CHAPTER 7

Circulating miRNAs in serum of patients with classical Hodgkin Lymphoma

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INTRODUCTION
In cHL there is a need for biomarkers that can predict prognosis of individual patients at diagnosis. Circulating miRNAs are remarkably stable and may serve as non-invasive biomarkers. We aimed to identify miRNAs in serum of cHL patients that could aid in prognostication.

MATERIALS AND METHODS
We performed miRNA microarray profiling on serum samples from eight non-responsive patients, eight responsive patients and eight healthy controls. Validation was performed for a selection of five miRNAs based on the array results and five miRNAs with high expression in cHL cell lines or tissues. In addition, we tested the circulating levels of miR-135a, for which tissue levels were previously shown to correlate with prognosis.

RESULTS
Microarray profiling resulted in detection of 124 out of 1348 miRNAs. Only miR-718 was detected with significantly higher levels in serum of responsive patients compared to controls and non-responsive patients with multiple testing correction. This difference could not be validated by qRT-PCR on the same set of samples. MiR-451a, miR-494, miR-575, miR-718 and miR-3648 were significantly different without multiple testing correction and had more than two fold expression change between the groups. Of these only miR-451a had reliable levels on qRT-PCR but was not significantly different between the groups. Of the five miRNAs with high expression in cHL cell lines or tissue and miR-135a only miR-155 showed significantly lower levels among non-responsive patients compared to controls. Responsive patients showed a trend similar to the non-responsive patients.

CONCLUSION
In conclusion, we were unable to identify miRNAs in serum of cHL patients that could predict prognosis. MiRNAs highly expressed in cHL tissue and cell lines and the previously reported prognostic miR-135a are not suitable as prognostic circulating biomarkers in cHL.
Introduction

The introduction and further development of multi-agent chemotherapy regimens combined with major improvements in radiotherapy transformed classical Hodgkin lymphoma (cHL) from an incurable to a curable disease in at least 80% of patients. Current treatment decisions are based on a combination of accurate staging, non-specific laboratory factors and patient factors such as presence of systemic symptoms and comorbidity. This risk-stratification cannot reliably distinguish patients with refractory disease from responsive patients on individual patient level. As a consequence, patients might be undertreated and suffer from life-threatening refractory or relapsed disease or overtreated and suffer from serious treatment toxicity. There is a need for (bio)markers predicting treatment response either before or early during treatment to overcome over- and under-treatment.

MicroRNAs (miRNAs) are single stranded non-coding RNAs of ~21 nucleotides that regulate gene expression through targeting of messenger RNAs. MiRNAs can be found in serum or plasma and are remarkably stable making them suitable candidates for non-invasive biomarkers. During recent years circulating miRNAs have been studied as diagnostic or prognostic markers. A frequently applied approach in studies aiming at the identification of disease specific circulating miRNAs is to measure a set of pre-selected miRNAs by qRT-PCR. This has resulted in the identification of elevated or decreased circulating miRNAs levels in many different tumor types including diffuse large B-cell lymphoma. Another frequently applied approach is to apply a miRNA profiling technique of serum or plasma samples to identify disease specific alterations in circulating miRNA levels.

Jones et al. recently studied circulating miRNAs as disease response biomarkers in cHL and found that miR-494, miR-1973 and miR-21 were elevated in plasma of cHL patients before treatment and showed that levels returned to normal after treatment. The aim of this study was to profile miRNAs from serum samples of both responsive and non-responsive advanced stage cHL patients and healthy controls to identify miRNAs that can aid in prognostication.
Materials and Methods

Patient selection and controls
Serum from cHL patients was collected in the Centre for Lymphoid Cancer, BC Cancer Agency, Vancouver, British Columbia, Canada. We selected 8 non-responsive based on the following criteria: (1) refractory to first line treatment or relapsed; (2) uniform treatment with ABVD; (3) treatment failure within 1 year; (4) patients with stage II bulky or advanced stage only. This selection resulted in 9 available samples of which the 8 with shortest freedom from treatment failure were chosen for RNA isolation. For responsive patients, patients were selected based on the following criteria: (1) persistent complete remission on first line treatment; (2) uniform treatment with ABVD; (3) stage II bulky or advanced stage patients only; and (4) recorded freedom from treatment failure >5 years. This selection resulted in serum samples available from 39 patients. We selected eight patients based on matching age, sex and stage with the selected non-responsive patients. We subsequently collected serum from eight age and sex matched healthy controls.

Serum isolation
Serum was obtained by venapuncture in yellow top serum separator tubes (BD Vacutainer®). According to collection protocol, samples were left at room temperature to allow clotting for 30-45 minutes. Serum was subsequently isolated by centrifuging the sample at 1940g for 10 minutes at room temperature after which the supernatant was pipetted carefully into aliquots that were immediately stored at -20°C. Hemolytic samples were excluded from analysis.

RNA isolation
Total RNA was extracted from 150ul of serum using the miRNeasy Micro Kit (Qiagen), following the manufacturer’s instructions, with minor modifications: (1) after adding 750ul Qiazol serum was left at room temperature for 20 minutes to guarantee optimal RNAse inhibition before adding spike-in; (2) 5ul synthetic C. elegans miR-39 (250pM) was added for data normalization; (3) the first RPE buffer volume was increased from 500ul to 700ul for optimal column washing; (4) an additional elution step was performed with RNAse free water and stored separately. All isolations were performed in duplicate to minimize variation in isolation efficiency (total serum volume used for isolation is 300ul) and elutions were merged. RNA concentrations were quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Microarray analysis and statistics
A volume of 4ul or 8ul of the total elution volume of 24ul was dephosphorylated, desalted, labeled and hybridized to the Agilent Human miRNA Microarray version 16. This array contained 1368 probesets for control, human and viral miRNAs (according miRBase version
Arrays were scanned using an Agilent scanner according to the manufacturer’s instructions (Agilent Technologies). Array images were analyzed using Agilent feature extraction software (v10.7.3.1). All further analyses were performed using GeneSpring GX version 12.5 software (Agilent Technologies). First raw signals were thresholded to 1.0, followed by a 97-percentile shift normalization. One control sample and one patient sample were excluded from further analysis since principal component analysis showed aberrant variance compared to the other samples. MiRNAs detected above background signals in all samples were used for hierarchical clustering using distance metric Pearson’s correlation in Genesis software (Graz University of Technology). Differential expression analysis between the three groups was performed using an analysis of variance test (ANOVA) with the Benjamini–Hochberg correction for multiple testing.

**qRT–PCR**

Serum miRNA levels were analyzed by qRT–PCR using Taqman MicroRNA assays. RNA was first reverse transcribed using Taqman microRNAs Reverse Transcription kit in combination with multiplex reverse transcription primers for Taqman microRNAs Assays (Life Technologies, Carlsbad, CA, USA). In addition to the significantly differentially expressed miRNAs, we also tested serum levels of miRNAs that were reported to be highly expressed in cHL cell lines, microdissected Hodgkin Reed-Sternberg (HRS) cell and total tissue sections (Landgraf et al. 2007; Navarro et al. 2008; Gibcus et al. 2009; Van Vlierberghe et al. 2009; Jones et al. 2013). In total, we analyzed five miRNAs based on the array results, five miRNA based on the cell/line tissue expression (miR-9, miR-16, miR-20a, miR-21 and miR-155). one miRNA with previously shown prognostic value in tissue (miR-135a) and cel-miR-39 as exogenous control.

The cDNA reaction was performed using 2.5ul of eluted RNA diluted to a volume of 5ul. Together with the reagents the total cDNA volume was 15ul. cDNA was subsequently diluted 15 times and 2.5ul of cDNA was mixed with qPCR MasterMix Plus (Eurogentec, Liege, Belgium) and Taqman Gene expression assay to a total reaction volume of 10ul. All reactions were performed in triplicate on the Lightcycler 480 Real-Time PCR machine (Roche Life Sciences). Cycle threshold (Cp) values were calculated using the fit point method. Synthetic cel-miR-39 spike in was used to correct for differences in efficiency of RNA isolation and qRT-PCR procedures. Relative expression levels were calculated using the $2^{-\Delta Ct}$ formula.

**Statistical analyses**

Statistical analysis of qRT–PCR data was performed with the GraphPad Prism software (La Jolla, CA, USA). Comparisons of multiple unpaired samples were performed using ANOVA method with Bonferroni’s multiple comparison post-test. All statistical analyses were two sided at the significance level of p<0.05.
Results

Patient characteristics
The main characteristics of the 16 selected patients and 8 healthy controls are demonstrated in Table 1. Median age was 31 years and 30 years in both patient groups and 30 years in controls. Half of the samples were derived from male individuals. All patients were selected for having advanced stage disease and receiving ABVD treatment. Median freedom from treatment failure was 116 months in the responsive patient group and 9 months in the non-responsive patient group (p<.001).

<table>
<thead>
<tr>
<th>Table 1. Basic characteristics of the selected patients.</th>
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<tr>
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<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Median age (range)</td>
</tr>
<tr>
<td>Male sex (n (%))</td>
</tr>
<tr>
<td>NS subtype</td>
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<tr>
<td>Median survival (months)</td>
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FFTF, Freedom from treatment failure is defined as time from diagnosis until progression; OS, Overall survival is the time from diagnosis until death or censoring for follow-up. NS, nodular sclerosis

miRNA microarray analysis
A total of 124 (9.2%), including 106/1206 (8.8%) human and 18/142 (12.7%) viral, miRNAs were flagged present in all serum samples. Unsupervised hierarchical clustering did not result in a distinctive clustering pattern for cHL or responsive and non-responsive patients (data not shown). Only one miRNA, i.e. miR-718, showed a significantly lower expression value in non-responsive patients and controls compared to responsive patients (p = .00039). 12 miRNAs were significantly different without multiple testing correction, of which five showed a >2 fold difference, including miR-718 (Table 2). Unsupervised hierarchical clustering for these 12 miRNAs did not reveal a distinctive clustering pattern (Figure 1).

qRT-PCR results
The five human miRNAs with fold changes > 2.0, i.e. miR-451a, miR-494, miR-575, miR-718 and miR-3648, were selected for qRT-PCR validation. Four of the five miRNAs showed Ct values above 35 and these were considered to be unreliable. MiR-451a resulted in reliable Ct values, but levels did not significantly differ between the three groups (Figure 2).
Figure 1. Unsupervised hierarchical clustering of the 12 miRNAs that had differential expression without multiple testing correction. Clustering did not result in a distinctive clustering pattern between responsive patients, non-responsive patients or controls. NR indicates non-responsive patients, R indicates responsive patients and C indicates healthy controls.

Table 2. Differentially expressed miRNAs in the three subgroups as determined by ANOVA without multiple testing correction.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Fold change</th>
<th>p-value</th>
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<tbody>
<tr>
<td></td>
<td>control vs.</td>
<td>control vs.</td>
</tr>
<tr>
<td></td>
<td>non-responsive</td>
<td>responsive</td>
</tr>
<tr>
<td>hsa-miR-718</td>
<td>2.03</td>
<td>1.97</td>
</tr>
<tr>
<td>hsa-miR-4281</td>
<td>1.46</td>
<td>↓</td>
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<tr>
<td>hsv2-miR-H22</td>
<td>1.51</td>
<td>↓</td>
</tr>
<tr>
<td>hsa-miR-2276</td>
<td>1.26</td>
<td>↓</td>
</tr>
<tr>
<td>hsv1-miR-H16</td>
<td>4.55</td>
<td>↓</td>
</tr>
<tr>
<td>hsa-miR-4327</td>
<td>1.43</td>
<td>↓</td>
</tr>
<tr>
<td>hsa-miR-3648</td>
<td>4.92</td>
<td>↓</td>
</tr>
<tr>
<td>hsa-miR-223</td>
<td>1.79</td>
<td>↑</td>
</tr>
<tr>
<td>hsa-miR-575</td>
<td>2.05</td>
<td>↑</td>
</tr>
<tr>
<td>hsa-miR-451a</td>
<td>3.28</td>
<td>↑</td>
</tr>
<tr>
<td>hsa-miR-3679</td>
<td>1.32</td>
<td>↑</td>
</tr>
<tr>
<td>hsa-miR-494</td>
<td>4.16</td>
<td>↑</td>
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↓ = downregulated; ↑ = upregulated

miRNAs displayed in bold were selected for testing with qRT-PCR.
We also performed qRT-PCR for miR-9, miR-16, mir-20a, miR-21 and miR-155 that were selected based on their known high expression in cHL tissue or cell lines. MiR-9 showed Ct values above 35 and was considered unreliable. MiR-16, miR-20a, miR-21 and miR-155 showed reliable Ct values. Significantly lower levels were detected only for miR-155 in non-responsive patients compared to controls (Figure 3). Responsive patients showed a similar trend, but this difference did not reach significance. In addition, we tested circulating miR-135a because a previous study showed prognostic value of this miRNA in whole tissue samples. Ct values for this miRNA were also above 35 and thus regarded as unreliable.

![miR-451a array expression](image1)

![miR-451a qRT-PCR](image2)

**Figure 2.** MiR-451a levels based on the microarray and on qRT-PCR. Of the five miRNAs that were identified as possibly differentially expressed only miR-451a could reliably be detected by real-time polymerase chain reaction (qRT-PCR). On the array, mean expression was 3.28 fold higher in controls compared to responsive patients (A). With the qRT-PCR platform, mean levels were not significantly different between all groups (B).
Figure 3. Relative serum levels of miRNAs with high expression in cHL tissues and/or cell lines. MiR-16, miR-20a and miR-21 were not significantly different between patient groups, while miR-155 was significantly lower in non-responsive patients compared to controls (p = .019). MiR-9 and miR-135a were undetectable by qRT-PCR (not displayed).
Discussion

During recent years, there has been increasing attention to the value of circulating miRNAs as non-invasive biomarkers for disease detection or prognosis. In our study we aimed to identify miRNAs in serum of responsive- and non-responsive cHL patients that could aid in prognostication before start of treatment. Using a hybridization-based miRNA microarray profiling platform we identified significantly higher miR-718 levels in responsive patients as compared to controls and non-responsive patients. Among significantly different miRNA without multiple testing we found four additional miRNAs with a fold change more than two between the groups. However, qRT-PCR based validation on the same samples did not yield reliable results for miR-718 as well as three of the other four selected miRNAs. The five previously reported miRNAs with high expression in cell lines and/or tissues were all detectable in serum with Ct values in the range of 19 to 33. A significant difference was found only for miR-155, with unexpectedly significantly lower levels in non-responsive patients compared to controls.

Since not all tissue miRNAs can usually be detected in serum we hypothesized that direct profiling of serum would be the most appropriate approach to identify a set of miRNAs that could discriminate between responders and non-responders. Hybridization-based miRNA profiling has a higher throughput than PCR-based assays and was therefore considered advantageous in the discovery setting. A previous study showed that the Agilent miRNA microarray platform was the most sensitive and specific hybridization-based platform to measure tissue derived miRNAs. It was also considered to be a reproducible method for analyzing circulating miRNAs from archival plasma samples. However, four out of five miRNAs that were selected based on our array results were undetectable by qRT-PCR. This made us wonder whether the hybridization-based profiling method had sufficient specificity using low RNA input such as RNA isolated from serum samples. Our set of 124 detectable miRNAs contained 18 (12.7%) viral miRNAs, which was very similar to the proportion of viral (11.5%) probesets present on the array. This might indicate non-selective detection of miRNAs. Moreover, miRNAs that have extensively shown to be present in serum samples at high levels like miR-16, miR-20a and miR-21 were not detected on the microarray but showed high levels using qRT-PCR. A previous paper mentioned that for the Agilent miRNA microarray the number of samples in which each probeset was detected depended on its GC content (Callari, 2013). Consistent with this notion, we indeed observed the highest signal for miR-4281, a miRNA with a GC percentage of 89%. The Agilent miRNA microarray platform has been used in over 15 miRNA profiling studies in serum samples with variable results. Some have identified sets of miRNAs related to disease or prognosis and others failed to identify differentially present miRNAs. For example, in an explorative canine study, all of the differentially expressed miRNAs could be detected at reliable levels using qRT-PCR but no significant changes were not found. A comparative paper studied accuracy and sensitivity of hybridization-based, qRT-PCR-based and sequencing-based platforms to study miRNA levels in...
cells, tissue or serum. In line with our results they found that the Agilent hybridization-based platform has very low accuracy and sensitivity when using low RNA input samples like RNA isolated from serum or plasma. They also showed that platforms based on sequencing have lower accuracy and sensitivity compared to qRT-PCR based platforms when using low input RNA samples.

In our study, 12 miRNAs were found to be differentially expressed between the three groups using ANOVA without multiple testing correction. Only miR-718 remained significant with appropriate multiple testing correction. Of the group of 12 miRNAs we selected five human miRNAs with a fold change >2.0 and reasonable expression levels based on the microarray data. Using qRT-PCR, we observed differences in circulating miRNA levels for 1 out of 11 miRNAs tested. A trend similar to the array result was observed for miR-451a, albeit not significant. For miR-155 we found a decrease in levels in non-responsive cHL patients, with a similar trend in responsive cHL patient samples. The only miRNA showing a significant difference after multiple testing correction, could not be validated by qRT-PCR due to unreliably high Ct values. The lower levels of miR-155 observed among cHL patients are not likely to result from depletion from the circulation by the tumor cells that are known to express high levels of miR-155, but more likely result from an active immune state secondary to the malignancy.

Our results are not in line with the results of Jones et al. In their study, analysis of five miRNAs selected based on high expression in whole tissue sections in pre-treatment plasma revealed elevated levels of miR-494 and miR-1973 in samples of cHL patients compared to controls. These levels decreased after successful treatment. We also identified miR-494 as differentially expressed based on our serum microarray data, but could not confirm this finding by qRT-PCR due to unreliably high Ct values. Jones et al. showed elevated mir-16, miR-21 and miR-155 plasma levels in patient samples with miR-21 levels returning back to normal levels after successful treatment. We also studied these miRNAs, but could not confirm elevated levels in our cohort. In contrast we found lower levels of miR-155 in non-responsive cHL patients compared to controls with a similar trend in responsive patients. The two main differences between the two studies are (1) the differences in study design including patient selection and (2) the use of plasma in their study compared to serum in our study. They did not select patients based on treatment outcome or tumor load. In contrast, we selected patients based on prognosis and tumor load. Plasma used in the study of Jones et al. has the risk of platelet admixture depending on the method of isolation. Platelets and other blood cells contain a wide range of miRNAs and have been identified as a likely source of elevated miRNA levels in many circulating miRNA studies. Jones et al. found a correlation between elevated LDH and miR-494 and miR-21 levels and also between elevated leukocytes and miR-16, miR-21 and miR-155 levels. They applied the same normalization strategy as we did, i.e. relative to cel-miR-39 spike-in. However, the differences found by Jones et al were neutralized when normalized to cellular RNA U6 instead of cel-miR-39
spike-in. These findings suggest significant cellular admixture that might have influenced results. Indeed, miR-16, miR-21 and miR-155 levels were demonstrated to be high in blood cells and have been proposed as the likely source of these elevated levels in other studies.\textsuperscript{11,12}

Low miR-135a expression was previously reported to correlate with adverse prognosis in whole tissue of cHL patients.\textsuperscript{13} We were not able to detect miR-135a in our serum samples by qRT-PCR among both patients and controls indicating that at least circulating miR-135a is not a suitable biomarker for prognostication.

In conclusion, we could not identify circulating miRNAs that aid in prognostication of cHL patients before treatment. Moreover, miRNAs with high expression in cHL tumor cells like miR-9, miR-16, miR-20a, miR-21 and miR-155 or the prognostic tissue miR-135a are not suitable as circulating biomarkers in cHL patients in our cohort.
References


SUMMARY AND DISCUSSION