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

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

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by AS, CA, SR, LM; MI performed statistical analysis. The draft of the manuscript was written by CA, SA, CG. All authors read and approved the final manuscript. This work was partially presented as poster presentation (#1091) at the 111th Meeting of the United States and Canadian Academy of Pathology in Los Angeles , CA , USA, 19-24 March, 2022.

Acknowledgements and Funding: The publication of this article was supported by the "Ricerca Corrente" funding from the Italian Ministry of Health to SA. This work was supported by funds for selected research topics from the Fondazione CARISBO Project (#19085) to SA.

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

correlate the biologic profiles CNS-DLBCL to clinical findings and explore new potential therapeutic targets.

Methods

Patients' selection and tumour samples collection. From the review of the medical records of the Department of Neurosurgery of IRCCS Istituto Delle Scienze Neurologiche, Bologna, we identified 107 pathologically confirmed cases of CNS-DLBCL diagnosed between 2005 and 2020. Of those, sixty-one cases with formalin fixed paraffin embedded (FFPE) adequate material were included in the study. The study was approved by local ethics committee (620/2020/OSS/AUSLBO) and it was performed in accordance with the Declaration of Helsinki. Thirty patients (49.2%) were male and 31 females (50.8%), with an age range between 32 and 82 years and a median age of 66 years (IQR: 57–72).

Immunohistochemistry. Paraffin-embedded sections were deparaffinized in HistoClear and dehydrated through graded ethanol. The antigen retrieval was performed in the PT-Link (Agilent Dako, Santa Clara, CA, USA, code PT100/ PT 101), for 5 min at 92°C in EnVision Flex Target retrieval solution High pH (Agilent Dako, code K 8004). Then, tissue samples were incubated at RT for 30 minutes with the following antibodies: CD20 (Agilent Dako 1:300, clone L26, code M0755), CD10 (Leica NewCastle, UK, 1:30, clone 56C6 code CD10-270-L), BCL2 (Abcam, Cambridge , UK, 1:100, clone E17, code Ab32124), BCL6 (kindly provided by Prof. Falini, indiluted, clone PG-B6p), cMYC (Epitomics, Burlingame, CA 1:80, clone Y69, code 1472-1,), IRF4 (kindly provided by Prof. Falini, 1:3, clone IRF4). Immunostaining has been completed using the Alkaline REAL Detection System Alkaline Phosphatase/RED Rabbit/Mouse (Agilent Dako, code K5005) and chromogen (Fast red), provided with the kit. Finally, slides were counterstained with Hematoxylin, mounted in Glycerine and observed and analysed by Olympus microscope. Slides were observed by 2 expert pathologists (CA, SA). Results were recorded as percentage of positive cells and graded as follows: + if >30% positive neoplastic cells, for CD10/BCL6/IRF4 according to Hans' algorithm.

[11] A case was defined as cMyc or BCL2 positive if ≥40% and ≥50% cells were stained respectively.[6]

Genetic analysis.

Fluorescence in situ hybridization (FISH). Paraffin embedded tissue were deparaffinized and then pre-treated with two different antigen retrievals. The slides were before incubated in 1mM EDTA buffer (pH 8) in a pressure cooker (9 minutes) and after in Pepsin solution (Sigma Aldrich code P7012) for 14 minutes at 38°C [12]. The probes and the samples were denatured at 80 °C for 22 min and then hybridizated at 38°C for 22 hours in a hybridizer (Agilent Dako, Santa Clara, CA USA). The experiments were conducted using the probes LSI MYC Dual Color Break Apart Rearrangement (Vysis, Abbott, Downers Grove, Illinois, USA, code 01N63-020, cut-off 3,8%), LSI BCL2 Dual Color Break Apart Rearrangement (Vysis Abbott, code 05N51-020, cut-off 4,7%) and LSI BCL6 dual color Break apart Rearrangement t(3q27) (Vysis Abbott, code 01N23-020, cut off 5,5%). Following a stringency washes (0,4XSSC/0,03%NP-40 pH 7,4 solutions at 73°C for 2 min and with 2XSSC/0,1% NP-40 pH 7 solutions at room temperature for 1 min), the slides were mounted and counterstained with DAPI I (Vysis Abbott, code 06J49-001). Microscopical analysis were carried out with an Olympus BX61 microscope and images were recorded using the Cell^F program. *BCL6* and *BCL2* rearrangements were investigated only in cases with translocation of the *cMYC* gene.

Next Generation Sequencing. DNA from FFPE PCNSL tissue samples was purified by Quick Extract FFPE DNA Extraction Kit (Epicentre, Madison, WI) with some modifications described by Ricci C et al.[13] DNA mutations were detected using the protocol described previously[14] analysing the following gene panel: *MYD88, CD79B, PIM1, GNAS, NOTCH1, KRAS, PIK3CA, EGFR, CASP8, AIP, PTEN*. In brief, after target enrichment by multiplex PCR, libraries with tagged primers were generated using Nextera adapters. Each run on MiSEQ platform (Illumina, Palo Alto, CA) was designed to allocate at least 2K reads/region aimed to have a depth of coverage of at least 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 $\chi 2$ 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.

Fish analysis. FISH analysis was successful in 59 of 61 (96.7%) samples; *cMYC* rearrangements were present in 2 (3.4%) of 59 cases and in one was associated to *BCL6* translocation. This latter harboured *MYD88* mutated (p.L265P).

Next generation sequencing showed *MYD88* mutations in 24 (54.5%) of 44, all carrying p.L265P except one case with p.A260T. Both mutations are located in the Toll/Il-1 Receptor (TIR) domain of the protein.

Twenty-three cases carried a mutation in proto-oncogene with serine/threonine kinase *PIM1 (23/43, 53.5%)*, with a great variety of different mutations, mostly missense mutations, occurring at protein kinase domain, of which the p.E135K was the most common involving four cases. In 9 samples 2 or 3 different *PIM1* mutations were simultaneously present. *CD79B* missense mutations were found in 13 cases (of 43, 30.2%), located within immunoreceptor tyrosine-based activation motif (ITAM), 5 of which had p.Y196H, 2 cases p.Y196N, 2 p.Y196S, 2 p.Y196C, 1 p.Y196D, and one the rare p.E198G. Concurrent mutations were found: *MYD88*, *PIM1* and *CD79B* in 4 cases, *MYD88* and *PIM1* in 13 cases, *MYD88* and *CD79B* in 9 cases, and *CD79B* and *PIM1* in 8.

Seven cases carried missense mutations in *TP53* gene (7 of 38, 18.4%). Additional mutations were found: *NOTCH1 in* 4 of 35 (11,4%), *AIP in* 9 of 39 (23,1%), *GNAS in* 3 of 37 (8.1%) and *CASP8 in* 3 of 38 (7.9%).

PI3KCA missense mutations were found in 6 of 40 (15%); in 5 cases they produced an amino acid substitution in the catalytic domain (p.A1046V, p.D1045N, p.G1049S and p.G1049D in two cases). *PTEN and EGFR* genes mutations were found in 2 of 39 (5.1%) and 2 of 31 (6.4%) cases respectively, while p.G12D and in CIS p.G13D mutations of *KRAS* gene co-occurred in a single patient (1/39). Table 2 summarizes all these sequencing data.

Clinical findings. The disease presented with a single lesion in 25 of 61 (41%) of patients and with multiple lesions in 36 of 61 (59%). On MRI, most patients showed deeply located lesions with homogeneous contrast-enhancement (36 of 50, 72%). Multiple enhancing was seen in 53%. Only one of 34 patients with DWI performed showed a high ADC value, all others presented

hyperintense diffusion signal. Perfusion T2-w studies was available in 24 patients, 54% with 2 to 7 times higher value of rCBV. No significant statistical correlations were observed between biological and clinical parameters and neuroradiological features.

Gross total resection was achieved in 13 of 61 (21%), subtotal resection in 21 (35%) and biopsy in 27 (44%). Twenty-four patients, whose treatment was known, had undergone the matrix protocol MTX + cytarabine or MATRix regimen. Of the 61 patients included in the study, 57 died during the follow-up, with a median follow-up time of 3.3 months (95% CI: [2.1 - 5.0]). The longest follow-up was 69.2 months. Figure 1 shows the Kaplan-Meier survival curve. One-month, 3-month, 6-month overall survival rates were 88.5% (95% CI: [77.4% - 94.4%]), 52.5% (95% CI: [39.3% - 64.1%]), and 37.7% (95% CI: [25.7% - 49.6%]), respectively.

No statistically significant correlation was found between cell of origin GCB/non-GCB, BCL2 and cMYC expression and survival nor between DE or non-DE status and prognosis. Survival was significantly longer for patients with mutated *MYD88* (8.7 months vs. 1.7 months; log-rank test = 5.43; p = 0.020) compared to those without *MYD88* mutation, and for patients with mutated *CD79B* (10.8 months vs. 2.5 months; log-rank test = 4.64; p = 0.031) compared to those without *CD79B* mutation.

Discussion

CNS-DLBCL, together with vitreoretinal and testicular DLBCL, is now grouped in a new category called large B-cell lymphoma of the immune-privileged sites.[16] The prognosis of CNS-DLBCL remains poor and new therapeutic approaches are needed to improve patient survival. OS is lower in our series than prior studies, this could be due to patient selection bias, which in our study had a median older age (56 vs 64) and a much higher frequency of multiple lesions (59% vs 30%).[1]

Similar to previously reported, based on our data, single and double hit rearrangements of MYC gene were rare .[1,6] Differently from a recent report by Asano et al and Kim et al DE status for BCL2 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

inhibiting aryl hydrocarbon receptor was proposed as potential therapeutic target.[39-41] Recently *AIP* was found to be a positive regulator of BCL6 expression in germinal centers cells, protecting BCL6 from ubiquitin-mediated proteasomal degradation, and deletion of *AIP* in B cells decrease BCL6 expression, reducing germinal center B cells and diminishing adaptive immune responses.[42] Furthermore AIP was required for optimal AKT signaling in response to BCR stimulation and seems to be highly expressed in primary DLBCL compared to healthy tissue with implications for the pathobiology of this disease.[42]

The NOTCH signalling pathway is widely involved in cellular proliferation, differentiation, and apoptosis.[43] We *NOTCH1* mutations in 11,4% of the samples: *NOTCH1* mutations are distinctive drivers of systemic DLBCL and were not previously described in CNS-DLBCL.[8]

GNAS and *CASP8* were mutated in 8.1% and 7.9% of the cases, respectively. *GNAS* is the most frequently mutated G-protein in human cancers and activating mutations in the gene GNAS have been found in pituitary, thyroid, pancreatic, biliary tract and intestine tumors as well as in Burkitt lymphoma, systemic DLBCL and Hodgkin lymphoma.[44-47] Zhou et al reported *GNA13* mutations to be associated with a shorter PFS and overall survival in primary central nervous system lymphoma patients, however we did not find any correlation with prognosis.[48] We also identified somatic mutations *CASP8* for which a functional role has not been previously suspected in CNS-DLBCL, although p.R472* nonsense mutation detected in one of our samples was previously reported in systemic DLBCL.[49] *CASP8* encodes a member of the cysteine-aspartic acid protease (caspase) family and sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. In addition, caspase-8 participates in maintenance of genomic integrity and loss of caspase-8 in B lymphocytes leads to B-cell malignancies; moreover, *CASP8* mutations were found in mantle cell lymphoma.[50]

PI3KCA missense mutations, previously described in several types of carcinomas,[1] were found in 6 of 40 (15%), while *PTEN* mutations were detected in 2 of 39 samples (5.1%). The PI3K/AKT/mTOR is an important pro-survival pathway which plays a pivotal role in the development of malignant tumours being often aberrantly activated in different types of cancer also including lymphomas. Zang et al recently showed that PI3K/AKT/mTOR signaling is aberrantly activated in CNS-DLBCL and correlated with a poor prognosis.[51] They found that p-mTOR expression was an independent risk factor in terms of PFS in patients with CNS-DLBCL. Moreover, Takashima et al found that copy number losses in the PTEN-PI3K-AKT proapoptotic pathway are associated with poor prognosis in CNS-DLBCL patients while PTEN mutation was related to shorter OS in the study of Todorovic et al.[52,53] PI3K/mTOR inhibitors has also become a potential therapeutic target in CNS-DLBCL. A phase II trial of 37 relapsed/refractory CNS-DLBCL patients demonstrated that temsirolimus had a positive effect with 54% overall response rate, while lower response rate of 25% was seen in a clinical trial targeting using the pan-PI3K inhibitor buparlisib.[54,55] The use of dual pan-PI3K/mTOR inhibitor bimiralisib (PQR309) in a multicenter phase I/II trial (NCT02669511) as well as BAY80-6946 (copanlisib) in a phase Ib/II clinical trial (NCT03581942) are still under investigation in relapsed/refractory CNS-DLBCL. Moreover, Inhibition of the PI3K isoforms p110α/p110δ or mTOR synergized with ibrutinib to induce cell death in CD79B-mutant CNS-DLBCL cells suggesting that combined inhibition of BTK and PI3K/mTOR could overcome the resistance of lymphoma cells to ibrutinib.[56] So far, no PI3K mutations were described in CNS-DLBCL and our data provide a strong rational for a clinical application of inhibitors targeting the PI3K/ AKT/mTOR signalling pathway at least in a subset of patients.

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

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