Contribution of cerebrospinal fluid sCD19 levels to the detection of CNS lymphoma and its impact on disease outcome

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**KEY POINTS**

Increased levels of soluble CD19 protein in the CSF are associated with CNS disease in DLBCL and BL patients at risk of CNS lymphoma

Presence of lymphoma cells by FCM and/or increased CSF sCD19 levels are related with a poorer EFS and/or OS in DLBCL and BL patients

**ABSTRACT**

Flow cytometry (FCM) is more sensitive than conventional cytology for detection of occult leptomeningeal lymphoma; however, some FCM-negative patients show central nervous system (CNS) recurrence. Here we evaluated the cerebrospinal fluid (CSF) levels of 13 B-cell associated markers and their contribution to the diagnosis of CNS lymphoma in 91 diffuse large B-cell lymphomas (DLBCL) and 22 Burkitt lymphomas (BL). From all markers tested, CD19 was the most informative. Thus, higher soluble CD19 levels (sCD19) were associated with a greater frequency of neurological symptoms in DLBCL and BL and with parenchymal CNS lymphoma in DLBCL, sCD19 emerging as a powerful predictor of event-free and overall survival in DLBCL and BL, particularly when combined with FCM detection of CNS disease. These results support the utility of combined FCM detection of lymphoma cells and assessment of sCD19 levels in CSF, for more accurate identification of CNS disease in DLBCL and BL patients.

**Keywords:** Central nervous system lymphoma, cerebrospinal fluid, CD19, flow cytometry, Burkitt lymphoma, diffuse large B-cell lymphoma
INTRODUCTION

Primary or secondary central nervous system (CNS) involvement by B-cell non-Hodgkin’s lymphoma (NHL) is a relatively rare condition associated with a very poor prognosis. Currently, it is well-established that flow cytometry (FCM) has a greater sensitivity than conventional cytology (CC) for the detection of cerebrospinal fluid (CSF) infiltration in Burkitt lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL) patients at risk of CNS disease. However, still a significant proportion of FCM/CC cases (~10%) presents with highly suspicious neurological symptoms and/or parenchymal CNS disease. Moreover, recent data indicates that some FCM-negative cases may develop overt CNS disease early after diagnosis, particularly when CNS-directed prophylaxis had not been administrated.

Altogether, these findings support the notion that parenchymal involvement of CNS by aggressive B-NHL could go undetected by both CC and FCM because, in the absence of leptomeningeal disease, lymphoma cells would not reach the CSF in detectable numbers. Although recirculation of tumor cells through CSF might still exist in such cases, it could be hypothesized that if an increased turnover of lymphoma cells occurs inside the brain, tumor cell products (e.g. proteins, RNA, DNA and microvesicles) would be released at abnormally high rates into the extracellular medium. Under such circumstances, the lymphoma cell components could potentially reach the CSF. Actually, higher levels of specific miRNAs (e.g. miR-19b, miR-21 and miR-92a) and proteins (e.g. CD27) have been reported in CSF from CNS lymphoma vs. CNS lymphoma-free patients. However, in these studies, no B-cell specific markers expressed by every lymphoma case, have been investigated.
Herein, we analyzed the CSF levels of a large panel of B-cell associated proteins in 113 B-NHL (DLBCL and BL) at risk of CNS disease, to determine their potential utility as surrogate markers for CNS lymphoma.

MATERIALS AND METHODS

Patients and samples. A total of 113 patients diagnosed with DLBCL (n=91) and BL (n=22) using the WHO criteria, at the hospitals of the Spanish CNS Lymphoma Study Group (Spain), were studied. Only BL patients plus aggressive DLBCL who met ≥1 of the following criteria entered the study: infiltration of extranodal sites, elevated serum LDH and/or neurological symptoms. The study was approved by the Ethics Committees of the participating centers, following the Declaration of Helsinki protocol.

Patients were treated according to their institutional standards with CHOP or CHOP-like regimens including rituximab for DLBCL, and standard regimens for BL. All but 6 DLBCL patients received intrathecal prophylaxis (n=82) or active treatment (n=25). Median follow-up was of 30 months (range: 1-54 months); 21 patients had recurrent disease (18 DLBCL and 3 BL) from which two DLBCL had CNS relapse.

Cerebrospinal fluid analyses. Conventional cytomorphological analysis of CSF was performed on fresh samples at each center, while FCM was done in Transfix™-stabilized CSF samples (<24h) at the University Hospital of Salamanca (Spain), as described elsewhere3,15 (Supplementary text).

Quantification of soluble CD19, CD21, CD22, CD24, CD38, CD44, CD72, free light chain (FLC)-kappa, FLC-lambda, IgA, IgG, IgM and β2-microglobulin was
performed in 100µL of thawed, freshly-frozen CSF supernatants by standard ELISA, FLC and β₂-microglobulin assays (Supplementary text).

**Statistical methods.** Receiver operating curve (ROC) analysis was performed in a subgroup of 40 CSF samples to establish the most accurate cut-off values for each CSF marker, including sCD19 (≥1.18ng/mL); sCD19 was further evaluated in another 73 CSF samples using the same (most informative) cut-off (n=113). Event-free survival (EFS) and overall survival (OS) were determined by the Kaplan-Meier method and compared by the log-rank test. Multivariate Cox proportional hazard models (stepwise regression) were developed to explore the independent effect of different parameters on EFS and OS.

**RESULTS AND DISCUSSION**

First, we analyzed the CSF levels of a panel of 13 B-cell associated markers in 40 B-NHL patients at risk of CNS disease, classified according to the presence vs. absence of CNS disease by FCM and/or presence of highly suspicious neurological symptoms. From all markers investigated in these 40 cases, only sCD19 (≥1.18ng/mL) and to a lesser extent also β₂-microglobulin (≥2.56ng/mL), showed a significant association (p<.05) with CNS disease, with an accuracy of 88% and 78%, respectively (vs. <70% for all the other markers) (Supplementary Table 1 and supplementary Figure 1). Of note, CD19 was the only protein investigated which is both a pan-B cell marker and a B-cell specific protein. All other markers display either a pattern of expression restricted to specific B-cell maturation stages and to some subtypes of B-NHL - including BL and DLBCL- where they are expressed at variable percentages (e.g. CD21
and CD24)\textsuperscript{17-19}, and/or they are also expressed by cells other than B-cells from brain tissues (e.g. neurons, astrocytes, glial and glioma cells)\textsuperscript{20-22}. Overall, this could contribute to explain why sCD19 emerged as the marker with the highest degree of association with CNS lymphoma. However, the biological significance of sCD19 remains to be fully elucidated.

Further analysis of sCD19 in the whole cohort of 113 patients (Table 1), showed that higher sCD19 CSF levels (≥1.18 ng/mL) were more frequently found among those DLBCL cases displaying overt (FCM\textsuperscript{+}/CC\textsuperscript{+}; p=.05) and occult (FCM\textsuperscript{+}/CC\textsuperscript{−}; p<.001) leptomeningeal disease, as well as among DLBCL and BL patients who presented with highly suspicious neurological symptoms (p<.001 and p<.02, respectively) and DLBCL cases with parenchymal lymphoma by MRI (p=.007). DLBCL cases with higher sCD19 levels also showed older age (p=.04), higher International Prognostic Index (p=.05), and poorer performance status (p=.04). These results indicate that sCD19 could be a sensitive marker for the detection of CNS involvement in patients with BL and DLBCL at risk of CNS disease in routine diagnostics, particularly if combined with FCM. At the same time, they suggest that sCD19 CSF levels in DLBCL and BL are not directly impacted by systemic disease since no association (p>.05) of sCD19 with peripheral blood, lymph node and bone marrow involvement, was observed. Then, we further investigated the potential impact of sCD19 CSF levels on patient survival.

Several studies have shown a clear association between occult leptomeningeal disease detected by FCM and a shorter CNS relapse-free survival\textsuperscript{23}. Despite a similar frequency of occult leptomeningeal disease was found in these and our series, only two of our patients had CNS recurrence, which is probably due to the administration of active therapy to most (71%) of our FCM\textsuperscript{+} cases. Consequently, it was not possible to establish here, the impact of sCD19 on CNS relapse-free survival. However, previous
studies have also recurrently shown that occult leptomeningeal disease by FCM has a clear impact on OS, of both DLBCL and BL. In this regard, we also found an association between higher sCD19 CSF levels and a shorter EFS and OS among DLBCL (p=.02 and p=.002, respectively; Figure 1A-B) and BL patients (p=.09 and p=.04, respectively; Figure 1G-H). Even more, once FCM-negative cases were specifically considered, FCM-negative BL patients with higher sCD19 showed a shorter EFS (p=.02) and OS (p=.02) than FCM-/sCD19low cases, the survival rates of FCM+/CC- /sCD19high cases being similar (p>.05) to those of FCM+/CC- BL patients (Figure 1I-J); a similar tendency (p>.05) was also observed for DLBCL (Figure 1C-D). The greater impact of sCD19 than FCM on BL patient survival supports the notion that in high proliferative tumors with increased apoptosis such as BL, the measurement of sCD19 could be particularly informative. Further studies are required to validate our findings and determine whether sCD19 CSF measurements should become routine in BL and high, as well as low risk, DLBCL. In turn, our results suggest that despite the absence of CNS recurrence, minimal CNS involvement (e.g. CC- but FCM+ and/or sCD19high) may contribute to systemic disease recurrence, particularly when no CNS-directed therapy is used. Most interestingly, sCD19 CSF levels, when combined with FCM, emerged as a powerful independent CSF-associated prognostic factor for OS (p=.007) in DLBCL and for both EFS (p=.03) and OS (p=.05) in BL (Supplementary Tables 2 and 3).
Acknowledgements/Funding:

This work was supported in part by grants RD06/0020/0035 and RD12/0036/0048 from RETICS (Instituto de Salud Carlos III, Ministerio de Economía y Competitividad, Madrid, Spain and Fondos FEDER) and an unrestricted grant from Mundipharma Pharmaceuticals, SL.

Footnotes:

The online version of the article contains a data supplement.

Authorship Contributions:

C.M. and L.M.M. performed experiments, analyzed results, made figures and contributed to the writing of the manuscript, A.L. and J.A. analyzed results, A.O. designed the research, interpreted data and wrote the manuscript, the other authors enrolled patients and collected clinical data; all authors read and agreed with the contents of the manuscript.

Disclosure of Conflicts of Interest:

The authors declare that there are no conflicts of interest to disclose.
REFERENCES

FIGURE LEGEND

Figure 1. Prognostic impact of soluble (s) CD19 levels, conventional cytology (CC), flow cytometry immunophenotyping (FCM) and their combination, in cerebrospinal fluid (CSF) samples from diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma (BL) patients screened for central nervous system disease. Event-free survival (panels A, C, E, G, I and K) and overall survival (panels B, D, F, H, J and L) curves are separately shown for DLBCL (panels A to F) and BL (panels G to L) cases, classified according to their CSF status as defined by sCD19 levels in both the whole patient group (panels A, B, G and H) and among only FCM cases (panels C, D, I and J), and by CC and FCM in combination with sCD19 (panels E, F, K and L).
Table 1. Soluble CD19 protein (sCD19) levels in cerebrospinal fluid (CSF) samples from diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma (BL) patients screened for central nervous system (CNS) disease: association with other prognostic factors of the disease.

<table>
<thead>
<tr>
<th>Disease features</th>
<th>Total cases (n=113)</th>
<th>DLBCL</th>
<th>BL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female)</td>
<td>66%/34%</td>
<td>59</td>
<td>13</td>
</tr>
<tr>
<td>Age at diagnosis ≥60 years</td>
<td>43%</td>
<td>41%</td>
<td>8%</td>
</tr>
<tr>
<td>Stage III/IV</td>
<td>74%</td>
<td>66%</td>
<td>77%</td>
</tr>
<tr>
<td>IPI ≥2</td>
<td>75%</td>
<td>66%</td>
<td>77%</td>
</tr>
<tr>
<td>ECOG ≥2</td>
<td>49%</td>
<td>41%</td>
<td>38%</td>
</tr>
<tr>
<td>Neurological symptoms*</td>
<td>21%</td>
<td>5%</td>
<td>15%</td>
</tr>
<tr>
<td>Adenopathies</td>
<td>80%</td>
<td>81%</td>
<td>62%</td>
</tr>
<tr>
<td>Hepato and/or splenomegaly</td>
<td>30%</td>
<td>36%</td>
<td>23%</td>
</tr>
<tr>
<td>Extraneal involvement</td>
<td>83%</td>
<td>80%</td>
<td>92%</td>
</tr>
<tr>
<td>Extraneal sites involved ≥2</td>
<td>38%</td>
<td>27%</td>
<td>38%</td>
</tr>
<tr>
<td>PB involvement</td>
<td>6%</td>
<td>2%</td>
<td>23%</td>
</tr>
<tr>
<td>BM involvement</td>
<td>34%</td>
<td>27%</td>
<td>67%</td>
</tr>
<tr>
<td>Parenchymal CNS disease</td>
<td>8%</td>
<td>2%</td>
<td>8%</td>
</tr>
<tr>
<td>Hemoglobin &lt;100 g/L</td>
<td>25%</td>
<td>22%</td>
<td>38%</td>
</tr>
<tr>
<td>N. of platelets &lt;100 x10^9/L</td>
<td>12%</td>
<td>5%</td>
<td>23%</td>
</tr>
<tr>
<td>N. of lymphocytes &gt;5 x10^9/L</td>
<td>19%</td>
<td>10%</td>
<td>33%</td>
</tr>
<tr>
<td>Total proteins &lt;70 g/L</td>
<td>73%</td>
<td>73%</td>
<td>85%</td>
</tr>
<tr>
<td>Serum LDH ≥450 IU/L</td>
<td>62%</td>
<td>61%</td>
<td>69%</td>
</tr>
<tr>
<td>CRP &gt;0.5 mg/dL</td>
<td>81%</td>
<td>86%</td>
<td>78%</td>
</tr>
<tr>
<td>Serum β2-M ≥3 mg/L</td>
<td>48%</td>
<td>50%</td>
<td>30%</td>
</tr>
<tr>
<td>Immune suppression</td>
<td>14%</td>
<td>14%</td>
<td>15%</td>
</tr>
<tr>
<td>HIV+</td>
<td>9%</td>
<td>10%</td>
<td>15%</td>
</tr>
<tr>
<td>Intrathecal prophylaxis</td>
<td>73%</td>
<td>88%</td>
<td>77%</td>
</tr>
<tr>
<td>Active intrathecal treatment</td>
<td>22%</td>
<td>5%</td>
<td>23%</td>
</tr>
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CSF parameters

| Glucose >120 mg/dL              | 4%      | 2%    | 0%  |
| Total proteins ≥0.45 g/L        | 29%     | 25%   | 33% |
| FCM+/CC+                        | 8%      | 2%    | 8%  |
| FCM+/CC-                        | 13%     | 0%    | 8%  |

NS, not statistically significant; IPI, international prognostic index; ECOG, eastern cooperative oncology group; PB, peripheral blood; BM, bone marrow; LDH, lactate dehydrogenase; CRP, c-reactive protein; β2-M, β2-microglobulin; HIV, human immunodeficiency virus; FCM, flow cytometry; CC, conventional cytology.

*15/24 (63%) patients with neurological symptoms received active intrathecal therapy; †Cases with CNS relapse from acute lymphoblastic leukemia.
Figure 1

Diffuse Large B-Cell Lymphoma

A. CSF sCD19 < 1.18
   CSF sCD19 ≥ 1.18

B. FCM CSF sCD19 < 1.18
   FCM CSF sCD19 ≥ 1.18

C. CC FCM CSF sCD19 < 1.18
   CC FCM CSF sCD19 ≥ 1.18

Burkitt Lymphoma

G. CSF sCD19 < 1.18
   CSF sCD19 ≥ 1.18

H. FCM CSF sCD19 < 1.18
   FCM CSF sCD19 ≥ 1.18

I. CC FCM CSF sCD19 < 1.18
   CC FCM CSF sCD19 ≥ 1.18

J. CC FCM CSF sCD19 ≤ 1.18 ng/mL
   CC FCM CSF sCD19 ≥ 1.18 ng/mL

K. CC FCM CSF sCD19 ≤ 1.18 ng/mL
   CC FCM CSF sCD19 ≥ 1.18 ng/mL

L. CC FCM CSF sCD19 ≤ 1.18 ng/mL
   CC FCM CSF sCD19 ≥ 1.18 ng/mL
   CC+ (n= 4)
Contribution of cerebrospinal fluid sCD19 levels to the detection of CNS lymphoma and its impact on disease outcome

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