**Plasma microRNA are disease response biomarkers in classical Hodgkin lymphoma**

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Running Title: Plasma miRNA disease response biomarkers in cHL

Key Words: miRNA, Hodgkin Lymphoma, biomarker, plasma

Financial support: Work in the Clinical Immunohaematology laboratory is sponsored by the National Health and Medical Research Council (Australia), Leukaemia Foundation (Australia), Cancer Council of Queensland and Queensland Office of Health and Medical Research. K.J and C.K. are supported by the Leukaemia Foundation (Australia), K.J. is also supported by QIMR, M.K.G. by the Cancer Council of Queensland and Queensland Health and Medical Research, and J.P.N. by the Cancer Cure Foundation.

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Conflict-of-interest disclosure: The authors report no potential conflict of interests.

Statement of translational relevance word count: 136; Abstract word count: 242; Text word count: 4,058; References: 50; Figures: 4; Tables: 2.
Statement of Translational Relevance

Optimal treatment for classical Hodgkin lymphoma (cHL) involves accurately identifying patients for risk-stratified therapy. Those with a rapid response to initial treatment may benefit from truncated treatment regimens, thus the need for more accurate measures of disease response. Cell-free microRNA (miRNA) are highly stable in blood, are overexpressed in cancer and are quantifiable within the diagnostic laboratory. Therefore they are a rational target for further investigation. We demonstrate that plasma miR-21, miR-494 and miR-1973 are promising disease response biomarkers in cHL. Furthermore, plasma miRNA biomarkers have distinct kinetics during therapy, with miR-494 and miR-1973 best reflecting interim therapy response. Circulating miRNA have the potential to greatly assist clinical decisions making and aid interpretation of PET/CT. A further advantage is they can also be performed at each consultation to assess disease response and detection of early relapse.
Abstract

Purpose: Although microRNA show potential as diagnostic biomarkers in cancer, their role as circulating cell-free disease response biomarkers remains unknown. Candidate circulating microRNA biomarkers for classical Hodgkin Lymphoma (cHL) might arise from Hodgkin-Reed-Sternberg (HRS) cells and/or non-malignant tumor-infiltrating cells. HRS-cells are sparse within the diseased node, embedded within a benign microenvironment, the composition of which is distinct from that seen in healthy lymph nodes.

Experimental Design: Microarray profiling of >1000 human microRNA in 14 cHL primary tissues and 8 healthy lymph nodes revealed a number of new disease node-associated microRNAs, including miR-494 and miR-1973. Using qRT-PCR, we tested the utility of these, as well as previously identified disease node-associated plasma microRNA (including miR-21 and miR-155), as disease response biomarkers in a prospective cohort of 42 cHL patients. Blood samples were taken in conjunction with radiological imaging at fixed time-points prior to, during and after therapy. Absolute quantification was used so as to facilitate implementation in diagnostic laboratories.

Results: Levels of miR-494, miR-1973 and miR-21 were higher in patients than control (n=20) plasma (P=0.004, P=0.007, P<0.0001, respectively). MiR-494 and miR-21 associated with Hasenclever scores ≥3. Strikingly, all three microRNA returned to normal at remission (P=0.0006, P=0.0002, P<0.0001 respectively). However, only miR-494 and miR-1973 reflected interim therapy response with reduction being more pronounced in patients achieving complete versus partial responses (P=0.043, P=0.0012, respectively).

Conclusion: Our results demonstrate that in patients with cHL, circulating cell-free microRNA can reflect disease response once therapy has commenced.
Introduction

Long-term disease control of classical Hodgkin Lymphoma (cHL) is relatively high (1). Thus the emerging issue is to minimize treatment related complications such as secondary cancer, cardiopulmonary complications, stroke and infertility (2, 3). Paradoxically, there remains a significant minority with refractory disease. In these patients prolonged exposure to first-line agents can induce chemo-resistance and unnecessary toxicity, and alternate rescue strategies should be instituted early. The challenge remains to accurately predict and monitor response to therapy, so that a risk-stratified approach can be commenced. Although positron emission tomography combined with computerized tomography (PET/CT) has a high negative predictive value, its positive predictive value is more modest (4). Furthermore, it is impractical to perform PET/CT prior to each follow-up visit. New approaches such as blood biomarkers, might assist interpretation of conventional measures of disease response, to better identify those that could be spared excessive treatment, and those where change in therapy should be expedited (5, 6). Circulating disease response biomarkers have the added advantage of being non-invasive and practical for frequent testing. Should easily measurable blood biomarkers be identified, they have the potential to assist therapeutic decision-making both when PET/CT is and isn’t available.

MicroRNA (miRNA) are small non-coding RNA molecules that play key regulatory roles in numerous biological processes, and are ubiquitously dysregulated in malignancies including lymphoma (7, 8). They are remarkably stable in blood, are resistant to multiple freeze-thaw cycles and are present in elevated levels within the cell-free compartment of a variety of cancers (9-12). Much circulating miRNA biomarker research has focused on diagnostic signatures, for use as non-invasive assays in situations where screening biomarkers would be clinically beneficial (e.g. prostate...
and lung cancer). In contrast the potential role of miRNA's as disease response cancer biomarkers has been relatively neglected. To our knowledge, of the few studies on elevated circulating miRNA in lymphoma, all have been in the setting of non-Hodgkin lymphoma prior to treatment. Lawrie et al. found serum levels of miR-155, miR-210 and miR-21 were higher in diffuse large B-cell lymphoma (DLBCL) patients than control sera, and high expression was associated with reduced relapse-free survival (8). Elevated serum levels of miR-155 in DLBCL were validated in a subsequent study (13) and circulating miR-221 was raised pre-therapy in extra nodal Natural Killer/T-cell Lymphoma (14). However, in these three studies no sequential samples were taken to investigate whether serial monitoring may have clinical utility as disease response biomarkers. To date, little published data exists on the relative kinetics of circulating microRNAs following the commencement of chemotherapy in any malignancy. Although circulating miRNA may be elevated pre-therapy, concerns remain that the indiscriminate cell lysis induced by chemotherapy may cause non-specific elevation of miRNA and hence preclude their value as disease response biomarkers.

In cHL, the malignant Hodgkin–Reed–Sternberg (HRS) cells are scant within the diseased node, surrounded by a benign microenvironment composed primarily of lymphocytes and macrophages (15, 16). These tumor-infiltrating cells are known to have a functional role in the pathology of cHL but may also be important as biomarkers of disease. Gene-expression profiling of the diseased node in cHL has recently been shown to predict overall survival (17). We recently demonstrated that circulating cell-free biomarkers of both tumor-infiltrating cells (CD163) and HRS-cells (TARC and EBV-DNA) reflect disease response in cHL, but that relative to each other, HRS and tumor-infiltrate associated protein biomarkers had distinct kinetics following initiation of therapy (5).
Investigators had previously profiled up to 360 miRNAs in HRS cell-lines and/or microdissected primary HRS cells (18, 19). In these studies, several miRNA (including miR-21, miR-155, miR-16) had been identified as being preferentially over-expressed in HRS cells as compared to transformed B-cells or Burkitt Lymphoma cell-lines. However, by in-situ hybridization, these miRNA are observed within both HRS and non-malignant tumor infiltrating cells including lymphocytes and macrophages (20). Therefore they are best deemed disease node-associated and not cHL specific. Circulating cell-free miRNA originating from HRS cells hold the promise of high specificity, whereas those from the microenvironment may be more sensitive owing to the relative abundance of stroma. Other factors which will influence circulating miRNA levels include ability for the miRNA to be released into the circulation.

In this study, microarray profiling of >1000 human miRNA in a discovery cohort of 14 cHL primary tissue and 8 healthy lymph nodes revealed new disease node-associated miRNA, including miR-494 and miR-1973. We tested the utility of plasma miR-494 and miR-1973 as well as miR-155, miR-21 and miR-16 as disease response biomarkers in an independent prospective cohort of 42 cHL patients and 20 healthy participants. Blood samples were taken at fixed time-points prior to, during and after therapy. Results were compared with disease response at each time-point.
Materials and methods:

Patients

Forty-two newly diagnosed cHL patients were enrolled, with exclusion criteria limited to HIV positivity, active Hepatitis B or C infection. All patients were enrolled before commencement of therapy and serial blood samples were taken at three time-points: pre-therapy, immediately pre-third therapy and six months post-therapy. Plasma was cryopreserved, thawed and tested in batches as previously outlined (21). Tissues from diagnostic tumor biopsies were tested when available. Clinical parameters including the Hasenclever prognostic score were prospectively recorded (22, 23). Early stage disease was defined according to the Southwest Oncology Group (SWOG) and Cancer and Leukemia Group B (CALGB) previously published definition (Ann Arbor stage I or II without any B symptoms, infradiaphragmatic presentations or mediastinal masses greater than one third the maximum thoracic diameter) (24).

This was a multi-center, Australia-wide, observational study conducted under the auspices of the Australasian Leukaemia and Lymphoma Group (ALLG). Therapy was applied as per clinician’s preference (Table 1). The majority of our patient cohort (85%) was treated with ‘ABVD’ [adriamycin, bleomycin, vinblastine, dacarbazine] combination chemotherapy (25). Other regimens used were ‘BEACOPP’ [bleomycin, etoposide, adriamycin, cyclophosphamide, procarbazine and prednisolone] (26), ABVD followed by BEACOPP and ‘ChlVPP’ [chlorambucil, procarbazine, prednisolone and vinblastine] (27).

Initial staging and re-staging one month after completion of therapy was by PET and CT scans. Interim disease response (generally after the third cycle of therapy), and re-staging after completion of therapy was assessed by CT, typically in combination with PET (70% had interim treatment restaging PET). Complete and partial response
(CR and PR) were defined as per the International Harmonization response criteria (28), or when applicable (i.e. patients that had interim treatment CT scans only) the International Working Group response criteria (29).

Twenty healthy age and sex matched participant plasma samples were also used. In addition, a separate retrospective cohort of 14 cHL tissues were obtained form a previous ALLG study and 8 non-malignant lymph node tissues (uninvolved auxiliary node dissections from patients with breast cancer) (30). This study conformed to the Declaration of Helsinki and written informed consent was provided by all participants and was approved by all participating hospitals/research institute Human Research Ethics Committees.

**MiRNA extraction and qRT-PCR**

Tissue miRNA was extracted from all available formalin-fixed, paraffin-embedded (FFPE) tumor biopsies using RecoverAll™ Total Nucleic Acid Isolation kit (Ambion). Plasma miRNA was extracted from plasma (600ul) using mirVana™ Paris™ kit (Ambion) and DNase I treated using TURBO DNA-free™ kit (Ambion). As previously described, *C. elegans* miR-39 synthetic oligonucleotide RNA (25fmol in a 5ul total volume) was added to the 600ul of plasma after addition of denaturing solution to control for extraction efficiency and all subsequent procedural steps including reverse transcription and qRT-PCR amplification (10). All kits were used as per manufacturer’s instructions.

**MiRNA microarray**

Over 1000 human miRNA were quantified by the Ramaciotti Centre (Sydney, Australia) using miRNA Microarray (Agilent Technologies, version 16.0, Gene
Expression Ominubus Accession: GSE45264. This array tested for numerous miRNA that had not previously been examined in cHL. Assays were performed on 14 cHL diagnostic biopsy tissue (8 nodular sclerosing, 6 mixed cellularity) and 8 non-malignant lymph node biopsies. Expression data was quantile normalized using Genespring GX software and then analyzed using Genepattern software (Broad Institute).

MiRNA quantification by qRT-PCR

Using the Qiagen miScript PCR system, including miScript reverse transcription kit, SYBR® Green and universal primer, plasma and tissue miRNA were quantified on a Rotorgene 3000 qRT-PCR (Corbett Research). Each reaction contained the equivalent of either 3ng tissue RNA or 0.2ul plasma cDNA, all run in duplicate 20ul reactions. Two Qiagen miScript primers were used: *C. Elegans* miR-39 (cel-miR-39) and miR-16. In-house primers were used for miR-21 (5’-CGTAGCTTATCAGACTGATGTTGA A-3’), miR-155 (5’-TTAATGCTAATCGTGATAGG GGTAA-3’), miR-494 (5’-GAAACATACACGGGAAACCTCAAA-3’), miR-638 (5’-CGGGTGCGGGCCGCTAATAA-3’), miR-1976 (5’-ACCGTGCAAAGGTAGCATAAA-3’). Initially, comparative quantification was used to determine relative quantities of miRNA. Two standards, one for tissue and one for plasma, were prepared on mass and stored in aliquots to avoid freeze/thawing (31). The same peripheral blood mononuclear cells (PBMC) cDNA were used for both standards, with the plasma standard at a 2-fold dilution and containing the spike-in control cel-miR-39 cDNA (10). Absolute quantification using standard curves was done on select miRNA. For this, standard curves were made from Qiagen miScript reverse transcribed RNA oligonucleotides (Sigma-Aldrich) specific for each miRNA of interest as well as cel-miR-39 and U6. Results are reported as miRNA copy number per ul of
plasma, calculated based on the known copy number of cel-miR-39 spike-in per plasma volume (25fmol per 600ul plasma is equivalent to $2.508 \times 10^7$ copies/μl of plasma).

**EBV-tissue positivity, plasma EBV-DNA and human genomic DNA quantification**

EBV-tissue positivity was determined by EBV encoded RNA *in situ* hybridization (EBER-ISH) in conjunction with hematoxylin and eosin staining (32). EBV-DNA (BALF5) and human genomic DNA (Albumin) were quantified in plasma by qRT-PCR as previously described (21). A threshold of 200 EBV genomes/ml was used.

**Statistics**

Microarray data was quantile normalized using Genespring GX and analyzed using GenePattern (Broad Institute). Comparative marker selection analysis was performed using GenePattern to identify significantly different microRNAs between Hodgkin and healthy lymph nodes. Wilcoxon matched-pairs signed rank T-tests were used to compare all matched samples. This includes all analysis comparing miRNA levels between different time-points. Otherwise the Mann Whitney T-test was used. Correlations were determined using the Spearman test. Receiver Operating Characteristic (ROC) curve analysis was used to determine sensitivity and specificity. Statistical analysis was performed using Graphpad Prism 5.0 (Graphpad Software Inc, California).
Results

Patient Characteristics

Forty-two cHL patients were accrued (mean age: 36 years, range: 18-79; female: male ratio 20:22). Patient characteristics are provided in Table 1. Interim therapy (immediately prior to third therapy) samples were available for 38 of these patients and post-therapy samples were available for 37. Of these 37 cHL patients, 32 achieved CR by the six months post-therapy time-point. Matching biopsy tissue was available for 26 cHL patients. As controls, 20 healthy participant blood samples were used (mean age: 42 years, range: 22-68; female: male ratio 8:12). In addition, an independent miRNA discovery cohort of 14 cHL diagnostic biopsy (6 mixed cellularity and 8 nodular sclerosing) and 8 non-malignant lymph node tissues were used.

Differential expression of human miRNA in cHL primary tissue compared to normal lymph nodes by microarray

Over one thousand human miRNA were quantified in our discovery cohort of 14 cHL patient biopsy tissue and 8 non-malignant lymph nodes using Agilent microarray, version 16. The data was quantile normalized using Genespring GX software, analyzed using GenePattern (Broad Institute) and ranked based on comparative marker selection analysis. Comparing cHL from normal lymph node tissue, there were 474 differentially expressed human miRNA (false discover rate, FDR, <5%), 238 of these had elevated expression in cHL. Figure 1A shows unsupervised clustering of the top 50 differentially expressed human miRNA. From the ranked data, we selected the top five miRNA with elevated expression in cHL tissue for further analysis by qRT-PCR (miR-2861, miR-638, miR-494, miR-663b and miR-1973, P<0.003, FDR <0.0048). In addition to these five miRNA, we also selected miR-155, miR-21 and miR-16, known to be over-expressed in
HRS cells and to have a functional role in lymphomagenesis (18-20, 33, 34). MiR-16 has also been used as a reference miRNA in previous studies (8). None of these three miRNA were significantly elevated in cHL nodes compared to healthy nodes by our microarray analysis.

**qRT-PCR tissue miRNA analysis correlates with microarray results**

Using comparative quantification qRT-PCR and adjusting to the levels of the house-keeping small RNA U6, we quantified miR-2861, miR-638, miR-494 and miR-1973, as well as miR-155, miR-21 and miR-16 in 14 cHL biopsy tissue and 8 normal lymph nodes. MiR-663b was dropped from the analysis, as we were unable to amplify it by qRT-PCR with high-specificity. The qRT-PCR results correlated with matched microarray results for all seven miRNA (Spearman r 0.64-0.89, all P-values <0.001), validating our qRT-PCR technique. We then quantified these seven miRNA in 26 cHL tissue from the prospective cHL cohort. As shown in Figure 1 and consistent with the microarray results, miR-494 (P=0.0001, Fig. 1B), miR-1973 (P=0.0035, Fig. 1C) miR-2861 (P=0.0002, Fig. 1D), and miR-638 (P=0.0027, Fig. 1E) were significantly elevated in these cHL tissues compared to controls and miR-155 (Fig. 1G) was not. However, both miR-21 (Fig. 1F) and miR-16 were elevated above normal lymph node levels, although only miR-16 reached significance (P=0.0247, Figure 1H), which was not observed by microarray in the discovery cohort.

**Circulating miRNA are elevated in plasma of patients with cHL at diagnosis and are associated with Hasenclever Score**

Plasma miRNA were quantified in all pre-therapy cHL patient samples and in healthy participant plasma by comparative quantification qRT-PCR (miR-2861, miR-
638, miR-494, miR-1973, miR-155, miR-21 and miR-16, as well as controls U6 and cel-
miR39). In this analysis of plasma miRNA, results were normalized to the spike-in
control cel-miR-39 (but not U6). Five of the seven miRNA were significantly elevated in
CHL pre-therapy plasma compared to healthy participants (miR-494 P= 0.0041, miR-
1973 P= 0.0144, miR-155 P= 0.0025, miR-21 P<0.0001, miR-16 P=0.0007; Fig. S1). The
small RNA U6 was also elevated (P= 0.0117).

Pre-therapy levels of miRNA were analyzed for associations with all clinical
characteristics listed in Table 1. Interestingly, Hasenclever scores ≥3 were associated
with increased levels of mir-494 (P= 0.031), miR-2861 (P= 0.034), miR-21 (P= 0.007),
miR-155 (P= 0.031), and miR-16 (P= 0.044). Lactate dehydrogenase (LDH) levels above
the normal range were associated with higher levels of miR-494 (P= 0.023) and miR-21
(P= 0.020) while patients with leucocytes ≥15x10⁹/L had increased levels of miR-21 (P=
0.001), miR-155 (P= 0.004), and miR-16 (P= 0.006). Patients with Ann Arbor stage ≥3
were also associated with increased levels of miR-494 (P=0.0368). No correlation was
found between pre-therapy plasma miRNA levels and the 26 matched biopsy tissue
miRNA levels. In our previous study we found an association between circulating CD163
and EBV (5, 35). In contrast, in this study we found no association between any
circulating miRNA and EBER-ISH status or (in those with EBV-related cHL) with plasma
EBV-DNA.

**MiR-494, miR-1973, and miR-21 are biomarkers of disease response in cHL.**

We then performed a match-paired analysis of pre-therapy, interim therapy and
post-therapy samples in cHL patients in CR at the six month post-therapy time-point.
Strikingly miR-494, miR-1973, and miR-21 significantly differentiated diseased pre-therapy
plasma from matched 6 month CR plasma (P=0.0082, P=0.0003 and P<0.0001, respectively,
Supplementary Fig. S1). The 6 month CR plasma levels were equivalent with healthy controls. Plasma miR-16 CR post-therapy levels also significantly decreased compared to pre-therapy (P=0.0314), however these remained elevated compared to healthy control levels. No other miRNAs reflected disease response.

In order to quantify exact copy number per volume of plasma, consistent with standard practice for circulating cell-free protein (5, 36) and DNA (21, 37, 38) biomarker studies, we next determined the absolute quantities of circulating miR-494, miR-1973, miR-21, miR-16, U6 and spike-in cel-miR-39. Using reverse-transcribed miRNA oligonucleotide standard curves and known spike-in cel-miR-39 copy numbers, results were calculated to copies per μl plasma. As it has been shown to be dysregulated in HRS cells, absolute quantities of miR-155 were also determined, however, as with the relative quantification, levels remained elevated throughout therapy, and thus did not reflect disease response (data not shown). The relative and absolute quantification values highly correlated for all miRNA at all time-points (r=0.87-0.94, p<0.001) with absolute values having similar significance between all pre-therapy patients with cHL and healthy controls (mir-494 P=0.004, miR-1973 P=0.007, miR-21 P<0.0001, miR-16 P=0.0107; Supplementary Fig. S2) and in match-paired analysis of pre-therapy compared to 6 month post-therapy complete remission levels (miR-494 P=0.0006, miR-1973 P=0.0002, miR-21 P<0.0001, miR-16 P=0.0101; Fig. 3A-C). We performed receiver operating curve (ROC) analysis to determine the sensitivity and specificity at defined time-points. Although ROC analysis of miR-16 showed it significantly delineated pre-therapy cHL from healthy participants (AUC 0.70, P=0.011, 95% C.I. 0.5693 to 0.8355), it did not reach significance and had poor specificity and sensitivity for distinguishing pre-therapy from remission samples at six months post-therapy and was excluded from further analysis as a potential disease response biomarker. In contrast, we found that
miR-494, miR-1973, and miR-21 are sensitive and specific markers for delineating pre-therapy cHL from healthy participants (miR-494: AUC 0.73, P=0.004, 95% CI 0.60 to 0.85; miR-1973: AUC 0.71, P=0.007, 95% CI 0.57 to 0.85; miR-21: AUC 0.92, P<0.0001, 95% CI 0.84 to 0.99; Fig. 2A-C) and pre-therapy from remission samples at six months post-therapy (miR-494: AUC 0.65, P=0.037, 95% CI 0.52 to 0.77; miR-1973: AUC 0.75, P=0.0004, 95% CI 0.63 to 0.86; miR-21: AUC 0.86, P<0.0001, 95% CI 0.77 to 0.95; Fig. 2D-F). In order to maximize both sensitivity and specificity compared to healthy controls, cut-off values for miR-494, miR-1973 and miR-21 were defined as follows: 3.0x10^5 miR-494 copies/ul plasma with 85% sensitivity and 60% specificity, 1.6x10^6 miR-1973 copies/ul plasma with 75% sensitivity and 67% specificity, 1.0x10^6 miR-21 copies/ul plasma with 95% sensitivity and 86% specificity. The associations with clinical prognosticators and pre-therapy absolute levels of miR-494, miR-1973 and miR-21 are shown in Table 2.

The time range of sample processing from venipuncture was 4-36 hours in our samples (typically 18-24 hours). The risk of hemolysis increases the longer the time from collection. Hemolysis has been shown to greatly increase circulating levels of some miRNA but not others (39, 40). In order to evaluate this in our miRNAs of interest, we conducted a time-course analysis on two blood samples; a patient with advanced cHL with blood taken at an interim therapy time-point and a healthy control. Plasma was collected from the blood sample at 4 time-points from venipuncture (0h, 18h, 24h and 40 hours). All miRNA evaluated (miR-494, miR-1973, miR-21, miR-16 and U6) remained constant over time (Supplementary Fig. S3).

**Varying kinetics of plasma miRNA biomarkers**

Figure 3A-C illustrates the differing kinetics of miR-494, miR-1973, and miR-21
throughout therapy. In patients who achieved CR by six-months post-therapy, both miR-494 and miR-1973 drop to levels equivalent with healthy controls by the interim time-point. In contrast, miR-21 interim therapy levels remain equivalent to pre-therapy and elevated compared to healthy controls, dropping to normal levels by six months post-therapy. In order to determine how the different interim kinetics of miR-494, miR-1973, or miR-21 associate with interim therapy response, we compared pre-therapy samples with paired interim samples delineated as either CR or PR by radiological assessment, using match-paired analysis. Interestingly, for both miR-494 and miR-1973 there was no significant difference between pre-therapy samples and those in PR at the interim time-point. However, there was a significant difference for miR-494 and miR-1973 between pre-therapy and those in CR at the interim time-point (P=0.0438, P=0.0012, Fig. 3D,E). In contrast, pre-therapy miR-21 levels were equivalent to both interim PR and CR levels (Fig. 3F). Of the patients who attained CR at the interim time-point, only one patient relapsed by six months post-therapy. This patient had elevated miR494 levels (above the cut-off) at interim whereas miR-1973 and miR-21 were not elevated. Interestingly, four of the patients in PR at the interim time-point had relapsed/refractory disease post-therapy. Of these, miR-494 and miR1973 levels were elevated in one patient, while three had elevated miR-21 levels.

Interestingly, miR-494 strongly correlated with miR-1973 pre, interim and post-therapy (r=0.77, P<0.0001; r=0.62, P=0.0002; r=0.52, P=0.0002, respectively) while miR-21 had no correlation to these miRNA at any time-point. However, miR-21 did strongly correlate with miR-155 and miR-16 pre-therapy (r=0.72, P<0.0001 and r=0.76, P<0.0001 respectively).

Of those patients who achieved CR post-therapy, 65% of patients had miR-494 levels above the cut-off pre-therapy, 64% had interim levels lower than pre-therapy and 76%
had post-therapy levels lower than pre-therapy. Both interim therapy and post-therapy levels were lower than pre-therapy in 55%. A reduction in levels from pre-therapy to interim, and interim to post-therapy was observed in 26%. For miR1973, 65% of patients had levels above the cut-off pre-therapy, 67% of patients had interim levels lower than pre-therapy and 85% had post-therapy levels lower than pre-therapy. Both interim therapy and post-therapy levels were lower than pre-therapy in 61%. A reduction in levels from pre-therapy to interim, and interim to post-therapy was observed in 19%. For miR21, 84% of patients had levels above the cut-off pre-therapy, 42% of patients had interim levels lower than pre-therapy and 94% had post-therapy levels lower than pre-therapy. Both interim therapy and post-therapy levels were lower than pre-therapy in 42%. A reduction in levels from pre-therapy to interim, and interim to post-therapy was observed in 39%.

**Circulating miRNA relative to cellular RNA**

Plasma levels of the small RNA U6 may be used to represent levels of cellular RNA in the plasma. However, there is no current consensus on the use of U6 as a reference gene for qRT-PCR miRNA analysis. Plasma U6 levels were elevated pre-therapy compared to healthy controls (P<0.0001, Fig. 4A) and remained elevated throughout therapy despite patients achieving CR. Pre-therapy U6 levels were strongly associated with LDH levels greater than 250U/L (P=0.0003). To examine this further, we compared U6 with cell-free human genomic DNA levels (Albumin DNA, Fig. 4B). Notably values were correlated (r=0.6, p<0.0001). When we analyzed our miRNA results relative to U6, we found that the significantly increased levels of this non-specific cellular RNA, neutralized the elevated levels of plasma miRNA in pre-therapy samples compared to controls. However, reporting our patient results relative to U6 enhanced the decrease of miR-494, miR-1973, and miR-21 levels interim therapy in
match-paired analysis (Fig. 4 C-E; pre vs. interim therapy: miR494 $P<0.0001$, miR1973 $P=0.0007$, miR21 $P=0.0063$).
Discussion

We present the first study of circulating cell-free miRNA as disease response biomarkers in any lymphoma. Seven cHL-associated miRNA were tested for utility as disease response biomarkers in a prospective cohort of cHL patients. Following testing in serial plasma samples, in comparison with healthy samples and with reference to radiological assessment, we assessed the kinetics of these miRNA during therapy. We found three miRNA (miR-494, miR-1973 and miR-21) showed promise as disease response biomarkers. In contrast to the majority of circulating miRNA studies in cancer, the focus of this study was evaluation of circulating markers of disease response and not prognosis. However it is interesting that miR-494 and miR-21 both associated with Hasenclever score ≥3. Future studies should test for the ability of circulating miRNA to serve as biological prognosticators in cHL.

We hypothesized that elevated miRNA in the diseased tissue are elevated in plasma and that this level would normalize once disease resolved. Alternatively, it is possible that underrepresented miRNA in the diseased tissue may be reflected by decreased levels in the plasma of cHL patients. Further studies are required to evaluate underrepresented miRNA. We selected five novel miRNA that we identified as over-expressed, in the diseased node relative to healthy nodes. The miRNA were evaluated in unison with previously identified cHL-associated miRNAs. Of these, levels of miR-494, miR-1973 and miR-21 miRNA were higher in patients than healthy control participants’ plasma and all three miRNA returned to normal at remission. Initially, comparative quantification was used to identify promising miRNA biomarkers. Once identified, absolute quantification was used to determine exact miRNA copy number per volume of plasma. Reporting results in absolute terms will be critical for inter-laboratory comparisons and standardization, both important for this test to be implemented in the clinical setting. Normalizing
results to copy number per volume of plasma/serum, and not a housekeeper, is consistent with standard practice for circulating cell-free DNA (21, 37, 38), protein (5, 36) and numerous miRNA studies (10, 41). MiR-494 and miR-1973 levels were strongly correlated with each other (but not with miR-21) and both reflected interim therapy response with reduction being more pronounced in patients achieving complete versus partial responses. By contrast, miR-21 showed no relationship to radiological response during therapy.

No correlation was found between pre-therapy plasma miRNA levels and matched biopsy tissue miRNA. Similarly, we previously found no correlation between circulating cell-free TARC and CD163 and tissue expression by qRT-PCR (5). There are two likely explanations: firstly that circulating levels only partially reflect the disease node, with contribution also coming from non-diseased tissue. Secondly, qRT-PCR measures tissue expression within a given volume of diseased tissue but does not factor the total volume of disease (tumor burden). The only work to date accurately assessing tumor burden was in a single centre study of 60 cHL patients (6). This showed that pre-therapy plasma TARC levels directly correlated with the metabolic tumour volume. Future studies are required to determine whether circulating plasma miRNA also correlate with metabolic tumor volume.

There is currently no consensus on a reference gene for circulating miRNA. MiR-16 has been used, however, some studies found it to be inconsistent (8, 9, 42). Circulating miR-16 levels need to be interpreted with caution as miR-16 is highly expressed in red blood cells and hemolysis increases miR-16 plasma levels by up to 30-fold (39, 40, 43). Unsurprisingly, given that we confirmed that miR-16 was disease node-associated, we found miR-16 to be an inappropriate cell-free house-keeping gene for cHL. MiR-16 values were significantly elevated pre-therapy compared to healthy control participants and gradually declined to normal levels by six months post-therapy.
Within the field, an exogenous technical control for RNA extraction efficiency is frequently utilized. We selected cel-miR-39 based on published literature that normalizing to the mean of 3 exogenous miRNAs did not improve precision, as compared with normalizing to cel-miR-39 alone (43). The small RNA U6 has also been advocated as a reference for miRNA. However, in our study pre-therapy U6 levels were strongly associated with LDH and correlated with cell-free albumin DNA levels. We have previously shown the latter is elevated at pre-therapy in lymphomas, but remains elevated during and following therapy (21). Thus our results imply that U6, as with albumin-DNA, appears to be a marker of cell-integrity and is unsuitable as a house-keeper in comparisons between cHL patients and healthy individuals. It’s use in patients with known cHL remains uncertain. Notably in our cohort, the kinetics of U6 normalized assays were similar, although interim levels decreased more rapidly when this normalization was performed.

Relative to miR-21, both miR-494 and miR-1973 appear to have a relatively restricted tissue distribution. MiR-1973 is newly identified and has no known validated targets, but is expressed in B-cell acute lymphoblastic leukemia cells (44). MiR-494 is over-expressed in follicular lymphoma tissue and functionally contributes to cancer persistence (45-47). Specifically, miR-494 is implicated in chemo-resistance and is required for the accumulation and function of tumor-expanded granulocytic and monocytic myeloid-derived suppressor cells (46, 47). Interestingly, CD163+ M2 macrophages are enriched within the microenvironment of the cHL diseased node and we have shown CD163+ monocytes are elevated in the peripheral blood of patients with cHL (5, 48). MiR-21 is ubiquitously expressed in a variety of cell-types and miR-155 is known to be up-regulated in hematopoietic cells (49). Both miR-21 and miR-155 are dysregulated in a variety of cancers, however, in a study of miRNA expression in solid
tumors, only miR-21 was up-regulated in all cancers evaluated (34). Both miR-155 and miR-21 are involved in B-cell activation and, in two separate studies, induction of miR-21 and miR-155 in mouse-models resulted in lymphoma development (50-53). We confirm the findings of Navarro and colleagues that miR-155 was not over-expressed in cHL patient versus healthy nodes (20). In contrast to that group, we did not find miR-21 was elevated (values were approximately two-fold higher in cHL nodes but this did not reach significance).

Given the unique nature of cHL nodes and the numerous cell-types that express miRNA, it must be emphasized that miRNA tumor specificity is not absolute, and is more accurately described as a spectrum. HRS cells and the microenvironment represent different aspects of cHL biology, which is reflected in the relative distribution of miRNA within cell-types. Consistent with this and with our previous study of serum proteins in cHL (5), the three miRNA tested had distinct kinetics following initiation of therapy. As with circulating cell-free CD163 and TARC, it is likely that disease response is best served by analyzing multiple miRNA simultaneously. Future risk-adapted treatment algorithms combining circulating protein and miRNA biomarkers with interim-PET/CT should be evaluated.
Acknowledgements

This was an Australasian Leukaemia and Lymphoma Group sponsored study. The authors would like to thank Susan Arnold for her assistance with data collection.

Grant Support

Work in the Clinical Immunohaematology laboratory is sponsored by the National Health and Medical Research Council (Australia), Leukaemia Foundation (Australia), Cancer Council of Queensland and Queensland Office of Health and Medical Research. K.J and C.K. are supported by the Leukaemia Foundation (Australia), K.J. is also supported by QIMR, M.K.G. by the Cancer Council of Queensland and Queensland Health and Medical Research, and J.P.N. by the Cancer Cure Foundation.


Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Patient Characteristic</th>
<th>No. Patients (Total N=42)</th>
<th>% Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age median (range)</td>
<td>35.6 (18-79)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>22</td>
<td>52%</td>
</tr>
<tr>
<td>Ann Arbor Stage III-IV</td>
<td>21</td>
<td>50%</td>
</tr>
<tr>
<td>Serum Albumin, less than 40g/L</td>
<td>30</td>
<td>71%</td>
</tr>
<tr>
<td>LDH, greater than 250U/L</td>
<td>24</td>
<td>57%</td>
</tr>
<tr>
<td>Hemoglobin, less than 105g/L</td>
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<td>21%</td>
</tr>
<tr>
<td>Lymphocyte Count, at least 0.6x10^9/L</td>
<td>38</td>
<td>90%</td>
</tr>
<tr>
<td>White blood cell count, at least 15x10^9/L</td>
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<td>17%</td>
</tr>
<tr>
<td>Hasenclever Score, at least 3</td>
<td>15</td>
<td>36%</td>
</tr>
<tr>
<td>B symptoms</td>
<td>16</td>
<td>38%</td>
</tr>
<tr>
<td>EBER-ISH Positive</td>
<td>12</td>
<td>28%</td>
</tr>
<tr>
<td>EBER-ISH Unavailable</td>
<td>3</td>
<td>7%</td>
</tr>
<tr>
<td>Early stage</td>
<td>9</td>
<td>21%</td>
</tr>
<tr>
<td>Advanced stage</td>
<td>33</td>
<td>79%</td>
</tr>
<tr>
<td>Histological Subtype</td>
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<td></td>
</tr>
<tr>
<td>Nodular Sclerosing</td>
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<td>62%</td>
</tr>
<tr>
<td>Mixed cellularity</td>
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<tr>
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<tr>
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<td>16%</td>
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<tr>
<td>Treatment</td>
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<td></td>
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<tr>
<td>ABVD</td>
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</tr>
<tr>
<td>BEACOPP</td>
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<tr>
<td>ABVD + BEACOPP</td>
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</tr>
<tr>
<td>ChlVPP</td>
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<td>5%</td>
</tr>
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</table>
Table 2. Associations with clinical prognosticators and pre-therapy absolute levels of miR-494, miR-1973 and miR-21

<table>
<thead>
<tr>
<th>Patient Characteristic</th>
<th>miR-494 P-value (Mean± SEM)</th>
<th>miR-1973 P-value (Mean± SEM)</th>
<th>miR-21 P-value (Mean± SEM)</th>
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<tbody>
<tr>
<td>Age Less than 45 years</td>
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<td>NS</td>
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<td>Gender</td>
<td>NS</td>
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<td>Ann Arbor Stage</td>
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<td>I-II (5.8x10^5±1.4x10^5)</td>
<td>NS</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>III-IV (1.2x10^6±2.7x10^5)</td>
<td>NS</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Serum Albumin Less than 40g/L</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Lactate Dehydrogenase</td>
<td>0.0214</td>
<td>NS</td>
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<td>At most 250U/L</td>
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<td>(2.2x10^5±9.7x10^5)</td>
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<tr>
<td>Greater than 250U/L</td>
<td>(1.2x10^6±2.4x10^5)</td>
<td>(3.1x10^6±7.4x10^5)</td>
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<tr>
<td>Hemoglobin Less than 105g/L</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphocyte Count</td>
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<td>NS</td>
<td>NS</td>
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<td>NS</td>
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<td>NS</td>
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<tr>
<td>At least 15x10^9/L</td>
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<td>(7.4x10^6±2.9x10^6)</td>
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<td>Hasenclever Score</td>
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<td>Less than 3</td>
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<td>At least 3</td>
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<td>(4.7x10^6±1.5x10^6)</td>
<td></td>
</tr>
<tr>
<td>B symptoms</td>
<td>NS</td>
<td>NS</td>
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<td>Yes vs. No</td>
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<tr>
<td>Nodular sclerosing vs.</td>
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<td>NS</td>
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</tr>
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<td>other specified</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>EBER-ISH</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Negative vs. Positive</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma EBV-DNA†</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>&lt;200 genomes/ml</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

SEM: standard error of the mean; Mean± SEM values are given as copies/μl of plasma; NS: not significant; Other specified: mixed cellularity, lymphocyte-rich, and lymphocyte-depleted cHL subtypes. †In patients with EBV-related cHL.
Figure Legends

Figure 1. Differential expression of human miRNA in cHL primary tissue.

(A) Unsupervised clustering of the top 50 differentially expressed human miRNA by microarray in a discovery cohort of 14 cHL diseased nodes (MC: mixed cellularity, NS: nodular sclerosing) versus 8 non-malignant lymph nodes (LN). Red denotes high expression. (B-H) Comparison of miRNA expression in prospective cohort of 27 cHL diseased nodes versus 8 non-malignant lymph nodes by qRT-PCR. Error bars represent mean with SEM.

Figure 2. Plasma miR-494, miR-1973 and miR-21 are disease response biomarkers in cHL. (A-C) Receiver Operating Characteristic (ROC) plots demonstrate high sensitivity and specificity of pre-therapy cHL versus healthy participant; (D-F) pre-therapy cHL versus CR six months post-therapy; AUC, area under the curve.

Figure 3. Kinetics of circulating miRNA disease response biomarkers.

Results are reported by absolute quantification as copies/ul of plasma. Error bars represent mean with SEM. (A-C) Plasma miRNA levels throughout therapy in cHL patients in CR at six months post-therapy. (D-F) Comparison of interim therapy treatment response. cHL patients, restricted to those with paired interim samples that matched interim radiological assessment. Lines identify paired samples. (D,E) miR-494 and miR-1973 levels show a significant difference between paired pre-therapy and CR interim therapy (P=0.0438, P=0.0012 respectively) while no significant difference was seen between paired pre-therapy and PR interim therapy (P=NS for both). (F) miR-21 levels show no significant difference between paired pre-therapy versus CR or PR interim therapy. P<0.001=***, P<0.01=**, P<0.05=*, P>0.05=NS.
Figure 4. Circulating miRNA relative to cellular RNA (U6)

(A) Plasma U6 levels and (B) plasma albumin-DNA levels throughout therapy in cHL patients in CR at six months post-therapy. (C-E) Plasma miRNA levels relative to plasma cellular RNA (U6) levels. Error bars represent mean with SEM. P<0.001=***, P<0.01=**, P<0.05=*, P>0.05=NS.
**Figure 2**

**pre-therapy vs healthy**

- **A**: miR-494, AUC=0.73, P=0.004
- **B**: miR-1973, AUC=0.71, P=0.007
- **C**: miR-21, AUC=0.92, P<0.0001

**complete responders: pre-therapy vs post-therapy**

- **D**: miR-494, AUC=0.65, P=0.034
- **E**: miR-1973, AUC=0.74, P=0.0006
- **F**: miR-21, AUC=0.86, P<0.0001
miR-494

A. pre-therapy

B. interim

C. post-therapy

D. healthy

miR-1973

E. pre-therapy

F. interim

G. post-therapy

H. healthy

miR-21

I. pre-therapy

J. interim

K. post-therapy

L. healthy

Figure 3

[Graphs showing expression levels of miR-494, miR-1973, and miR-21 across pre-therapy, interim, post-therapy, and healthy conditions.]

[Research. on April 15, 2017. © 2013 American Association for Cancer Research.]

[Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited. Author Manuscript Published OnlineFirst on November 12, 2013; DOI: 10.1158/1078-0432.CCR-13-1024]
Figure 4

A. U6

B. Albumin-DNA

C. miR-494

D. miR-1973

E. miR-21

Plasma miRNA levels relative to plasma cellular RNA
Plasma microRNA are disease response biomarkers in classical Hodgkin lymphoma

Kimberley L Jones, Jamie P Nourse, Colm Keane, et al.

Clin Cancer Res Published OnlineFirst November 12, 2013.

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