Genomic Profiling of Primary Diffuse Large B-Cell Lymphoma of the Central Nervous System Suggests Novel Potential Therapeutic Targets

(Article begins on next page)

24 September 2024

1 **Genomic profiling of CNS-DLBC suggests novel potential therapeutic targets.**

2

3 Claudio Agostinelli[1,2], Luca Morandi[3,4], Simona Righi[2], Luigi Cirillo[4], Marica Iommi[3], Caterina

4 Tonon[3,4], Diego Mazzatenta[5], Matteo Zoli[3,4], Maura Rossi[1], Gianmarco Bagnato[2,6], Alessandro

5 Broccoli[2,6], Raffaele Lodi[3,4], Pier Luigi Zinzani[2,6], Elena Sabattini[1], Caterina Giannini[3,7], Sofia

6 Asioli[3,5].

7

8 [1] Haematopathology Unit, IRCCS Azienda Ospedaliero-Universitaria of Bologna, Via Massarenti 9,

9 40138, Bologna, Italy.

10 [2] Department of Medical and Surgical Sciences, University of Bologna, 40138 Bologna, Italy

11 [3] Department of Biomedical and Neuromotor Sciences, University of Bologna, 40138 Bologna,

12 Italy.

13 [4] IRCCS Istituto delle Scienze Neurologiche di Bologna, Functional and Molecular Neuroimaging

14 Unit, Bologna, Italy.

15 [5] IRCCS Istituto delle Scienze Neurologiche di Bologna, Center for the Diagnosis and Treatment of

16 Hypothalamic-Pituitary Diseases, Pituitary Unit

17 [6] IRCCS Azienda Ospedaliero-Universitaria di Bologna Istituto di Ematologia "Seràgnoli" Bologna

18 Italy.

19 [7] Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota, USA

20

21 **Correspondence:** Sofia Asioli, MD; Department of Biomedical and Neuromotor Sciences, Alma

22 Mater Studiorum - University of Bologna; IRCCS Istituto delle Scienze Neurologiche di Bologna

23 Via Altura No. 3, 40126 Bologna; Tel 0516225006; Fax 0516225759; Email: sofia.asioli3@unibo.it

24

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

ABSTRACT

Primary diffuse large B-cell lymphoma of the CNS (CNS-DLBCL) is an aggressive disease, with dismal prognosis despite the use of high dose methotrexate (MTX)-based polychemotherapy. Our study aimed to expand the biologic profiles of CNS-DLBCL and to correlate them with clinical/imaging findings to gain diagnostic insight and possibly identify new therapeutic targets. We selected 61 CNS-DLBCL whose FFPE samples at first diagnosis were available. These were investigated by immunohistochemistry, cMYC rearrangements were explored by fluorescence in situ hybridization and CNS-DLBCL mutated genes were evaluated by Next Generation Sequencing. CD10, BCL6 and IRF4 were observed in 16%, 83.6% and 93% of cases respectively. As typical of CNS-lymphoma 10/61 (16.4%) cases were classified as germinal center (GCB) and type and 51/61 (83.6%) as non-germinal center (non-GCB) type according to the Hans algorithm. Double expression (DE) status for BCL2 and cMYC was detected in 36/61 (59%) cases while 25/61 (41%) were non DE. Rearrangement of the *cMYC* gene was detected in 2 cases, associated with *BCL6* translocation only 1 case. *MYD88, PIM1*, *CD79B* and *TP53* were mutated in 54.5%, *53.5%*, 30.2% and 18.4% cases respectively. Novel mutations not previously reported in CNS-DLBCL were found: *AIP* in 23,1%, *PI3KCA* in 15%, *NOTCH1 in* 11,4%, *GNAS* 8.1%, *CASP8 in* 7.9%, *EGFR* in 6.4% *PTEN* in 5.1 and *KRAS* in 2.6%. Survival was significantly longer for patients with mutated *MYD88* (8.7 months vs. 1.7 months; log-rank test = 5.43; p = 0.020) and for patients with mutated *CD79B* (10.8 months vs. 2.5 months; log-rank test = 4.64; p = 0.031).*MYD88* and *CD79B* predicted a longer survival in patients affected by CNS-DLBCL. Notably, we identified novel mutations that enrich mutational landscape of CNS-DLBCL, suggest a role of PTEN-PI3K-AKT and RTK-RAS-MAPK signalling in a subset of CNS-DLBCL and provide new potential therapeutic targets.

Introduction

Diffuse large B cell lymphoma of the primary central nervous system (CNS-DLBCL) accounts for <3% of brain tumor with a peak incidence between the 5th and 6th decade[1]. The most frequent (60%) localization is the supra-tentorial frontal region. Ocular lesions develop concurrently in 20% of patients,[1,2] while extra-neural dissemination is rare.[1,3] CNS-DLBCL lesions may be single or multiple, with distinct margins or poorly defined with diffuse parenchyma infiltration .[1] The neoplastic population consists of medium/large proliferating blasts, with a mature B phenotype.[1] Most CNS-DLBCL are BCL6^{+}/IRF4^{+} and[1,4] approximately 80% of CNS-DLBCL are BCL2^{+}/cMYC[+] (double expressors), a protein profile that confers poor clinical prognosis; double-hit or triple-hit high grade B-cell lymphomas, with MYC and BCL2 and/or BCL6 rearrangements, are rare.[5,6] Despite high-dose polychemotherapy and methotrexate (MTX), the prognosis remains poor, with a median overall survival (OS) of about 3 years and a median progression free survival (PFS) of 12 months.[7] At the bio-pathological level, proliferation and survival of the neoplastic clones are supported by the constitutive activation of the B-cell receptor (BCR), Toll-like receptor (TLR) and NF-kB signaling pathways caused by mutations of *MYD88* and/or *CD79B*.[1] Recently, the landscape of recurrent genetic drivers in DLBCL has been expanded by Schmitz G et al and Chapuy B et al. that uncovered genetic subtypes of DLBCL with distinct clinical characteristics, providing a potential nosology for precision-medicine strategies in DLBCL.[8,9] These subtypes showed distinct outcomes after immunochemotherapy with the potential to affect the selection of targeted therapies owing to their distinct oncogenic abnormalities. Wright et al revealed a high prevalence of the sub-type termed MCD (based on the co-occurrence of *MYD88* p.L265P and *CD79B* mutations) in primary CNS-DLBCL, defined by gene aberrations involving *MYD88*, *CD79B* and *PIM1*.[10] Their combined genetic, phenotypic, functional, and clinical data suggest that MCD-subtype may be sensitive to BTK, PI3K, BET, BCL2, and JAK inhibitors.[11]

Despite all attempts, the prognosis of CNS-DLBCL remains poor and new therapeutic approaches are needed to improve patient survival. With this background, our aim was to characterize and

105 correlate the biologic profiles CNS-DLBCL to clinical findings and explore new potential

106 therapeutic targets.

107 **Methods**

108 *Patients' selection and tumour samples collection.* From the review of the medical records of the

109 Department of Neurosurgery of IRCCS Istituto Delle Scienze Neurologiche, Bologna, we identified

110 107 pathologically confirmed cases of CNS-DLBCL diagnosed between 2005 and 2020. Of those,

111 sixty-one cases with formalin fixed paraffin embedded (FFPE) adequate material were included in

112 the study. The study was approved by local ethics committee (620/2020/OSS/AUSLBO) and it was

113 performed in accordance with the Declaration of Helsinki. Thirty patients (49.2%) were male and

114 31 females (50.8%), with an age range between 32 and 82 years and a median age of 66 years (IQR:

115 57–72).

116 *Immunohistochemistry.* Paraffin-embedded sections were deparaffinized in HistoClear and

117 dehydrated through graded ethanol. The antigen retrieval was performed in the PT-Link (Agilent

118 Dako, Santa Clara, CA, USA, code PT100/ PT 101), for 5 min at 92°C in EnVision Flex Target

119 retrieval solution High pH (Agilent Dako, code K 8004). Then, tissue samples were incubated at RT

120 for 30 minutes with the following antibodies: CD20 (Agilent Dako 1:300, clone L26, code M0755),

121 CD10 (Leica NewCastle, UK, 1:30, clone 56C6 code CD10-270-L), BCL2 (Abcam, Cambridge ,

122 UK, 1:100, clone E17, code Ab32124), BCL6 (kindly provided by Prof. Falini, indiluted, clone PG-

123 B6p), cMYC (Epitomics, Burlingame, CA 1:80, clone Y69, code 1472-1,), IRF4 (kindly provided

124 by Prof. Falini, 1:3, clone IRF4). Immunostaining has been completed using the Alkaline REAL

125 Detection System Alkaline Phosphatase/RED Rabbit/Mouse (Agilent Dako, code K5005) and

126 chromogen (Fast red), provided with the kit. Finally, slides were counterstained with Hematoxylin,

127 mounted in Glycerine and observed and analysed by Olympus microscope. Slides were observed by

128 2 expert pathologists (CA, SA). Results were recorded as percentage of positive cells and graded as

129 follows: + if >30% positive neoplastic cells, for CD10/BCL6/IRF4 according to Hans' algorithm.

5

130   [11] A case was defined as cMyc or BCL2 positive if ≥40% and ≥50% cells were stained

131   respectively.[6]

132   *Genetic analysis.*

133   *Fluorescence in situ hybridization (FISH).* Paraffin embedded tissue were deparaffinized and then

134   pre-treated with two different antigen retrievals. The slides were before incubated in 1mM EDTA

135   buffer (pH 8) in a pressure cooker (9 minutes) and after in Pepsin solution (Sigma Aldrich code

136   P7012) for 14 minutes at 38°C [12]. The probes and the samples were denatured at 80 °C for 22 min

137   and then hybridized at 38°C for 22 hours in a hybridizer (Agilent Dako, Santa Clara, CA USA).

138   The experiments were conducted using the probes LSI MYC Dual Color Break Apart

139   Rearrangement (Vysis, Abbott, Downers Grove, Illinois, USA, code 01N63-020, cut-off 3,8%), LSI

140   BCL2 Dual Color Break Apart Rearrangement (Vysis Abbott, code 05N51-020, cut-off 4,7%) and

141   LSI BCL6 dual color Break apart Rearrangement t(3q27) (Vysis Abbott, code 01N23-020, cut off

142   5,5%). Following a stringency washes (0,4XSSC/0,03%NP-40 pH 7,4 solutions at 73°C for 2 min

143   and with 2XSSC/0,1% NP-40 pH 7 solutions at room temperature for 1 min), the slides were

144   mounted and counterstained with DAPI I (Vysis Abbott, code 06J49-001). Microscopical analysis

145   were carried out with an Olympus BX61 microscope and images were recorded using the Cell^F

146   program. *BCL6* and *BCL2* rearrangements were investigated only in cases with translocation of the

147   *cMYC* gene.

148   *Next Generation Sequencing*. DNA from FFPE PCNSL tissue samples was purified by Quick

149   Extract FFPE DNA Extraction Kit (Epicentre, Madison, WI) with some modifications described by

150   Ricci C et al.[13] DNA mutations were detected using the protocol described previously[14] analysing

151   the following gene panel: *MYD88, CD79B, PIM1, GNAS, NOTCH1, KRAS, PIK3CA, EGFR,*

152   *CASP8, AIP, PTEN*. In brief, after target enrichment by multiplex PCR, libraries with tagged

153   primers were generated using Nextera adapters. Each run on MiSEQ platform (Illumina, Palo Alto,

154   CA) was designed to allocate at least 2K reads/region aimed to have a depth of coverage of at least

155   2000×. FASTQ files were filtered with PHRED quality score > Q30 and length > 100 bp, and reads

were mapped in a Galaxy Project environment to the hg38 human reference genome with BWA-MEM, GATK local realignment, HaplotypeCaller and Picard MarkDuplicates.[15] The BAM files were visualized using the Integrative Genomic Viewer (IGV) to identify mutations with Variant Allele Frequency (VAF) of at least 10%; only bidirectional variant calls with more than 10 reads were reported. [15] Next generation sequencing analysis was available in 46 cases, as fifteen FFPE specimens were over-fixed and not amplifiable. Protein sequence and functional information were obtained by Uniprot database.

Statistical analysis. Demographic and clinical features were described using absolute frequencies and percentages for categorical variables, mean and standard deviation for quantitative symmetrical variables or median and interquartile range (IQR) for quantitative asymmetrical variables. The associations between immunohistochemistry and radiology, between immunohistochemistry and mutated genes, and between radiology and mutated genes were investigated using the χ2 test, or Exact Fisher's test when expected cell count is less than 5. The Kaplan–Meier product limit was used to estimate the overall survival (OS) curve. Differences in survival between subgroups were assessed with the log-rank test. Statistical analysis was performed with IBM SPSS version 25.0 and Stata 13. The significance level was set at $p<0.05$.

Results

Phenotypic profile. By immunohistochemistry, diffuse and strong expression of CD20 characterized all 61 (100%) samples. CD10, BCL6 and IRF4 were observed in 10 (16%), 51 (83.6%) and 57 (93%) of 61 cases respectively. As typical of CNS-lymphoma 10 of 61 (16.4%) cases were classified as GCB type and 51/61 (83.6%) as non-GCB type according to the Hans algorithm. BCL2 and cMYC proteins were expressed in 51 (83.6%) and 41 (67.2%) of 61 samples respectively. Double expression (DE) status for BCL2 and cMYC was detected in 36 (59%) cases while 25 (41%) were non DE. The immunohistochemical data is summarized in Table 1.

180 *Fish analysis.* FISH analysis was successful in 59 of 61 (96.7%) samples; *cMYC* rearrangements

181 were present in 2 (3.4%) of 59 cases and in one was associated to *BCL6* translocation. This latter

182 harboured *MYD88* mutated (p.L265P).

183 Next generation sequencing showed *MYD88* mutations in 24 (54.5%) of 44, all carrying p.L265P

184 except one case with p.A260T. Both mutations are located in the Toll/Il-1 Receptor (TIR) domain

185 of the protein.

186 Twenty-three cases carried a mutation in proto-oncogene with serine/threonine kinase *PIM1 (23/43,*

187 *53.5%)*, with a great variety of different mutations, mostly missense mutations, occurring at protein

188 kinase domain, of which the p.E135K was the most common involving four cases. In 9 samples 2 or

189 3 different *PIM1* mutations were simultaneously present. *CD79B* missense mutations were found in

190 13 cases (of 43, 30.2%), located within immunoreceptor tyrosine-based activation motif (ITAM), 5

191 of which had p.Y196H, 2 cases p.Y196N, 2 p.Y196S, 2 p.Y196C, 1 p.Y196D, and one the rare

192 p.E198G. Concurrent mutations were found: *MYD88*, *PIM1* and *CD79B* in 4 cases, *MYD88* and

193 *PIM1* in 13 cases, *MYD88* and *CD79B* in 9 cases, and *CD79B* and *PIM1* in 8.

194 Seven cases carried missense mutations in *TP53* gene (7 of 38, 18.4%). Additional mutations were

195 found: *NOTCH1 in* 4 of 35 (11,4%), *AIP in* 9 of 39 (23,1%), *GNAS in* 3 of 37 (8.1%) and *CASP8*

196 *in* 3 of 38 (7.9%).

197 *PI3KCA* missense mutations were found in 6 of 40 (15%); in 5 cases they produced an amino acid

198 substitution in the catalytic domain (p.A1046V, p.D1045N, p.G1049S and p.G1049D in two cases).

199 *PTEN and EGFR* genes mutations were found in 2 of 39 (5.1%) and 2 of 31 (6.4%) cases

200 respectively, while p.G12D and in CIS p.G13D mutations of *KRAS* gene co-occurred in a single

201 patient (1/39). Table 2 summarizes all these sequencing data.

202 *Clinical findings.* The disease presented with a single lesion in 25 of 61 (41%) of patients and with

203 multiple lesions in 36 of 61 (59%). On MRI, most patients showed deeply located lesions with

204 homogeneous contrast-enhancement (36 of 50, 72%). Multiple enhancing was seen in 53%. Only

205 one of 34 patients with DWI performed showed a high ADC value, all others presented

206   hyperintense diffusion signal. Perfusion T2-w studies was available in 24 patients, 54% with 2 to 7

207   times higher value of rCBV. No significant statistical correlations were observed between

208   biological and clinical parameters and neuroradiological features.

209   Gross total resection was achieved in 13 of 61 (21%), subtotal resection in 21 (35%) and biopsy in

210   27 (44%). Twenty-four patients, whose treatment was known, had undergone the matrix protocol

211   MTX + cytarabine or MATRix regimen. Of the 61 patients included in the study, 57 died during the

212   follow-up, with a median follow-up time of 3.3 months (95% CI: [2.1 - 5.0]). The longest follow-up

213   was 69.2 months. Figure 1 shows the Kaplan-Meier survival curve. One-month, 3-month, 6-month

214   overall survival rates were 88.5% (95% CI: [77.4% - 94.4%]), 52.5% (95% CI: [39.3% - 64.1%]),

215   and 37.7% (95% CI: [25.7% - 49.6%]), respectively.

216   No statistically significant correlation was found between cell of origin GCB/non-GCB, BCL2 and

217   cMYC expression and survival nor between DE or non-DE status and prognosis. Survival was

218   significantly longer for patients with mutated *MYD88* (8.7 months vs. 1.7 months; log-rank test =

219   5.43; p = 0.020) compared to those without *MYD88* mutation, and for patients with mutated *CD79B*

220   (10.8 months vs. 2.5 months; log-rank test = 4.64; p = 0.031) compared to those without *CD79B*

221   mutation.

222   **Discussion**

223   CNS-DLBCL, together with vitreoretinal and testicular DLBCL, is now grouped in a new category

224   called large B-cell lymphoma of the immune-privileged sites.[16] The prognosis of CNS-DLBCL

225   remains poor and new therapeutic approaches are needed to improve patient survival. OS is lower in

226   our series than prior studies, this could be due to patient selection bias, which in our study had a

227   median older age (56 vs 64) and a much higher frequency of multiple lesions (59% vs 30%).[1]

228   Similar to previously reported, based on our data, single and double hit rearrangements of MYC

229   gene were rare .[1,6] Differently from a recent report by Asano et al and Kim et al DE status for BCL2

230   and cMYC expression did not impact on prognosis in our cohort of patients.[5,17]

231 Genomic studies suggest that cell proliferation and survival in CNS-DLBCL are driven by

232 deregulated TLR and BCR signaling pathways inducing constitutive NFκB activation, with a high

233 frequency of somatic non-synonymous mutations in *MYD88* and *CD79B* genes.[18-24] Therefore

234 inhibitors of TLR/BCR signaling such as ibrutinib, blocking Bruton's tyrosine kinase (BTK), was

235 proposed as alternative therapeutic target and seem to be effective in CNS-DLBCL.[25-28] We found

236 *MYD88* mutations in 54.5% of the cases resulting the most frequently mutated gene in our series;

237 *CD79B* mutations considered one of the hallmark of CNS-DLBCL mutational signature, were

238 demonstrated in 30.2% of the cases. This gene encodes the Ig-beta protein of BCR multimeric

239 complex and its activating mutations reinforce BCR signalling contributing to sensitivity to

240 Ibrutinib. Interestingly, survival was significantly longer for patients with mutated *MYD88* and

241 *CD79B* compared to those with wild type genes. Our findings confirm data reported by Curran et al

242 and Zhou et al respectively, and are in contrast with two studies reporting a poor prognosis

243 associated with the MYD88 mutation. [29-32] These conflicting results might reflect a selection bias

244 among small study populations, given the rarity of CNS-DLBCL.

245 *PIM1* was found to be the second most frequently mutated gene (53.5%), with a great variety of

246 different mutations. PIM1 protein belongs to the Ser/Thr protein kinase family which is

247 overexpressed in hematopoietic malignancies and in prostate and breast cancers where it was

248 proposed as new therapeutic target.[33-35] Although *PIM1* mutation status was reported to impact the

249 outcome, we did not find any correlation with prognosis.[32]

250 *TP53* alterations seem to play a minor role in CNS-DLBCL. Zorofchian et al (26.7%) and other

251 authors have suggested that disruption of the p53-pathway may be associated with poor PFS.[37,38]

252 In our series *TP53* mutations were not significantly related to prognosis.

253 Notably, we detected for the first time *AIP* mutations in CNS-DLBCL (23,1% of the cases). Aryl

254 hydrocarbon receptor-interacting protein (*AIP*) is a co-chaperone to heat shock proteins and nuclear

255 receptors which behaves as tumor suppressor gene. In colorectal, pancreatic and gastric cancer high

256 expression of AIP is associated with tumour development and more aggressive disease and

257 inhibiting aryl hydrocarbon receptor was proposed as potential therapeutic target.[39-41] Recently *AIP*

258 was found to be a positive regulator of BCL6 expression in germinal centers cells, protecting BCL6

259 from ubiquitin-mediated proteasomal degradation, and deletion of *AIP* in B cells decrease BCL6

260 expression, reducing germinal center B cells and diminishing adaptive immune responses.[42]

261 Furthermore AIP was required for optimal AKT signaling in response to BCR stimulation and

262 seems to be highly expressed in primary DLBCL compared to healthy tissue with implications for

263 the pathobiology of this disease.[42]

264 The NOTCH signalling pathway is widely involved in cellular proliferation, differentiation, and

265 apoptosis.[43] We *NOTCH1* mutations in 11,4% of the samples: *NOTCH1* mutations are distinctive

266 drivers of systemic DLBCL and were not previously described in CNS-DLBCL.[8]

267 *GNAS* and *CASP8* were mutated in 8.1% and 7.9% of the cases, respectively. *GNAS* is the most

268 frequently mutated G-protein in human cancers and activating mutations in the gene GNAS have

269 been found in pituitary, thyroid, pancreatic, biliary tract and intestine tumors as well as in Burkitt

270 lymphoma, systemic DLBCL and Hodgkin lymphoma.[44-47] Zhou et al reported *GNA13* mutations to

271 be associated with a shorter PFS and overall survival in primary central nervous system lymphoma

272 patients, however we did not find any correlation with prognosis.[48] We also identified somatic

273 mutations *CASP8* for which a functional role has not been previously suspected in CNS-DLBCL,

274 although p.R472* nonsense mutation detected in one of our samples was previously reported in

275 systemic DLBCL.[49] *CASP8* encodes a member of the cysteine-aspartic acid protease (caspase)

276 family and sequential activation of caspases plays a central role in the execution-phase of cell

277 apoptosis. In addition, caspase-8 participates in maintenance of genomic integrity and loss of

278 caspase-8 in B lymphocytes leads to B-cell malignancies; moreover, *CASP8* mutations were found

279 in mantle cell lymphoma.[50]

280   *PI3KCA* missense mutations, previously described in several types of carcinomas,[1] were found in 6

281   of 40 (15%), while *PTEN* mutations were detected in 2 of 39 samples (5.1%). The

282   PI3K/AKT/mTOR is an important pro-survival pathway which plays a pivotal role in the

283   development of malignant tumours being often aberrantly activated in different types of cancer also

284   including lymphomas. Zang et al recently showed that PI3K/AKT/mTOR signaling is aberrantly

285   activated in CNS-DLBCL and correlated with a poor prognosis.[51] They found that p-mTOR

286   expression was an independent risk factor in terms of PFS in patients with CNS-DLBCL.

287   Moreover, Takashima et al found that copy number losses in the PTEN-PI3K-AKT proapoptotic

288   pathway are associated with poor prognosis in CNS-DLBCL patients while PTEN mutation was

289   related to shorter OS in the study of Todorovic et al.[52,53] PI3K/mTOR inhibitors has also become a

290   potential therapeutic target in CNS-DLBCL. A phase II trial of 37 relapsed/refractory CNS-DLBCL

291   patients demonstrated that temsirolimus had a positive effect with 54% overall response rate, while

292   lower response rate of 25% was seen in a clinical trial targeting using the pan-PI3K inhibitor

293   buparlisib.[54,55] The use of dual pan-PI3K/mTOR inhibitor bimiralisib (PQR309) in a multicenter

294   phase I/II trial (NCT02669511) as well as BAY80-6946 (copanlisib) in a phase Ib/II clinical trial

295   (NCT03581942) are still under investigation in relapsed/refractory CNS-DLBCL. Moreover,

296   Inhibition of the PI3K isoforms p110α/p110δ or mTOR synergized with ibrutinib to induce cell

297   death in CD79B-mutant CNS-DLBCL cells suggesting that combined inhibition of BTK and

298   PI3K/mTOR could overcome the resistance of lymphoma cells to ibrutinib.[56] So far, no PI3K

299   mutations were described in CNS-DLBCL and our data provide a strong rational for a clinical

300   application of inhibitors targeting the PI3K/ AKT/mTOR signalling pathway at least in a subset of

301   patients.

302   Finally, mutations in the *EGFR* and *KRAS* genes were found in in 6.4% and 2.6% of the cases

303   respectively that could be promising therapeutic targets. Although copy number alterations with

amplifications in RTK-RAS-MAPK signalling has been correlated to a poorer prognosis in CNS-DLBCL we didn't find any association with survival.[52]

Conclusion

MYD88 and *CD79B* predict a longer survival in patients affected by CNS-DLBCL. The *rearrangements* of the *MYC* gene are rare as well as double hit events involving BCL6 and/or BCL2 genes in the present series of CNS-DLBCL. COO and DE status does not affect the prognosis of the 61 CNS-DLBCL cases. Notably, we identified novel mutations that enrich the mutational landscape of CNS-DLBCL, suggests a role of PTEN-PI3K-AKT and RTK-RAS-MAPK signalling in a subset of CNS-DLBCL and that provides new potential therapeutic targets.

References

1. Kluin PM, et al. Primary diffuse large B-cell lymphoma of the CNS. In: Swerdlow SH, Campo E, Harris NL, et al. (eds). WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon, France: IARC Press; 2017; 300-2.

2. Schlegel. Primary CNS lymphoma. *Ther Adv Neurol Disord* **2**:93-104 U (2009).

3. Deckert M, Engert A, Brück W, Ferreri AJ, Finke J, Illerhaus G, et al. Modern concepts in the biology, diagnosis, differential diagnosis and treatmentof primary central nervous system lymphoma. *Leukemia* **25**:1797-807 (2011)

4. Marcus C, Maragkos GA, Alterman RL, Uhlmann E, Pihan G, Varma H. GCB-type is a favorable prognostic factor in primary CNS diffuse large B-cell lymphomas. *J Clin Neurosci* **83**: 49-55 (2021)

5. Kim S, Nam SJ, Kwon D, Kim H, Lee E, Kim TM, et al. MYC and BCL2 overexpression is associated with a higher class of Memorial Sloan-Kettering Cancer Center prognostic model and poor clinical outcome in primary diffuse large B cell lymphoma of the central nervous system. *BMC Cancer* **16**:363 (2016)

329   6. Pina-Oviedo S, Bellamy WT, Gokden M. Analysis of primary central nervous system large B-

330   cell lymphoma in the era of high-grade B-cell lymphoma: Detection of two cases with MYC and

331   BCL6 rearrangements in a cohort of 12 cases. *Ann Diagn Pathol* **48**: 151610 (2020)

332   7. Abrey LE, DeAngelis LM, Yahalom J. Long-term survival in primary CNS lymphoma. *J Clin*

333   *Oncol* **16**:859–63 (1998)

334   8. Schmitz R, Wright GW, Huang DW, Johnson CA, Phelan JD, Wang JQ, et al. Genetics and

335   Pathogenesis of Diffuse Large B-Cell Lymphoma. *N Engl J Med* **378**, 1396–1407 (2018).

336   9. Chapuy B, Stewart C, Dunford AJ, Kim J, Kamburov A, Redd RA,, et al. Molecular subtypes of

337   diffuse large B cell lymphoma are associated with distinct pathogenic mechanisms and outcomes.

338   *Nat Med* **24**, 679–690 (2018)

339   10. Wright GW, Huang DW, Phelan JD, Coulibaly ZA, Roulland S, Young RM, et al. A

340   Probabilistic Classification Tool for Genetic Subtypes of Diffuse Large B Cell Lymphoma with

341   Therapeutic Implications. *Cancer Cell* **37**(4):551-568 (2020)

342   11. Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, et al. Confirmation of

343   the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a

344   tissue microarray. *Blood* **1**;103(1):275-82 (2004)

345   12. Ventura RA, Martin-Subero JI, Jones M, McParland J, Gesk S, Mason DY, et al. FISH analysis

346   for the detection of lymphoma-associated chromosomal abnormalities in routine paraffin-embedded

347   tissue. *J Mol Diagn* **8**(2):141-51 (2006)

348   13. Ricci C, Morandi L, Ambrosi F, Righi A, Gibertoni D, Maletta F, et al. Intron 4-5 hTERT DNA

349   Hypermethylation in Merkel Cell Carcinoma: Frequency, Association with Other Clinico-

350   pathological Features and Prognostic Relevance. Multicenter Study. *Endocr Pathol* **32**(3):385-395

351   (2021)

352   14. Gabusi A, Gissi DB, Montebugnoli L, Asioli S, Tarsitano A, Marchetti C, et al. Prognostic

353   impact of intra-field heterogeneity in oral squamous cell carcinoma. *Virchows Arch* **476**(4):585-595

354   (2020)

355   15. Afgan E, Baker D, van den Beek M, Blankenberg D, Bouvier D, Čech M, et al. The Galaxy

356   platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. *Nucleic*

357   *Acids Res* **8**;44(W1):W3-W10 (2016)

358   16. Alaggio R, Amador C, Anagnostopoulos I, Attygalle AD, Araujo IBO, Berti E, et al Leukemia.

359   The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours:

360   Lymphoid Neoplasms. 2022 Jul;36(7):1720-1748.

361   17. Asano K, Yamashita Y, Ono T, Natsumeda M, Beppu T, Matsuda K et al . Clinicopathological

362   risk factors for a poor prognosis of primary central nervous system lymphoma in elderly patients in

363   the Tohoku and Niigata area: a multicenter, retrospective, cohort study of the Tohoku Brain Tumor

364   Study Group. *Brain Tumor Pathol* **39**(3):139-150 (2022)

365   18. Braggio E, McPhail ER, Macon W, Lopes MB, Schiff D, Law M, et al. Primary central nervous

366   system lymphomas: a validation study of array-based comparative genomic hybridization in

367   formalin-fixed paraffin-embedded tumor specimens. *Clin Cancer Res.***17**:4245–53 (2011)

368   19. Montesinos-Rongen, M., Schafer, E., Siebert, R. & Deckert, M. Genes regulating the B cell

369   receptor pathway are recurrently mutated in primary central nervous system lymphoma. *Acta*

370   *Neuropathol.* **124**, 905–906 (2012).

371   20. Bruno A, Boisselier B, Labreche K, Marie Y, Polivka M, Jouvet A, et al. Mutational analysis of

372   primary central nervous system lymphoma. *Oncotarget* **5**:5065–75 (2014)

373   21. Vater I, Montesinos-Rongen M, Schlesner M, Haake A, Purschke F, Sprute R, et al. The

374   mutational pattern of primary lymphoma of the central nervous system determined by whole-exome

375   sequencing. *Leukemia* **29**:677–85 (2015)

376   22. Radke J, Ishaque N, Koll R, Gu Z, Schumann E, Sieverling L, et al. The genomic and

377   transcriptional landscape of primary central nervous system lymphoma. *Nat Commun*

378   **10**;13(1):2558 (2022)

379   23. Yamada, S., Ishida, Y., Matsuno, A, Yamazaki, K. Primary diffuse large B-cell lymphomas of

380   central nervous system exhibit remarkably high prevalence of oncogenic MYD88 and CD79B

381   mutations. *Leuk. Lymphoma* **56**, 2141–2145 (2015)

382   24. Nayyar N, White MD, Gill CM, Lastrapes M, Bertalan M, Kaplan A, et al. MYD88 L265P

383   mutation and CDKN2A loss are early mutational events in primary central nervous system diffuse

384   large B-cell lymphomas. *Blood Adv* **3,** 375–383 (2019)

385   25. Grommes, C., Nayak, L., Tun, H. W, Batchelor, T. T. Introduction of novel agents in the

386   treatment of primary CNS lymphoma. *Neuro-Oncol* **21**, 306–313, (2019)

387   26. Soussain C, Choquet S, Blonski M, Leclercq D, Houillier C, Rezai K, et al. Ibrutinib

388   monotherapy for relapse or refractory primary CNS lymphoma and primary vitreoretinal

389   lymphoma: Final analysis of the phase II 'proof-of-concept' iLOC study by the Lymphoma study

390   association (LYSA) and the French oculo-cerebral lymphoma (LOC) network. *Eur. J. Cancer* **117**,

391   121–130 (2019)

392   27. Lionakis MS, Dunleavy K, Roschewski M, Widemann BC, Butman JA, Schmitz R, et al.

393   Inhibition of B cell receptor signaling by Ibrutinib in primary CNS lymphoma. *Cancer Cell* **31**,

394   833–843 e835 (2017)

395   28. Grommes C, Pastore A, Palaskas N, Tang SS, Campos C, Schartz D, et al. Ibrutinib unmasks

396   critical role of Bruton tyrosine kinase in primary CNS lymphoma. *Cancer Discov* **7**, 1018–1029

397   (2017)

398   29. Curran OE, Poon MTC, Gilroy L, Torgersen A, Smith C, Al-Qsous W. MYD88 L265P

399   mutation in primary central nervous system lymphoma is associated with better survival: A single-

400   center experience. *Neurooncol Adv* **7**;3(1):vdab090 (2021)

401   30. Zhou J, Zuo M, Li L, Li F, Ke P, Zhou Y, Xu Y, et al. PIM1 and CD79B Mutation Status

402   Impacts the Outcome of Primary Diffuse Large B-Cell Lymphoma of the CNS. *Front Oncol*

403   **9**;12:824632 (2022)

404   31. Takano S, Hattori K, Ishikawa E, Narita Y, Iwadate Y, Yamaguchi F, et al. MyD88 Mutation in

405   Elderly Predicts Poor Prognosis in Primary Central Nervous System Lymphoma: Multi-Institutional

406   Analysis. *World Neurosurg* **112**:e69-e73 (2018)

407   32. Hattori K, Sakata-Yanagimoto M, Okoshi Y, Goshima Y, Yanagimoto S, Nakamoto-Matsubara

408   R, et al. MYD88 (L265P) mutation is associated with an unfavorable outcome of primary central

409   nervous system lymphoma. *Br J Haematol* **177**(3):492-494 (2017)

410   33. Mahata S, Sahoo PK, Pal R, Sarkar S, Mistry T, Ghosh S, et al. PIM1/STAT3 axis: a potential

411   co-targeted therapeutic approach in triple-negative breast cancer. *Med Oncol* **15**;39(5):74 (2022)

412   34. Zhong S, Peng S, Chen Z, Chen Z, Luo JL. Choosing Kinase Inhibitors for Androgen

413   Deprivation Therapy-Resistant Prostate Cancer. *Pharmaceutics* **24**;14(3):498 (2022)

414   35. Zhao Y, Aziz AUR, Zhang H, Zhang Z, Li N, Liu B. A systematic review on active sites and

415   functions of PIM-1 protein. *Hum Cell* **35**(2):427-440 (2022)

416   36. Lacy SE, Barrans SL, Beer PA, Painter D, Smith AG, Roman E, et al. Targeted sequencing in

417   DLBCL, molecular subtypes, and outcomes: A Haematological Malignancy Research Network

418   report. *Blood* **135**, 1759–1771 (2020).

419   37. Zorofchian S, El-Achi H, Yan Y, Esquenazi Y, Ballester LY. Characterization of genomic

420   alterations in primary central nervous system lymphomas. *J Neurooncol* **140**(3):509-517 (2018)

421   38. Munch-Petersen HD, Asmar F, Dimopoulos K, Areškevičiūtė A, Brown P, Girkov MS, et al.

422   TP53 hotspot mutations are predictive of survival in primary central nervous system lymphoma

423   patients treated with combination chemotherapy. *Acta Neuropathol Commun* **22**;4:40 (2016)

424   39. Solís-Fernández G, Montero-Calle A, Sánchez-Martínez M, Peláez-García A, Fernández-

425   Aceñero MJ, Pallarés P, et al. Aryl-hydrocarbon receptor-interacting protein regulates tumorigenic

426   and metastatic properties of colorectal cancer cells driving liver metastasis. *Br J Cancer*

427   **126**(11):1604-1615 (2022)

428   40. Fernández-Aceñero MJ, Barderas R, Peláez García A, Martínez-Useros J, Díez-Valladares L,

429   Pérez-Aguirre, et al. Aryl hydrocarbon receptor interacting protein (AIP) significantly influences

430   prognosis of pancreatic carcinoma. *Ann Diagn Pathol* **53**:151742 (2021)

431   41. Díaz Del Arco C, Estrada Muñoz L, Barderas Manchado R, Peláez García A, Ortega Medina L,

432   Molina Roldán E, et al. Prognostic Role of Aryl Hydrocarbon Receptor Interacting Protein (AIP)

433   Immunohistochemical Expression in Patients with Resected Gastric Carcinomas. *Pathol Oncol Res*

434   **26**(4):2641-2650 (2020)

435   42. Sun D, Stopka-Farooqui U, Barry S, Aksoy E, Parsonage G, Vossenkämper A, et al. Aryl

436   Hydrocarbon Receptor Interacting Protein Maintains Germinal Center B Cells through Suppression

437   of BCL6 Degradation. *Cell Rep* 30;27(5):1461-1471 (2019)

438   43. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal

439   integration in development. *Science* **30**;284(5415):770-6 (1999)

440   44. O'Hayre M, Vazquez-Prado J, Kufareva I, Stawiski EW, Handel TM, Seshagiri S, et al. The

441   emerging mutational landscape of G proteins and G-protein-coupled receptors in cancer. N*ature*

442   *reviews Cancer* **13**(6):412–24 (2013)

443   45. Love C, Sun Z, Jima D, Li G, Zhang J, Miles R, et al. The genetic landscape of mutations in

444   Burkitt lymphoma. *Nature genetics* **44**(12):1321–5 (2012)

445   46. Lohr JG, Stojanov P, Lawrence MS, Auclair D, Chapuy B, Sougnez C, et al. Discovery and

446   prioritization of somatic mutations in diffuse large B-cell lymphoma (DLBCL) by whole-exome

447   sequencing. *Proc Natl Acad Sci U S A* **6**;109(10):3879-84 (2012)

448   47. Wienand K, Chapuy B, Stewart C, Dunford AJ, Wu D, Kim J, et al. Genomic analyses of flow-

449   sorted Hodgkin Reed-Sternberg cells reveal complementary mechanisms of immune evasion. *Blood*

450   *Adv* **10**;3(23):4065-4080 (2019)

451   48. Zhou Y, Liu W, Xu Z, Zhu H, Xiao D, Su W, et al. Analysis of Genomic Alteration in Primary

452   Central Nervous System Lymphoma and the Expression of Some Related Genes. *Neoplasia*

453   **20**(10):1059-1069 (2018)

454 49. Hakem A, El Ghamrasni S, Maire G, Lemmers B, Karaskova J, Jurisicova A, et al. Caspase-8 is

455 essential for maintaining chromosomal stability and suppressing B-cell lymphomagenesis. *Blood*

456 **12**;119(15):3495-502 (2012)

457 50. Yang P, Zhang W, Wang J, Liu Y, An R, Jing H. Genomic landscape and prognostic analysis of

458 mantle cell lymphoma. *Cancer Gene Ther* **25**(5-6):129-140 (2018)

459 51. Zhang X, Wu Y, Sun X, Cui Q, Bai X, Dong G, et al.The PI3K/AKT/mTOR signaling pathway

460 is aberrantly activated in primary central nervous system lymphoma and correlated with a poor

461 prognosis. *BMC Cancer* **20**;22(1):190 (2022)

462 52. Takashima Y, Sasaki Y, Hayano A, Homma J, Fukai J, Iwadate Y, et al. Target amplicon

463 exome-sequencing identifies promising diagnosis and prognostic markers involved in RTK-RAS

464 and PI3K-AKT signaling as central oncopathways in primary central nervous system lymphoma.

465 *Oncotarget* **8**;9(44):27471-27486 (2018)

466 53. Todorovic BM, Jelicic J, Mihaljevic B, Kostic J, Stanic B, Balint B, et al. Gene mutation profles

467 in primary difuse large B cell lymphoma of central nervous system: next generation sequencing

468 analyses. *Int J Mol Sci* **17**(5):683 (2016)

469 54. Korfel A, Schlegel U, Herrlinger U, Dreyling M, Schmidt C, von Baumgarten L,, et al. Phase II

470 trial of temsirolimus for relapsed/refractory primary CNS lymphoma. *J Clin Oncol* **34**(15):1757–

471 1763 (2016)

472 55. Grommes C, Pentsova E, Nolan C, Wolfe J, Mellinghoff IK, Deangelis L. Phase II study of

473 single agent buparlisib in recurrent/refractory primary (PCNSL) and secondary CNS lymphoma

474 (SCNSL). *Ann Oncol* **27**(suppl 6):335 (2016)

475 56. Grommes C, Pastore A, Palaskas N, Tang SS, Campos C, Schartz D,, et al. Ibrutinib unmasks

476 critical role of bruton tyrosine kinase in primary CNS lymphoma. *Cancer Discov* **7**: 1018–1029

477 (2017)

478

479

480

481

482

483

484

485

486

487

488

489

490

491

492