A 220-Gene Targeted Next-Generation Sequencing Panel for the Detection of Variants in Diffuse Large B-Cell Lymphoma, Follicular Lymphoma, and Mantle Cell Lymphoma: Application to a Cohort of 85 Formalin-Fixed Paraffin-Embedded Diffuse Large B-Cell Lymphoma Biopsies

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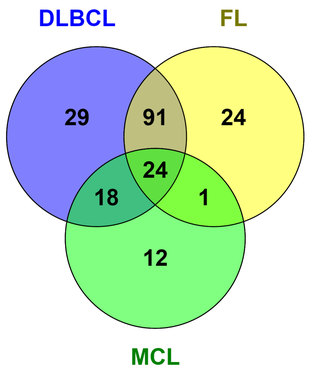
Introduction

- A 220 gene panel was designed using Nimblegen target capture probes to sequence exonic regions of genes reported to carry somatic mutations in DLBCL, FL, MCL, and CLL.
- Gene selection for the panel was based on published reports or from public databases including Catalog of Somatic Mutations in Cancer (COSMIC).[1-6]
- For final inclusion on the panel, the following considerations were made; role in B-cell lymphomagenesis, reported role in B-cell lymphomagenesis, reported role in prognostication in the target diseases, frequency in each disease type, targets of novel therapies, genomic localization within a site of genomic imbalance in the target diseases, and member of either BCR signaling, WNT, or PI3K pathway.

Focus::Lymphoma™ Targeted NGS Panel Features

ABCA13	BIRC3	CDKN2B	EPHA7	HIST1H1C	KDM6B	NLK	PIK3R2	RB1	TBL1XR1
ABCA3	BLK	CIITA	ERBB3	HIST1H1E	KDR	NOTCH1	PIKFYVE	RGS4	TCF3
ABCC4	BLNK	CNOT6L	ETS1	HIST1H2BC	KIT	NOTCH2	PIM1	RHOA	TCF4
ABCC9	BRAF	COL16A1	EZH2	HIST1H3B	KLHL6	NOX4	PLCB1	ROBO2	TEC
ABL1	BRD4	CREBBP	FAS	HIST1H4I	KRAS	NR3C1	PLCB4	ROCK2	TENM4
ACTB	BTG1	CSMD3	FAT2	HIST2H2BE	LRP1	NRAS	PLCD1	ROS1	TET2
ACTN1	BTG2	CSNK1D	FAT4	HRAS	LRP6	NTRK1	PLCD3	RRAGC	TLR2
ADAM10	BTK	CTBP2	FBXO11	ID3	LRRC7	OGDHL	PLCE1	S1PR2	TMEM30A
AHR	CARD10	CTNNA2	FGFR1	IGSF1	LYST	P2RX5	PLCG2	SALL3	TNF
AKT	CARD11	DCC	FLT1	IKBKB	MALT1	P2RY8	PLCZ1	SEMA6C	TNFAIP3
APC	CARM1	DCP1B	FOXO1	IKZF1	MAP2K1	PARP1	POT1	SF3B1	TNFRSF11A
APC2	CCND1	DDX21	FOXP1	IKZF3	MED12	PDCD1	POU2AF1	SGK1	TNFRSF14
AQR	CCND3	DGKZ	FYN	INPP5B	MEF2B	PDGFRA	POU2F2	SI	TNIK
ARID1A	CD22	DHDH	GCC2	IRF4	MLL2	PDK1	PRDM1	SLC17A6	TNRC6B
ATM	CD36	DLC1	GNA13	IRF8	MLL3	PIK3AP1	PRKCA	SLC30A4	TP53
ATP11C	CD58	DSEL	GNAI2	ITGB3	MLLT6	PIK3C2A	PRKCB	SMARCA4	TRAF2
ATP6AP1	CD70	DTX1	GPR112	ITPK1	MRGPRF	PIK3C2G	PRKDC	SOCS1	TRIM8
B2M	CD79A	DUSP2	GRB2	ITPKB	MTOR	PIK3C3	PTEN	SP140	UBR5
BAP1	CD79B	DUSP27	GRIN2A	ITPR1	MYC	PIK3CA	PTPN13	STAT3	VAV1
BCL10	CDIPT	EBF1	GSK3B	ITPR2	MYD88	PIK3CD	PYHIN1	STAT6	WHSC1
BCL2	CDK4	EIF2AK4	HCK	JAK1	NFKBIA	PIK3CG	RAF1	SYK	XPO1
BCL6	CDKN2A	EP300	HIST1H1B	JAK2	NFKBIB	PIK3R1	RAPGEF1	SYNJ2	ZMYM3

Clinical Targets	*BCR/PI3K/WNT Pathway*	*Outcome/COO*	*Genomic gain/loss*
BRAF	BLNK	BTG1	B2M
BTK	CSNK1D	CD79A/B	BCL2
JAK1/2	CTNNA2	FOXO1	BCL6
EZH2	GRB2	ID3	CCND3
MTOR	GSK3B	IRF4	FAS
PIK3CD	PI3KCA	PRDM1	MALT1



Conflicts of Interest

JF, AG, CM, VT, JH are employees of Cancer Genetics, Inc., and are stock/stock option holders.

SK and IS have no conflicts of interest.

Materials and Methods

- Nimblegen hybrid-capture design encompassing 4099 targets (exonic regions only)
- Applicable to fresh frozen (FF) and formalin-fixed, paraffin embedded (FFPE) material
- Starting material required->30 ng FF, >100 ng FFPE
- Libraries prepared using Kapa Hyper or HyperPlus kit
- Sequencing performed on an Illumina sequencing platform (Miseq or Hiseq2500)

Alignment and Somatic Variant Calling
- CLC Biomedical Genomics Workbench
- Human ref genome hg19/GRCh37

Initial Filtering
- dbSNP, 1000 Genomes, HapMap MAF >0.1%

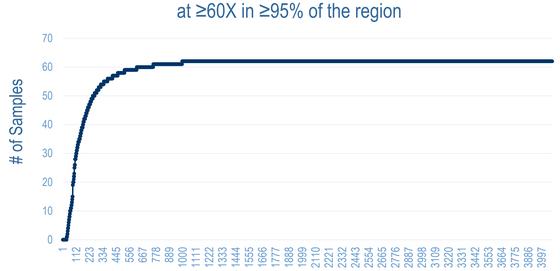
Final Filtering
- Non-synonymous and noncoding
- Homopolymer regions
- SIFT and PolyPhen prediction T and B

Results-Quality Control

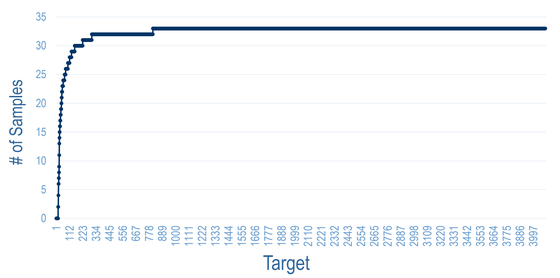
Individual Target Performance

Target performance was assessed in a group of 62 FFPE and 33 FF samples. Individual targets were assessed by calculating percent of the total target region that achieved a coverage of 60X or greater. In the above graph, the individual target is plotted against the number of samples which met that criteria.

FFPE, N = 62



FF, N = 33



Of the 4099 total targeted regions, 39 in FFPE and 15 in FF failed to make ≥ 95% of the region covered at 60X or greater. Out of the targets not achieving the cutoff in any samples, 3 have reported somatic variants in that target region for the applicable diseases.

Target	Somatic variant reported in	Achieved cutoff in FFPE	Achieved cutoff in FF
FOXO1	DLBCL, FL	No	Yes
APC2	CLL	No	Yes
KMT2D	DLBCL, FL	No	Yes

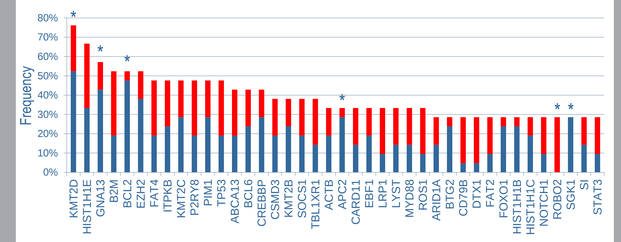
Results-Variant Detection

Application of Panel to a Set of 85 FFPE DLBCL Specimens

85 FFPE DLBCL(>70% tumor burden) specimens for which aCGH was performed → 26 samples were excluded for poor quality; 4 samples did not have enough material → 55 samples subjected to NGS using Focus::Lymphoma™ Panel

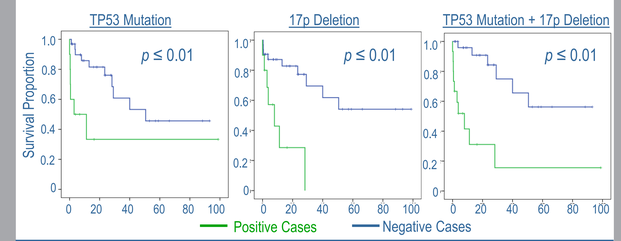
Clinico- and Pathogenomic Associations with Detected Variants

After final filtering, each specimen was scored present or absent for a mutation in a gene. Given below are genes found mutated in greater than 25% of the cohort, and the subtype (GCB vs. non-GCB, Hans algorithm) of the sample for which that variant is detected in is also plotted.



Specimens were scored positive or negative for TP53 mutation, 17p deletion, or either one or the other and plotted using log-rank statistic.

Type	Cases+	Cases-
TP53 Mutation	10	32
17p Deletion	10	32
TP53 Mut or 17p Deletion	15	27



Reproducibility of Low Level Variants

Below are all of the somatic variants that were detected below an AVF of 20% in several specimens from this cohort. Each specimen was run twice in independent library preparations and independent Miseq runs.

Case 66 Variants	Chr.	Region	Type	Run 1 (%AVF)	Run 2 (% AVF)
BCL2	18	60,985,892	SNV	8.7	19.3
BCL2	18	60985542..60985543	MNV	9.4	18.8
BCL2	18	60,985,333	SNV	3.7	15.6
ROBO2	3	77,147,295	SNV	10.0	13.8
EP300	22	41,565,529	SNV	6.0	13.4
ITPKB	1	226,924,726	SNV	7.8	13.1
STAT3	17	40,477,036	SNV	7.6	12.4
BTG1	12	92,538,182	SNV	7.1	11.9
ROS1	6	117,730,774	SNV	3.2	8.5

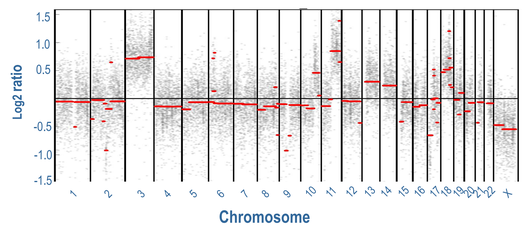
Case 84 Variants	Chr.	Region	Type	Run 1 (%AVF)	Run 2 (% AVF)
TP53	17	7,577,124	SNV	6.9	6.0

Case 85 Variants	Chr.	Region	Type	Run 1 (%AVF)	Run 2 (% AVF)
NOTCH1	9	139,412,376	Insertion	5.7	5.3

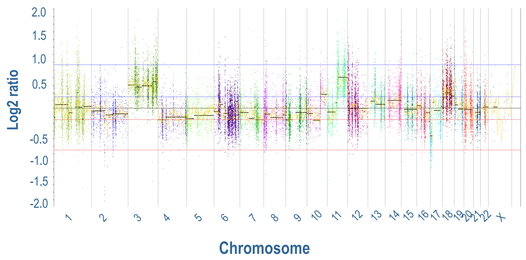
Results-Copy Number Analysis

Copy number for each specimen was determined using either aCGH or CNVkit from NGS results. For CNVkit analysis, on-target and off-target sequencing reads were partitioned into bins. Normalization for binned reads was done using a set of 8 HapMap controls to determine copy number.

CNVkit



Targeted aCGH



Results-Average Coverage of Regions Reported to Harbor Resistance Mutations in Ibrutinib Treated Patients

Gene	Target Region/ Reported Resistance Mutation[2]	Average Coverage
BTK	Exon 15/C481S	341X
	Exon 16/L528W	383X
PLCG2	Exon 11/D334H	568X
	Exon 18/R665W	492X
	Exon 19/S707Y	425X
	Exon 20/R742P	598X
	Exon 23/L845Y	535X
	Exon 29/D1140G	618X

Conclusions

- Using a hybrid capture panel, we are able to establish target performance metrics using a set of 62 FFPE and 32 FF samples. For almost all of the FF samples, less than 2% of the targets did not reach a coverage of at least 60% in 95% of the region. For FFPE, less than 5% of the targets did not reach this same metric.
- Pilot study showed correlation between variants associated with GCB and non-GCB subtypes, thus validating the clinical robustness of the panel.
- Panel can be used to assess copy number changes using off-target reads and CNVkit algorithm
- Sites of clinically relevant resistance mutations achieve an average coverage high enough to detect low level variants

References

1. Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html
2. Maddocks, K.J., et al. 2015. Etiology of Ibrutinib Therapy Discontinuation and Outcomes in Patients With Chronic Lymphocytic Leukemia. JAMA Oncol. 2015;1(1):80-87.