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RESEARCH ARTICLE

Genome-wide characterization of copy number variations in diffuse large B-cell lymphoma with implications in targeted therapy

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Abstract

Diffuse large B-cell lymphoma (DLBCL) is the aggressive form of haematological malignancies with relapse/refractory in ~40% of cases. It mostly develops due to accumulation of various genetic and epigenetic variations that contribute to its aggressiveness. Though large-scale structural alterations have been reported in DLBCL, their functional role in pathogenesis and as potential targets for therapy is not yet well understood. In this study we performed detection and analysis of copy number variations (CNVs) in 11 human DLBCL cell lines (4 activated B-cell–like [ABC] and 7 germinal-centre B-cell–like [GCB]), that serve as model systems for DLBCL cancer cell biology. Significant heterogeneity observed in CNV profiles of these cell lines and poor prognosis associated with ABC subtype indicates the importance of individualized screening for diagnostic and prognostic targets. Functional analysis of key cancer genes exhibiting copy alterations across the cell lines revealed activation/disruption of ten potentially targetable immuno-oncogenic pathways. Genome guided *in silico* therapy that putatively target these pathways is elucidated. Based on our analysis, five CNV-genes associated with worst survival prognosis are proposed as potential prognostic markers of DLBCL.

Key words: Copy number variations; diffuse large B-cell lymphoma; oncogenic pathways; therapy

Background

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid malignancy in adults, accounting for ~35% of non-Hodgkin's lymphoma cases worldwide. It is a highly heterogeneous cancer and several classification systems have been proposed. The most common classification based on cell-of-origin (COO): (i) germinal centre B-cell (GCB) and (ii) activated B-cell (ABC), is responsible for the heterogeneous behaviour, with the ABC subtype identified with poor prognosis[1]. Though various immunostaining and gene expression profiling methods have been proposed, distinguishing the two subtypes has not been easily achieved in clinical setting[2]. It is proposed that genetic alterations may also be responsible for the observed heterogeneity and a comprehensive analysis of genetic alterations may help

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Table 1. Some known CNV loci and CNV-genes characterized as molecular biomarkers for DLBCL.

CNV Type	Subtype Specific		Common to GCB & ABC Subtypes
	GCB	ABC	
Gain	2p13.3–25.3, 2p16.1 (REL), 3q27.3 (BCL6), 9q21.32–22.31, 12q13.12, 12q15 (MDM2), 13q31.3 (mir-17-92)	3p13 (FOXP1), 11q24.3, 18q21 (BCL2), 19q13 (SPIB)	1q23.3 (FCGR2B, FCGR2C), 12q12 (ARID2)
Loss	1p36, 10q23.3 (PTEN, FAS), 11q24, 13q33–34 (ING1), 15q21.2	6q21 (PRDM1), 6q23.2 (SGK1), 9p21 (CDKN2A/2B), 17p13.1	1p13.1 (CD58), 6p21 (MHC-I, MHC-II), 16p13.3 (CIITA)

in identifying biomarkers for distinguishing between the two subtypes.

Among various genetic alterations, copy number variations (CNVs) are known to play a significant role in cancer initiation and progression as these encompass large genomic regions (50 bp–5 Mbp) and exhibit high alteration rates (100–10,000×) compared to small sequence variations (SNVs and Indels). Several studies have shown CNVs to affect protein-coding and non-coding genes leading to activation/disruption of important pathways in DLBCL Table 1[3,4]. For example, copy loss of tumour suppressor gene PTEN at 10q23.3, often observed in GCB cases, is associated with activation of PI3K-AKT-mTOR signalling pathway essential for metabolism, angiogenesis, tumour cell proliferation and survival[5]. This pathway is also activated by alternate molecular mechanisms, e.g., by amplification of 13q31.3 (mir-17-92) in some GCB cases[6], clearly indicating alternate routes to cancer initiation and progression. Similarly, deletion at 9p21 that encodes tumour suppressor genes (CDKN2A and CDKN2B) involved in regulating cell cycle, is another hallmark of ABC subtype.

Recent comprehensive analysis of genomic and transcriptomic alterations in DLBCL has revealed novel copy variant genes and chromosomal regions. However, their clinical significance is not yet well understood. In this study we analyzed tumour cell lines that serve as model systems for cancer cell biology to understand the role of structural variations in diagnosis and prognosis. Here we propose to investigate stratification of the two subtypes based on CNVs and CNV-genes, identify novel prognostic markers, and propose a pipeline for genome-guided *in silico* prescription based on CNV-profiles and pathways affected.

Methods

Whole-genome next generation sequence data of 12 DLBCL cell lines (read length: 100 bp) were downloaded from Sequence Read Archive (SRA project ID: SRP020237[7]). Paired-end reads with base quality < 30 were trimmed using NGS QC-Toolkit[8] and aligned to human reference assembly hg19 using Bowtie2[9]. Alignment files were then submitted to our CNV detection and annotation pipeline, iCopyDAV[10], with bin size = 1000 bp, GC correction by Loess regression and mappability cut-off = 0.8 (for handling low-complexity regions). CNVs were detected using two segmentation approaches, Total Variation Minimization and Circular Binary Segmentation combined. The number of CNVs identified were roughly the same across all cell lines (except OCI-LY3) and ranged in size from 20–245 Mb (median: 101 Mb), summarized in Table 2. Cell line OCI-LY3 (ABC subtype) exhibited a 7-fold increase in CNVs (compared to others) and was discarded. Thus, all together eleven DLBCL cell lines (7 GCB, 4 ABC) were included for this study (complete CNVs are listed in CNVs in Supplementary Table 1).

To identify common CNVs in the two subtypes, copy number variant regions (CNVRs) were first constructed by merging CNVs at each locus based on 1 bp criteria and considering farthest boundaries of overlapping regions for each subtype. The CNVRs present in at least 3 GCB and 2 ABC cell lines, respectively, are referred to as recurrent variants of each subtype. From this set of recurrent variants, common CNVs between the two subtypes were identified based on 50% reciprocal overlap criteria; remaining variants unique to each subtype are referred as subtype-specific CNVs. To assess the functional significance of these copy variant regions, genes and regulatory elements overlapping the CNVs (1 bp overlap criteria) were extracted using annotation module in iCopyDAV. Oncogenes and tumour suppressor genes exhibiting copy gain or copy loss events were identified using MSigDB[11]. Functional enrichment analysis of CNV-genes was carried out using STRING database[12] and g:Profiler[13].

The pre-computed CNV-profiles in 48 DLBCL patient samples from The Cancer Genome Atlas (TCGA)[14] were extracted using cBioPortal for Cancer Genomics[15] and recurrent CNVs across these samples were similarly identified (as discussed above). No subtype information was available for these samples. The genetic landscape and survival information of patients was extracted from cBioPortal. Kaplan-Meier overall/progression-free survival plots with and without copy alterations were generated for gene(s) of interest using 'survival' option in cBioPortal.

Results

To establish prediction accuracy of iCopyDAV, CNVs predicted in 8 of the 11 cell lines were compared with the pre-computed CNVs reported in Cancer Cell Line

Table 2. Number of CNVs detected in 12 DLBCL cell lines using iCopyDAV.

Subtype	Sample ID	Cell line	Sequence Coverage	Age (yrs)	CNVs	Gain	Loss
GCB	SRR1236466	OCI-LY1	35×	44	769	508	261
	SRR1236467	NU-DHL-1	37×	73	752	505	247
	SRR1236468	DB	27×	45	724	436	238
	SRR1236470	OCI-LY7	33×	48	606	397	209
	SRR1236474	DOHH-2	39×	60	649	450	199
	SRR1236475	WSU-DLCL2	31×	41	647	477	170
	SRR1236476	SU-DHL-6	37×	43	659	442	217
ABC	SRR1236469	NU-DUL-1	30×	43	820	526	294
	SRR1236471	OCI-LY3	30×	52	4983	4707	276
	SRR1236472	MD903	33×	38	706	509	197
	SRR1236473	SU-DHL-9	33×	64	740	470	270
	SRR1236477	OCI-LY19	31×	27	675	416	259

Encyclopaedia (CCLE)[16] (Supplementary Table 2). High recall values, ~68% to 89%, were observed for seven cell lines (for OCI-LY19 ~ 38%) using 1 bp overlap criteria. As shown in Fig. 1, the density distribution of CNVs indicated wide variation in CNV events with higher copy gain events compared to copy loss events (thick black horizontal lines) across all the chromosomes. In particular, chromosomes 1, 2, 4, 7, 9, 16, 17 and 21 exhibited above average (dotted horizontal line) copy gain events, while chromosomes 1, 2, 4, 7 and 8 exhibited above average copy loss events. It was noted that chromosomes 1, 11 and 16 were enriched with large copy gain events (few of which have been previously implicated in DLBCL[17]), chromosomes 4, 6, 8 and 20 with copy loss events, while chromosomes 7, 15, 18 and 21 exhibited similar number of gene-enriched copy gain and copy loss events (Fig. 2). Subtype-specific CNV events were also observed, e.g., chromosome 10 that was densely populated with copy gain events mostly in GCB subtype.

Copy number alterations in the genomes of cell lines and TCGA samples

Wide variations in CNV profiles of 11 cell lines was noted (Fig. 2). Only few CNV loci, *viz.*, 1p12 (gain), 6p21 (loss), 8p21.2 (loss), 21p11.1 (gain) were observed in all the cell lines, while the majority of loci were altered in the few cell lines (e.g., 9p21 (loss) in GCB: DOHH-2, NU-DHL-1, ABC: MD903, etc.), irrespective of their COO subtype. Many of these CNVs were observed in TCGA samples (number of samples given in parenthesis): 1p12 (1), 8p21.1 (2) and 9p12 (1) and/or associated with DLBCL in earlier studies, *viz.*, 6p21.32, 6q15, 6q21 and 6q27 (Table 1). Deletion at 6p21.32-6p21.33 was associated with loss of immune genes such as Human Leukocyte Antigen genes (HLA-I and HLA-II), affecting T-cell infiltration, while deletions in large arm of chromosome 6 at 6q15, 6q16 and 6q21 are involved in cellular processes resulting in cancer development. Similarly, three CNV loci 8p11.22, 8p22 and 8p23.3 were observed to be deleted 11, 2 and 9 cell lines respectively, and in 3 TCGA samples. Deletion at 8p11.22, observed in all 11 cell lines, spanned a pseudogene, RNF5P1, while genes NAT1 and NAT2 on 8p22 were involved in detoxifying carcinogens and participate in N-/O-acetylation of carcinogens, affecting cancer susceptibility. Deletion at 8p23.3 encompassed gene ERICH1 which has been associated with tumour suppressor activity in pancreatic cancers[18].

Few subtype-specific alterations observed across the cell lines were: 1q21.3 (loss), 4q35.2 (gain), 13q31.1 (gain) in GCB subtype and 8q23.3 (loss), 19q13.3 (loss), 21q22.3 (gain) in ABC subtype. Of these, some known aberrations are amplification of MIR17HG at 13q31.1 (GCB-specific) and loci 21q22.3 (ABC-specific)[17,19]. These are also present in ≥1 TCGA samples (Supplementary Table 3). We also identified gene enriched CNVs with possible role in cancer development and proliferation, though not yet directly implicated in DLBCL, e.g., gain of PRKCA at 17q24.2 (both subtypes), loss of CRNN at 1q21.3 (GCB), and loss of TRPS1 at 8q23.3 (ABC).

Analysis of 48 DLBCL patient samples from TCGA also revealed large variations in their CNV profiles. The most recurrently aberrated region, 9p21.3 (loss) was altered in only 27% samples (13/48), followed by 2p16 (gain) altered in ~20% samples. Genes on CNV loci altered in ≥3 samples were compared with those in DLBCL cell lines to analyse common copy alterations (Fig. 3). Large scale copy loss events in chromosomes 6 and 8 were also observed in TCGA samples. Few subtype-specific CNV loci observed in both TCGA samples and cell lines are gain at 10p15.3 (ZMYND11), loss at 10q23.31 (PTEN and FAS), specific to GCB subtype, and gain at 6q15 (MAP3K7 and BACH2) and 6q24 (PLAGL1 and HIVEP2), specific to ABC subtype.

CNVs contribute towards molecular heterogeneity in DLBCL

Functional analysis of CNV profiles revealed a varying set of oncogenes and tumour suppressor genes across

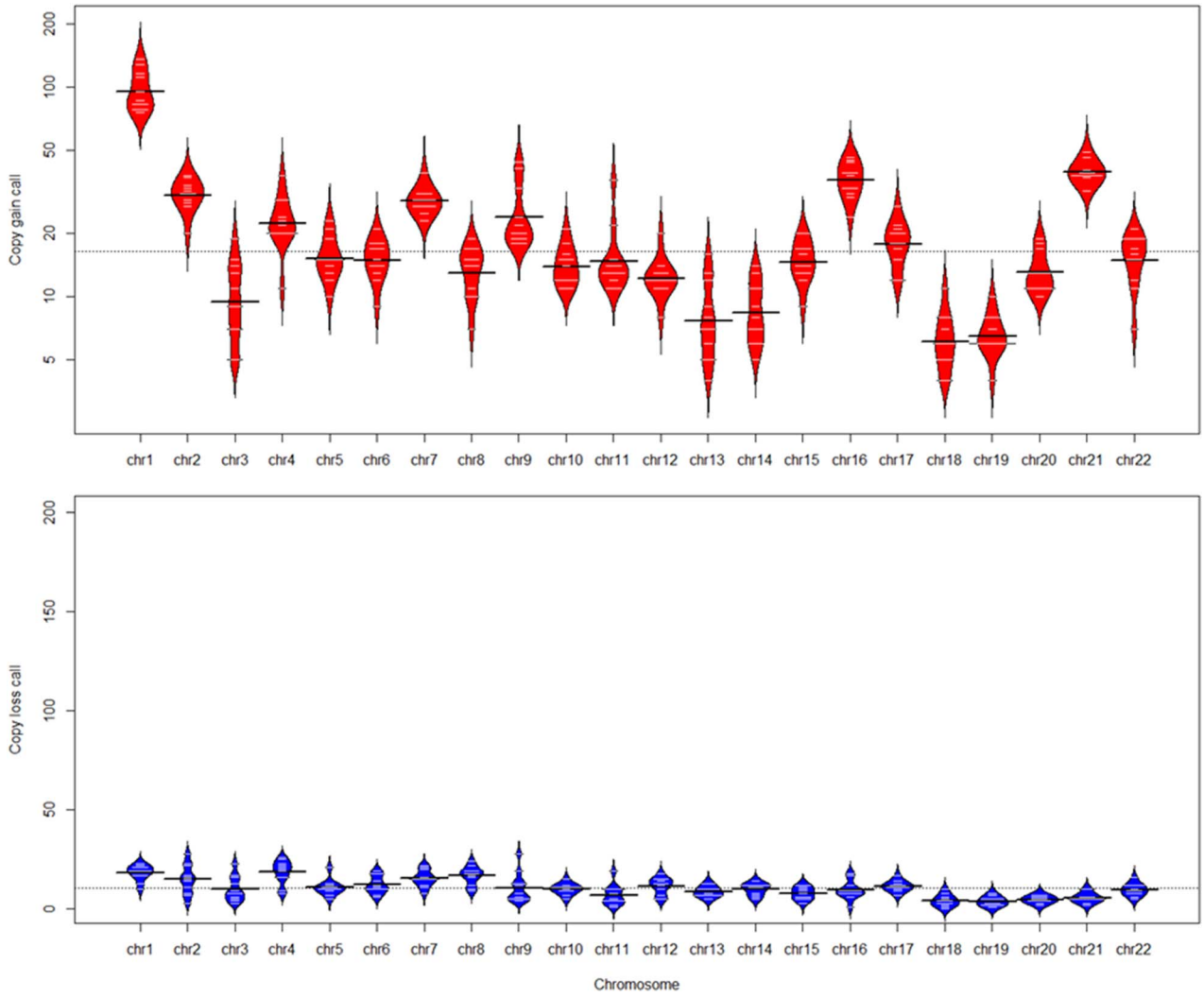

Figure 1. An asymmetric Beanplot (ver 1.2) depicting the distribution of copy gain (top) and copy loss (bottom) calls chromosome-wise in 11 DLBCL cell lines. The short white horizontal lines: CNV calls per chromosome in each cell line; thick black horizontal line: average number of CNV calls; dotted horizontal line: overall average across all cell line genomes.

11 cell lines, with few notable exceptions (Table 3). For example, amplification of oncogenes NOTCH2 (1p12) and deletion of 6p21 loci encompassing HLA genes were observed in all 11 cell lines. However, amplification of oncogenes FOXO1, CCND3, IRF4 and HSP90AB1 and deletion of tumour suppressor gene CDKN2A were observed in only few cell lines and TCGA samples (Supplementary Table 3). Functional analysis of these copy altered oncogenes and tumour suppressor genes revealed few common pathways altered across the cell lines. For example, PI3K-AKT-mTOR pathway was observed to be affected as a consequence of different CNV events in 8 DLBCL cell lines: by gain of onco-MIR, mir-17-92 (13q31.3) in DB, OCI-LY7 and SU-DHL-6, gain of oncogene HSP90AB1 (6p21.1) in SU-DHL-6 and NU-DUL-1, gain of RPTOR (17q25.3) in MD903 and SU-DHL-9, and loss of PTEN in OCI-LY1 and WSU-DLCL2 cell lines. In ABC cell lines, gain of FCGR2B (1q23.3) affected BCR signalling and gain of TRK oncogenes; TPM3, NTRK1, and TPR (1q21.3) affected MAPK pathway in NU-DUL-1. On the other hand, gain of transcription factor FOXP1 (3p13) regulated BCR signalling and loss of MAP2K4 (17p12) affects MAPK pathway in SU-DHL-9 and OCI-LY19 cell lines. Similarly, inactivation of tumour suppressor gene CDKN2A (9p21) in cell lines NU-DHL-1, DOHH-2 and MD903, amplification of CCND3 (6p21.1) in SU-DHL-6 and SU-DHL-9, inactivation of TP53 (17p13.1) in OCI-LY19, respectively, contribute to uncontrolled growth of tumour cells by losing the ability to arrest cell cycle/apoptosis. Similar heterogeneity in CNV events was observed in TCGA samples as well, with very few key genes such as CDKN2A (9p21.3), XPO1 (2p15), REL and BCL11A (2p16.1) altered in $>15\%$ patients, leading to lymphomagenesis through alterations in cell cycle (CDKN2A, REL and XPO1) and B cell differentiation (BCL11A). These observations indicated the role of genomic alterations in contributing towards molecular heterogeneity in DLBCL.

Table 3. Copy number variations observed in key genes and their associated pathways in 11 DLBCL cell lines.

	BCR	Cell cycle/Apoptosis	Chromatin modifications	Immune responses	JAK-STAT	MAPK	NF-κB	NOTCH Signalling	PI3K-AKT	RTK	Proposed Therapy (gene/pathway)
OCI-LY1	-	FAS (L), RUNX1T1 (G), ZBTB16 (G)	JAK2 (G), KDM5A (G), NCOA2 (G), PRDM16 (G)	HLA (L), CD274/CD273 (G), TRIM26/31 (L)	JAK2 (G)	-	ERC1 (G)	NOTCH2 (G)	PTEN (L)	-	LY294002 (PI3K-inhibitor)*, Rapamycin + MK-2206 (AKT-mTOR), Cerdulatinib (JAK-STAT)
NU-DHL-1	CDK6 (G), CCND2 (G), RAF1 (G), KRAS (G)	CDKN2A (L), CDK6 (G), CCND2 (G)	-	HLA (L), CD274/CD273 (L), CD4 (G), TRIM26/31 (L)	CCND2 (G), LIFR (G), RAF1 (G)	KRAS (G), RAF1 (G)	ERC1 (G)	NOTCH2 (G)	-	-	Cerdulatinib
DB	BCL6 (G)	BCL6 (G)	-	HLA (L), TRIM26/31 (L)	-	-	-	NOTCH2 (G)	mir-17-92 (G)	-	Pterostilbene*, RI-BPI (BCL6)
OCI-LY7	-	-	-	HLA (L), TRIM26/31 (L)	-	-	-	NOTCH2 (G)	mir-17-92 (G)	-	Rapamycin + MK-2206
DOHH-2	-	CDKN2A (L)	-	HLA (L), TRIM26/31 (L)	-	-	-	NOTCH2 (G)	-	-	Ilorasertib (CDKN2A)
WSU-DLCL2	-	FOXO1 (G), HSP90AA1 (G), FAS (L), RB1 (L)	-	HLA (L), TRIM26/31 (L)	-	-	LCP1 (G)	NOTCH2 (G)	PTEN (L), FOXO1 (G)	EGFR (G)	LY294002, Rapamycin + MK-2206, Cetuximab (EGFR)
SU-DHL-6	-	CCND3 (G), HSP90AB1 (G), PIM1 (G)	DEK (G), multiple CNV-genes	HLA (L), TRIM26/31 (L)	-	-	-	NOTCH2 (G)	mir-17-92 (G), HSP90AB1 (G)	FGFR3 (G)	LY294002, Rapamycin + MK-2206*, PD173074 (FGFR3)
NU-DUL-1	FCGR2B (G)	FOXO1 (G), HSP90AB1 (G), NCOA1 (G)	-	HLA (L), TRIM26/31 (L)	-	TPM3 (G), NTRK1 (G), TPR (G)	-	NOTCH2 (G)	HSP90AB1 (G)	-	DCZ3301*, Cereblon-aided lenalidomide (IRF4)
MD903	-	CDKN2A (L)	-	HLA (L), CD4 (G), PTPN6 (L), TRIM26/31 (L)	-	-	-	NOTCH2 (G)	-	FLT3 (G)	Ilorasertib, Midostaurin (FLT3)
SU-DHL-9	FOXP1 (G)	CCND3 (G)	-	HLA (L), TRIM26/31 (L)	-	-	-	NOTCH2 (G)	-	-	Ibrutinib (BCR)
OCI-LY19	-	TP53 (L)	-	HLA (L), TRIM26/31 (L)	-	MAP2K4 (L)	-	NOTCH2 (G)	-	-	AZD6738 (TP53)

*Drug(s) experimentally tested in the respective cell line. G: gain; L: loss.

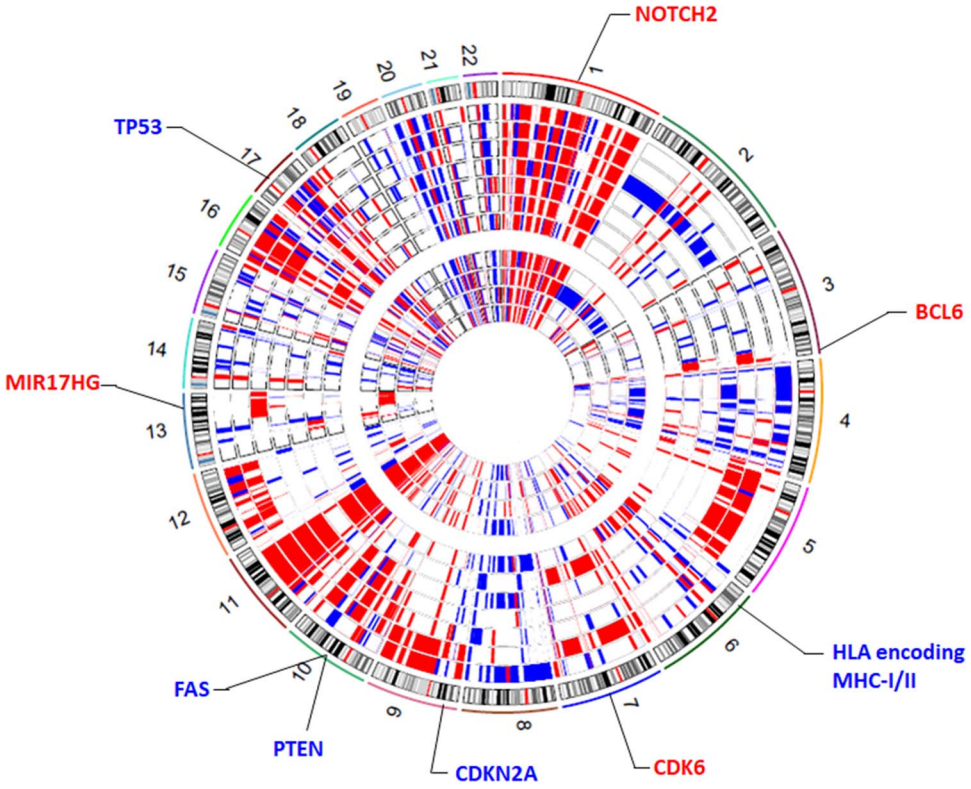

Figure 2. Distribution of gene enriched CNVs across 11 cell lines (GCB: 7 outer circles; ABC: 4 inner circles). Few significant CNV-genes implicated in DLBCL are highlighted. Red: copy gain events, Blue: copy loss events.

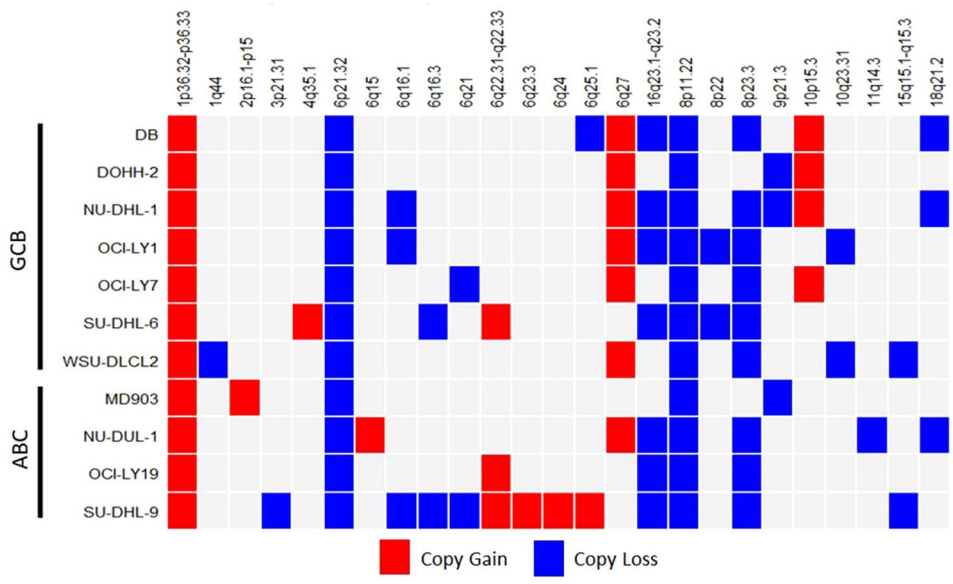

Figure 3. Comparison of frequently altered genomic regions in 48 DLBCL patient samples from TCGA with those predicted in 11 DLBCL cell lines.

Therapeutic interventions based on pathway analysis

Using cell lines as experimental cancer models, we see that analysis of genomic alterations can help in identifying genes/pathways that may be targeted for therapy. The variation in copy number of some key genes across the 11 cell lines that participate in targetable immuno-oncogenic pathways is shown in Fig. 4. Below we discuss probable therapy for each cell line based on their CNV profile, by extracting information from available literature on probable drugs (case study, preclinical, clinical or

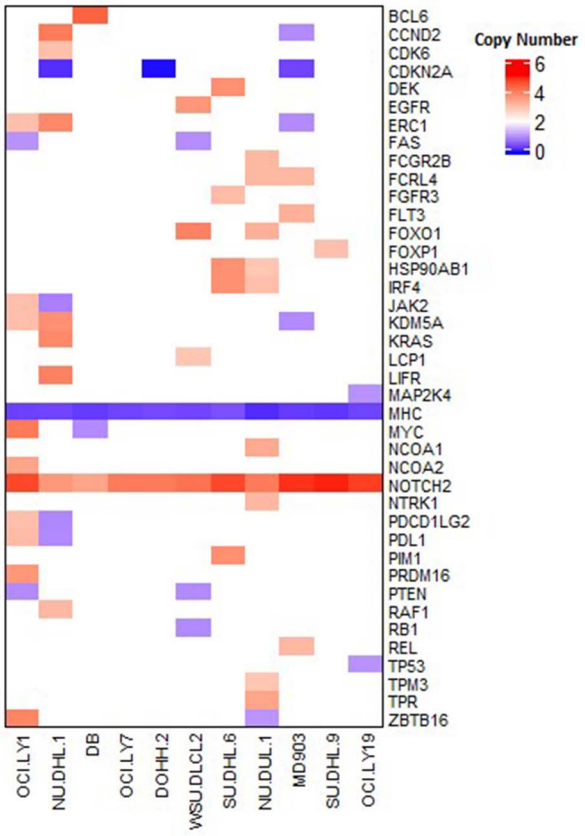

Figure 4. Heatmap showing the variation in absolute copy number of key CNV-genes across 11 DLBCL cell lines.

FDA approved) and experimental evidence on cell lines (if available).

Genetic alterations affecting common immuno-oncogenic pathways involved in cancer development and progression in the cell lines are identified and summarized in Table 3. Amplification of NOTCH2 gene (1p12), loss of HLA genes (6p21) and alteration of genes involved in interferon-gamma (IFNγ) signalling pathway were observed in all 11 cell lines (and few TCGA samples). In a recent study[20] a highly potent NOTCH inhibitor, *LY3039478*, demonstrated significant decrease in Notch signalling, suggesting its use in improving the survival of DLBCL patients. Loss of HLA is associated with prevention of antigen presentation, an immune eradication evading strategy by tumour cells and often observed in haematological malignancies. Novel immunotherapies such as chimeric antigen receptor (CAR) T cells or T cell engager (TCE) antibodies that target CD19 antigens present on the tumour cell surface and activates immune response against tumour cells in an MHC-independent manner. Studies have shown high overall response rate (43%–71%) and complete response (19%–57%) in refractory DLBCL patients with immunotherapies[21,22], indicating this to be a promising treatment. The IFNγ pathway is involved in the regulation of several immune processes such as pro-inflammatory responses, activation of macrophages and cytokines, *etc*[23]. In cancers including DLBCL, IFNγ is also known to activate JAK-STAT signalling cascade and consequently, overexpression of ligands binding PD-1 (PD-L1/2) which are essential for tumour progression and survival. The programmed death 1 (PD-1) signalling pathway (p-value <0.001) was observed to be enriched in 9 cell lines (except SU-DHL-6 and NU-DUL-1). Clinical trials are already underway to check the efficacy of blocking PD-1/PD-L1 pathway in DLBCL patients and monoclonal antibodies, *nivolumab* and *pembrolizumab*, are identified to be efficient checkpoint inhibitors targeting PD-1 with high affinity[24]. Thus, combination of NOTCH inhibitor, *LY3039478*, and *nivolumab* inhibitor targeting PD-1 along with immunotherapies such as *CAR T cells* would be the recommended therapy in DLBCL patients, irrespective of the COO subtype.

Few important pathways observed to be affected in some cell lines and involved in DLBCL tumorigenesis are summarized in Table 3. Loss of tumour suppressor PTEN suggests hyperactivation of PI3K-AKT-mTOR pathway leading to angiogenesis, tumour cell proliferation and survival. Pfeifer *et al* have shown efficacy of a potential anti-tumour drug, pan-PI3K inhibitor, *LY294002*, in inhibiting PI3K-AKT-mTOR pathway in OCI-LY1 cell line[5]. Another study by Petrich *et al* have shown reduction in tumour cell viability in SU-DHL-6 cell line using mTOR inhibitor (*Rapamycin*) along with AKT inhibitor (*MK-2206*)[25]. Various drugs, such as *Everolimus*, *Sirolimus* (*Rapamycin*) and *Temsirolimus*, have also been proposed to inhibit mTOR signalling pathway by targeting RPTOR of mTOR complex 1[26]. Thus, based on the alterations of various elements of this pathway, corresponding therapeutics may be used (Table 3). Inactivation of tumour suppressor protein CDKN2A leading to tumorigenesis through dysregulation of cell cycle (RB1 and TP53 pathways), angiogenesis and cell invasion, mediated via cyclin D/CDKs or vascular endothelial growth factor (VEGF), is well characterized. A recent study[27] on cancers with CDKN2A loss reported the efficacy of *Ilorasertib*, an inhibitor of aurora kinase and VEGF, suggesting its proposed use in DLBCL patients CDKN2A loss. Other significant immuno-oncogenic pathways, such as BCR, JAK-STAT, MAPK, NF-κB, RTK that are altered in various DLBCL cell lines are summarized in Table 3 along with respective drugs.

Apart from this, various cell line-specific genomic alterations observed indicate the need for individualized therapy. For example, amplification of gene (3q27.3) encoding for a transcription factor, B-cell lymphoma 6, was observed in DB cell line and in $\sim$8% of TCGA samples. Other mutational events (e.g., gene fusion and somatic mutations) have been commonly observed in BCL6 gene which is considered a hallmark for GCB subtype. Oncogene BCL6 plays a key role in B cell differentiation in germinal centre and aberrations in its expression have been implicated in DLBCL tumorigenesis in mouse models[28]. It also acts as a

repressor factor inhibiting IRF4 expression, a key gene involved in pro-tumour NF-κB signalling pathway. In such cases, BCL6 inhibitors, e.g., Retro-Inverso BCL6 Peptide Inhibitor (*RI-BPI*) that target the interacting domain, BTB and its lateral repressors to inhibit survival of tumour cells is proposed[29]. Deletion of TP53 was observed in cell line OCI-LY19 and 1 TCGA sample. Cancers with TP53 loss are often associated with higher DNA replication stress and dependent on S and G2/M checkpoints. An *in vivo* study carried out on chronic lymphocytic leukaemia (type B-cell lymphoma) cell lines with TP53 defects showed ATR as a potential tumour-specific DNA damage response (DDR) target and its inhibitor, *AZD6738* resulted in tumour cell death[30], suggesting its applicability in DLBCL cases with TP53 loss.

CNVs as novel markers for DLBCL

A detailed analysis of CNVs not yet reported to be associated with DLBCL in literature was carried out to identify novel markers for DLBCL tumorigenesis and/or prognostics. First, CNVs overlapping with functional elements such as protein-coding genes, regulatory elements (enhancers, promoters) and noncoding RNA (miRNA, lncRNA), were identified using the annotation module of iCopyDAV. Next, these were filtered based on their recurrence in cell lines. Presence of these CNV events in some of the TCGA samples further confirmed their significance in DLBCL (Supplementary Table 4). Novel CNVs and their biological significance are summarized in Table 4 and putative role of few novel CNV genes (in pentagon shapes) is shown in Fig. 5.

A key pathway affected in GCB subtype involves regulation of histone modification (p-value: 0.00017). Genes PAX5 at 9p13.2 (copy gain), USP17L2 at 8p23.1 (copy gain) and their interacting proteins are involved in this pathway (predicted using HIPPIE v2.0[31]). Gene PAX5 is an oncogene and B cell marker, crucial for generating and maintaining B-cell identity from pro-B cell to mature B-cell stage. It interacts with histone acetylases and chromatin-remodelling complexes and contributes to regulating DNA methylation and histone modifications. It has been shown to play a role in transcription misregulation of various cancers (leukaemia, lymphoma, sarcoma, prostate, *etc.*), but is not yet implicated in DLBCL. Various other GCB-specific CNV-genes observed, *viz.*, amplification of MICAL3 and MAPK8IP3 and deletion of DLEU1, FKBP5, CNOT1 and MAPT are involved in distinct cellular processes such as PI3K-AKT-mTOR, immuno-regulation, cell migration and proliferation, *etc.*, indicating their probable role in DLBCL tumorigenesis. Another GCB marker is the enhancer hs1715 at 8p12 deleted in 3/7 GCB cell lines and is shown to affect the expression of DUSP26 gene (which may act as oncogene or tumour suppressor depending on cellular location) in mouse models. In ABC subtype, copy gain of RPTOR (17q25.3), which is part of MTOR1 complex, participates

Table 4. Some novel CNV-genes observed in DLBCL cell lines and their biological significance (number of cell lines given in parenthesis).

GCB (7)		ABC (4)		Common to GCB &ABC (11)	
Variant-Gene	Significance	Variant-Gene	Significance	Variant-Gene	Significance
DLEU1, **13q14.2 (Loss, 6)**	Tumour suppressor, putative prognostic marker	TRPS1 (5′ end), **8q23.3 (Loss, 4)**	Cell proliferation	SYNGAP1, **6p21.32 (Loss, 11)**	MAPK pathway
MICAL3, **22q11.21 (Gain, 5)**	Cell migration	WNK1, **12p13.33 (Loss, 3)**	WNT pathway	mir-663b, **2q21.2 (Gain, 11)**	Cell proliferation
FKBP5, **6p21.31 (Loss, 5)**	Immuno-regulation	RPTOR, **17q25.3 (Gain, 2)**	Cell growth	TRIO, **5p15.2 (Gain, 11)**	Cell cycle progression
MAPK8IP3, **16p13.3 (Gain, 4)**	JNK pathway, cell differentiation	RORA, **15q22.2 (Loss, 2)**	Immunity, NF-κB pathway	PRKCA, **17q24.2 (Gain, 11)**	Cell adhesion, cell cycle checkpoint
hs1715*- DUSP26-UNCD5D, **8p12 (Loss, 3)**	Cell proliferation	NONHSAG113422.1^{+}, NONHSAG095919.2^{+}, NONHSAG045263.2^{+}, EZR, **6q25.3 (Gain, 2)**	Apoptosis, MAPK activity, JAK-STAT pathway	SPIDR, **8q11.21 (Loss, 10)**	Translocation of immunoglobulin heavy chain
CNOT1 (5′ end), **6q21 (Loss, 3)**	Cell cycle arrest	TNN, **1q25.1 (Gain, 2)**	WNT pathway	NCOR1, **17p12 (Gain, 9)**	Promotes chromatin condensation
PAX5, **9p13.2 (Gain, 3)**	Maintaining B cell identity	CDK11A, **1p36.33 (Gain, 2)**	Cell cycle control	mir-650, **22q11.22 (Loss, 7)**	B-cell proliferation
MAPT, **17q21.31 (Loss, 3)**	Cell-cell signalling	MITF, **3p13 (Gain, 2)**	Oncogene, resistance to chemotherapy	Promoter of HLF, **17q22 (Loss, 6)**	Key transcriptional regulator

*indicates enhancer and $^{+}$ indicates long non-coding RNA.

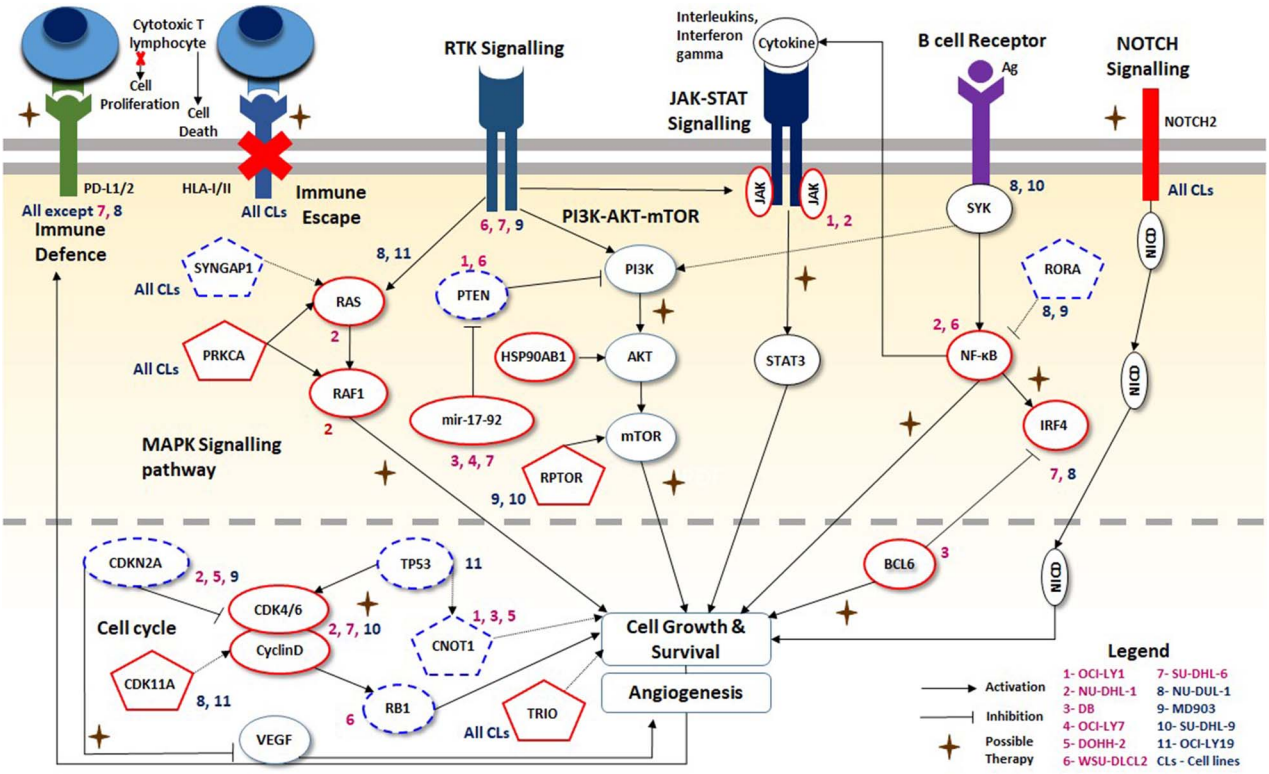

Figure 5. Key immuno-oncogenic pathways affected due to copy alterations in 11 DLBCL cell lines and the possible therapeutic targets. Solid shapes: copy gain; dashed shapes: copy loss; pentagons: novel CNV-genes identified; dotted lines: indirect effect of CNV-genes in activation/repression of pathways. Immune escape (deletion of HLA genes) and defence (activation of PD-1 pathway), and activation of NOTCH signalling (amplification of NOTCH2 gene) are observed in all 11 cell lines. RTK signalling was observed to trigger different pathways, *viz.*, MAPK, PI3K-AKT-mTOR and JAK-STAT pathways in cell lines WSU-DLCL-2, SU-DHL-6 and MD903, leading to tumour cell growth and survival. Alternately, in cell lines OCI-LY1 and NU-DHL-1, JAK-STAT pathway is activated by cytokines such as interleukins and INFγ. Constitutive activation of BCR signalling and NF-κB pathways is observed in cell lines, SU-DHL-6, NU-DUL-1 and SU-DHL-9. Key components of the cell cycle, CDKN2A, CDK6, CCND2/3, RB1 are either amplified or deleted leading to uncontrolled growth.

in mTOR signalling pathway (p-value: 0.0028). Higher expression of RPTOR is observed in several cancer types (including DLBCL). ABC-specific CNVs, namely, amplification of TNN, CDKN11A and MITF, and deletion of TRPS1, WNK1 and RORA are observed to be involved in oncogenic pathways NF-κB, WNT, cell growth and proliferation. Overexpression of MITF is associated with resistance to chemotherapy in melanoma patients[32] and is possibly responsible for poor outcome of ABC subtype patients after chemotherapy. Amplification of certain long non-coding RNAs and gene EZR at 6q25.3 are observed with possible role in cancer through activation of MAPK and JAK-STAT signalling pathways. Few novel CNVs common to GCB and ABC cell lines, e.g., gain of TRIO, NCOR1 and PRKCA genes, and loss of SPIDR and SYNGAP1 genes participate majorly in cell growth and proliferation. Certain recurrent CNVs with regulatory RNAs such as miRNAs that may contribute to DLBCL development are also observed. For example, amplification of mir-663b and deletion of mir-650 associated with tumour cell proliferation and B cell proliferation, respectively, are recurrently present across the cell lines.

Based on Kaplan-Meier survival analysis across 48 TCGA patient samples, we proposed five novel prognostic markers, namely, ERICH1, DLEU1, BMPR1A, DEK and SUFU, that would help in identifying high-risk DLBCL patients. These are oncogenes and tumour suppressors that exhibit copy number variation in cell lines as well as TCGA samples but have not yet been associated with DLBCL. The overall survival plots of these CNV-genes and their prognostic significance (p-value) are given in Fig. 6. Subset of DLBCL patients with deletions in tumour suppressors ERICH1 (~10 months), BMPR1A, DLEU1 and SUFU (~20 months) demonstrated poor outcome compared to the patients without such genomic lesions. Similarly, patients with amplification of oncogene DEK is associated with worst survival outcome (~2 months) compared to those without this aberration (~211 months). Deletion of ERICH1 has been previously implicated in pancreatic ductal adenocarcinoma, however, the molecular mechanism through which this lesion induces cancer is still unknown[18]. Tumour suppressor activity of long non-coding RNA, DLEU1, is reported in chronic lymphocytic leukaemia (CLL), but not yet characterized in DLBCL[33]. Tumour suppressor

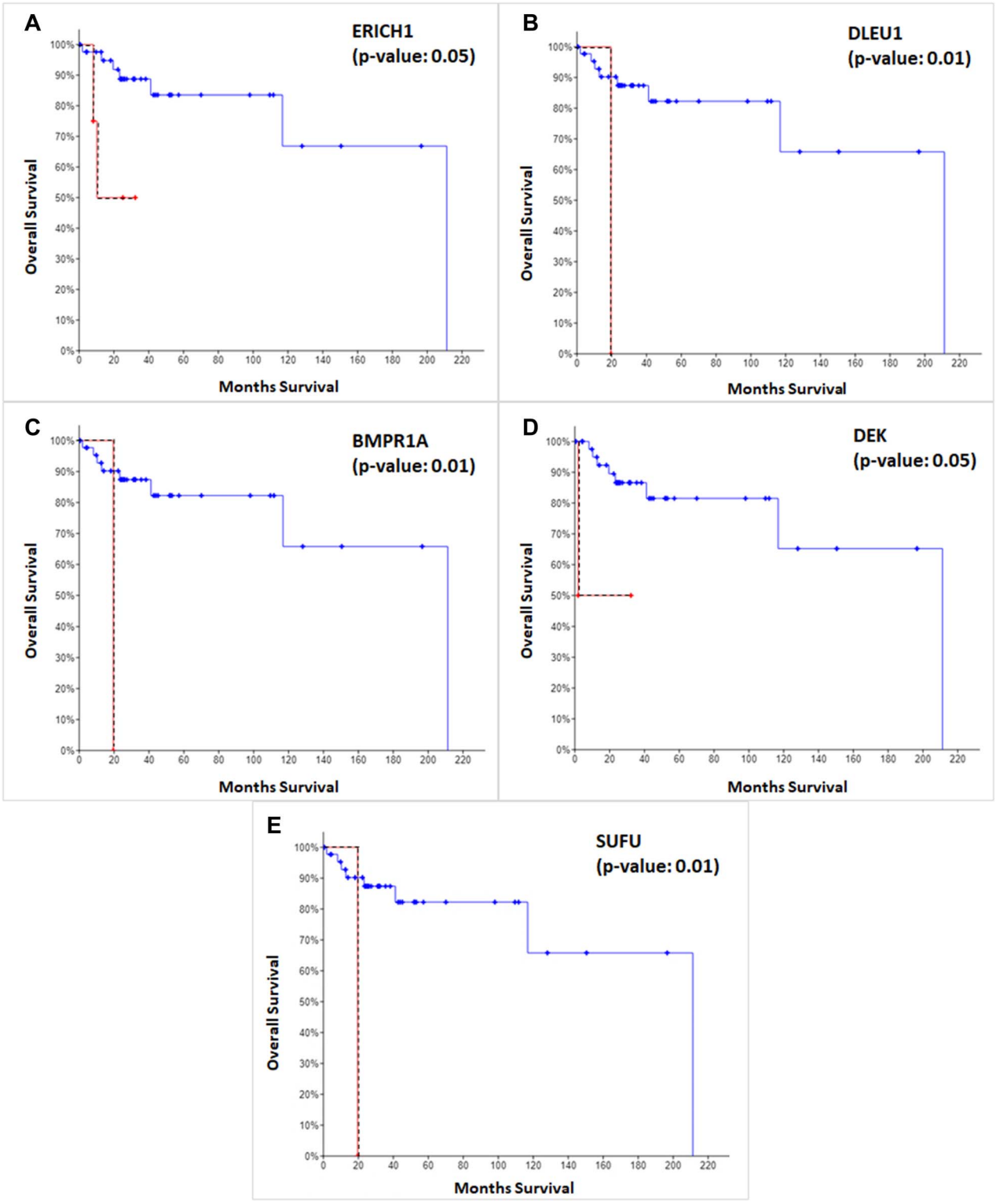

Figure 6. Kaplan-Meier plots illustrating overall survival for patients with (dashed line) and without (solid line) copy number alterations in genes. **A**. ERICH1 (loss, 9/11 cell lines); **B**. DLEUI (loss, 6/7 GCB cell lines); **C**. BMPR1A (loss, WSU-DLCL2); **D**. DEK (gain, SU-DHL-6); **E**. SUFU (loss, OCI-LY1).

BMPR1A belongs to a family of bone morphogenetic protein (BMP) receptors that bind to members of TGF-beta family and is involved in apoptosis and NF-κB signalling pathways. However, the exact mechanism through which BMPR1A initiates GCB lymphomagenesis is not clear. Tumour suppressor SUFU acts as a negative

regulator of Hedgehog pathway, essential for cell growth and differentiation, and is implicated in pathogenesis of medulloblastoma[34]. Overexpression of proto-oncogene, DEK is often a result of 6p22.3 locus amplification and is commonly observed in solid tumours such as breast cancer, bladder cancer and multiple myeloma[35].

Discussion

Genome-wide detection and analysis of copy alterations revealed heterogeneous CNV-profiles across the cell lines and patient samples (Table 3). Our analysis revealed 85 key genes lying on CNVs in these cell lines, of which 76 exhibited OncoScore[36] > 21 (threshold score), indicating their cancer associations. As expected, their frequency of occurrence was very low across the cell lines and TCGA samples. Similar behaviour was observed across the TCGA samples with most frequently altered genomic region (9p21.3) spanning tumour suppressor CDKN2A represented in 13/48 patient samples (and 3/11 cell lines). In this study we discuss our analysis of large structural variations and their impact on immuno-oncogenic pathways to gain insight into molecular heterogeneity in DLBCL and propose individualized therapeutic options based on the pathways altered in each cell line (Fig. 5).

Immune system is capable of recognizing cancer cells, inhibit their growth and remove malignant cells. However, cancer cells evolve to escape the attack and surveillance by multiple immune response evasive mechanisms. Here we understand that genetic aberrations leading to loss of MHC-II or activation of INFγ and PD-1 signalling pathways are some of the ways by which lymphoma cells 'hide' or 'defend' an immune attack from host T cells[37]. Normal B cells present antigen through HLA class I genes, while presence of neo-antigen (modified due to mutation) by cancer B cells triggers anti-tumour immune responses. The T cell surface molecule, PD-1, on binding to ligands PD-L1/PD-L2 often present on tumour cells, act as an immune checkpoint and regulate the adaptive immune system by inhibiting T cell proliferation, cytokine production and cytolytic function[38]. In all the 11 cell lines irrespective of the subtype, CNV events overlapping genes involved in immune system surveillance, *viz*., B2M, HLA-cluster, IFIT3, IRF2, PSMB8, *etc*. were observed (Fig. 4). Copy alterations affecting immune response pathways were also observed in 35/48 TCGA samples. Recent improved understanding of PD-1/PD-L1 signalling pathway has led to the development of immunotherapy. Phase I and II trials using anti-PD-1 antibodies (*Nivolumab* and *Pembrolizumab*) have shown promising results in patients with relapsed or refractory DLBCL[24]. Another pathway commonly varying across the 11 cell lines (1 TCGA sample) is NOTCH signalling pathway, which plays a role in several cancer pathways including angiogenesis, genomic instability, sustained proliferative signalling and resisting cell death (Fig. 4)[39]. Alterations in NOTCH signalling pathway and cell cycle dysregulation due to TP53/CDKN2A deletions has been shown to be associated with poor overall survival in DLBCL patients by Chapuy et al[17] and Karube et al[19].

Apart from these various other pathways involved in cell growth and apoptosis were found to be enriched with CNV genes revealing possible mechanisms for lymphomagenesis in DLBCL cell lines (Fig. 5). Activation of PI3K-AKT-mTOR signalling pathway is affected in few GCB-DLBCL cell lines, by differing mechanisms, e.g., through deletion of PTEN (OCI-LY1 and WSU-DLCL2), indirectly through amplification of mir-17-92 (DB, OCI-LY7 and SU-DHL-6) which inhibits the expression of PTEN, or gain of HSP90AB1 (SU-DHL-6 and NU-DUL-1). Tumour suppressor PTEN controls the activation of phosphatidylinositol-3-kinase (PI3K) under normal conditions; upon its deletion, PI3K gets accumulated, activating downstream AKT and mTORC1. The repression of PTEN promoter, leading to loss of PTEN expression, is observed to occur through amplification of oncoMIR, mir-17-92 in two cell lines. Alternatively, PI3K-AKT-mTOR signalling pathway is also activated through alteration in RTK signalling cascades, which also affects other downstream pathways, MAPK and JAK-STAT signal transductions (WSU-DLCL2, SU-DHL-6 and MD903).

In NU-DHL-1 cell line, amplification of Ras molecules (KRAS), a key component of MAPK signalling results in activating subsequent kinases in the cascade, MAP3K (RAF1), MAP2K and finally, MAPK. Activated MAPK is known to regulate several transcriptional factors including those involved in cell cycle and progression. Increased JAK-STAT signal transduction and transcription regulation observed in OCI-LY1 and NU-DHL-1 cell lines is often associated with DLBCL lymphomagenesis. Binding of cytokines such as interleukins and interferons to cytokine receptors dimerizes the receptors bringing the JAKs in proximity, activating them and subsequently dissociating STAT3 from the receptor complex. Activated STAT3 migrates to the nucleus and participates in several cell activities including uncontrolled cell division and reduced apoptosis.

The B cell receptor (BCR) signalling pathway is involved in initiation and maintenance of pathogenic B cells in numerous B cell malignancies including DLBCL. Alterations in BCR complex/signalling components (such as gain of FOXP1 or FCGR2B genes in NU-DUL-1 and SU-DHL-9 cell lines, respectively) lead to uncontrolled cell growth and prevents apoptosis partly through crosstalk with PI3K-AKT-mTOR signalling pathway via spleen tyrosine kinase (SYK). The BCR signalling pathway also triggers the downstream pro-survival pathways such as NF-κB in the malignant cells. Increased activity of NF-κB signalling is mainly observed in ABC-DLBCL cases. Upon activation NF-κB family of transcription factors (through ERC1 or LCP1 in NU-DHL-1 and WSU-DLCL2 cell lines), a part of NF-κB complex is transferred to nucleus through a non-canonical pathway cascade activating downstream gene-transcription, cytokine production, immune response and cell survival. Higher

NF-κB activity is also known to be associated with increased JAK-STAT activity in DLBCL. Alterations in the various signalling pathways observed indicate alternate mechanisms leading to dysregulation of cell cycle and reduced apoptosis in DLBCL. Thus, knowledge of the pathways affected in DLBCL patients is desirable for designing targeted therapy.

In our analysis we also observed few CNVs directly affecting cell cycle regulating proteins, *viz.*, CCND2/CCND3, TP53, etc. Deletion of CDKN2A in NU-DHL-1, DOHH-2 and MD903 cell lines affects cell cycle regulation by increased activity of cyclin D (CCND2/CCND3) and bound cyclin dependent kinases (CDK4/CDK6), inhibiting RB1 signalling pathway essential for blocking G1 to S phase transition. Alternately, gain of CCND2, CCND3 and CDK6 observed in NU-DHL-1, SU-DHL-6 and SU-DHL-9 cell lines results in the uncontrolled growth of tumour cells. Additionally, loss of RB1 observed in WSU-DLCL2 cell line also results in cell cycle progression. In OCI-LY19 cell line, deletion of tumour suppressor TP53 disrupts cell cycle regulation, apoptosis and DNA repair, contributing to tumour cell growth and survival. A majority of these copy alterations in key genes were present in 17/48 TCGA samples confirming their significance in DLBCL. In such cases, use of pro-apoptotic drugs, *Pterostilbene* and *DCZ3301*, to arrest cell cycle is proposed in combination with standard R-CHOP treatment. The efficacy of these drugs has been confirmed in DB and NU-DUL-1 cell lines[40,41].

Our analysis also revealed few known copy variant genes associated with poor prognosis. These include amplification of IRF4 and FOXP1, and deletion of CDKN2A, FAS and TP53 genes. The analysis also revealed 5 novel markers with significant prognostic value (Fig. 6). These include ERICH1 (GCB and ABC), DLEU1 (GCB), BMPR1A (GCB), DEK (GCB) and SUFU (GCB) with possible implications in stratifying the low/high-risk groups in DLBCL patients. To assess somatic/germline status of these markers, we extracted 'Masked Copy Number Segment' files from 48 TCGA patient samples. These files contain filtered set of CNVs after removing probe sets previously indicated to be associated with frequent germline CNVs. Interestingly, all the five proposed prognostic markers were found to be somatic variants. The exact mechanism through which these alterations affect the DLBCL lymphomagenesis and their role as potential prognostic markers need to be assessed using a large patient cohort.

The CNVs may influence gene expression through gene dosage imbalances, disruption, fusion and/or change in cis/trans-regulatory sequences. The role of CNVs in cancer initiation and as stable biomarkers is well known as these provide an advantage of easier, faster and reliable (low noise) predictions compared to other commonly studied genetic variations, e.g., SNVs. Here we have presented an *in silico* genomic-guided approach to identify and target various pathways triggered/disrupted by copy number alterations. Our work provides a list of copy gain and copy loss events encompassing functional elements in 11 DLBCL cell lines, which would serve as a valuable resource for the cancer research community. Functional analysis of CNV-profiles revealed variations in cancer genes and pathways affected across the cell lines, indicating the need for genome-wide screening of genetic alterations for personalized therapy. A novel set of CNV-genes are also observed, which would help in stratifying DLBCL patients into low/high-risk groups and design appropriate therapy. To take this approach to clinical setting, there clearly is a need for a comprehensive resource that provides annotations associating tumour alterations to genes, pathways and potential drug targets. Characterization of other genetic variants such as SNVs, INDELs, etc., would provide a complete variation profile for assessing their impact on molecular mechanisms of lymphomagenesis. Furthermore, with the availability of matched-control samples, tumour specific features such as tumour purity and heterogeneity can be handled, improving the reliability of predictions.

Supplementary data

Supplementary data is available in *Precision Clinical Medicine* online at https://doi.org/10.1093/pcmedi/pbz024.

Conflict of interest

None declared.

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