Clinical Laboratory Implementation of the Detection of Genomic Aberrations in Formalin-fixed, Paraffin-Embedded Small Lymphocytic Lymphoma Specimens by aCGH

CANCER GENETICS
Empowering Personalized Cancer Treatment

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Introduction

Chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL) are the same clonal B-cell disorder diagnostically distinguished based on disease distribution. Risk stratification for patients diagnosed with CLL/SLL is essential due to the highly variable clinical course of the disease. Current prognostication in CLL encompasses clinical features such as disease stage together with various molecular biomarkers including the presence of specific chromosomal aberrations. In SLL, implementation of routine detection of the same biomarkers in a clinical diagnostic setting is hampered by the solid tissue type which often requires working with thin sections taken from formalin-fixed, paraffin-embedded (FFPE) specimens with highly inconsistent sample qualities. Here, we established appropriate laboratory procedures for the clinical implementation of array comparative genomic hybridization (aCGH) using SLL FFPE samples.

MatBA®

Agilent 4x44K format with 35kb resolution
Duplicate probes
80 regions covering 0.3-21.3 Mbp (CLL/SLL regions in red)
1 Mbp resolution backbone probes
5 x 301 control probes

Region	Size (Mbp)	Region	Size (Mbp)	Region	Size (Mbp)
1p36.32-p36.23	7.9	6p21.31-p21.2	2	11q25 (*ATM*)	2
1p21	13	6p21.1	2	12p13.1	2.2
1p13.2-p13.1	6	6q12	0.3	12q13.1-q13.2	11.9
1q21	10.9	6q16	12.8	12q15	3.8
1q31	19.7	6q21	2.5	13q14 (*DLEU2*)	12.7
1q41-q44	8	6q22	16.5	13q31	16
2p25.3	1.7	6q23.3-q24	11.9	13q33-q34	13.6
2p16.1-p15	4.6	6q25	1	14q12	9
2p11.2-q11.2	2	7p22	7.2	14q32	15
2q13-2q14.1	0.6	7p21.3-p21.2	1.7	15q21.1	1.5
2q24	14.9	7q31	19.7	15q23-q24	10
3p22	9.9	8p23	12.7	16p13.3	6.3
3p14.1-p13	3.8	8p21.3	4.5	16p13.13	2
3q12.2-q12.3	1.2	8p12-p11.23	2	16p11.1-p11.2	10.6
3q21.2	0.3	8q21.2	0.3	16q24	5.2
3q22	8.9	8q24.21	4.2	17p13 (*TP53*)	11.2
3q26.1-q26.2	11.3	9p24.2-p24.1	2	17q22-q23.1	2
3q26.31	2	9p21	12.9	17q24.2-25.1	5.8
3q27	5.2	9q22	12	18p11	16.1
4p15	9.8	9q33.2-q34.1	10	18q21	18
4q11-q12	4	10p14	5.6	18q23	0.3
4q24	2.8	10p12.31-p12.2	2.5	19p13.3-p13.2	11
4q34.3-q35	11.6	10q23.2	1.9	19q13.33-q13.43	10
5p15	10	11p13	1.5	20q13	21.3
5q13.2-5q13.3	3	11q13	13.6	21q21	15.2
5q31.3	1	11q22.1-q22.2	1.4	22q12	15.9
6p25	7	11q22.3-q23	14		

Materials and Methods

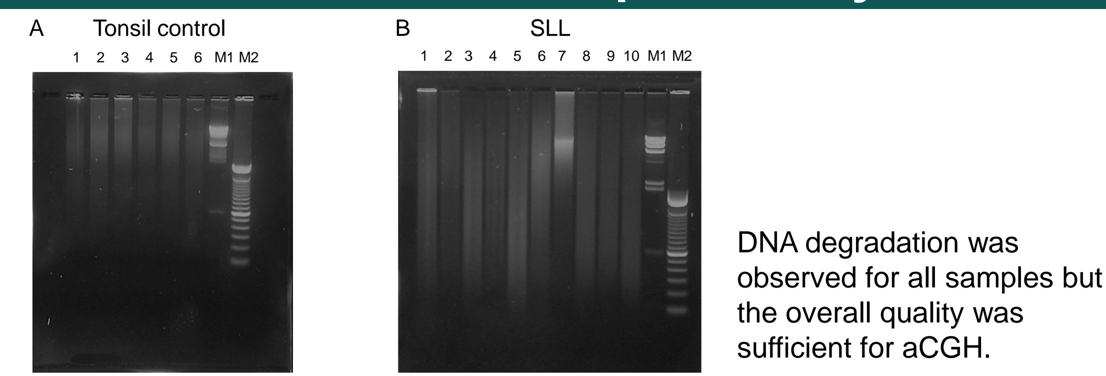
FFPE specimen and DNA extraction
Six FFPE tonsil specimen and ten FFPE SLL specimens were available for the study. All SLL samples contain greater than 90% tumor cells except for SLL5 which contains approximately 70% based on H&E staining. Genomic DNA was extracted using a modified DNA extraction procedure based on the Agilent aCGH Sample Preparation protocol for FFPE samples (L. Dias *et al.*, in preparation). QC criteria:OD260/280 between 1.6 and 2.0 and OD260/230>1.9.

Targeted aCGH
Array processing procedures were performed essentially based on Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis protocol except genomic DNA was first heat-fragmented to 400-800bp in length and 1ug of each DNA sample was then labeled using Enzo CGH Labeling Kit for Oligo Arrays. An equal mixture of normal male/female DNAs from Promega was used as reference. For analysis, the log2 ratios for duplicate probes were first combined then the Aberration Detection Method 2 (ADM-2) algorithm at thresholds of 6.0 with a filter against aberrations that contain fewer than 8 probes and a log2 ratio average less than ± 0.4 was used to identify genomic aberrations.

Quantitative PCR (QPCR)
Taqman-based QPCR was performed with copy-number assay primers/probes selected from the Applied Biosystems Inc. (ABI) copy-number assay library. Samples with a relative ratio lower than 0.75 (deletion) or higher than 1.25 (amplification) when compared to the average of two reference genes were determined to contain authentic copy number change as calculated based on the $\Delta\Delta C_t$ method. When there was potential copy number change in one of the two reference genes, then one reference gene was used and the experiment was repeated as validation.

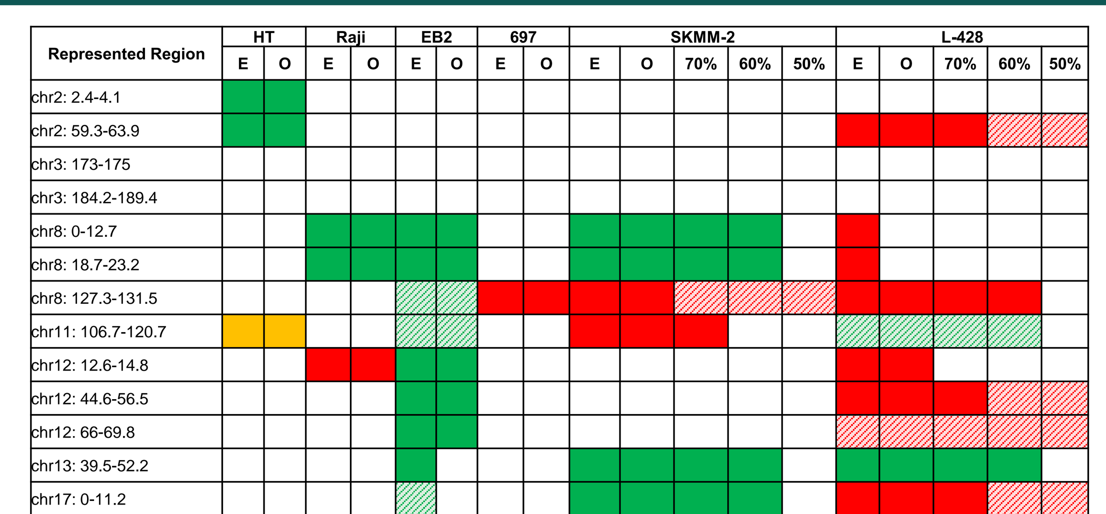
Fluorescence *in situ* hybridization (FISH)
The procedure for processing FFPE samples for FISH analysis was carried out essentially as described (IHC Staining Methods, Dako North America, 2009; pp. 67-73). The three color probe panel for *DLEU2* was purchased from Abbott Laboratories while for *TP53*, home-brew, BAC-based probes were used. For analysis, the ratio of R/A or R/G signals per 100 cells were calculated for either 200 cells (*DLEU2*) or 400 cells (*TP53*) from different regions in each sample. Ratio decreases greater than 2 standard deviations from controls were taken as positive.

FFPE DNA Sample Quality



DNA degradation was observed for all samples but the overall quality was sufficient for aCGH.

Accuracy and Sensitivity



E = expected, O = observed
Red: gain, Green: loss, Yellow: both, Hatched: partial overlap

Array accuracy was assessed by using DNA extracted from six cell lines with publicly available genomic copy number profiles based on an independent platform (http://www.sanger.ac.uk/cgibin/genetics/CGP/cghviewer/CghHome.cgi). Table above shows the expected versus observed results for the six cell lines. For four cell lines concordance was found for all regions. For EB2 and L-428, a few aberrations were not detected. Overall, the aberrations detected were mostly comparable to those obtained using a different platform.

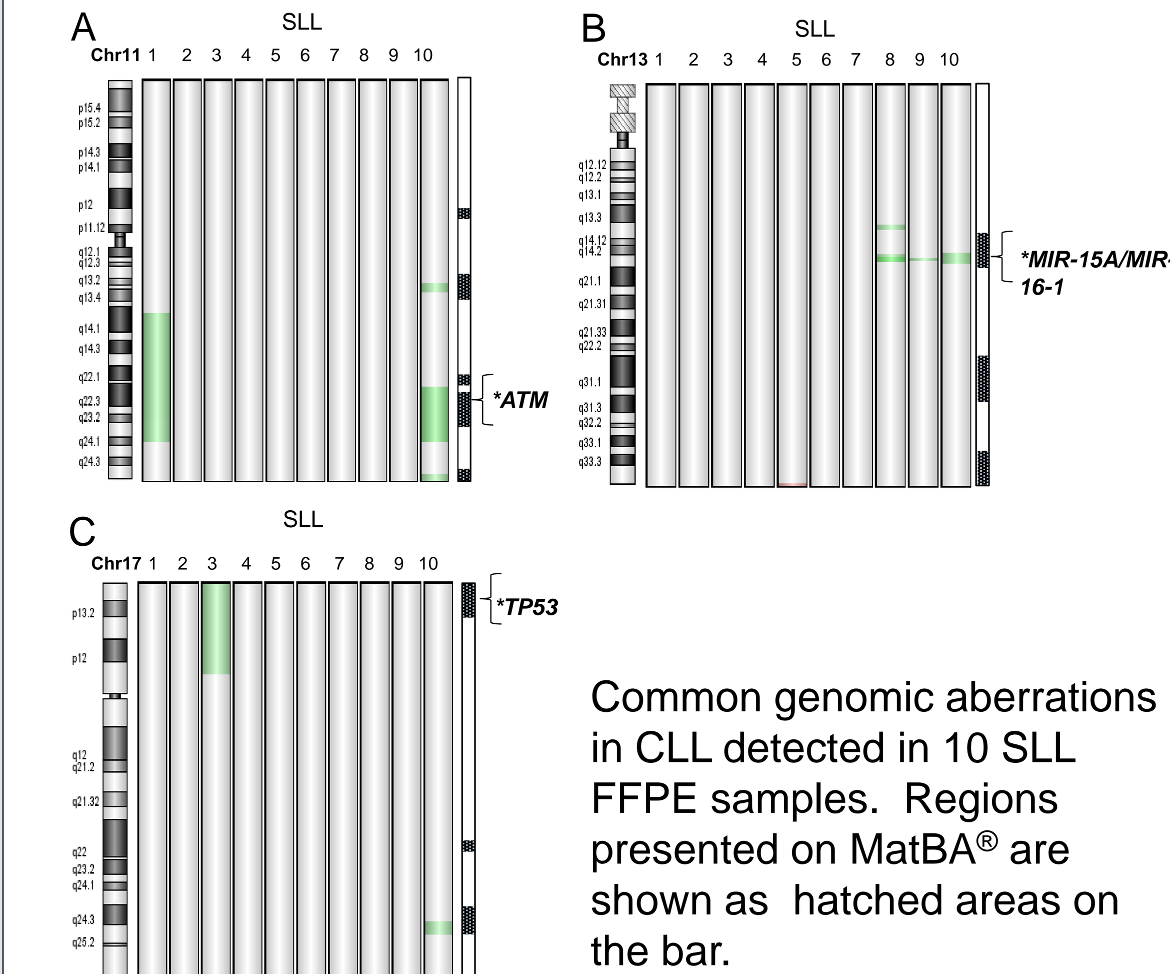
The limit of detection was assessed by dilutions of DNAs from two cell lines from a normal individual. For the two cell lines, aberrations were routinely observed at 70% dilution using the established detection criteria (ADM2 threshold=6.0, 8 and 0.4 filter). Fewer were observed at 60% but did comprise both gains and losses. Thus, the sensitivity for this assay is around 70%.

Specificity

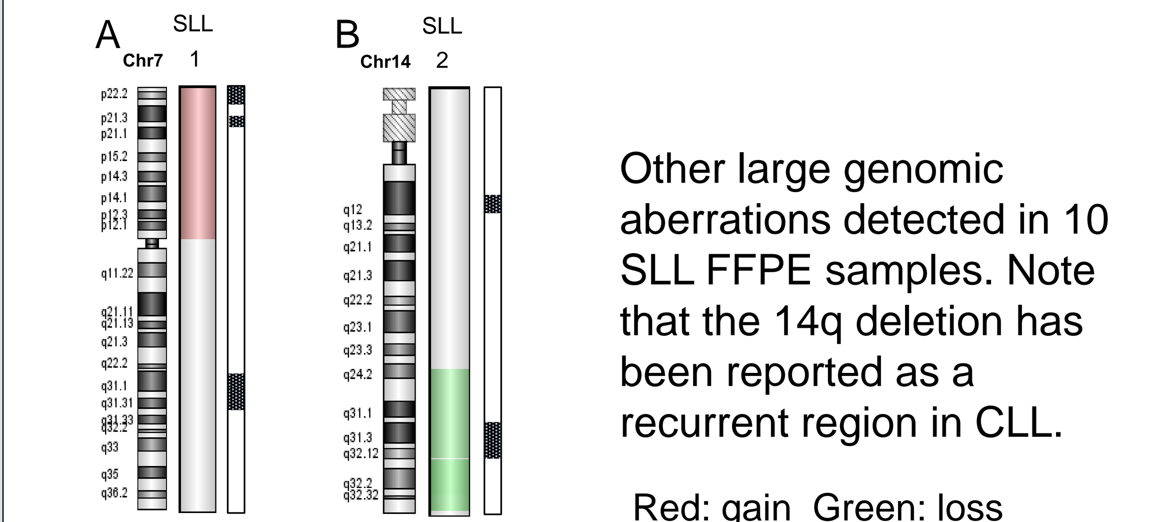
Array specificity was assessed by submitting DNAs extracted from six FFPE samples of normal tonsil tissues to aCGH according to the standard procedures in duplicate. Only a few gains/losses were observed in regions represented on MatBA®, but not in the CLL regions. The aberrations detected in most cases were small and represent CNVs observed within normal populations as according to the recurrent variants recorded in the Database of Genomic Variants (http://projects.tcag.ca/variation/).

Genomic Aberrations Detected by aCGH

Using aCGH, we detected aberrations commonly found in CLL in 5/10 SLL FFPE samples. In addition, two large aberrations were also found . Array reproducibility was evaluated by repeat hybridization of all samples used in the study on different days with different slides at least twice. Same aberrations were observed for each sample after independent runs.



Common genomic aberrations in CLL detected in 10 SLL FFPE samples. Regions presented on MatBA® are shown as hatched areas on the bar.



Other large genomic aberrations detected in 10 SLL FFPE samples. Note that the 14q deletion has been reported as a recurrent region in CLL.

Red: gain Green: loss

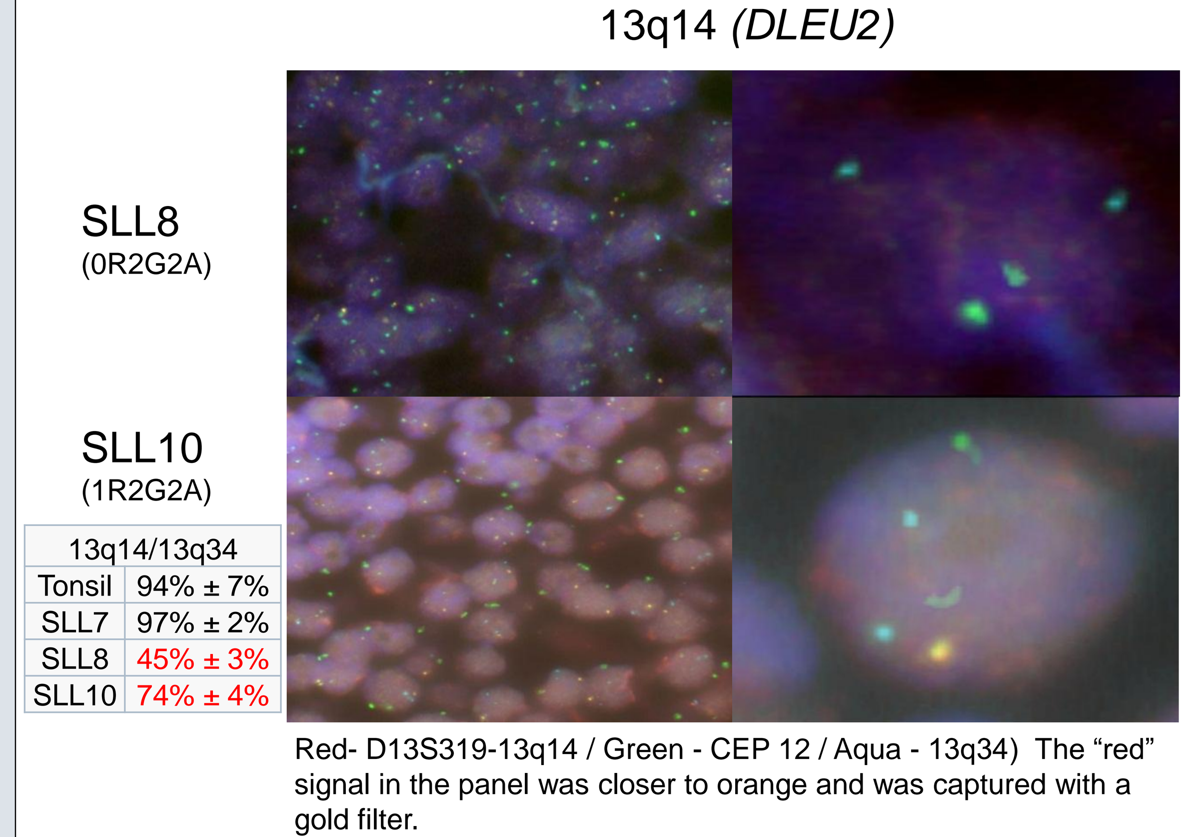
Confirmation by QPCR

Locus		SLL1	SLL3	SLL8	SLL9	SLL10
ATM	Intra	0.27				0.21
		0.32				0.33
	Inter	0.58				0.33
	Inter					0.34
TP53	Intra		0.46			
			0.53			
	Inter		0.4			
	Inter		0.56			
DLEU2	Intra			0.35	0.59	0.51
				0.34	0.6	0.65
					0.7	0.62
					0.66	0.55
	Inter			0.28	0.58	0.54
	Inter			0.29	0.6	
	Inter			0.29	0.69	

Based on the criteria stated above, we observed concordance between aCGH and QPCR. Each validation was run either two times within one experiment (intra) or independently (inter) to ensure data reproducibility.

Confirmation by FISH

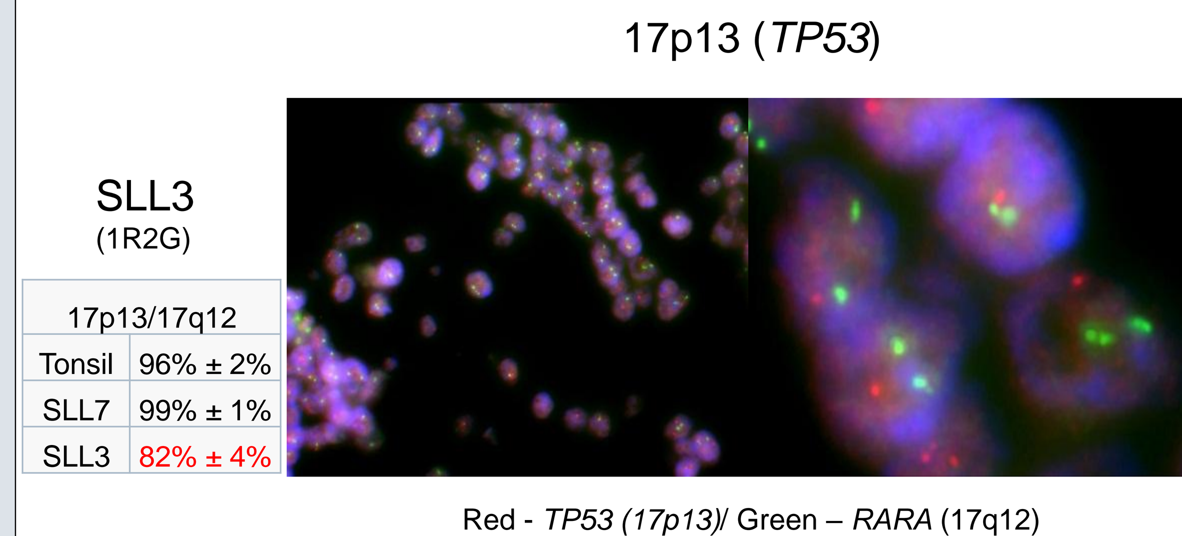
13q14 (*DLEU2*)



13q14/13q34	
Tonsil	94% ± 7%
SLL7	97% ± 2%
SLL8	45% ± 3%
SLL10	74% ± 4%

Red- D13S319-13q14 / Green - CEP 12 / Aqua - 13q34) The "red" signal in the panel was closer to orange and was captured with a gold filter.

17p13 (*TP53*)



17p13/17q12	
Tonsil	96% ± 2%
SLL7	99% ± 1%
SLL3	82% ± 4%

Red - *TP53* (17p13)/ Green – *RARA* (17q12)

A selected set of genomic aberrations detected in the 10 SLL FFPE samples were subjected to independent confirmation by FISH. For the three cases studied, we observed concordance between aCGH data and FISH results.

Conclusion

Using DNA extracted from FFPE samples, aCGH was carried out to detect aberrations in SLL patient genomes. The detected genomic aberrations are commonly found in CLL patients and they could be independently authenticated by QPCR and FISH. Commonly detected genomic aberrations in CLL /SLL have significant prognostic values and have been used together with other biomarkers such as *IGHV* mutation status to predict patient overall survival and time to first treatment length. Taken together, our data extended the usage of aCGH to the detection of genomic aberrations in SLL patient samples and suggested that aCGH can be used as a clinical diagnostic tool in SLL patient prognosis. We have implemented this procedure as a routine diagnostic method in our laboratory.