Plasma MicroRNA Are Disease Response Biomarkers in Classical Hodgkin Lymphoma

Kimberley Jones¹,², Jamie P. Nourse¹, Colm Keane¹,³,⁴, Atul Bhatnagar¹, and Maher K. Gandhi¹,²,³

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 chemoresistance and unnecessary toxicity, and alternate rescue strategies should be instituted early. The challenge remains to interpret conventional measures of disease response accurately predict and monitor response to therapy, so that a risk-stratified approach can be commenced. Although PET/CT has a high negative predictive value, its positive predictive value is more modest (4). Furthermore, it is impractical to perform PET/CT before 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 in which change in therapy should be expedited (5, 6). Circulating disease response biomarkers have the added advantage of being noninvasive 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 is not available.

MicroRNAs (miRNA) are small noncoding RNA molecules that play key regulatory roles in numerous biologic 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 noninvasive assays in situations in which PET/CT is impractical to perform before each follow-up visit.
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 microRNAs (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 miRNAs have the potential to greatly assist clinical decision making and aid interpretation of positron emission tomography combined with computerized tomography (PET/CT). A further advantage is they can also be performed at each consultation to assess disease response and detection of early relapse.

which screening biomarkers would be clinically beneficial (e.g., prostate and lung cancer). In contrast, the potential role of miRNAs as disease response cancer biomarkers has been relatively neglected. To our knowledge, of the few studies on circulating miRNAs in lymphoma, all have been in the setting of non-Hodgkin lymphoma before treatment. Lawrie and colleagues found that serum levels of miR-155, miR-210, and miR-21 were higher in patients with diffuse large B-cell lymphoma (DLBCL) 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 pretherapy 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 exist on the relative kinetics of circulating miRNAs following the commencement of chemotherapy in any malignancy. Although circulating miRNAs may be elevated pretherapy, concerns remain that the indiscriminate cell lysis induced by chemotherapy may cause nonspecific elevation of miRNAs 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 Epstein–Barr virus (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 miRNAs (including miR-21, miR-155, and miR-16) had been identified as being preferentially overexpressed in HRS cells as compared with transformed B-cells or Burkitt lymphoma cell lines. However, by in situ hybridization, these miRNAs are observed within both HRS and nonmalignant tumor-infiltrating cells including lymphocytes and macrophages (20). Therefore they are best-deemed disease node–associated and not cHL specific. Circulating cell-free miRNAs 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 that will influence circulating miRNA levels include ability for the miRNAs to be released into the circulation.

In this study, microarray profiling of more than 1,000 human miRNAs in a discovery cohort of 14 cHL primary tissue and eight healthy lymph nodes revealed new disease node–associated miRNAs, 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 patients with cHL and 20 healthy participants. Blood samples were taken at fixed time points before, during, and after therapy. Results were compared with disease response at each time point.

Materials and Methods

 Patients

Forty-two newly diagnosed patients with cHL 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: pretherapy, immediately prethird therapy, and 6 months posttherapy. 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, infra-diaphragmatic presentations, or mediastinal masses greater than one-third the maximum thoracic diameter; ref. 24).

This was a multicenter, 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, vincristine, and dacarbazine)
combination chemotherapy (25). Other regimens were used. “BEACOPP” (bleomycin, etoposide, adriamycin, cyclophosphamide, procarbazine, and prednisolone; ref. 26), ABVD followed by BEACOPP and “ChlVPP” (chlorambucil, procarbazine, and prednisolone; ref. 27).

Initial staging and restaging 1 month after completion of therapy was by PET and CT scans. Interim disease response (generally after the third cycle of therapy), and restaging 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 was obtained from a previous ALLG study and eight nonmalignant lymph node tissues (uninvolved auxiliary node dissections from patients with breast cancer; ref. 30). This study conformed to the Declaration of Helsinki and written informed consent was provided by all patients and was approved by all participating hospitals/research institute Human Research Ethics Committees.

**MiRNA extraction and quantitative real-time 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 (600 μL) 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 (25 fmol in 5 μL total volume) was added to the 600 μL of plasma after addition of denaturing solution to control for extraction efficiency and all subsequent procedural steps including reverse transcription and quantitative real-time PCR (qRT-PCR) amplification (10). All kits were used as per manufacturer’s instructions.

**MiRNA microarray**

More than 1,000 human miRNAs 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 miRNAs that had not previously been examined in cHL. Assays were performed on 14 cHL diagnostic biopsy tissue (eight nodular sclerosing and six mixed cellularity) and eight nonmalignant lymph node biopsies. Expression data were 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 RT kit, SYBR Green and universal primer, plasma and tissue miRNAs were quantified on a Rotor-Gene 3000 qRT-PCR (Corbett Research). Each reaction contained the equivalent of either 3 ng tissue RNA or 0.2 μL plasma cDNA, all run in duplicate 20 μL reactions. Two Qiagen miScript primers were used: C. Elegans miR-39 (cel-miR-39) and miR-16. Inhouse primers were used for miR-21 (5'-CGTAGCTTATCA-GCTGATGTTGAA-3'), miR-155 (5'-TATGTCAATCTGGA CAGGGGCTAA-3'), miR-499 (5'-AAACATAACGCGGAA ACCCTCAAA-3'), miR-638 (5'-CCCGTGCGCGGCCCTAA-3'), miR-1976 (5'-ACCCGCAGGATGAGACACGAA-3'), miR-2861 (5'-GGCGGGCCGAGGCAAA-3'), and U6 (5'-GCAGGTTGGTACACGCA-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) cDNAs 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 miRNAs. 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

<table>
<thead>
<tr>
<th>Table 1. Patient characteristics</th>
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<tr>
<td><strong>Patient characteristic</strong></td>
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</table>
| **Age median (range)**          | 35.6 (18–79)%
| **Male**                        | 22 (52)%
| **Ann Arbor Stage III–IV**      | 21 (50)%
| **Serum albumin, less than 40 g/L** | 30 (71)%
| **LDH, greater than 250 U/L**   | 24 (57)%
| **Hemoglobin, less than 105 g/L** | 9 (21)%
| **Lymphocyte count, at least 0.6 × 10⁹/L** | 38 (90)%
| **White blood cell count, at least 15 × 10⁹/L** | 7 (17)%
| **Hasenclever score, at least 3** | 15 (36)%
| **B symptoms**                  | 16 (38)%
| **EBER-ISH positive**           | 12 (28)%
| **EBER-ISH unavailable**        | 3 (7)%
| **Early-stage**                 | 9 (21)%
| **Advanced-stage**              | 33 (79)%
| **Histologic Subtype**          |  |
| **Nodular sclerosing**          | 26 (62)%
| **Mixed cellularity**           | 4 (10)%
| **Lymphocyte-rich**             | 4 (10)%
| **Lymphocyte-depleted**         | 1 (2)%
| **cHL, unspecified**            | 7 (16)%
| **Treatment**                   |  |
| **ABVD**                        | 36 (85)%
| **BEACOPP**                     | 2 (5)%
| **ABVD + BEACOPP**              | 2 (5)%
| **ChlVPP**                      | 2 (5)%

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Plasma miRNA Disease Response Biomarkers in cHL

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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.

Statistical analysis

Microarray data were quantile-normalized using GeneSpring GX and analyzed using GenePattern (Broad Institute). Comparative marker selection analysis was performed using GenePattern to identify significantly different miRNAs 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.).

Results

Patient characteristics

Forty-two patients with cHL were accrued (mean age, 36 years; range, 18–79; female:male ratio, 20:22). Patient characteristics are provided in Table 1. Interim therapy (immediately before third therapy) samples were available for 38 of these patients and posttherapy samples were available for 37 patients. Of these 37 patients with cHL, 32 achieved CR by the 6-month posttherapy time point.

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

More than 1,000 human miRNAs were quantified in our discovery cohort of 14 cHL patient biopsy tissues and eight nonmalignant lymph nodes using Agilent microarray, version 16. The data were quantile-normalized using GeneSpring GX software, analyzed using GenePattern (Broad Institute), and ranked on the basis of comparative marker selection analysis. Comparing cHL from normal lymph node tissue, there were 474 differentially expressed human miRNAs (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 miRNAs. From the ranked data, we selected the top five miRNAs 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 miRNAs, we also selected miR-155, miR-21, and miR-16, known to be overexpressed 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 miRNAs were significantly elevated in cHL nodes compared with 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 housekeeping 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 eight 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 miRNAs (Spearman r = 0.64–0.89; all P values < 0.001), validating our qRT-PCR technique. We then quantified these seven miRNAs in 26 cHL tissues from the prospective cHL cohort. As shown in Fig. 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 with 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, Fig. 1H), which was not observed by microarray in the discovery cohort.

Circulating miRNAs are elevated in plasma of patients with cHL at diagnosis and are associated with Hasenclever score

Plasma miRNAs were quantified in all pretherapy 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-miR-39). In this analysis of plasma miRNA, results were normalized to the spike-in control cel-miR-39 (but not U6). Five of the seven miRNAs were significantly elevated in cHL pretherapy plasma compared with 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; Supplementary Fig. S1). The small RNA U6 was also elevated (P = 0.0117).

Pretherapy 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 ($P = 0.020$), whereas patients with leucocytes $\geq 15 \times 10^9/L$ 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 $\geq$III were also associated with increased levels of miR-494 ($P = 0.0368$). No correlation was found between pretherapy 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 pretherapy, interim therapy, and posttherapy samples in patients with cHL in CR at the 6-month posttherapy time point. Strikingly, miR-494, miR-1973, and miR-21 significantly differentiated diseased pretherapy 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 posttherapy levels also significantly decreased compared with pretherapy ($P = 0.0314$); however, these remained elevated compared with healthy control levels. No other miRNAs reflected disease response.

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 $\mu$L plasma. As it has been shown to be dysregulated in HRS cells, absolute

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**Figure 1.** Differential expression of human miRNAs in cHL primary tissue. A, unsupervised clustering of the top 50 differentially expressed human miRNAs by microarray in a discovery cohort of 14 cHL diseased nodes (MC, mixed cellularity; NS, nodular sclerosing) versus eight nonmalignant lymph nodes (LN). Red, high expression. B–H, comparison of miRNA expression in prospective cohort of 27 cHL diseased nodes versus eight nonmalignant lymph nodes by qRT-PCR. Error bars, mean with SEM. ns, not significant.
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 miRNAs at all time points ($r = 0.87–0.94$; $P < 0.001$) with absolute values having similar significance between all pretherapy 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 pretherapy compared with 6-month posttherapy CR 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 ROC analysis to determine the sensitivity and specificity at defined time points. Although ROC analysis of miR-16 showed it significantly delineated pretherapy cHL from healthy participants [area under the curve (AUC), 0.70; $P = 0.011$; 95% confidence interval (CI), 0.5693–0.8355], it did not reach significance and had poor specificity and sensitivity for distinguishing pretherapy from remission samples at 6 months posttherapy (Fig. 2A–C) and pretherapy from remission samples at 6 months posttherapy (miR-494: AUC, 0.65, $P = 0.037$, 95% CI, 0.52–0.77; miR-1973: AUC, 0.75, $P = 0.0004$, 95% CI, 0.63–0.86; miR-21: AUC, 0.86, $P < 0.0001$, 95% CI, 0.77–0.95; Fig. 2D–F). To maximize both sensitivity and specificity compared with healthy controls, cutoff values for miR-494, miR-1973, and miR-21 were defined as follows: $3.0 \times 10^5$ miR-494 copies/mL plasma with 85% sensitivity and 60% specificity, $1.6 \times 10^6$ miR-1973 copies/mL plasma with 75% sensitivity and 67% specificity, and $1.0 \times 10^6$ miR-21 copies/mL plasma with 95% sensitivity and 86% specificity. The associations with clinical prognosticators and pretherapy 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 to 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 miRNAs but not others (39, 40). 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 four time points from venipuncture (0, 18, 24, and 40 hours). All miRNAs evaluated (miR-494, miR-1973, miR-21, miR-16, and U6) remained constant over time (Supplementary Fig. S3).

Figure 2. Plasma miR-494, miR-1973, and miR-21 are disease response biomarkers in cHL. A–C, ROC plots demonstrate high sensitivity and specificity of pretherapy cHL versus healthy participant; D–F, pretherapy cHL versus CR 6 months posttherapy.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>AUC Value</th>
<th>P Value</th>
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<tbody>
<tr>
<td>miR-494</td>
<td>0.73</td>
<td>0.004</td>
</tr>
<tr>
<td>miR-1973</td>
<td>0.71</td>
<td>0.007</td>
</tr>
<tr>
<td>miR-21</td>
<td>0.92</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>miR-494</td>
<td>0.65</td>
<td>0.034</td>
</tr>
<tr>
<td>miR-1973</td>
<td>0.74</td>
<td>0.006</td>
</tr>
<tr>
<td>miR-21</td>
<td>0.86</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Note: AUC = area under the curve; CI = confidence interval; CR = complete response; miR = microRNA.
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 6 months posttherapy, 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 pretherapy and elevated compared with healthy controls, dropping to normal levels by 6 months posttherapy. To determine how the different interim kinetics of miR-494, miR-1973, or miR-21 associate with interim therapy response, we compared pretherapy samples with paired interim samples delineated as either CR or PR by radiologic assessment, using matched-paired analysis. Interestingly, for both miR-494 and miR-1973, there was no significant difference between pretherapy samples and those in PR at the interim time point. However, there was a significant difference for miR-494 and miR-1973 between pretherapy and those in CR at the interim time point (P = 0.0438, P = 0.0012; Fig. 3D and E). In contrast, pretherapy 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 1 patient relapsed by 6 months posttherapy. This patient had elevated miR-494 levels (above the cutoff) at interim, whereas miR-1973 and miR-21 were not elevated. Interestingly, 4 of the patients in PR at the interim time point had relapsed/refractory disease posttherapy. Of these, miR-494 and miR1973 levels were elevated in 1 patient, whereas 3 patients 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), whereas miR-21 had no correlation to these miRNAs at any time point. However, miR-21 did strongly correlate with miR-155 and miR-16 pretherapy (r = 0.72, P < 0.0001 and r = 0.76, P < 0.0001, respectively).

Of those patients who achieved CR posttherapy, 65% of patients had miR-494 levels above the cutoff pretherapy, 64% had interim levels lower than pretherapy, and 76% of patients had posttherapy levels lower than pretherapy. Both interim therapy and posttherapy levels were lower than pretherapy in 55% of patients. A reduction in levels from pretherapy to interim, and interim to posttherapy was observed in 26% of patients. For miR1973, 65% of patients had levels above the cutoff pretherapy, 67% of patients had interim levels lower than pretherapy, and 85% of patients had posttherapy levels lower than pretherapy. Both interim therapy and posttherapy levels were lower than pretherapy in 61% of patients. A reduction in levels from pretherapy to interim, and interim
to posttherapy was observed in 19% of patients. For miR-21, 84% of patients had levels above the cutoff pretherapy, 42% of patients had interim levels lower than pretherapy, and 94% of patients had posttherapy levels lower than pretherapy. Both interim therapy and post-therapy levels were lower than pretherapy in 42% of patients. A reduction in levels from pretherapy to interim, and interim to posttherapy was observed in 39% of patients.

Circulating miRNAs 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 pretherapy compared with 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 250 U/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 nonspecific cellular RNA neutralized the elevated levels of plasma miRNA in

<table>
<thead>
<tr>
<th>Patient characteristic</th>
<th>miR-494 Mean ± SEM</th>
<th>miR-1973 Mean ± SEM</th>
<th>miR-21 Mean ± SEM</th>
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<tbody>
<tr>
<td>Age</td>
<td>NS</td>
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<tr>
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<td>NS</td>
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<td>Gender</td>
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<tr>
<td>Ann Arbor stage</td>
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<td>NS</td>
</tr>
<tr>
<td>I–II</td>
<td>($5.8 \times 10^5 \pm 1.4 \times 10^5$)</td>
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<td>NS</td>
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<tr>
<td>III–IV</td>
<td>($1.2 \times 10^6 \pm 2.7 \times 10^5$)</td>
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</tr>
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<td>NS</td>
</tr>
<tr>
<td>Less than 40 g/L</td>
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<td>NS</td>
<td>NS</td>
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<td>At most 250 U/L</td>
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<td>NS</td>
<td>($2.2 \times 10^6 \pm 9.7 \times 10^5$)</td>
</tr>
<tr>
<td>Greater than 250 U/L</td>
<td>($1.2 \times 10^6 \pm 2.4 \times 10^5$)</td>
<td>($3.1 \times 10^6 \pm 7.4 \times 10^5$)</td>
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<tr>
<td>Hemoglobin</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Less than 105 g/L</td>
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<td>NS</td>
<td>NS</td>
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<tr>
<td>Lymphocyte count</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>At least 1 × 10^9/L</td>
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<td>NS</td>
</tr>
<tr>
<td>White blood cell count</td>
<td>0.0019</td>
<td>NS</td>
<td>0.0014</td>
</tr>
<tr>
<td>Less than 15 × 10^9/L</td>
<td>($1.8 \times 10^6 \pm 2.4 \times 10^5$)</td>
<td>($7.4 \times 10^6 \pm 2.9 \times 10^9$)</td>
<td></td>
</tr>
<tr>
<td>At least 15 × 10^9/L</td>
<td>($4.7 \times 10^6 \pm 1.8 \times 10^5$)</td>
<td>($4.7 \times 10^6 \pm 1.8 \times 10^5$)</td>
<td></td>
</tr>
<tr>
<td>Hasenclever score</td>
<td>0.0224</td>
<td>NS</td>
<td>0.0314</td>
</tr>
<tr>
<td>Less than 3</td>
<td>($5.5 \times 10^6 \pm 1.0 \times 10^6$)</td>
<td>($1.6 \times 10^6 \pm 1.8 \times 10^5$)</td>
<td></td>
</tr>
<tr>
<td>At least 3</td>
<td>($1.5 \times 10^6 \pm 3.5 \times 10^5$)</td>
<td>($4.7 \times 10^6 \pm 1.5 \times 10^5$)</td>
<td></td>
</tr>
<tr>
<td>B symptoms</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Yes vs. no</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Stage</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Early vs. advanced</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Histology</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Nodular sclerosing vs. 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&lt;, &lt;200 genomes/mL</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

NOTE: Mean ± SEM values are given as copies/μL of plasma. Abbreviation: NS, not significant.

$^aP < 0.05$.

$^b$Other specified: mixed cellularity, lymphocyte-rich, and lymphocyte-depleted cHL subtypes.

$^c$In patients with EBV-related cHL.
pretherapy samples compared with 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. 4C–E; pre vs. interim therapy: miR-494 \( P < 0.0001 \), miR-1973 \( P = 0.0007 \), and miR-21 \( P = 0.0063 \)).

**Discussion**

We present the first study of circulating cell-free miRNAs as disease response biomarkers in any lymphoma. Seven cHL-associated miRNAs were tested for utility as disease response biomarkers in a prospective cohort of patients with cHL. Following testing in serial plasma samples, in comparison with healthy samples and with reference to radiologic assessment, we assessed the kinetics of these miRNAs during therapy. We found that three miRNAs (miR-494, miR-1973, and miR-21) showed promise as disease response biomarkers. In contrast with 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 \( \geq 3 \). Future studies should test for the ability of circulating miRNAs to serve as biologic prognosticators in cHL.

We hypothesized that elevated miRNAs in the diseased tissue are elevated in plasma and that this level would normalize once disease resolved. Alternatively, it is possible that underrepresented miRNAs in the diseased tissue may be reflected by decreased levels in the plasma of patients with cHL. Further studies are required to evaluate underrepresented miRNAs. We selected five novel miRNAs that we identified as overexpressed in the diseased node relative to healthy nodes. The miRNAs were evaluated in unison with previously identified cHL-associated miRNAs. Of these, levels of miR-494, miR-1973, and miR-21 miRNAs were higher in patients than healthy control participants’ plasma and all three miRNAs 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 interlaboratory 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 CRs versus PRs. In contrast, miR-21 showed no relationship with radiologic response during therapy.

No correlation was found between pretherapy 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: first, that circulating levels only partially reflect the disease node, with contribution also coming from nondiseased tissue. Second, 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 center study of 60 patients with cHL (6). This showed that pretherapy plasma TARC levels directly correlated with the metabolic tumor volume. Future studies are required to determine whether circulating plasma miRNAs also correlate with metabolic tumor volume.

There is currently no consensus on a reference gene for circulating miRNAs. 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 housekeeping gene for cHL. MiR-16 values were significantly elevated pretherapy compared with healthy control participants and gradually declined to normal levels by 6 months posttherapy.

Within the field, an exogenous technical control for RNA extraction efficiency is frequently used. We selected cel-miR-39 on the basis of the published literature that normalizing to the mean of three 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, pretherapy U6 levels were strongly associated with LDH and correlated with cell-free albumin DNA levels. We have previously shown that the latter is elevated at pretherapy in lymphomas, but remains elevated during and following therapy (21). Thus, our results imply that U6, as with albumin-DNA, seems to be a marker of cell integrity and is unsuitable as a housekeeper in comparison between patients with cHL and healthy individuals. Its 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 seem 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 overexpressed in follicular lymphoma tissue and functionally contributes to cancer persistence (45–47).

Specifically, miR-494 is implicated in chemoresistance 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 that 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 upregulated 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 upregulated 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 overexpressed in cHL patient versus healthy nodes (20). In contrast to that group, we did not find miR-21 was elevated (values were ~2-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 miRNAs, 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 miRNAs within cell types. Consistent with this and with our previous study of serum proteins in cHL (5), the three miRNAs 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 miRNAs simultaneously. Future risk-adapted treatment algorithms combining circulating protein and miRNA biomarkers with interim PET/CT should be evaluated.

Disclosure of Potential Conflicts of Interest

K. Jones, C. Keane, and M.K. Gandhi have ownership interest in a provisional patent. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: K. Jones, M.K. Gandhi
Development of methodology: K. Jones, J.P. Nourse, C. Keane, M.K. Gandhi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Jones, A. Bhatnagar, M.K. Gandhi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Jones, M.K. Gandhi
Writing, review, and/or revision of the manuscript: K. Jones, J.P. Nourse, C. Keane, M.K. Gandhi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Jones, A. Bhatnagar, M.K. Gandhi
Study supervision: M.K. Gandhi

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References
