is a cell surface marker for activated B cells which plays an important role in the pathophysiology of allergic diseases. Celiksoy *et al*. demonstrated reduction of the activated B-cell population in patients with allergic rhinitis/bronchial asthma following immunotherapy (12), suggesting that CD23 population is useful in not only diagnosis but also evaluation of therapeutic effects. The limitation of this study is that the tumor tissues were isolated only at an initial presentation; however, the tumor tissues were not available after treatments. Therefore, it was impossible to look into CD23 alteration before and after treatments in ocular adnexal lymphoproliferative disorders. Another limitation is that this study only contained definitive IgG4-ROD whilst probable/possible IgG4-ROD was not examined. Therefore, further studies are required to clarify the difference of cell surface markers between definitive and probable/possible IgG4-ROD.

In conclusion, quantification of cell surface markers by FCM is useful to differentiate benign lymphoproliferative disorders from EMZL in the ocular adnexa. Sensitivity of immunoglobulin light chain restriction was not high in this method.

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