Array-based Quantitative Nuclease Protection Assay Can Reproducibly Identify Prognostic mRNA Biomarkers in Archival Mantle Cell Lymphoma Specimens

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Abstract

Background: Gene expression profiling has identified several potential prognostic biomarkers in mantle cell lymphoma (MCL), a B-cell lymphoma with a variable clinical course ranging from indolent to aggressive. However, technical limitations have hampered the translation of these findings into the clinical laboratory. Array based quantitative nuclease protection assay (qNPA) can assess gene expression in formalin-fixed paraffin-embedded (FFPE) tissue in a simple, robust manner and has potential for clinical assay development. We determined the ability of qNPA to identify prognostic mRNA biomarkers in routinely processed FFPE biopsies of MCL.

Design: Expression of 42 genes with potential prognostic significance was analyzed using qNPA in a plate based, low density format on FFPE tissue on a discovery cohort of 57 patients. Gene expression was normalized to two housekeeping genes, TBP and B2M. Cox proportional hazards models were used to assess association between individual gene expression and progression free survival (PFS). Results were validated on an independent cohort of 32 patients with a meta-analysis approach for combined results between discovery and replication cohorts.

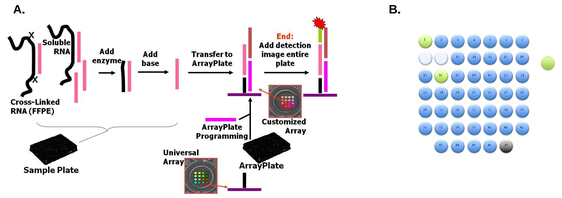
Results: Expression of the 42 genes on the panel distinguished MCL from control cases representing a variety proliferative indices using unsupervised clustering (Figure 1). 11 genes (26%) were associated with PFS (p<0.1) in the discovery cohort (Table 1). Three of these genes (MYC, HPRT1, CDKN2A) were prognostically significant in the validation cohort (p<0.05) and an additional 3 genes (TNFRSF10B, ASPM, and SOX11) were significant in the pooled meta-analysis.

Conclusions: Array based qNPA can be utilized to develop a prognostic MCL gene expression assay with potential for adoption by the clinical laboratory.

Table 1. Patient characteristics

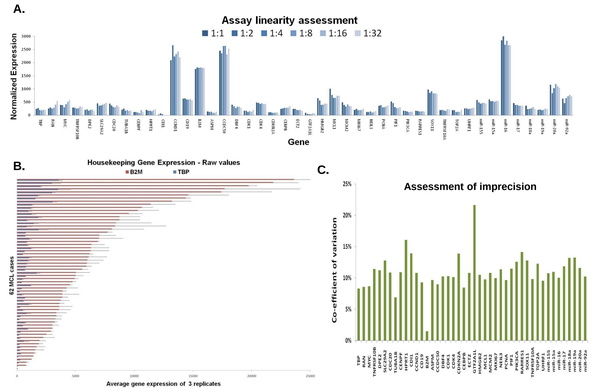
Characteristics of patients at diagnosis	Cohort 1 n = 60	Cohort 2 n = 32
Age (years)		
Median	62	63
Range	42 - 91	48 - 84
Gender		
Male	48 (80%)	27 (84%)
Female	12 (20%)	5 (16%)
ECOG Performance Status		
0-1	35 (80%)	32 (100%)
>=2	9 (20%)	0
Ann Arbor Stage		
I - II	6 (11%)	4 (13%)
III - IV	49 (89%)	28 (87%)
WBC count		
0 – 10 x 10^3/uL	41 (75%)	23 (82%)
>10 x 10^3/uL	14 (25%)	5 (18%)
LDH		
<= normal	26 (55%)	18 (69%)
> normal	21 (45%)	8 (31%)
MIPI risk		
Low-intermediate	33 (61%)	32 (100%)
High	21 (39%)	0
Treatment regimen		
Frontline Adriamycin-containing polychemotherapy	43/60 (73%)	19/32 (59%)

Figure 1. Gene expression analysis of archival formalin-fixed paraffin-embedded (FFPE) tissue by qNPA™ (High Throughput Genomics, Tucson, AZ)



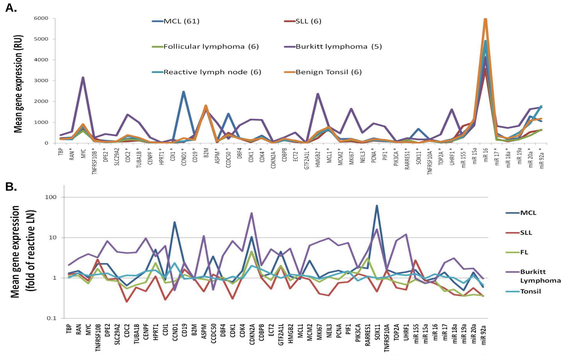
(**A**) FFPE sample is first dissolved in lysis buffer. 25-mer probes specific for the genes of interest are incubated with the samples, forming specific probe-mRNA duplexes and unhybridized probes were digested by subsequent addition of S1 nuclease. Then, alkaline hydrolysis destroys the mRNA in the duplexes, leaving intact probes in stoichiometric concentrations proportional to the amounts of specific mRNA originally present. Samples were transferred to ArrayPlates for probe detection. The ArrayPlate contains a universal array of 47 unique, covalently bound, 25-mer 'anchor' oligonucleotides spotted on a grid on the bottom of each well. The universal array is customized to bind 25-mer probes for the genes of interest at preselected positions by exposing the array to a mixture of programming linker oligonucleotides that contain a sequence to bind a specific probe at one end and an anchor at the other end. Finally, a mixture of detection linker oligos is added, followed by detection probe that contains bound HRP. Upon the addition of chemiluminescent peroxidase substrate, each array element gives off light proportional to the amount of sample probe bound at that position. **(B)** Graphic depiction of the array utilized to assess mRNA expression of 45 genes (including 2 housekeepers marked in green and negative control in black.

Figure 2. Cohort 1 qNPA™ assay characteristics



(A) Assessment of assay linearity was performed by serial dilution of 3 representative MCL cases and average R^2 for 44 genes was 0.992. Chart depicts normalized expression of each serial dilution for targeted genes of one representative MCL case. **(B)** Expression of the housekeeping genes TBP and B2M was adequately detected in 61 of 62 (98%) samples tested. **(C)** The co-efficient of variation (CV = SD / mean x 100%) was determined by analysis of each gene in triplicate for 90 cases including 61 MCL cases and 29 controls. Overall reproducibility was excellent with 42 of 44 genes (95%) having an average CV of less than 15%.

Figure 3. Cohort 1 gene expression patterns of MCL vs controls



(A) Graphic representation of average expression patterns of target genes between MCL and 5 controls. By Kruskal-Wallis, a significant difference (P<0.05) in gene expression distributions between the 6 diagnoses was found in 35 of 42 (83%) genes marked with *, but no significant difference was detected in the expression of the housekeepers TBP and B2M. **(B)** Target gene expression in MCL, SLL, FL, Burkitt lymphoma, and tonsil relative to reactive lymph node. Tonsil demonstrates a similar pattern of expression compared to reactive lymph node, while Burkitt lymphoma and SLL show relatively higher and lower expression of most genes respectively. MCL demonstrates distinct peaks in CCND1 and SOX11 relative to reactive lymph node.

Figure 4. Unsupervised clustering of gene expression from cohort 1 and controls

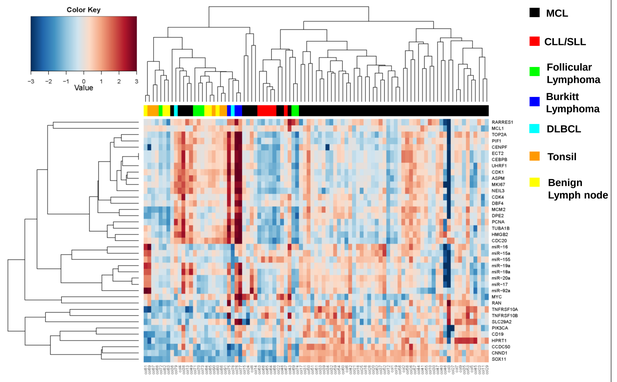


Table 2. Association of gene expression and clinical outcomes (PFS) in cohort 1 (test) and cohort 2 (validation)* by Cox proportional hazard model adjusted for MIPI.

	Cohort 1 (PFS)				Cohort 2 (PFS)				Cohort 1 and 2 pooled			
Gene	Hazard Ratio	Lower CI	Upper CI	p-value	Hazard Ratio	Lower CI	Upper CI	p-value	Hazard Ratio	Lower CI	Upper CI	p-value
MYC	1.92	1.17	3.16	***0.01***	3.18	1.44	7.01	***0.0041***	2.21	1.45	3.37	***2.13E-04***
TNFRSF10B	0.31	0.14	0.68	***<0.01***	0.58	0.17	1.99	0.39	0.37	0.19	0.72	***0.0036***
HPRT1	0.61	0.38	0.97	***0.04***	0.19	0.06	0.66	***0.0088***	0.52	0.34	0.81	***0.0036***
CD19	0.41	0.21	0.78	***0.01***	1.10	0.29	4.18	0.89	0.5	0.28	0.9	***0.020***
ASPM	1.53	1.00	2.33	***0.05***	1.48	0.57	3.80	0.42	1.52	1.03	2.24	***0.033***
CDKN2A	0.54	0.34	0.86	***0.01***	0.30	0.09	1.02	***0.055***	0.5	0.32	0.77	***0.0018***
SOX11	0.77	0.57	1.03	0.07	0.64	0.32	1.27	0.20	0.75	0.57	0.98	***0.036***
miR155	0.51	0.27	0.95	***0.03***	1.70	0.50	5.80	0.40	0.66	0.37	1.15	0.14
miR15a	0.45	0.25	0.81	***0.01***	1.01	0.32	3.21	0.99	0.53	0.31	0.9	0.018
miR16	0.42	0.18	0.97	***0.04***	1.23	0.41	3.71	0.71	0.62	0.32	1.22	0.17
miR20a	0.55	0.33	0.92	***0.02***	1.58	0.28	8.83	0.60	0.6	0.37	0.98	0.041

*** Test and validation cohorts were run at independent institutions utilizing the same qNPA array**

Conclusions

- qNPA can reliably and reproducibly assess the expression of specific genes from archival formalin fixed paraffin embedded MCL specimens
- For the targeted genes, distinct expression profiles can be identified for MCL, SLL, follicular lymphoma, Burkitt lymphoma, DLBCL, and benign lymphoid tissue
- A subset of prognostically significant genes can be validated by independent analysis performed on an independent cohort of MCL patients

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