


Original Article

Flow Cytometry Shows Added Value in Diagnosing Lymphoma in Brain Biopsies

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Background: To assess the sensitivity, specificity and turnaround time of flow cytometric analysis on brain biopsies compared to histology plus immunohistochemistry analysis in tumors with clinical suspicion of lymphoma.

Methods: All brain biopsies performed between 2010 and 2015 at our institution and analyzed by both immunohistochemistry and flow cytometry were included in this retrospective study. Immunohistochemistry was considered the gold standard.

Results: In a total of 77 biopsies from 71 patients, 49 lymphomas were diagnosed by immunohistochemistry, flow cytometry results were concordant in 71 biopsies (92.2%). We found a specificity and sensitivity of flow cytometry of 100% and 87.8%, respectively. The time between the biopsy and reporting the result (turnaround time) was significantly shorter for flow cytometry, compared to immunohistochemistry (median: 1 vs. 5 days).

Conclusions: Flow cytometry has a high specificity and can confirm the diagnosis of a lymphoma significantly faster than immunohistochemistry. This allows for rapid initiation of treatment in this highly aggressive tumor. However, since its sensitivity is less than 100%, we recommend to perform histology plus immunohistochemistry in parallel to flow cytometry. © 2018 The Authors. Cytometry Part B: Clinical Cytometry published by Wiley Periodicals, Inc. on behalf of International Clinical Cytometry Society

Key terms: brain biopsies; primary brain tumor; central nervous system lymphoma; immunohistochemistry; flow cytometry; diagnostic accuracy

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Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Primary central nervous system lymphoma (PCNSL) is a rare non-Hodgkin lymphoma confined to the brain, leptomeninges, eyes, or spinal cord (1). Approximately 3% of all brain tumors are PCNSL. Secondary central nervous system lymphoma, or CNS localization of systemic lymphoma, occurs most frequently in Burkitt lymphoma (up to 43%) or in diffuse large B-cell lymphoma (DLBCL) patients (5–14%, depending on its stage or risk factors) (2,3). Common presenting symptoms of a CNS lymphoma are focal neurological deficits, neuropsychiatric symptoms, headache, and less typically, seizures (4). MRI mostly shows single or multiple space occupying lesions, with homogeneous contrast enhancement. Before starting treatment, cytological, or histologic confirmation of the presence of a lymphoma is required. Since clinical deterioration is frequent in both primary and secondary CNS lymphoma, a rapid diagnosis is preferable. Sometimes a CNS lymphoma can be diagnosed by vitreous or cerebrospinal fluid (CSF) analysis (5). However, a spinal tap may be contraindicated in space occupying lesions and even if safely possible, PCNSL is diagnosed on CSF in about 30% of patients only (6). Consequently, a brain biopsy remains necessary in the majority of the patients. Similarly, systemic lymphoma may also present with intraparenchymatous lesions, and may present with diagnostic uncertainties requiring histological confirmation. Histology with immunohistochemistry (IHC) is considered the gold standard in the analysis of brain biopsies in diagnosing a lymphoma. Immunophenotyping by flow cytometry is an objective and quantitative method ideally suited to identify small populations of cells with aberrant phenotypes (7). It is particularly helpful for the detection of small clonal populations of B-lymphocytes. The technique has proven its value in the analysis of bone marrow, fine needle aspiration of lymph nodes and in cerebrospinal fluid (8–12). In cerebrospinal fluid, the sensitivity increases 2–3 times (13–17). However, few data defining the added value and diagnostic accuracy of flow cytometry in brain biopsies have been published. In our center immunophenotyping using eight-color flow cytometry has been utilized in addition to histology with IHC in brain biopsies since 2010 in brain tumor patients in whom a lymphoma was suspected, based on clinical and radiological features. The aim of this study was to determine the added clinical and diagnostic value of immunophenotyping by flow cytometry in brain biopsies. Furthermore, since analysis by flow cytometry is in general much faster than by immunohistochemistry, we also sought to investigate the difference in time needed to acquire a diagnosis by these two techniques.

METHODS

Patients

All brain biopsies performed at the Erasmus University Medical Center in Rotterdam, the Netherlands, between January 2010 and December 2015 were retrospectively extracted from patient and laboratory registries. See Supporting Information Figure S1 for the flowchart of

selecting biopsies. Only biopsies which were analyzed by both IHC and flow cytometry were included for statistical analysis. Flow cytometric analysis was routinely performed when a lymphoma was suspected on radiological grounds. In addition, HIV-status, use of corticosteroids, and immunomodulating medication, of all patients were collected. Turnaround time (time between biopsy and report of the analysis) was extracted from patient files or laboratory log. Preliminary results given to the clinician were not included in our statistical analysis. The size of the biopsies and the numbers of cells within the flow cytometric analysis were registered. As a check for lymphoma patients not included, all patients with CNS lymphoma diagnosed in the same period in our center were extracted from the national pathology database PALGRA. The study was approved of by the Independent Review Board of our institution.

Neurosurgical Procedures

Brain tissue was collected by image-guided stereotactic biopsies; when a high grade glioma was suspected patients went for open surgery. The stereotactic biopsies were framelessly performed using the Medtronic Stealth Treon™ Vertek® system until 2010 and the Brainlab® Varioguide neuronavigation system ever since (18,19). In general, four biopsies were obtained at the preoperatively determined target, as well as two to four more biopsies at a site proximal to the target on the same biopsy trajectory. Open biopsies were performed using image-guided navigation and the operation microscope. After surgery the collected biopsies were divided for histopathology and flow cytometry by the neurosurgeon, or by the pathologist if all material had initially been sent to the pathology laboratory. Intra-operative freeze sections were not performed in most patients to maximize available tissue for definitive pathology and flow cytometry.

Histology and Immunohistochemistry

All tumors were classified according to the World Health Organization (WHO) classification of Tumours of Haematopoietic and Lymphoid Tissues version 2008 by conventional histological assessment on 2 µm hematoxylin and eosin (H&E) stained sections and on 4 µm immunohistochemically stained sections. Sections were cut from formalin-fixed brain tumor tissues, embedded in paraffin blocks using standard pathology tissue processing procedures (20). For immunohistochemistry, the following primary antibodies were used: CD3, CD5, CD10, CD19, CD20, CD79a, Bcl-2, Bcl-6, and Mib-1. When appropriate this panel was extended with one or more of the following antibodies: BOB-1, MUM1, CD 15, cyclin D1, Smlgkappa, Smlglambda, CD21, CD23, CD68, CD138, CD4, GFAP, CD31, CD43, TIA-1, ALK-1, CD8, and PAX-5. All immunohistochemical procedures using primary and secondary antibodies and detection systems, were performed according to the manufacturer's recommendations on a Ventana Benchmark Ultra platform (Ventana Medical Systems Inc., Tucson, USA),

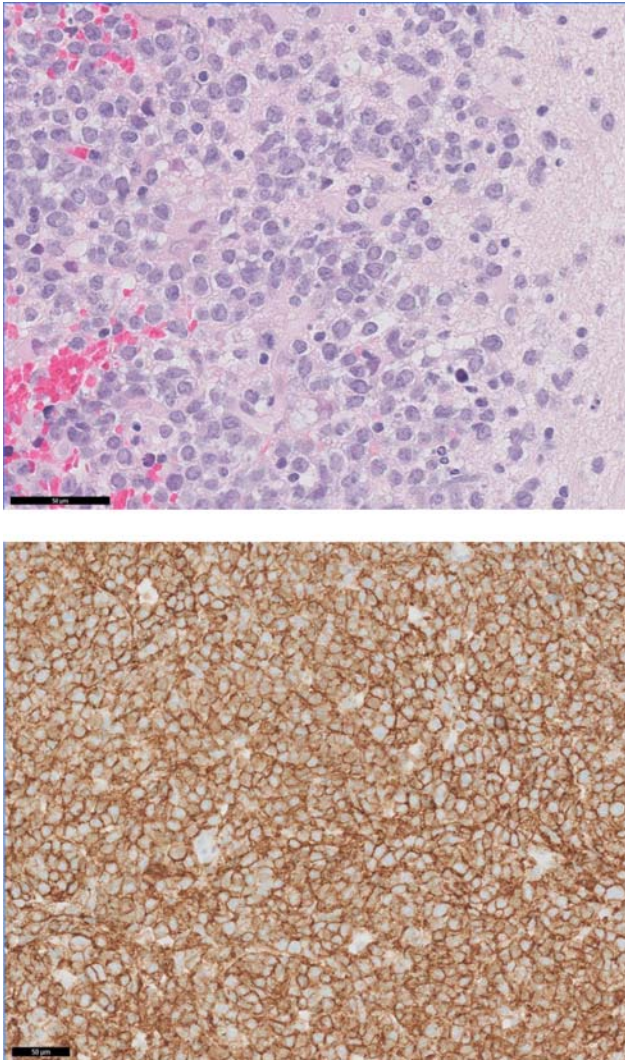


FIG. 1. Histology and immunohistochemistry analysis. HE-staining with diffuse large B-cell lymphoma, activated blast type: brain tissue (right) with infiltration of blastic cells with large vesicular nuclei with nucleoli (left). These tumor cells express CD20 (shown in the figure below), CD79a, BCL-2, BCL-6, and MUM1 and very weak expression of CD10. [Color figure can be viewed at wileyonlinelibrary.com]

tested and validated according to ISO 15189 standards. See Figure 1 for an example of a cerebral NHL, analyzed by histology with immunohistochemistry.

Flow Cytometry

Cell suspensions were generated from a single, unfixed brain biopsy by gentle manual disaggregation on a 100 μ m strainer using a 10 mL syringe plunger rod and wash buffer (PBS/BSA 0.5%; not using any enzymes). The released cells were collected by rinsing with a total volume of 10 mL wash buffer and washed twice in 10 mL wash buffer; centrifugation steps were for 5 minutes at 540g. After the last wash step, the supernatant was discarded and the pellet of cells was suspended in wash buffer. Fifty microliters of the cell suspension were stained using the EuroFlow

Lymphocytosis Screening Tube (LST), according to the EuroFlow protocol (21,22). The LST contains antibodies CD20-Pacific Blue (Clone: 2H7; Biolegend), CD4-Pacific Blue (RPA-T4; Biolegend), CD45-Pacific Orange (HI30; Invitrogen), CD8-FITC, Smlg λ -FITC, CD56-PE, Smlg κ -PE (SLPC mix; Cytognos), CD5-PerCP-Cy5.5 (L17F12, BD Biosciences), CD19-PC7 (J3-119; Beckman Coulter), SmCD3-APC (SK7, BD Biosciences), and CD38-APCH7 (HB7; BD Biosciences). Subsequently the suspension was acquired on a FACSCanto II flowcytometer (BD Biosciences, Erembodegem, BE) using EuroFlow settings (23). We aimed to acquire at least 5000 B-cells (with a minimum of 50.000 leukocytes); if this could not be reached we acquired all available cells in the tube. Appropriate instrument set-up and staining protocols were monitored by the EuroFlow QA scheme (24). After exclusion of debris, doublets and non-hematopoietic cells (CD45 negative, CD19 negative), which all together could add up to over 95% of acquired events in some samples, we defined the presence of a B-NHL population as a population with a marked shift in the SmlgKappa/SmlgLambda ratio (<0.7 or >2.8) and/or a clearly aberrant immunophenotype (e.g., abnormal expression of Ig, CD19, CD20, and/or CD38, abnormal (high) forward scatter). If a B-NHL was detected and sufficient cells were available, EuroFlow BCLPD tube 1 to 4 were stained as well. In all cases, the diagnosis of a B-NHL was based on the results of the LST tube only, the additional information resulting from the additional BCLPD tubes was used to further specify the immunophenotype and to hint to specific B-NHL subtypes. Even though pathologists and immunologists who evaluated the analyses were not blinded for each other's conclusion, the flow cytometry results were reported independently of histology plus IHC analysis. See Figure 2 for an example of a cerebral NHL, analyzed by flow cytometry.

Statistical Analysis

To determine the diagnostic value of flow cytometry, the reports of flow cytometry and immunohistochemistry were compared. Morphology plus IHC was considered the gold standard. In case the results were suspicious for a lymphoma but not conclusive, it was categorized as 'no lymphoma'. The turnaround time and whether the results were available within 24 hours, were compared between the two techniques using the Wilcoxon signed-ranks and a McNemar test, respectively. Differences with respect to use of dexamethasone and sample size, between concordant and discordant groups and between those who had multiple biopsies and who did not were analyzed by Mann-Whitney U or a Fisher's Exact test. All analyses were performed by SPSS Statistics 21.

RESULTS

Between January 2010 and December 2015 77 biopsies which have been analyzed by both histology and flow cytometry, were performed in 71 patients (59% male) with a median age of 63 (range 15–82). 10% of

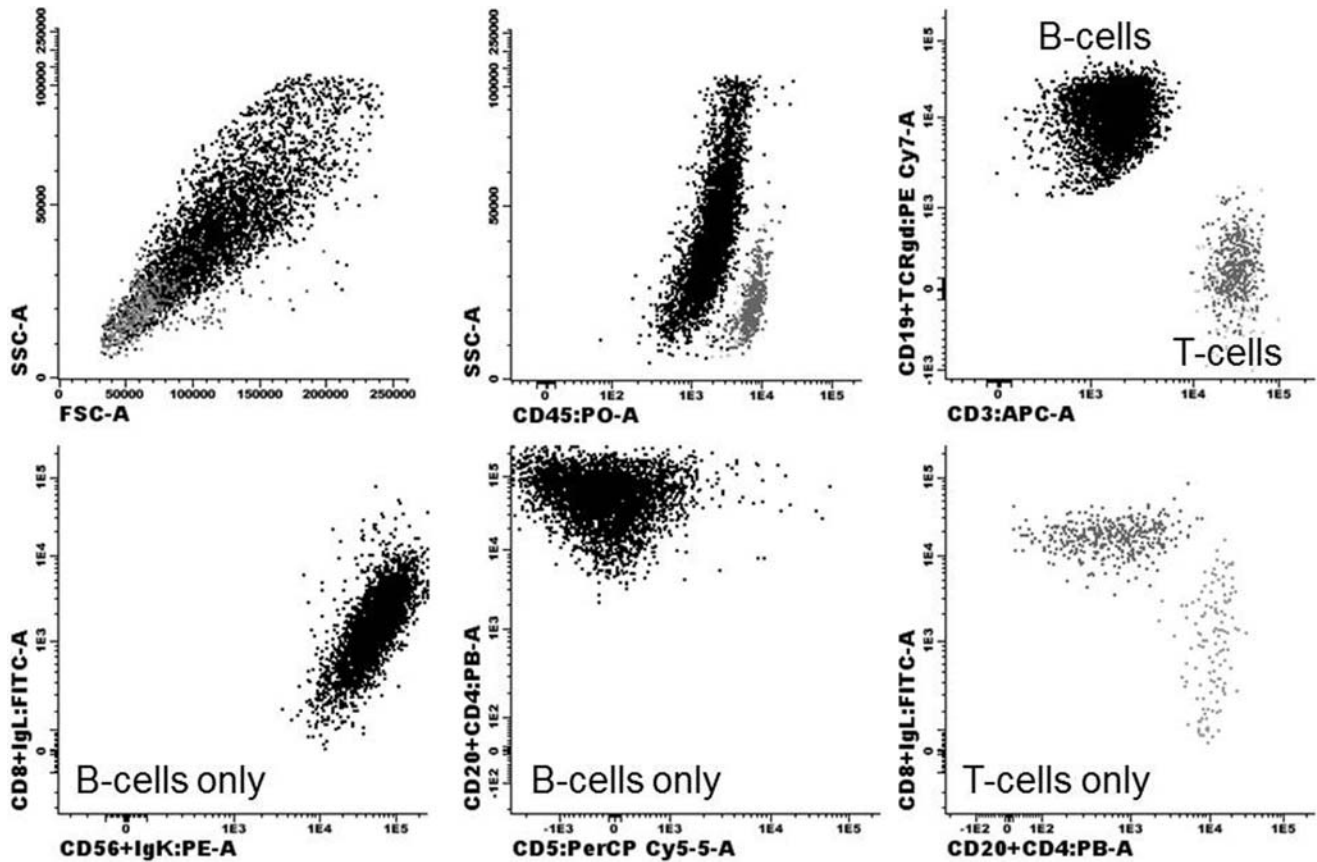


FIG. 2. Flow cytometry analysis. Flowcytometric analysis on brain biopsy, showing a cerebral NHL with the presence of T-cells (11% of leukocytes) and B-cells (89% of leukocytes). Whereas the T-cells (grey) showed a normal CD4/CD8 ratio (lower row, third plot) and a normal immunophenotype (CD3+/CD45+; upper row, second and third), the B-cells (CD19+; black) were clearly abnormal, with monotypic Immunoglobulin kappa expression, low expression of CD45, and light scatter characteristics (FSC and SSC; upper row, first plot) compatible with large cells. The biopsy was stained with the EuroFlow Lymphocytosis Screening Tube according to EuroFlow procedures.

the patients were immunocompromised, which was defined as being HIV-infected (one patient) or using systemic immunomodulating treatment (e.g., methotrexate, azathioprine). Of all CNS lymphoma patients diagnosed in our hospital between 2010 and 2015 by histology and IHC, only four were not sent for flow cytometric analysis. In two cases all material was immediately preserved in formalin which made the tissue no longer suitable for flow cytometry, in two additional cases lymphoma was not considered in the pre-operative differential diagnosis.

Forty-nine biopsies were diagnosed as brain lymphoma by histology and immunohistochemistry; 43 of these were also diagnosed as lymphoma by flow cytometry (Table 1). By flow cytometry, all identified cases were CD19+/CD20+; Ig light chain restriction was observed in most cases (38; 83%) whereas no Ig expression was detected in nine cases (17%). None of the 28 tissue samples not diagnosed as lymphoma by histology plus IHC were identified as lymphoma by flow cytometry. We thus found a concordance, specificity and sensitivity of immunophenotyping by flow cytometry in brain biopsies of 92.2% (71/77), 100% (28/28), and 87.8% (43/49), respectively. The positive predictive value was

100% (43/43) and the negative predictive value was 82.4% (28/34). Numbers of leukocytes (after exclusion of debris, doublets and non-hematopoietic cells) that could be analyzed by flow cytometric analysis ranged widely: 9425 (29–207,259), median (range). Although statistical analysis to compare biopsies with discordant and concordant results should be interpreted with caution due to small numbers, no significant differences were found with respect to sample size ($P = 0.06$), number of cells acquired by flow cytometry ($P = 0.62$), or corticosteroid use prior to biopsy ($P = 0.108$). All 6 discordant cases were DLBCL, without unusual evidence of necrosis. In 6/71 patients, a second biopsy and in 2/71

Table 1
Diagnostic Value of Flow Cytometry on Brain Biopsies

| Flow cytometry | Immunohistochemistry | | Total |
|----------------|----------------------|-----------------|-------|
| | Lymphoma | No lymphoma | |
| Lymphoma | 43 | 0 | 43 |
| No lymphoma | 6 | 28 ^a | 34 |
| | 49 | 28 | 77 |

^aIncluding 8 cases in which both results were "inconclusive."

Table 2
Time to Diagnosis

| | Immunohistochemistry <i>n</i> = 77 | Flow cytometry <i>n</i> = 76 | |
|--------------------------------------|---------------------------------------|------------------------------------|--------------|
| Turnaround time (days) | 5 (0–18) | 1 (0–7) | $P < 0.00^a$ |
| Diagnosis <24 hours (biopsies) | 7 (9%) | 41 (54%) | $P < 0.00^b$ |

Median time (range) in days between biopsy and diagnosis. Significance was calculated by ^aWilcoxon signed-ranks test and ^bMcNemar Test. In one biopsy the date of reporting was missing for flow cytometry analysis.

patients even a third biopsy was necessary to make a diagnosis, because of an inconclusive diagnosis in previous biopsies. Only those biopsies which were investigated by both techniques (6/8) were included in the statistical analysis. In the biopsies, analyzed by IHC only, two additional lymphoma were found. Use of corticosteroids prior to first biopsy ($P = 0.06$), size of the biopsy ($P = 0.68$) and/or number of cells for flow cytometric analysis ($P = 0.19$) were similar in patients with conclusive and inconclusive diagnoses. The 20 patients without a lymphoma were diagnosed with a myriad of diseases: 11 glioblastoma, 1 anaplastic astrocytoma, one germinoma, one stroke, five infections, and one CLIP-PERS syndrome (chronic lymphocytic inflammation with pontine perivascular enhancement responsive to steroids), a rare auto-immune disorder. We found a significantly shortened time to reporting of the results (turnaround time) for flow cytometry, compared to IHC (Table 2). Furthermore, in 54% of the biopsies the diagnosis was provided within 24 hours using flow cytometry, compared to 9% using histology plus IHC.

DISCUSSION

In this study, we compared flow cytometry with histology plus IHC on 77 brain biopsies, performed in patients clinically suspected of having a lymphoma. We found a high concordance between both techniques (92.2%) and a specificity and sensitivity of flow cytometry by immunophenotyping in brain biopsies of 100% and 88%, respectively. In six patients with histologically proven NHL, the presence of a lymphoma could not be identified by flow cytometry. No factors (e.g., sample size, use of corticosteroids prior to the biopsy) could be identified which could explain the missing diagnosis in flow cytometry. Unlike in CSF or bone marrow analysis no additional cases of brain lymphoma were identified by flow cytometry that had not been identified by immunohistochemistry. We found a significant difference in turnaround time for the two techniques. After biopsy a diagnosis was given with a median time of 5 days (range 0–18) for immunohistochemistry, compared to median of 1 day (range 0–7) for flow cytometry. In 54% of the biopsies the presence or absence of a lymphoma could be confirmed within 24 hours by flow cytometry,

compared to 9% for immunohistochemistry ($P < 0.00$), which means that correct treatment could be initiated within 24 hours. It should be noted that the preliminary results of the flow cytometric analysis were frequently reported to the clinician on the day of biopsy. Given the frequently rapid clinical deterioration in CNS lymphoma and the negative impact of a lower performance score on survival, according to the two largest validated prognostic models, (25,26) early diagnosis is may improve prognosis (27). Similar findings were reported in a much smaller cohort of 18 stereotactic biopsies recently (28). Cordone et al. found a significant agreement between flow cytometry and immunohistochemistry diagnosis ($P = 0.0034$). They described a sensitivity and specificity of flow cytometry by immunophenotyping of 89% and 100%, respectively. In the 2/18 PCNSL biopsies not identified by flow cytometry more central necrosis was present, compared to biopsies with concordant results and both patients used corticosteroids prior to the biopsy (28). We did not find more central necrosis in our discordant biopsies and corticosteroid use did not differ between concordant and discordant pairs. One other study analyzed flow cytometry on rinse fluid. Even though rinse fluid from the biopsy needle cannot be completely compared to brain tissue itself, this study showed similar results (29). In a small sample, a high specificity (100%) and sensitivity (75% on rinse fluid and 100% on tissue sample) of flow cytometry in detecting a brain lymphoma were found. The added value was again the time in which the flow cytometry could confirm the diagnosis (± 3 –20 hours, compared to 2–10 days for histopathological diagnosis). Because the diagnosis could be confirmed within 24 hours in 75% of the cases, the authors recommend to use both techniques, allowing chemotherapy to commence within 24 hours. In contrast with the results of our study and two comparable, though much smaller studies on brain biopsies, flow cytometry on bone marrow and CSF allowed identification of additional lymphoma cases over cytology. The sensitivity of cytological analysis of CSF for lymphoma cells is low (2–32%) (30). Several authors found that additional flow cytometry on CSF improves the sensitivity, up to 2–3 fold (13–15). In up to 80%, the lymphoma cells are detected in the first CSF sample, analyzed by flow cytometry (15). It is likely that this additional sensitivity of flow cytometry is a result of the low number of tumor cells available for diagnosis in CSF and bone marrow. Corticosteroids can induce apoptosis in lymphoma cells. This can mask the morphology and can even cause the tumor to vanish (31–33). In lymph nodes and CSF samples, flow cytometry can confirm a diagnosis on samples with a low cell count. We hypothesized that flow cytometry, being a more sensitive technique, may be able to recognize lymphoma in patients in whom, after steroid use, lympholysis had taken place and histology plus IHC was negative. Unfortunately, this was not the case in our series nor in the other two smaller studies available. Five patients who went for multiple biopsies and were diagnosed with brain lymphoma after

their second or third biopsy, used corticosteroids prior to their first (and second) biopsy. In none of these patients flow cytometry analysis was able to make the diagnosis when histology plus IHC were non-diagnostic. Clearly, immunohistochemistry as well as flow cytometry analysis can be compromised in patients using corticosteroids prior to the biopsy.

The strengths of this study are the comprehensive clinical and laboratory data in a large, unselected sample, allowing calculation of the diagnostic and clinical value of flow cytometry on brain biopsies. To the best of our knowledge, this is the largest cohort ever described comparing flow cytometry to immunohistochemistry in brain biopsies. Furthermore, due to our large population, we were able to show that the negative effect of corticosteroids on the diagnostic value of flow cytometry was similar to that on IHC. Even our series, however, still concerns a relatively small number of cases. The main drawback of our study is its retrospective nature: we may have missed some biopsies, even though we did a thorough search through all available databases in our hospital (neurosurgery, flow cytometry, pathology and neuro-oncology) and the immunologist and pathologist were not blinded for each other's results. Nevertheless the flowcytometric result was always reported without knowledge of the pathological evaluation. In addition, we did not perform freeze sections, so comparison with intraoperative diagnosis could not be made.

CONCLUSION

Flow cytometry analysis in brain biopsy is a feasible technique with 100% specificity to confirm the diagnosis of brain lymphoma in patients suspected for lymphoma on clinical grounds. The added clinical value is the speed by which flow cytometry can establish or confirm the diagnosis, enabling a faster initiation of treatment, while false positive cases were not identified. Flow cytometry is complementary to, but not more sensitive than, histopathology with immunohistochemistry analysis. We recommend to perform flow cytometry and immunohistochemistry in parallel in brain biopsies, suspected for a lymphoma.

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CONFLICT OF INTEREST

The authors have no conflict of interest to disclose

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