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Review Article

Molecular Pathogenesis of Diffuse Large B-Cell Lymphoma

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Diffuse large B-cell lymphoma (DLBCL) is divided into germinal center B-like (GCB) DLBCL and activated B-like (ABC) DLBCL. In recent years, whole genome sequencing (WGS), whole exome sequencing (WES), and transcriptome sequencing (RNA-seq) have been performed for samples from many patients with DLBCL. Here, I present a review of the results of next generation sequencing data for DLBCL. Somatic mutations show a low identity between studies with only 10–20% gene overlap. DLBCL is a disease that results from various molecular pathogeneses. Mutations in genes involved in chromatin remodeling were found in the GCB subtype. Mutations in members of B-cell receptor (BCR) signaling and the NF-κB pathway (*MYD88*) were found in the ABC subtype. The MYD88 L265P mutation was observed in 29% of ABC DLBCL cases. EZH2 mutations were observed in 21.7% of GCB DLBCL cases. WGS indicated that inactivating mutations in GNA13 (Gα protein) were prevalent in GCB DLBCL cases. In addition, *S1PR2* is a target of aberrant somatic hypermutation. In recent years, samples from patients with relapsed and refractory DLBCL were analyzed. The activation of the NF-κB pathway is associated with treatment resistance in DLBCL. Further clarification of the molecular pathogenesis of DLBCL is expected to lead to the development of individualized treatment for the disease. 〔*J Clin Exp Hematop 56(2):71-78, 2016*〕

Keywords: diffuse large B-cell lymphoma, next generation sequencing, *GNA13*, *EZH2*, *MYD88*

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common form of lymphoma. DLBCL has been thought to arise from the translocation of the *IGH* gene and subsequent enhanced expression of oncogenes (*BCL2*, *BCL6*, and *MYC*). In 2000, microarray analysis was performed on DLBCL samples which were divided into germinal center B-like (GCB) DLBCL and activated B-like (ABC) DLBCL.[1] In 2004, Hans *et al*.[2] divided DLBCL samples into GCB and non-GC using only immunostaining. The introduction of this criterion to clinical settings allowed for an easy form of identification of DLBCL molecular pathology. In 2005, next generation sequencing (NGS) made large outputs of DNA sequencing data possible. Whole exome sequencing (WES) analyzes the exon region that accounts for only 1 to 1.5% of the human genome. WES can be performed at a reduced cost compared to whole genome sequencing (WGS). WES can detect somatic mutations from tumor samples and normal samples such as blood. WGS can analyze splicing site mutations, copy number, and structure mutations. In addition, WGS can analyze allele frequency of intron and intergenic regions. Thus, WGS allows for a higher resolution analysis of the heterogeneity of tumors. Transcriptome sequencing (RNA-seq) quantifies the gene expression by sequencing RNA transcripts. RNA-seq can also produce sequencing data that can detect mutations and fusion genes. Targeted resequencing is a method of performing sequence analysis on a particular region of interest. Capture and amplicon sequencing can reduce technical error by sequencing the same position many times and allows for the detection of mutations in tumors with a smaller burden. WGS, WES, and RNA-seq have been performed on DLBCL samples from numerous patients. Sequencing has revealed single nucleotide variants (SNVs) in multiple cases.[3] It has become evident that NGS data is necessary to determine the molecular pathogenesis of individual DLBCL cases. In recent years, analysis of samples from patients with relapsed and refractory DLBCL detected mutations associated with treatment. Here, I present a review of the results of NGS data for DLBCL.

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WGS AND WES IN DLBCL

The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov/), which contains 48 DLBCL tumor samples, indicated that the median somatic mutation frequency in DLBCL is 3.3 mutations per Mb. I searched PubMed for the following search terms ("diffuse large B-cell lymphoma" OR "DLBCL") AND ("sequence" OR "sequencing" OR "sequenced"). I selected representative papers as original papers analyzing untreated DLBCL by WGS or WES. These representative papers are shown in Table 1.[3-9] WGS has been used to sequence more than 100 DLBCL samples, and the number of matches of gene mutations found between four research studies have been provided in Fig. 1.[3,6] Many somatic gene mutations differ between samples, and only 10–20% gene overlap was found between these studies. DLBCL is a disease that results from a variety of molecular pathogeneses. In addition, Fig. 2[4,6-9] shows the recurrent gene mutations found in at least 15% of DLBCL cases in any of the independent studies. The mutation rate in GCB DLBCL was calculated using the reports previously noted. In Fig. 2 is the same as in Fig. 1, but the mutation rate does not match that found in each paper. Due to the diversity of DLBCL, there remains the possibility the true mutation rate remains unknown. However, through our reanalysis of the published data on extranodal NK/T cell lymphoma, we have found new frequent aberrations.[10] It may be possible to identify new frequent genetic aberrations in DLBCL by reanalyzing the published data. Analysis of the NGS is still developing, and a number of problems with the analysis have been discovered. First, the sampling of tumors is an issue. In the case of tumors surrounded by inflammatory cells, the proportion of DNA from tumor cells and normal tissue is also an issue. In the future, it will be necessary to standardize the specimen by laser microdissection. Next, normal cell samples may contain a small number of tumor cells, and therefore, the detection of somatic mutation rate may be altered. In lymphoma studies, bone marrow biopsies or peripheral blood are often used as normal specimens. Lymphoma is a systemic disease; thus, compared with solid cancers, care should be taken to prevent contamination of normal specimens with tumor cells. Some analysis programs have already considered this. It will require the use of a detection program to detect tumor cells in normal specimens. Finally, there is a problem with the analyzers, such as MuTect[11] and VarScan,[12] which are used to detect cancer-causing somatic mutations. In the default setting, MuTect has a low power in low coverage area. VarScan detects many mutations in the default setting, but the settings may need to be adjusted depending on sample conditions. The adjustment of these settings is very difficult. In addition, the number of bioinformaticians who are able to perform cancer analyses is very small. In the papers that detected genes by WES in only a few specimens, there is a possibility that selection bias occurred. The analysis of a large sample size of standardized samples will allow for a picture of the molecular pathogeneses of DLBCL to come into view.

I put together a group of genes that showed recurrent somatic mutations in DLBCL (Table 2).[3] An *EZH2* (histone methyltransferase) mutation occurs in 1–22% of DLBCL cases. Recurrent mutations were found in *EZH2*, *MLL2*, *CREBBP*, and *MEF2B*. Mutations in these chromatin remodeling genes are thought to cause DLBCL. The distribution of the gene mutations depends on whether the DLBCL is GCB or ABC exclusively. Mutations in genes involved in chromatin remodeling are typically found in the GCB subtype. Mutations in members of BCR signaling and the NF-κB pathway (*MYD88*) were found in the ABC subtype. A report from China performed WES on 23 cases of primary DLBCL and eight cases of recurring DLBCL. *DTX1* mutations were observed in 10% or more of the samples. The author's reported that *DTX1* was involved in the activation of NOTCH pathway.[8]

Khodabakhshi *et al*.[13] analyzed regions of somatic hypermutations in 40 DLBCL cases that were already confirmed through RNA-seq and WGS. They selected 44 areas of somatic hypermutation from more than 46,000 areas (Table 3). They used the mutation pattern of the target and the

Table 1. Whole genome sequencing (WGS), whole exome sequencing (WES), and transcriptome sequencing (RNA-seq) in diffuse large B-cell lympoohoma (DLBCL)

Authors	WGS	WES	RNA-seq
Morin RD, *et al*. Nature 2011[5]	11 DLBCL	2 DLBCL	96 DLBCL, 10 DLBCL cell lines
Morin RD, *et al*. Blood 2013[4]	40 DLBCL and 13 cell lines	–	96 DLBCL
Zhang J, *et al*. Proc Natl Acac Sci U S A 2013[6]	73 DLBCL (34 with matched normal DNA)	21 DLBCL cell lines	–
Lohr JG, *et al*. Proc Natl Acac Sci U S A 2012[7]	–	55 DLBCL	–
de Miranda N, *et al*. Blood 2014[8]	–	31 DLBCL	–
Pasqualucci L, *et al*. Nat Genet 2011[9]	–	6 DLBCL	–

region of somatic hypermutation (the percentage of mutation in hot spot pattern WRCY (W: A or T, R: A or G, Y: C or T) or RGYW [reverse complement]), the mutation rate of CG/AT, and the ratio of transition and transversion. The somatic hypermutation (SHM) indicator was then calculated from this data. Morin *et al*.[4] further developed Khodabakhshi *et al*.'s research. They performed mutational and structural analyzes of DLBCL using WGS. They observed nonrandom patterns of SNVs distributed across the genomes with particular enrichment near transcription start sites, an observation that is consistent with aberrant somatic hypermutation (aSHM). They identified the recurrence of germinal center B-cell-restricted mutations that affected genes. An inactivating mutation in *GNA13* (Gα protein) is recurrent in DLBCL cases. In addition, *S1PR2* was found to be a target of aSHM[4,13] (Table 3). S1P2 (encoded by *S1PR2*) can couple to Gα proteins to regulate B-cell migration and homing.[14] The pattern of mutations affecting *GNA13* typically indicated inactivation of the protein, and confirmed that these mutations are enriched in GCB cases.[4] The researchers also found the fusion gene *TP63* by structural analysis. *TBL1XR1-TP63* was the only recurrent somatic novel gene fusion. This fusion gene was found to function in increasing the expression of *TP63*.[15]

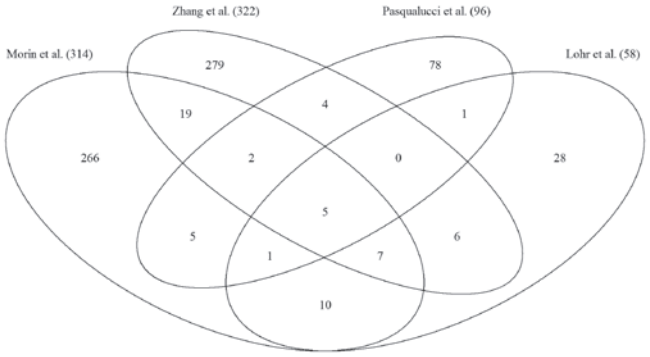

Fig. 1. Somatic gene mutations found in four independent studies. Four whole exome sequencing studies are shown in the Venn diagram. Approximately only 10-20% of the genes match between samples.

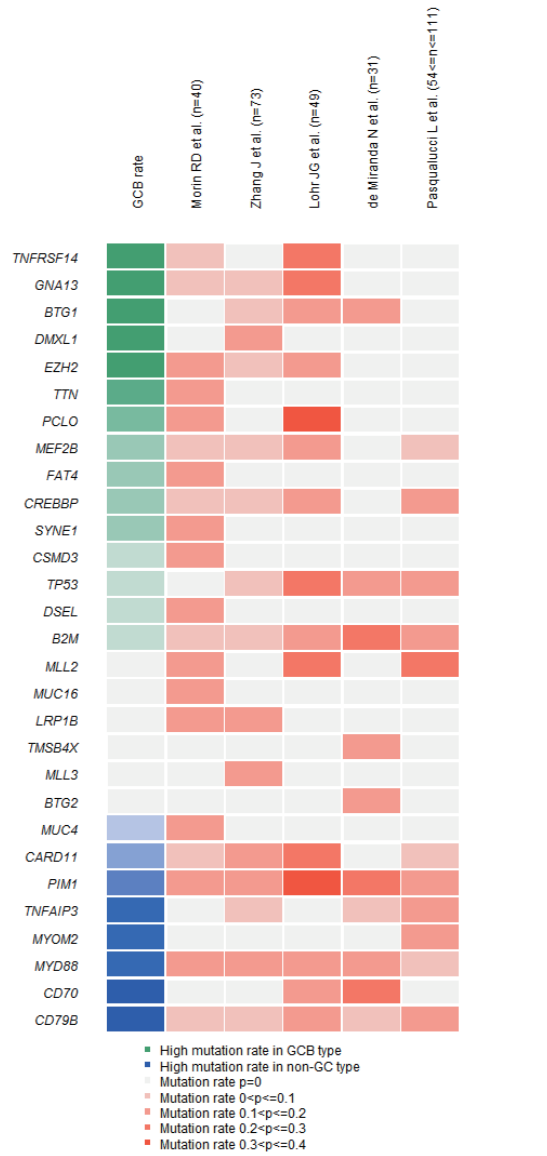

Fig. 2. Somatic gene mutations determined by five independent studies. Mutations that occur in more than 15% of the cases examined in the five whole exome sequencing studies are shown. *Red* indicates the mutation ratio. *Green* indicates that the gene mutation often occurs in germinal center B-like (GCB) type diffuse large B-cell lymphoma. *Blue* indicates that the gene mutation occurs primarily in non-GC type diffuse large B-cell lymphoma.

Table 2. Recurrent somatic mutations in diffuse large B-cell lympoohoma

Genes	Morin RD, *et al*.[5]	Zhang J, *et al*.[6]	Lohr JG, *et al*.[7]	de Miranda N, *et al*.[8]	Pasqualucci L, *et al*.[9]	Notes
EZH2	26%	1%	14%	3%	6%	Histone methyltransferase (HMT)
MLL2	28%	-	29%	6%	23%	
CREBBP	15%	4%	16%	10%	18%	Histone acetyltransferase (HAT) related genes
MEF2B	16%	3%	18%	-	8%	
MYD88	10%	15%	12%	19%	8%	NF-κB pathway activation

Table 3. The target of somatic hypermutation

Gene names	SHM indicator	Total SNVs	Mutated samples
ZFP36L1	0	52	16
SGK1	0	34	5
*CIITA**	0.0003	12	9
CD83	0.0006	34	8
CR607557	0.0008	13	9
HIST1H2AC	0.0009	19	9
CXCR4	0.0025	12	7
CD74	0.0032	14	8
DUSP2	0.004	9	4
*BCL7A**	0.0083	32	14
*PAX5**	0.0114	10	7
BTG2	0.0123	55	18
UBE2J1	0.014	6	3
*PIM1**	0.0146	12	7
S1PR2	0.0183	11	7
TMSB4X	0.0201	52	17
*IRF4**	0.0208	13	4
DMD	0.0239	10	3
*SOCS1**	0.0272	14	5
DTX1	0.0294	12	8
*RHOH**	0.0509	42	17
AK123543	0.0609	8	5
*MYC**	0.063	6	3
BTG1	0.0683	13	9
LTB	0.0794	23	10
GADD45B	0.1136	7	6
BIRC3	0.1158	21	12
SERPINA9	0.1296	36	7
*BCL6**	0.1389	179	27
GRHPR	0.1429	6	5
NCOA3	0.177	6	4
MALAT1	0.1786	11	7
ETS1	0.1877	9	8
MS4A1	0.1944	7	4
TCL1A	0.2012	17	8
*ST6GAL1**	0.2318	15	8
SPRED2	0.2356	11	6
IRF8	0.2448	13	9
LLT1	0.2591	10	3
*BCL2**	0.2642	146	11
*LRMP**	0.2823	13	7
P2RY8	0.3182	7	3
BACH2	0.5	30	8
POU2AF1	0.5	7	6

This table is a modified version of Table 1 from reference 13 (Khodabakhshi *et al*.). *GNA13* shows recurrent inactivating mutations in diffuse large B-cell lymphoma cases. *GNA13* and *S1PR2* function cooperatively, and *PIM1* and *BTG2* are high-frequency somatic mutation targets in primary central nervous system lymphoma.[30] SHM, somatic hypermutation; SNVs, single nucleotide variants. * indicates known somatic hypermutation targets.

MYD88 MUTATIONS

RNA-seq was performed on four cases of DLBCL with the ABC subtype. Sanger sequencing for *MYD88* was performed for 174 cases of ABC DLBCL. The *MYD88* L265P mutation was observed in 29% of cases. This mutation is rare in the GCB subtype and is likely a specific mutation to the ABC subtype. MYD88 is an adapter protein, which is involved in the Toll-like receptor or IL-1 receptor signaling pathways. The *MYD88* L265P somatic mutation is considered to contribute to the survival of the tumor by enhancing NF-κB and JAK-STAT3 signaling.[16] This mutation's target is the B-B loop of the TIR domain. The *MYD88* L265P mutation itself does not have a significant effect on prognosis. However, high MYD88 protein expression, which is independent of *MYD88* L265P, has an effect on tumor recurrence and disease-free survival.[17]

The *MYD88* L265P mutation has been detected in most cases of Waldenström's macroglobulinemia. The *MYD88* L265P mutation is used for disease discrimination and is known as distinctive variation in Waldenström's macroglobulinemia.[18]

EZH2 MUTATIONS

NGS data have reported overlapped recurrent somatic mutations between follicular lymphoma and the DLBCL GCB subtype. Exon15, which contains *EZH2*, has been analyzed for numerous DLBCL GCB subtype samples by Sanger sequencing. *EZH2* mutations were observed in 21.7%[19] of cases. High-level expression of EZH2 (EZH2 ≥ 70%), as detected by immunohistochemistry, was associated with good overall survival.[20] *EZH2* is a polycomb-group oncogene, which encodes a histone-lysine N-methyltransferase. It is the gene responsible for the trimethyl on Lys27 of histone H3 (H3K27). In the presence of *EZH2* wild-type, the variant *EZH2* Y641 increases the histone H3 Lys27-specific trimethylation (H3K27me3). The increase in H3K27me3 is believed to cause tumorigenesis, and allele-specific *EZH2* inhibitors are considered to be a future treatment strategy.[21] Bradley *et al*. found *EZH2* inhibitors to affect selectively, the turnover of trimethylated, but not monomethylated, histone H3 Lys27 at pharmacologically relevant doses. They also reported that these inhibitors are broadly efficacious in DLBCL models with wild-type *EZH2*.[22]

RELAPSED AND REFRACTORY PATIENTS

Jiang *et al*.[23] performed NGS sequencing of the VDJ junctions of 14 relapsed DLBCL cases and exome sequencing on seven of the samples. Ultra-deep sequencing of the rearranged IgH locus and exomes of diagnosis and relapse tumor pairs was used to identify two different scenarios of

clonal evolution in DLBCL. In one scenario, the relapse clones result from the diagnosis tumor. The mutation profiles between the relapse and original tumors are very similar and are limited to only a few different mutations. This scenario is termed 'late-divergent'. In the other scenario, the diagnosis and relapse tumors are from a common early progenitor cells, but evolve in parallel with a different somatic mutation pattern. After treatment, the major clone in the relapse tumor was present as a minor clone in the original diagnosis tumor. This scenario is termed 'early-divergent'. The researchers have indicated that mutations to epigenetic modifiers (*EP300*, *KMT2D*, and *SETDB1*) had occurred in the early stage lymphoma. These genes have a possibility of acting as driver genes of DLBCL. They also identified a frequent genetic mutation in the immune surveillance genes *B2M* and *CD58*. The researchers suggested that immune evasion had contributed to the lymphoma recurrence.

Morin *et al*.[24] used WES to analyze 38 samples from patients who had developed relapsed or refractory DLBCL after undergoing immunochemotherapy. Samples from patients with relapsed DLBCL were compared with 138 samples from unrelated patients with DLBCL for mutation analysis of relapsed DLBCL. *TP53*, *FOXO1*, *MLL3*, *CCND3*, *NFKBIZ*, and *STAT6* mutations were found to be associated with treatment resistance. *NFKBIE* and *NFKBIZ* mutations were also found. These two genes are regulatory genes of the NF-κB pathway. These genes could be new therapeutic targets in addition to *MYD88* and *CD79B* mutations. Moreover, the researchers found that the *STAT6* D419 mutation occurred in 36% of patients with the GCB type refractory DLBCL. This mutation increases the expression of the phospho-STAT6 protein and *STAT6* target genes via the JAK/STAT pathway. In addition, deep sequencing was performed on 12 biopsy samples of tumor pairs obtained from the tumors at the original time of diagnosis and at relapse. The presence of mutations in the relapse clones that were not detected at the time of diagnosis was confirmed. This indicates that it may be possible that mutations in the clones occurred during chemotherapy. These results suggest that the relapse clones with these mutations may have derived from a very small subpopulation in the diagnosis tumor or may have been acquired *de novo* in the time between diagnosis and recurrence.

In addition, Mareschal *et al*.[25] performed whole exome sequencing of 14 patients with relapsed or refractory DLBCL. In addition to well-known DLBCL mutation functions, the researchers reported that *TBL1XR1* mutations and activating mutations in *IRF4* or insulin regulatory pathways were observed. The analysis of copy number variations indicated that short-range recurrent copy number changes occurred in *REL*, *CDKN2A*, *HYAL2*, and *TP53*. In addition, the researchers also found that the activation of the NF-κB pathway is associated with treatment resistance of DLBCL.

PRIMARY LYMPHOMA OF THE CENTRAL NERVOUS SYSTEM (CNS)

Primary CNS lymphoma (PCNSL) is defined as DLBCL confined to the CNS. To date, research on PCNSL has been limited. PCNSL is diagnosed by stereotactic biopsy, and, therefore, the size of the specimen is very small. Vater *et al*.[26] and Bruno *et al*.[27] each performed WES on nine PCNSL cases. Vater *et al*. then validated the mutations using Sanger sequencing of 22 PCNSL cases. Bruno *et al*. targeted 37 genes during the sequencing of 28 PCNSL cases. Fig. 3 presents the data of the two papers[26,27] including the genes that were detected in three or more samples (in 18 PCNSL samples). *PIM1*, *IGLL5*, *MYD88*, *CD79B*, *MUC16*, *CSMD3*, *MPEG1*, and *OSBPL10* were identified among the most frequently mutated genes. Mutated genes are similar to the non-GC type of DLBCL. These mutated genes are important in the BCR, toll-like receptor, and NF-κB pathway signaling, as well as are involved in chromatin structure and modifications, cell-cycle regulation, and immune recognition. A median of 22.2% of somatic SNVs matched the RGYW motif targeted by SHM. In addition, a median of 7.9% in the SHM was found at the hotspot position, which is thought to play an important role in the pathogenesis of PCNSL. Mutations in *PIM1*, a well-known target of aSHM (Table 3), occurred most frequently. These studies revealed *OSBPL10* as a new target of aSHM. Mutations in *ODZ4* were observed in four cases of PCNSL. Mutations in *ODZ4* and *PIM1* were mutually exclusive. The ODZ4 gene is a known risk gene for bipolar disorder as determined by a genome-wide association study.[28] *ODZ4* encodes the teneurin transmembrane protein 4. It is highly expressed in the CNS in oligodendrocytes and neurons.[29] These findings may reveal the reason that DLBCL occurs in CNS and suggest a link between behavior and the progression of the disease. These may point to interesting mechanisms of CNS tropism in PCNSL. Recently, Fukumura *et al*.[30] performed WES for 41 PCNSL tumor tissues and paired normal specimens and RNA-seq for 30 PCNSL tumors. They found high frequencies of somatic mutations in *PIM1* (100%), *BTG2* (92.7%), and *MYD88* (85.4%). *PIM1* and *BTG2* are targets of aSHM (Table 3). Their results are different from those of Vater *et al*.[26] and Bruno *et al*.[27] *ODZ4* and *TBL1XR1* genes were not reported by Fukumura *et al*.; however, Fukumura *et al*. suggested that the small cohort size or lack of paired normal samples may explain the differences in the frequencies of somatic mutations. However, other genes also exhibit different frequencies, and analytical methods, diagnosis, and racial differences are likely to have caused these differences.

DLBCL, LEG-TYPE (DLBCL-LT)

Primary cutaneous large B-cell lymphoma, leg type is an

aggressive cutaneous lymphoma. It is a lymphoma typically seen in the elderly. In nodal ABC DLBCL, aberrant expression of members of the NF-κB pathway is considered an etiology. Therefore, *A20*, *CD79B*, *CARD11*, and *MYD88* were investigated in 10 cases of DLBCL-LT. Mutations were observed in *CD79B*, *MYD88*, and *CARD11*. The somatic *MYD88* L265P mutation in DLBCL-LT is also reported to occur at a high rate of 4/10.[31] A comprehensive analysis has not been done on DLBCL-LT, but additional analysis is expected.

LONG NON-CODING RNAS (LNCRNAS)

Gene expression analysis has compared DLBCL to normal B cells. Many studies have analyzed well known and annotated genes in DLBCL. Verma *et al*.[32] performed a systematic analysis to uncover novel unannotated long non-coding RNAs (lncRNAs) in DLBCL. They performed analysis of novel lncRNAs from the polyadenylated transcriptome of 116 primary DLBCL samples. The analysis was done using a *de novo* assembly pipeline. The researchers identified 2,632 novel lncRNAs, 2/3 of which were not found in normal B cells. More than one-third of lncRNAs are a differentially expressed in the ABC and GCB types of DLBCL. The researchers found that novel lncRNA potentially hold regulatory functions in DLBCL. They compared the novel lncRNA and H3K4me3 ChIP-seq data in cell lines.[33] The novel lncRNAs and ChIP-seq peak showed many overlaps. In addition, the group identified highly co-expressed protein-coding genes for at least 88% of the novel lncRNAs. These lncRNAs are considered to have a lymphoma maintenance function. These are considered to be future therapeutic targets.

CONCLUSION

I have summarized the NGS data to present the molecular pathogenesis of DLBCL. NGS techniques have been advanced in recent years, and sequencing has been performed on more samples. This has allowed for consistent reports of high frequency gene mutations. However, in the many specimens, the main gene mutations have not yet been found. In addition, profiling mutations by race and region may indicate differences. In particular, no *de novo* DLBCL sequence data have been reported for Japanese patients, and further studies are needed to obtain these data. Currently, we are still in the developmental stage of analytical methods for NGS. If accumulated samples could be analyzed using standardized analysis methods, this method could provide an overview of DLBCL molecular pathology using NGS data. Additionally, more studies are now underway to elucidate the molecular pathogenesis of DLBCL. Further clarification of the molecular pathogenesis of DLBCL is expected to lead to the development of individualized treatment for the disease.

CONFLICT OF INTEREST

The author declares no conflict of interest.

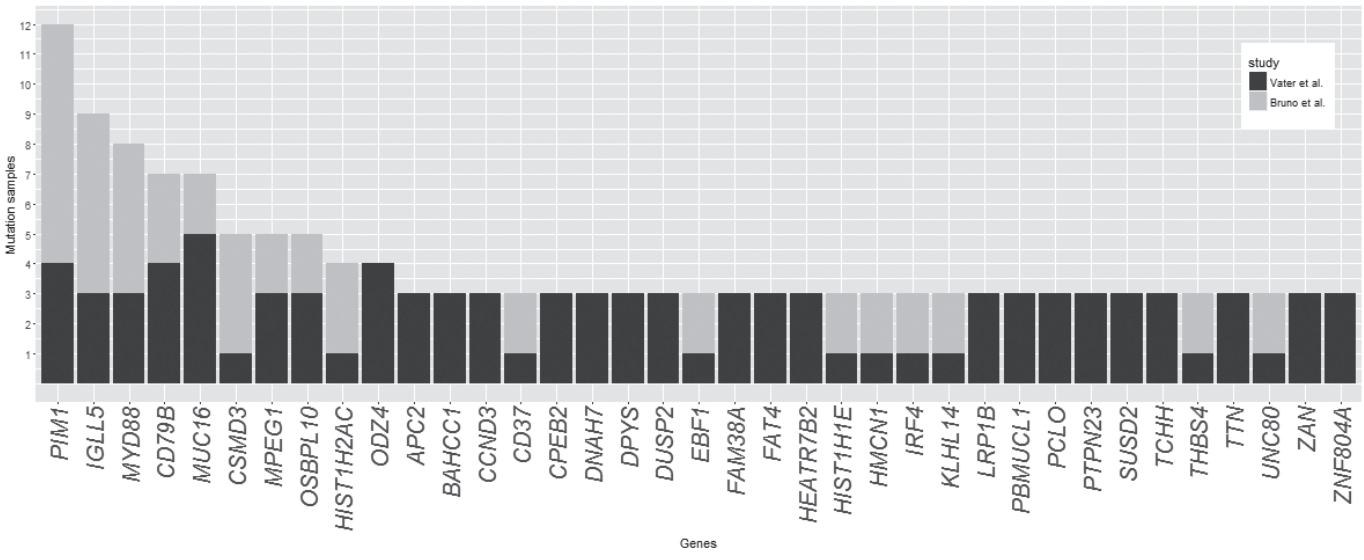

Fig. 3. Genes have mutations found in at least 3/18 primary central nervous system lymphoma (PCNSL) cases. Figure obtained by combining data from with Vater *et al*.[26] and Bruno *et al*.[27] Each study performed whole exome sequencing on nine PCNSL cases. I counted genes excluding registered dbSNPs or 1,000 genomes mutations from Supplementary Table 3 of reference 26 and Supplementary Table 1 of reference 27. I selected genes that three or more samples have mutations. *PIM1*, *IGLL5*, *MYD88*, *CD79B*, *MUC16*, *CSMD3*, *MPEG1*, and *OSBPL10* were identified among the most frequently mutated genes.

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