Next Generation Sequencing Demonstrates Clinical Utility and Increased Sensitivity in Detection of IgVH Somatic Hypermutation in Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma (CLL/SLL)

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Introduction

Chronic Lymphocytic Leukemia/Small lymphocytic lymphoma (CLL/SLL) is the most common leukemia diagnosed among adults in Western countries and is associated with heterogeneous clinical outcomes. IgVH somatic hypermutation (SHM) status is a primary component of the CLL International Prognostic Index (CLL-IPI) working group formulation for disease risk stratification.[1] Unmutated IgVH has been established as a strong and independent predictor of adverse clinical prognosis and reduced overall survival.

Clinical laboratory evaluation of IgVH SHM status traditionally involves rt-PCR followed by Sanger Sequencing using RNA extracted from patient peripheral blood or bone marrow aspirate samples, however RNA lability places significant burden on the submitting physician to ensure specimen transit time is minimized. Furthermore, Sanger Sequencing is time and labor intensive, and sensitivity of IgVH SHM detection may be limited for low abundance CLL/SLL clones. ZAP-70 expression by flow cytometry has therefore been widely utilized as a surrogate marker for IgVH SHM status. Positive ZAP-70 expression is usually associated with IgVH unmutated disease and an unfavorable clinical course.[2] Unfortunately, standardization for this marker is known to be poor; interpretation of the flow cytometry list mode data may be highly subjective, contributing to significant variability in clinical reporting.[3] Significant discordance between ZAP-70 expression patterns and expected IgVH SHM results may also be seen and has been attributed to pre-analytic sample processing factors in some studies.[4]

Herein we report on the clinical utility of a next generation sequencing (NGS) based approach to IgVH SHM testing using DNA derived from CLL/SLL patient samples.

Materials and Methods

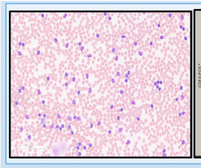
NGS: *IGH* framework 1 V-J amplicon libraries were prepared using Invivoscribe panel primers, PGM adapters, and patient sample DNA. Pooled libraries were quantified and sequenced on the Ion Torrent PGM™ instrument, with generated FASTQ files analyzed by LymphoTrack® PGM® Software (Version 2.0). A ≥ 4-fold difference between first and third most abundant *IGH* sequences defined clonal read data,[5] and IgVH somatic hypermutation status was resulted in accordance with recommended CAP reporting guidelines.[6]

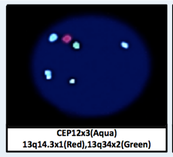
Sanger Sequencing (SS): Samples were previously tested at an outside national reference laboratory. Patient sample RNA was extracted and reverse transcribed into cDNA followed by rtPCR amplification using VH leader and JH primers. Identified clonal VH sequences were searched against a known database for homology. IgVH homologies were defined as follows: >98% = unmutated, 97%-98% = borderline, <97%=mutated. CLL clones were required to comprise at least 50% of total analyzed B-cells.

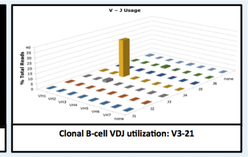
ZAP-70: Flow cytometry was performed on a Beckman Coulter Navios instrument utilizing ZAP-70 Clone 1E7.2 (Invitrogen). T and NK-cells served as internal control subpopulations. CD19+/CD5+ B-cell ZAP-70 results were defined as follows: >25% = positive; 20-25% = borderline; <20% = negative.

Results

- SS-PCR failed to detect a clonal population in 5/25 cases evaluated (20%), whereas, NGS detected clonal populations in all 25 cases studied.
  - V3-21 gene utilization was detected by NGS in 1 of the 5 cases for which SS-PCR failed to detect a B-cell clone.
- Accuracy: NGS demonstrated 100% concordance with SS-PCR among evaluable cases where SS-PCR did not fail to detect a clonal population.
- NGS Limit of detection: ~3% clonal B-cells (data not shown)
- NGS Precision: 100% (data not shown)
- Strength of agreement between NGS and ZAP-70 assays was 0.64 (Good), whereas, SS-PCR and ZAP-70 assays showed moderate agreement (0.535) (Kappa statistic).







54yo male with CLL/SLL presenting with hyperleukocytosis (WBC 302 k/uL, ALC 285.15 k/uL). Peripheral blood flow cytometry demonstrated a CD5+ monoclonal B-cell population with a CLL/SLL immunophenotype (CD45+, CD19+, CD20+, CD5+, CD10-, CD23+, FMC7-/+, CD38-, HLA DR+, slg kappa+, CD200+)(92% of sample, 98% of lymphocytes). FISH studies revealed trisomy 12 and del(13q). **SS IgVH SHM testing was performed at an outside national reference laboratory and was reported as *"No clonal population detected."* NGS IgVH SHM testing revealed 100% *IGH* sequence homology, confirming IgVH un-mutated status, and V3-21 gene utilization was also detected - a known adverse prognostic indicator in CLL/SLL.** Surrogate markers of IgVH SHM status including ZAP-70 and CD38 were negative in this case.

Sample Type	Absolute Lymphocyte Count (k/µL)	% Sample Involvement (Flow Cytometry)	FISH	CD38	ZAP-70	IgVH SHM status (Sanger Sequencing)	IgVH SHM status (NGS)	V_H Utilization (Sanger Sequencing)	V_H Utilization (NGS)	% V_H Homology (Sanger Sequencing)	% V_H Homology (NGS)
BM-EDTA	23.29	55	Trisomy 12	+	+	NEGATIVE	NEGATIVE	V1-45	V1-45	100	100
BM-EDTA	31.61	54	No alterations	+	+	NEGATIVE	NEGATIVE	V1-69	V1-69	100	100
PB-EDTA	40.9	83	Trisomy 12	ND	ND	NEGATIVE	NEGATIVE	V1-69	V1-69	99.65	100
PB-NAH	91.5	87	No alterations	-	-	BORDERLINE	BORDERLINE	V5-51	V5-51	97.92	97.35
PB-EDTA	47.76	NA	No alterations	ND	ND	NEGATIVE	NEGATIVE	V1-2	V1-2	100	100
PB-EDTA	61.6	84	Trisomy 12	+	+	NEGATIVE	NEGATIVE	V4-61	V4-61	100	100
BM-EDTA	49.97	53	ND (not done)	+	+	NEGATIVE	NEGATIVE	V4-30	V4-30	100	100
PB-EDTA	50.33	47	Trisomy 12, del(13q)	ND	+	No clonal population detected	POSITIVE	NA	V3-11	NA	100
PB-EDTA	Not Available (NA)	18.5	Low level *TP53* loss	ND	+	POSITIVE	POSITIVE	V3-23	V3-23	95.83	95.59
PB-EDTA	**285.15**	**92**	**Trisomy 12, del(13q)**	**-**	**ND**	**No clonal population detected**	**NEGATIVE**	**NA**	**V3-21**	**NA**	**100**
BM-EDTA	25.8	76	ND	-	Borderline	NEGATIVE	NEGATIVE	V3-11	V3-11	100	100
BM-EDTA	53.1	88	No alterations	+	ND	BORDERLINE	BORDERLINE	V3-48	V3-48	97.92	97.36
BM-NAH	73.8	91	11q23 *ATM* loss, del(6q), Trisomy 12, del(13q)	Borderline	+	NEGATIVE	NEGATIVE	V4-4	V4-4	100	100
BM-EDTA	16.2	48	Trisomy 12	+	+	NEGATIVE	NEGATIVE	V1-69	V1-69	100	100
PB-EDTA	20.3	63	del(13q)	-	ND	No clonal population detected	POSITIVE	NA	V3-23	NA	89
PB-EDTA	65	84	Trisomy 12	-	+	NEGATIVE	NEGATIVE	V1-69	V1-69	100	100
PB-EDTA	9.82	43	*TP53* loss, 11q23 *ATM* loss	-	-	POSITIVE	POSITIVE	V3-30	V3-30	90.28	88.99
PB-EDTA	NA	90	del(6q)	-	ND	NEGATIVE	NEGATIVE	V3-9	V3-9	100	100
PB-EDTA	20.29	64	del(13q)	-	+	NEGATIVE	NEGATIVE	V4-34	V4-34	100	100
BM-Heparin	1.00	4.7	ND	-	ND	No clonal population detected	POSITIVE	NA	V1-18	NA	98.67
BM-Heparin	12.4	28	ND	-	+	No clonal population detected	NEGATIVE	NA	V4-39	NA	94.32
PB-EDTA	5.94	14	No alterations	-	-	NEGATIVE	NEGATIVE	V1-69	V1-69	100	100
PB-EDTA	NA	30	No alterations	-	-	POSITIVE	POSITIVE	V4-34	V4-34	91.23	91.03
PB-NaH	6.68	40	ND	ND	-	POSITIVE	POSITIVE	V2-26	V2-26	96.56	96.81
PB-EDTA	5.62	17	del(13q)	-	-	POSITIVE	POSITIVE	V1-69	V1-69	94.79	91.59

Conclusions

Evaluation of IgVH SHM status using NGS is feasible for routine clinical testing in the private reference laboratory setting and confers significant advantages over traditional methodologies:

- NGS may show improved sensitivity in detection of clonal populations compared to Sanger Sequencing.
- DNA is an acceptable specimen substrate for NGS IgVH SHM detection, limiting practical constraints typically associated with RNA based testing.
- NGS significantly streamlines laboratory workflow:
  - Three day clinical reporting is feasible.
  - Assay hands on time is minimized (<5 hours).
  - Batch multiplexing of patient samples facilitates significant reductions in cost and labor.
- NGS sample input requirements are minimized. Clinically valid NGS results may be obtained using 1ml of peripheral blood or bone marrow, or 0.025 µg DNA.
- NGS data output is automated and non-subjective, and may include relative frequencies of clonal *IGH* reads, assessment of homology to most closely matched germline *IGH* reference sequences, and VH gene utilization profiles of patient read data.

References

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