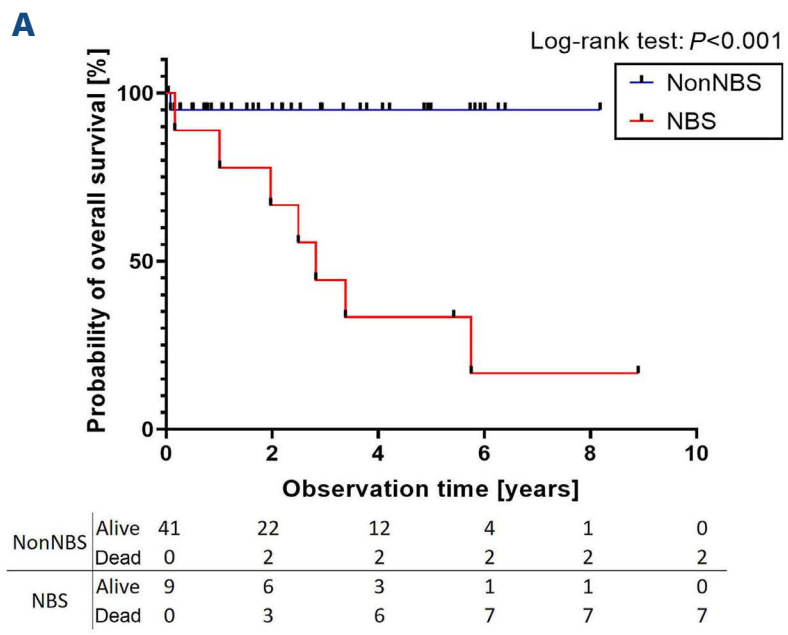
Clinical and laboratory diversity of diffuse large B-cell lymphomas in children with Nijmegen breakage syndrome

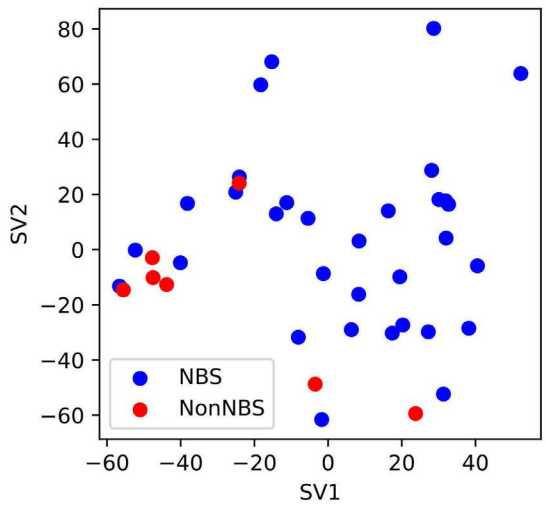
Nijmegen breakage syndrome (NBS) is an inherited DNA repair disorder characterized by a high predisposition to develop lymphoid malignancies during childhood, with diffuse large B-cell lymphoma (DLBCL) being one of the leading types.[1] Due to concomitant immunodeficiency and an increased risk of chemotherapy-induced toxicity, NBS patients often require modified and individualized cancer treatment.[2] Nevertheless, they rarely achieve progression- and relapse-free long-term survival rates without hematopoietic stem cell transplantation.[3] Unfavorable outcome of lymphomas in NBS may result not only from the reduction of drug dosages but could be associated with molecular aberrations occurring on the background of chromosomal instability. Therefore, we aimed to investigate clinical outcome, histopathological features, and genomic alterations of DLBCL in pediatric patients with NBS. The algorithm of the study is shown in the *Online Supplementary Figure S1A*.

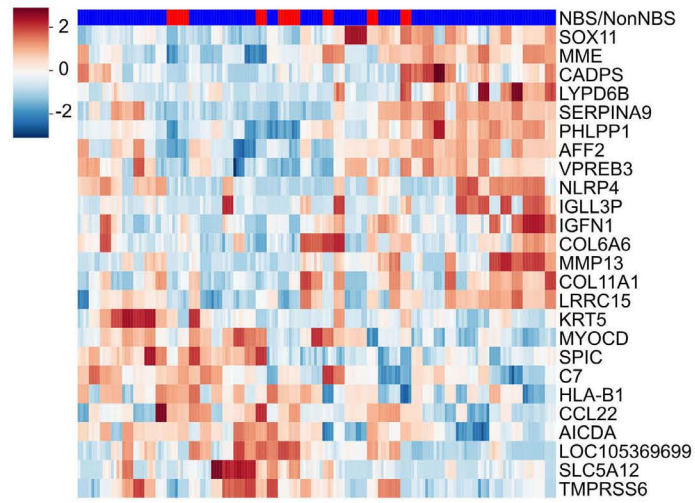
First, we estimated the probability of overall survival (OS) depending on NBS status in 50 children with newly diagnosed DLBCL, including nine (18%) NBS patients. They were treated in the 13 centers of the Polish Pediatric Leukemia/Lymphoma Study Group between 2014 and 2021 (NonNBS) and between 1993 and 2021 (NBS). All of the individuals with NBS carried the germline biallelic founder mutation c.657_661del5, p.Lys219Asnfs in the NBN gene (NM_001024688.3). The median age at diagnosis of lymphoma was 12.97 years (interquartile range [IQR], 7.98-15.64) and was similar in NBS and nonNBS individuals: 13.01 (IQR, 6.73-14.85) *versus* 12.94 (IQR, 8.39-15.64; *P*=0.11). The median observation time was 2.42 (IQR, 0.89-4.70) years. During observation time, nine children died: seven (78%) NBS and two (22%) NonNBS (*P*<0.001). The probability of 5-years OS was significantly lower in patients with NBS as compared to NonNBS individuals: 33.33% *versus* 95.12%, respectively (*P*<0.001) (Figure 1A). Regarding the causes of death in NBS patients, lymphoma progression was the most common (n=4) and was followed by: excessive toxicity (n=1), and infectious complications (n=2). While both NonNBS children experienced fatal treatment-related toxicity.

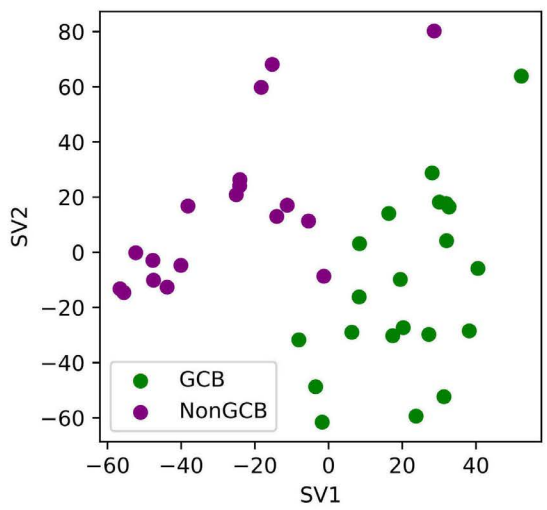
Next, we performed a two-step pathology review of tumor tissues, by the local and the central hematopathologists, following criteria for aggressive B-cell lymphomas included in the 4th World Health Organization Classification of Tumors of Hematopoietic and Lymphoid Tissues.[4] Sixteen pediatric patients with diagnosis of DLBCL had available formalin-fixed paraffin-embedded (FFPE) tissue blocks for extended pathological analysis including nine NonNBS (56.2%) and seven with NBS (43.8%). Our histopathological analysis demonstrated that NBS-positive lymphomas were more similar to adult-type than to pediatric-type DLBCL NOS. The tumors were predominantly classified as ABC and non-double expressors (NonDE) had a high Ki67 proliferation index and presented heterogenous morphology. Of note, none of the NBS lymphomas displayed positivity for CD10, and, in contrast, six (n=6/9, 66.7%) NonNBS tumors were positive for CD10; *P*=0.010. (Table 1A). CD10-negativity and other pathological features might be regarded as indicators of poor prognosis of NBS DLBCL when compared to non-NBS DLBCL.[5]

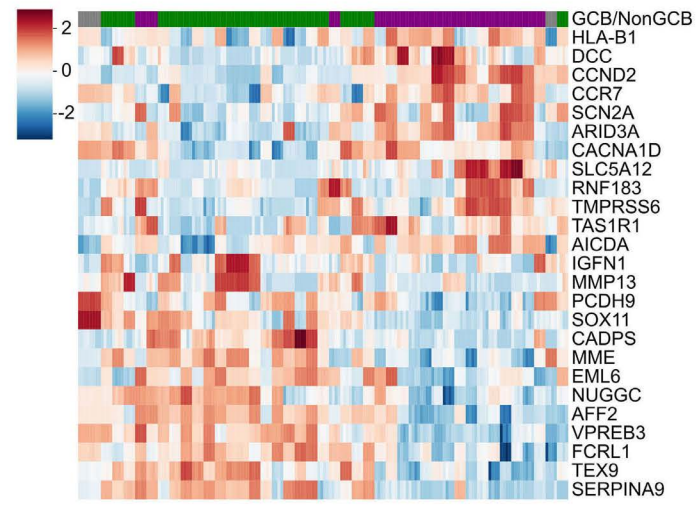
In order to delineate the entire spectrum of genetic alterations in DLBCL in NBS, whole genome sequencing (WGS) using Illumina TruSeq Nano 550 polymerase chain reaction (PCR)-free library preparation protocol was executed. The 150 bp paired-end sequences from the tumor and matched normal genomes were generated corresponding to an average per-base sequencing coverage of 107-fold and 32-fold, respectively. After applying a filtering algorithm, we detected an average of 8.763 somatic sequence alterations (median: 5.735; resulting in a tumor mutation burden [TMB] of 2.89 mutations/Mb/sample on average), including in total 39.021 single nucleotide variants (SNV) and 13.558 insertions and deletions, alongside 158 structural variants (SV). DLBCL in NBS patients shared a common predominant mutational signature (COSMIC SBS 9; see Figure 2), which has been frequently reported in malignant B-cell lymphomas.[6] It is associated with hyperactivation of AID enzyme and aberrant activity of DNA polymerase η during illegitimate somatic hypermutation events outside of immunoglobulin loci.[7] In two DLBCL cases an aggressive course of the disease could be explained by the presence of *TP53* lesions or high TMB concomitant with *PDL1* amplification, respectively. In PDL1-amplified sample we additionally observed kataegis at 7qter. The number of SNV and TMB in this DLBCL was n=22,334 and 7.9%, respectively compared to the median number of SNV and TMB n=1,999 (IQR, 1,328-6,016) and 1.97% (IQR, 1.145-2.6%) in the remaining genomes. *TP53* is affected in 8.9-31.7% of DLBCL and in the majority of studies predicted worse outcome.[8] *PD-L1* gene translocations and amplification, were observed in ~10% of DLBCL

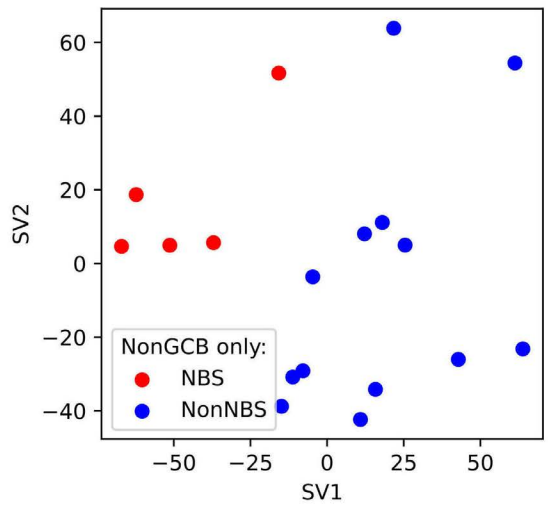












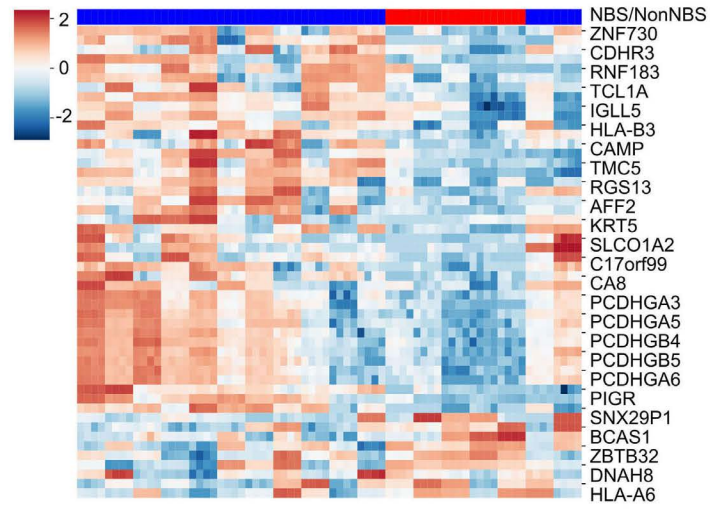


Figure 1. Patients with Nijmegen breakage syndrome, who developed diffuse large B-cell lymphoma (DLBCL), showed not only significantly lower 5-year overall survival, but also different tumor-gene expression profile when compared to non-syndromic DLBCL patients. (A) Kaplan–Meier curves displaying the probability of overall survival among pediatric Nijmegen breakage syndrome (NBS) patients with DLBCL (the red line) and non-syndromic individuals (the blue line): 33.33% *vs*. 95.12%, respectively (*P*<0.001). The comparison of tumor gene expression profile between (B) NBS *vs*. NonNBS, (C) germinal center B cell (GCB) *vs*. NonGCB, and (D) NBS *vs*. NonNBS within the subgroup of NonGCB DLBCL. Differences are shown with respect to the top of 25 differentially expressed genes sorted by weight*PLSDA coefficient for LV1 (group: NBS *vs*. NonNBS) and LV2 (molecular subtype: GCB *vs*. NonGCB) for the comparison between: (E) NBS *vs*. NonNBS, (F) GCB *vs*. NonGCB, and (G) NBS *vs*. NonNBS among NonGCB patients. These genes were further grouped based on their functions (see the *Online Supplementary Figure S1B*).

patients,[9,10] especially in patients less than 60 years old.[11] Although in all but one sample driver mutations were dissected, only a slight overlap of affected genes was present between the lymphoma samples, e.g., *STAT3, KMT2D, HIST1H1B* was found in two samples for each of the genes (Table 1B). Based on the knowledge that genomic instability is an intrinsic feature of NBS, we also searched for somatic SV that could have evolved due to inheritance.

Table 1. The comparison of pathological characteristics and pathogenic somatic alterations in patients with diffuse large B-cell lymphoma developed in the course of Nijmegen breakage syndrome and non-syndromic pediatric individuals.

(A) The pathological characteristic of DLBCL samples			
	NBS-positive DLBCL, NOS N=7/16, 43.8%	**NBS-negative, DLBCL, NOS N=9/16, 56.2%**	***P***
Morphology, N (%) Centroblastic Immunoblastic Anaplastic	 5 (71.4) 1 (14.3) 1 (14.3)	 7 (77.8) 0 (0.0) 2 (22.2)	0.487#
Cell of origin, N (%) GCB ABC	 2 (28.6) 5 (71.4)	 7 (77.8) 2 (22.2)	0.126^
BCL-2 positive, N (%)	4 (57.1)	4 (44.4)	1.000^
BLC-2, % (IQR)	50.0 (10.0-55.0)	30.0 (20.0-60.0)	0.873*
CD10 positive, N (%)	0 (0)	6 (66.7)	0.010^
c-MYC positive, N (%)	4 (57.1)	2 (22.2)	0.302^
C-MYC, % (IQR)	40.0 (5.0-55.0)	4.0 (2.0-15.0)	0.071*
Double expressor BCL2/MYC positive, N (%)	2 (28.6)	1 (11.1)	0.550^
Ki67 index (IQR)	80.0 (70.0-92.0)	75.0 (40.0-90.0)	0.456*

(B) Pathogenic somatic alterations detected in NBS DLBCL			
Sample ID	**Amplification**	**Genes with identified somatic pathogenic SNV/Indels**	**Somatic structural variants**
DLBCL _NBS1	seq[hg19]11 q24.2-q25(127130254-131045787)x5; seq[hg19]5p15.33(11881-1998109)x6; seq[hg19]7q21.3-q36.3(97119555-15512689)x7-8; seq[hg19]9p24.3-p23(1280059-9700448)x5-16 (amplification of 9p24.3-p23 involving locus PDL1)	*PTEN, KMT2D, BTG1, BTG2, PIK3CG, BTX, STAT3, FAT1, SMARCE1*	*STAT3* deletion; *PTPRD* truncating translocation
DLBCL _NBS2	duplication: seq[hg19]2p16.1(60753592-61234194)x3	not present	inverted duplication of *BCL11A*
DLBCL _NBS3	seq[hg19]3q12.1-q13.2(98567732-117599362)x3, seq[hg19]11p15.5-p11.2(48684843-48597576)x3	*LYN, KMT2D, FAS*	possible fusion *DOPEY2-TTC3*
DLBCL _NBS4	seq[hg19]11p22.1-q22.2 (100890992-102613285)x14-16 (possible hyperactivation of YAP1 and BIRC3); seq[hg19]18p21.2(48430066-49202094)x6; seq[hg19]Xq22.3-26.2(107296028-133033076)x5; seq[hg19]Xq28(148131415-154929412)x5	*TP53* (+ *TP53* LOH), *HIST1H1B, FAT4*	*SPEN* deletion Chromoanagenesis on chromosome 6q
DLBCL _NBS5	not identified	not identified	not identified
DLBCL _NBS7	not identified	*HIST1H1B*	*IRF4* translocation, *DLEU-KLF12* fusion Chromoanagenesis on chromosome 6p

(A) Differences regarding histopathological features of diffuse large B-cell lymphoma (DLBCL) between children with Nijmegen breakage syndrome (NBS) and non-syndromic pediatric individuals. The qualitative variables were presented as numbers followed by percentages of respective subgroups and quantitative variables were presented as medians followed by quartiles in brackets. #Pearson's X^2 test, ^two-tailed Fisher's X^2 test, *Mann Whitney U-test. (B) Pathogenic somatic alterations detected in DLBCL samples in patients with NBS. DLBCL NOS: diffuse large B-cell lymphoma not-otherwise specified. GCB: germinal center B cell; ABC: activated B cell-like; IQR: interquartile range; SNV: single nucleotide variant; Indels: insertions and deletions.

Beside of *PDL1* amplification, the following SV were detected: amplification within the chromosome 11p22.1-p22.2 leading to hyperactivation of *YAP1* and *BIRC3*, inverted duplication of *BCL11A*, and in two DLBCL samples the possible incidence of chromoanagenesis on chromosome 6. Complex structural variants events including chromothripsis, chromoplexy, and templated insertion has been observed in non-syndromic, newly diagnosed and relapsed DLBCL patients. However, only chromothripsis co-occurred with APOBEC signature and poor outcome.[12] We further investigated the difference in gene expression profiles between DLBCL in patients with NBS and non-syndromic individuals according to cell-of-origin (COO) subtype (germinal center B cell [GCB] or activated B cell-like [ABC]). In order to enrich the study group in ABC cases represented the minority among children without NBS, we additionally included 28 adult DLBCL samples representing both DLBCL subtypes (ABC n=13, GCB n=13) in the transcriptomic assessment. RNA sequencing was performed using SureSelect XT RNA Direct Human All Exon V6+UTR (Agilent Technologies, USA) and NextSeq™ 500/550 High Output Kit v2.5 (300 cycles; Illumina, USA). Differential expression analysis using DESeq2 revealed significant down- or upregulation of 195 (23.01%) genes between NBS *versus* NonNBS samples. However, the transcriptomic differences were partly affected by the representation of COO subtypes within both groups, GCB *versus* ABC DLBCL (n=524 genes, 22.45%). Therefore, we narrowed our analysis to

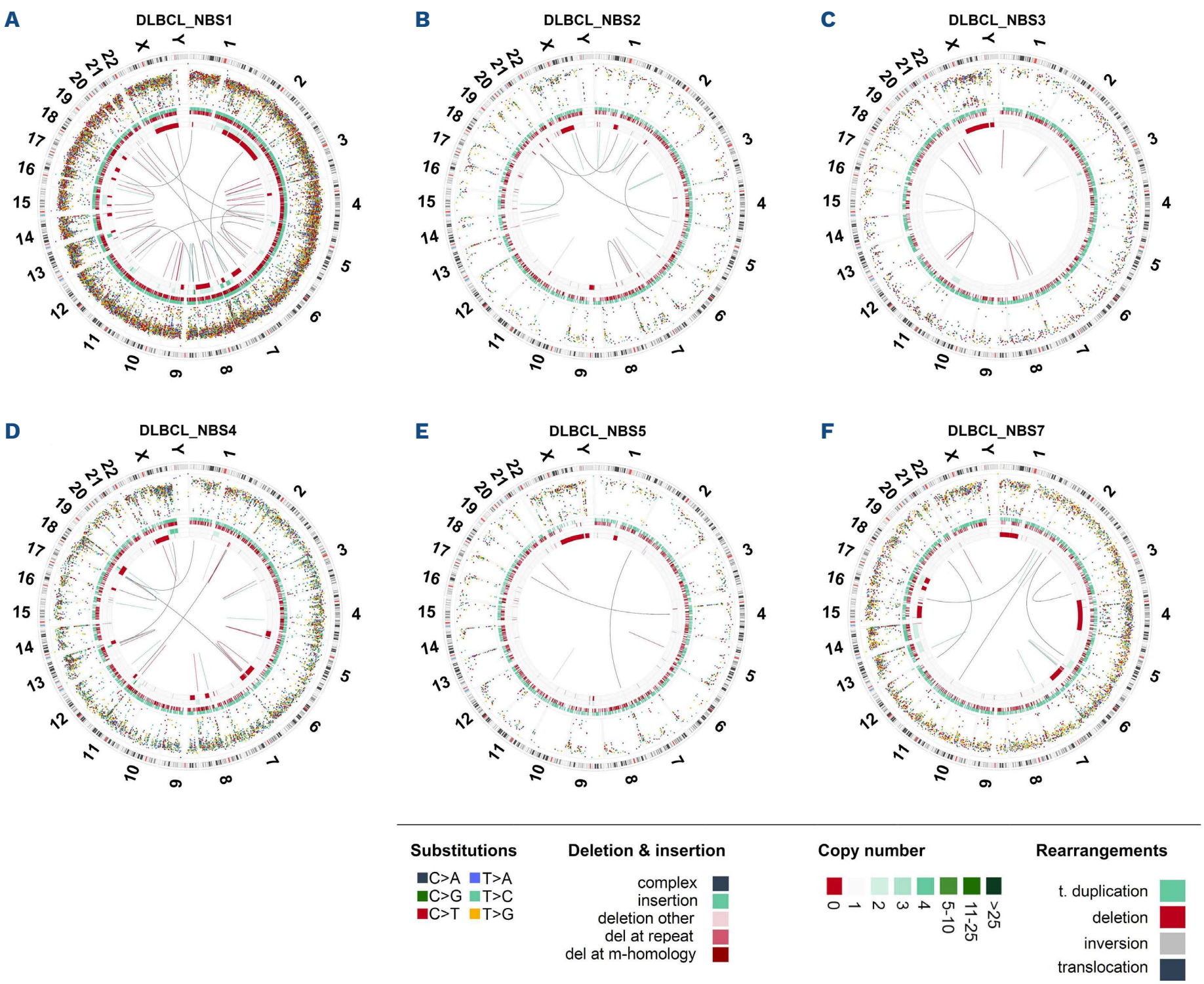


Figure 2. Whole genome circos plots displaying the types and location of genomic aberrations identified in diffuse large B-cell lymphoma in patients with Nijmegen breakage syndrome. The frequencies of the particular types of whole genome aberrations in diffuse large B-cell lymphoma (DLBCL) in patients with Nijmegen breakage syndrome are shown in the *Online Supplementary Figure S2;* del: deletion.

ABC patients, a frequent subtype in NBS, and found differential expression of 197 genes between NBS and non-syndromic tumors (11.24%). We employed the PLS-DA approach to visualize the differences between NBS *versus* NonNBS, GCB *versus* ABC, as well as NBS *versus* NonNBS within the group of ABC patients. The top 25 differentiating genes for these comparisons are shown in Figure 1B-G. We subsequently grouped these genes according to their cellular function (*Online Supplementary Figure S1B*). Among several groups of genes, NBS tumors showed downregulation of *cAMP (PRKAR1A)* which has been described as an aberrations promoting tumorgenesis in different types of malignancies, such as melanoma, lung cancer, and pancreatic tumors.[13] Additionally, targeting cAMP and its effectors may be a possible cancer treatment strategy.[14]

Considering relatively frequent incidence of Epstein Barr virus (EBV) infection or reactivation that precedes DLBCL development in NBS patients and the fact that EBV is one of the prominent infection-related causes of cancer, we aimed to investigate viral DNA incorporation into the tumor genomes.[15,16] Three of seven NBS DLBCL samples showed an enrichment of the number of EBV integration sites in somatic genomes with respect to non-cancer DNA, using the threshold of ten times difference between tumor and normal tissues. This reflected the median number of EBV-human split reads reaching n=2,047 (range, 62-6,743) in EBV-positive lymphomas as compared to n=12 (range, 9-19) in the remaining four EBV-negative DLBCL cases (P=0.05). Next, we searched for recurrent sites of EBV incorporation in the positive DLBCL samples and found five common EBV locations including three sites that were present in each of the tumor: on chromosome 2 (33141296-33141626, within the *LINC00486*), on chromosome 7 (105741882-105742671; within the intron 1 of *SYPL1*) and on chromosome 14 (99887280-99887288; within the intron 6 of SETD3) (*Online Supplementary Table S1A*). However, all of these recurrent EBV integration sites exclusively affected non-coding regions including simple repeats (polyG, polyT, polyA), introns, and Alu elements, the latter ones also being binding sites for several B-cell specific transcription factors (*data not shown*). This observation is in the line with WGS results obtained by Zapatka *et al.* who studied viral integration in more than 2,500 cancers across 38 tumor types in which it was found only 3.4% of integrations located in gene coding sequences.[16] Interestingly, one of the recurrent EBV incorporation in DLBCL genomes occurred at the short arm of chromosome 2 within the poly-G tract of *LINC00486*. We analyzed the incidence of similar long poly-G tracts (>100 nt and G content >85%) in the hg19 reference genome that could be incorrectly assigned to the site on chromosome 2. However, this genomic region showing preferential EBV incorporation turned out to be a unique sequence in the human genome regarding the length and high amount of G content. Moreover, it has been already described as an integration site of hepatitis B virus into the somatic genome of intrahepatic cholangiocarcinoma.[17] In order to investigate whether identified recurrent sites of EBV integration are unique for tumors in NBS patients, we performed deep WGS of genomes extracted from four non-syndromic and two NBS-positive EBV-immortalized cell lines. All of them showed the enrichment of viral incorporation in the previously observed chromosomal regions in NBS tumors (*Online Supplementary Table S1B*). These observations raise the question whether and why this region could be a preferential site of viral integration and what the functional consequences of the specific EBV incorporation could be.

To the best of our knowledge this is the first study providing a comprehensive analysis of tumor morphology and molecular aberrations that could contribute to tumor development in NBS patients. However, one of the essential limitations of this work is the limited number of DLBCL in NBS included in WGS due to unavailability of fresh frozen tumor tissue. This fact, combined with a significant molecular heterogeneity of the DLBCL, positions our results as a preliminary report requiring further investigation and improvements.

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https://doi.org/10.3324/haematol.2022.282325

Received: November 20, 2022.
Accepted: March 29, 2023.
Early view: April 6, 2023.

Disclosures

No conflicts of interest to disclose.

Contributions

AP and WM designed the research. AP and BSz analyzed the data and wrote the paper. JM, KW and BSz performed RNA sequencing experiments. PS and MW provided bioinformatic support in WGS analysis. DDW and RPK performed WGS and interpreted data. WG and MB conducted histopathological analysis. JCh and WF interpreted data analysis and visualization. WG, MB, HG, AW, BK and ZK provided patients samples and outcome data. WM supervised the project. All authors have read and agreed to the published version of the manuscript.

Funding

AP, BSz and WM were supported by the National Science Center grants 2017/26/D/NZ5/00811 (to AP) and 2020/39/O/NZ2/02954 (to BSz, WM). AP, ZK, RPK and WM were supported by the LEukemia GENe Discovery (LEGEND) by data sharing, mining and collaboration (COST Action - CA16223) 2017-2021. BSz was supported by the Ministry of Education and Science “Diamond Grant” (0136/DIA/2020/49). DDW is supported by grant KWF12090 from the Dutch Cancer Society.

Data-sharing statement

Data are available upon request to the corresponding author.

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