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STAT6 activation correlates with cerebrospinal fluid IL-4 and IL-10 and poor prognosis in Primary Central Nervous System Lymphoma

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ABSTRACT

Primary central nervous system lymphoma (PCNSL) is a rare and aggressive lymphoma with a dismal outcome in the majority of cases. In clinical practice, prognosis is estimated through clinical risk scoring, which can help in guiding the appropriate management. However, there is no established biomarker to track treatment response and relapse. Here, we showed that tumor expression of activated STAT6 (pSTAT6) correlates with cerebral spinal fluid (CSF) levels of its activator interleukins, IL-4 (r 0.62, p=0.013) and IL-10 (r 0.97, p= <0.001), suggesting their potential use as surrogate biomarkers of JAK/STAT activation. Elevated CSF cytokines levels were significantly associated with a shorter progression free survival (IL-4 HR 2.02, 95% CI 1.07-2.99; IL-10 HR 3.32, 95%CI 1.18-1.84), and presented a positive trend with inferior overall survival (IL-4 HR 1.75, 95% CI 0.93-2.45; IL-10 HR 2.09, 95%CI 0.89-1.43). In addition, CSF IL-4 and IL-6 levels correlated with response to therapy and relapsed disease. These results indicate that the CSF levels of IL-4 and IL-10 may be useful biomarkers of prognosis and disease activity in patients with PCNSL.

INTRODUCTION

Primary central nervous system lymphomas (PCNSL) are rare forms of diffuse large B cell lymphoma (DLBCL) that arise within the brain or the eyes[1]. Immunohistochemical analysis has shown that the majority of PCNSL express melanoma associated antigen 1 (MUM1)/interferon regulatory factor 4 (IRF4), resembling the activated B cell (ABC) immunophenotype[1]. Somatic mutations in *MYD88* and *CD79B* genes, coding proteins that induce NF-kB activation, are very common in PCNSL[2–4]. Several lines of evidence suggest that *JAK/STAT* pathway may also contribute to survival signaling in PCNSL, which is consistent with underlying activation of *MYD88* in this disease.

STAT proteins are transcription factors which play a crucial rule in proliferation and survival of B lymphocytes and are often deregulated in lymphomas[5]. In particular, expression and/or activation of STAT6[6,7] as well as amplification of the locus encoding STAT6 on chromosome 12 have been detected in more than 50% of PCNSL specimens[8]. Notably, the increased expression of activated STAT6 has been associated with early progression and short survival[6]. Interleukin-4 (IL-4) and IL-10, two activators of the JAK/STAT pathway, are also upregulated in PCNSL[6]. Similarly, changes in cerebral spinal fluid (CSF) concentration of IL-10 correlate with prognosis[9]. **Figure 1** shows a diagram of the IL-4, IL-10 and JAK/STAT signaling. Pharmacological inhibition or gene silencing of the STAT constitutive signal has shown anti-tumor activity[5]. Hence, STAT dependency is an exploitable target in patients with PCNSL with JAK/STAT somatic mutations. Here, we investigated the putative role of STAT6 phosphorylation (pSTAT6) and its activator cytokines IL-4 and IL-10 as biomarkers of prognosis and disease activity for PCNSL.

MATERIALS AND METHODS

Patients. From January 2009 to January 2016, 25 newly diagnosed human immunodeficiency virus (HIV)-negative patients affected by PCNSL were assessed at the University of Messina. Histologic diagnosis was performed according to the WHO criteria[1] by an expert pathologist. Patients were subdivided into 3 risk groups based on the International Extranodal Lymphoma Study Group (IELSG) scoring system. The score is based on five parameters, namely age >60 years, elevated serum lactate dehydrogenase (LDH), Eastern Cooperative Oncology Group score (ECOG) ≥2, involvement of deep brain structures, and raised CSF protein levels.[10] To examine the CSF sample, all the patients underwent lumbar puncture after informed consent, and IL-4 and IL-10 CSF levels were analyzed. Routine biochemical examination, including white cell counts, cytological examinations, and flow cytometry, was performed as well. The lumbar puncture was carried out in all patients at diagnosis and after the first line treatment and repeated in those with relapsed disease. All patients received high dose (HD) methotrexate (MTX)-based polychemiotherapy without whole brain radiotherapy (WBRT) consolidation according to the protocols of the *Association des Neuroncologues d'Expression Francaise*. Thirteen of them were treated with high dose cytarabine (Ara-C) combined to HD-MTX and rituximab, eight with MRT (HD-MTX, rituximab and temozolomide) regimen and three with the EORTC protocol (HD-MTX, lomustine, procarbazine, methylprednisolone, and intrathecal MTX and Ara-C). Response rate was assessed according to the International PCNSL Collaborative Group (IPCG) criteria.[11]

The study was conducted in accordance with the Declaration of Helsinki. Patients' written consent was obtained for sample collection, immunohistochemical and clinical analysis. The institutional ethics committee reviewed and approved the study. The data that support the findings of this study are available from the corresponding author upon reasonable request.

*Immunohistochemistry***.** Tumor samples were fixed in 4% formaldehyde and embedded in paraffin. Deparaffinized slides were antigen retrieved in citrate buffer pH 6.4 and endogenous peroxidase (HRP) activity was blocked by treating the sections with 3% hydrogen peroxide in methanol. Indirect immunohistochemistry was performed with antispecies-specific biotinylated secondary antibodies followed by avidin–horseradish peroxidase or avidin-AP, and developed by Vector Blue or DAB color substrates (Vector Laboratories). pSTAT6 antibody was purchased from Santa Cruz Biotechnology (#sc-374021). Photomicrographs were examined using a Zeiss Axioskop imaging microscope.

Enzyme-linked immunosorbent assay. To measure IL-4 and IL-10 levels in the CSF, 2–5 mL of CSF was obtained by lumbar puncture at the time of diagnosis, at the end of treatment and at relapse. The CSF samples were centrifuged and immediately stored at -80C. Determination of IL-4 and IL-10 concentration in CSF was performed in duplicate and quantified by standard curve. Enzyme-linked immunosorbent assay kits for IL-4 were supplied by BioLegend (#430301); for IL-10 by Aviva Systems Biology (#OKBB00193). The interleukins levels were considered as continuous variables. The kit range was 3.9-250 pg/mL for IL-4 and 7.8-500 pg/mL for IL-10.

Statistical analyses. Spearman's correlation was performed to evaluate the interdependency of CSF cytokines, tumor pSTAT6 expression and CSF cellularity. Overall survival (OS) and progression free survival (PFS) were estimated using the Kaplan–Meier method and defined according to the IPCG criteria.[11] Cox regression was used to identify independent prognosticators for PFS and OS. Multivariate analysis was performed only on the covariates that were significant at univariate analysis. All tests were 2 tailed, and $p<0.05$ was considered statistically significant. Statistical analyses were performed with MedCalc (version 11.0;

MedCalc Software Acacialaan, Ostend, Belgium) software and the GraphPad Prism (version 5.0; GraphPad Software, Inc., San Diego, CA, USA) package.

RESULTS

Patient characteristics at diagnosis. Overall, the median age at time of diagnosis was 52 years (range 41-69 years). A male predominance was observed (15/25, 60%). Most patients had elevated LDH (19/25, 76%), elevated CSF protein levels (18/25, 72%) and involvement of the deep cerebral structures (20/25, 80%). The median Karnofsky performance status (KPS) was 70% (range 50-90%). According to the IELSG risk scoring, nearly half of the patients were stratified into the intermediated risk group (12/25, 48%), six (24%) into the low risk and seven (28%) into the high risk. Similar to Rubenstein et al[6], immunohistochemical analysis presented three different patterns of pSTAT6 expression: protein absent with a low density of tumor cells (n =7, group A); scattered positivity of pSTAT6 and low cell density (n =8, group B); pSTAT6 in tumor cells as well as in the vascular endothelium, with a high cell density (n =10, group C) **(Figure 2)**. Notably, the IHC pattern largely corresponded with risk scoring. In detail, six patients in group A were in the low risk group and one in the intermediate, all the patients in group B were in the intermediate risk group, of the 10 patients in group C three were in the intermediate risk group and seven in the high risk group. The CSF levels of IL-4 and IL-10 were low in group A and B (8.5 ± 3.2 and 24.6 ± 12.8, respectively) and high in group C (29.8 ± 6.5 and 108.2 ± 15). To evaluate the interdependence of pSTAT6 expression and CSF cytokines in PCNSL patients, Spearman's rank coefficients were calculated pairwise. The pSTAT6 expression was correlated with increased CSF IL-10 (r 0.97, p= <0.001) and IL-4 levels (r 0.62, p=0.013). In addition, CSF cellularity was associated with increased CSF levels of IL-10 (r 0.68, p= 0.004) and IL-4 (r

0.56, p=0.029) as well. Taken together these data suggest that CSF IL-4 and IL-10 may be considered as surrogate biomarkers for JAK/STAT activation and potential diagnostic tools.

Response and survival. In group A and B, seven patients achieved a complete response (CR) (47%), six a partial response (PR) (40%) and two a stable disease (SD) (13%). In group C, no CR rates were observed, three patients obtained a PR (30%), two a SD (10%), and five patients were primary refractory to induction therapy (50%). At the time of analysis, the median follow-up was 38 months (range 11-50 months). Median PFS and OS were not reached in group A, 29 months (range 22-37 months) and 20 months (range 10-38 months) in group B, 4.5 months (2-12.5 months; p= < 0.001) and 6 months (range 3-15 months; p= 0.03) in group C. The most common cause of death was lymphoma-related. The post-therapy CSF concentration of IL-4 and IL-10 was lower than the pre-treatment measurement in all patients who achieved at least stable disease and correlated with treatment response. In particular, in group A and B the CSF levels of both cytokines dropped below the detection limit, whereas in group C both remained measurable in all cases and increased in the patients with refractory disease **(Figure 3** and **Supplementary Table 1)**. Similarly, seven patients who relapsed within 2 years from diagnosis presented raising levels of CSF IL-4 and IL-10 with median CSF concentration of 38 ± 18.2 and 185 ± 92 pg/mL, respectively. We also examined the impact of the CSF cytokines concentration on the outcomes of PCNSL patients. In univariate analysis, elevated CSF levels of IL-4 and IL-10 as well as elevated LDH and CSF proteins were significantly associated with a shorter PFS. Of these, only elevated CSF IL-4 and IL-10 levels demonstrated to be independent prognosticators. Even if not statistically significant, there was a positive trend between CSF IL-4 and IL-10 levels and shorter OS **(Table 1)**.

DISCUSSION

To the best of our knowledge this is the first study suggesting a correlation between tumor expression of pSTAT6 and CSF IL-4 and IL-10 levels, which ultimately seems to translate in poor outcome in PCNSL patients. Furthermore, we showed that measuring IL-4 and IL-10 in the CSF is a useful tool to monitor therapeutic response and predict disease relapse.

In this study, patients with high tumor expression of pSTAT6 presented with elevated CSF IL-4 and IL-10, whereas low-intermediate tumor expression was associated with lower levels of both cytokines. The CSF levels of IL-4 and IL-10 correlated with positive CSF cellularity as well, suggesting their possible use as diagnostic tool. In agreement with the literature[6,12], elevated levels of activated STAT6 as well as CSF IL-4 and IL-10 correlated with inadequate disease control after therapy and shorter PFS. In addition, trends were observed for patients having elevated CSF cytokines levels to inferior OS. These findings suggest that CSF IL-4 and IL-10 may reflect the severity of PCNSL and thus may facilitate the therapeutic decision-making process for individual patients. Similarly, the CSF IL-4 and IL-10 levels rose in the relapse/refractory setting, which may reflect the tumor burden or active disease and might be helpful to monitor response to therapy and predict relapse. However, the underlying molecular mechanism is unclear. Recent studies have indicated that a high frequency of MYD88 and CD79 mutations in PCNSL may be initiators of disease, leading to constitutive activation of NF-kB pathway and eventually resulting in increased secretion of IL-4 and IL-10. The existence of an amplification loop that links MYD88 and JAK/STAT signaling through IL-4 and IL-10[13] may be culprit in feeding this vicious mechanism, resulting in a more aggressive disease. Our data suggest that CSF IL-4 and IL-10 may function as surrogate biomarkers of JAK/STAT activation and might be used for selection of lymphoma patients if their predictive value will be confirmed. However, the study sample size limited the power of our conclusions and these results should be validated in a larger patient cohort. Targeting

JAK/STAT might be a therapeutic avenue in PCNSL with aberrant activation of this pathway. However, single agent regimens are unlikely to be curative in PCNSL, therefore it is important to understand the pathogenic mechanisms sufficiently well so to construct rationally designed combinatorial regimens. In accordance with the high frequency of mutations in the BCR and MYD88 pathways, the BTK inhibitor ibrutinib has demonstrated promising results in PCNSL[3,4,14] and it may yield a powerful synthetic lethal effect in combination with compounds interfering with JAK/STAT or with the mammalian target of rapamycin (mTOR) / phosphoinositide 3-kinases (PI3K). Indeed, active mTOR/PI3K pathway is common in PCNSL bearing CD79B mutations[3] and synergism is achieved in ABC-DLBCL models combining ibrutinib and inhibitors of the PI3K/mTOR[3,15–17] with a stronger inhibition, for example, of IL-4 production[15].

In conclusion, our study provides evidence that tumor expression of activated STAT6 and CSF IL-4 and IL-10 levels are potential prognostic biomarkers of PCNSL. Furthermore, CSF cytokines levels might be useful to monitor therapeutic response and predict disease relapse. However, future studies are warranted to confirm these promising results.

CONFLICT OF INTEREST

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Figure 1. Activation of the JAK/STAT pathway by cytokine receptor and its regulation on gene expression

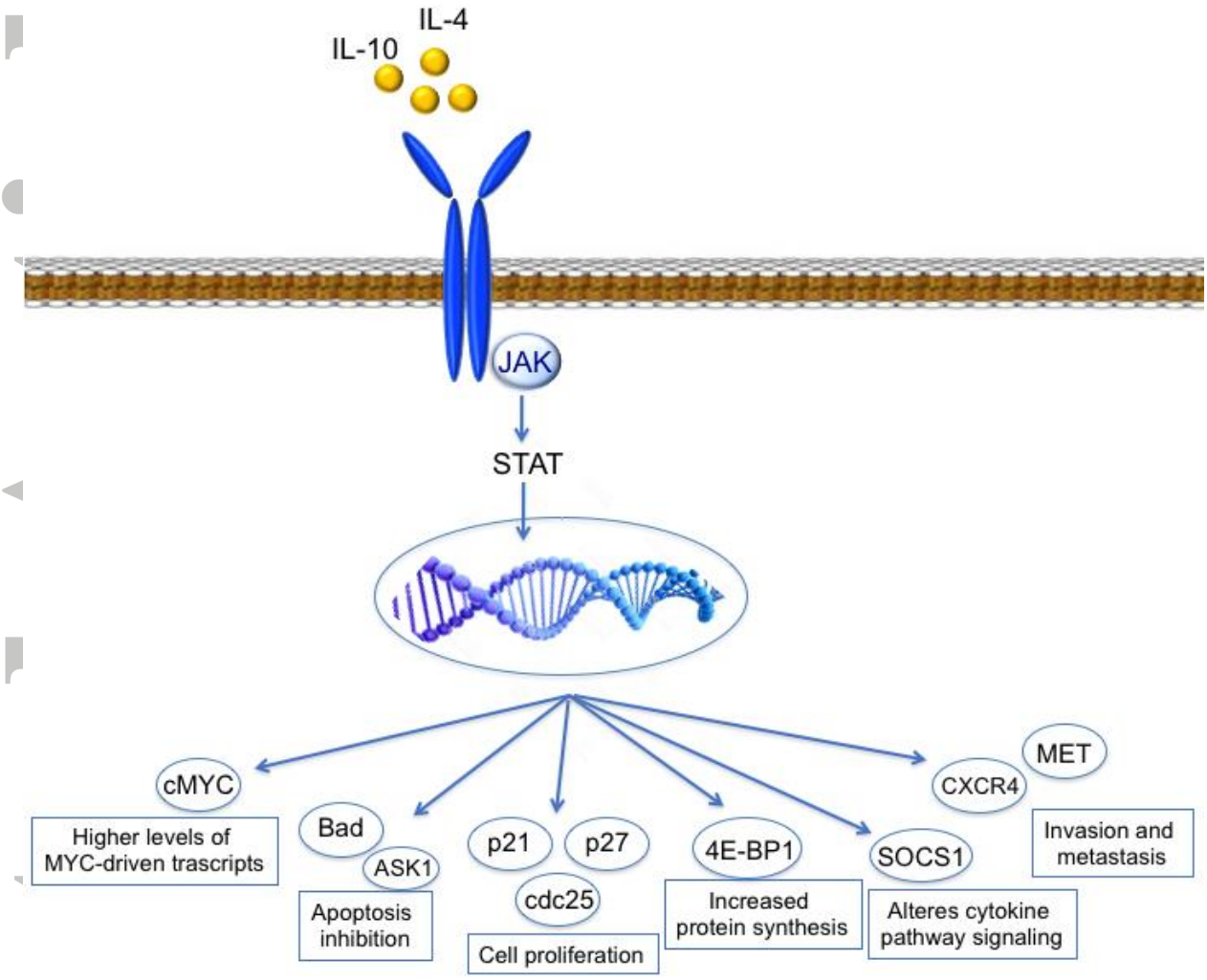

Figure 1. Activation of the JAK/STAT pathway by cytokine receptor and its regulation on gene expression.

Figure 2. Immunohistochemical pattern expression of pSTAT6 in PCNSL.

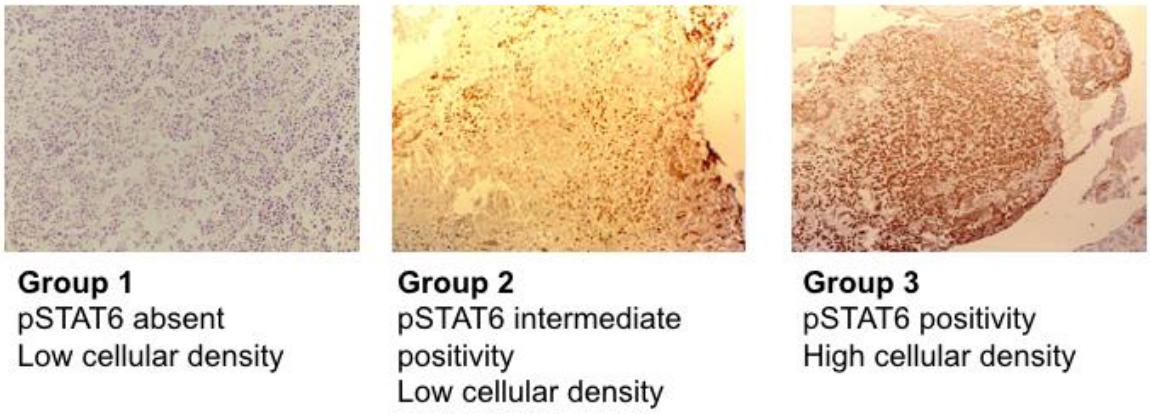

Figure 2. Immunohistochemical pattern expression of pSTAT6 in PCNSL.

Figure 3. CSF levels of IL-4 and IL-10 at diagnosis and after treatment in the patients with PCNSL.

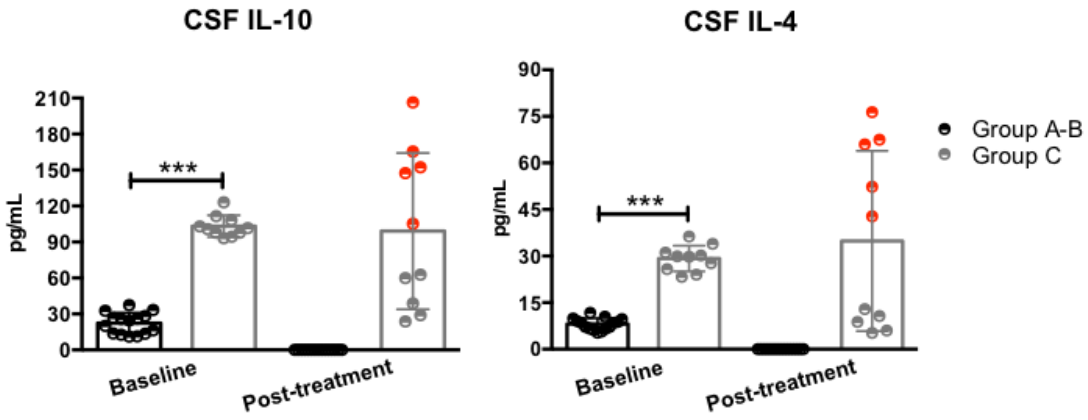

Figure 3. CSF levels of IL-4 and IL-10 at diagnosis and after treatment in the patients with PCNSL. Differences between groups were calculated with the Student *t* test. *** $p < 0.001$. Depicted in red patients with primary refractory disease.

Table 1. Cox's regression analysis for progression free survival and overall survival.

Univariate Analysis						
	PFS			**OS**		
	HR	95% CI	P	HR	95% CI	P
Sex	1.04	0.92-2.44	0.228	1.04	0.92-2.15	0.674
Age >60	1.10	0.98-1.02	0.181	1.02	0.98-1.01	0.925
Elevated LDH	1.52	1.00-1.32	0.037	1.46	0.96-1.29	0.352
Elevated CSF protein	2.01	1.03-1.21	0.016	1.93	0.82-1.50	0.052
Deep brain involvement	1.34	0.85-1.02	0.350	1.37	0.78-0.99	0.092
KPS	1.32	0.67-2.61	0.425	1.07	0.73-2.31	0.374
CSF levels of IL-4	2.93	1.07-2.99	0.023	2.16	1.03-1.99	0.051
CSF levels of IL-10	3.77	1.21-1.93	< 0.001	3.11	1.11-1.48	0.034
Multivariate Analysis						
	PFS			**OS**		
	HR	95% CI	P	HR	95% CI	P
Elevated LDH	1.33	1.02-1.41	0.099	1.31	0.32-1.77	0.851
Elevated CSF protein	1.88	1.00-1.39	0.045	1.42	0.55.2.56	0.329
CSF levels of IL-4	2.02	1.07-2.99	0.044	1.75	0.93-2.45	0.810
CSF levels of IL-10	3.32	1.18-1.84	0.029	2.09	0.89-1.43	0.482

Abbreviations: PFS, progression free survival; OS, overall survival; HR, hazard ration; CI, confidence interval; LDH, lactate dehydrogenase; CSF, cerebral spinal fluid; KPF, Karnofsky performance status, IL, interleukin.