

# MicroRNAs as Biomarkers of the Response to Treatment with ABVD Scheme in Hodgkin Lymphoma

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## Abstract

Hodgkin Lymphoma (HL) is a neoplasia characterized by the restrict number of malignant cells present in the lymph node. Actual criteria in diagnostic standards don't consider genetic and epigenetic alterations as risk factors in the development of this disease or in the response of the treatment. MicroRNAs (miRNAs) are important regulatory elements of genic expression that can be altered in the presence of cancer. New advances in the field suggest miRNAs as HL biomarkers. We evaluated the expression profiles of five miRNAs (hsa-miR-9, hsa-miR-20a, hsa-miR-21, hsa-miR-26a and hsa-miR-155) in the peripheral blood of three groups of patients: patients diagnosed with HL who had not received any radiologic or chemotherapeutic treatment; patients diagnosed with HL who had been treated with the Adriblastin, Bleomycin, Vinblastine and Dacarbazine (ABVD) chemotherapeutic scheme; and a control group consisting of healthy volunteers without HL. Our results showed that the expression profiles of hsa-miR-9, hsa-miR-21, hsa-miR-26a and hsa-miR-155 were able to significantly distinguish untreated HL patients from patients without the disease and that the hsa-miR-9, hsa-miR-21 and hsa-miR-155 expression profiles were altered by treatment with ABVD. These results suggest that miRNAs are promising blood biomarkers of HL and also a possible biomarkers of the response to ABVD treatment.

**Keywords:** MicroRNAs; Biomarkers; Response to treatment; ABVD scheme; Hodgkin lymphoma

## Introduction

Hodgkin lymphoma (HL) is a B-cell lymphoma that occurs in the lymph nodes (predominantly those in the cervical region) and is characterized by the presence of few cancer cells, usually representing 0.1 to 10% of the total number of cells in the tissue. HL is divided in classic Hodgkin Lymphoma (cHL) which are further subdivided according to its histology, being the nodular sclerosis, mixed cellularity, lymphocyte-rich and lymphocyte-depleted subtypes; and in cases of nodular lymphocyte-predominant HL [1].

HL is one of the most common types of lymphoma with an annual incidence of 5:100.000 persons globally and 3:100.000 persons in the western world [2,3]. Despite its incidence, HL mortality is low, with a cure rate of approximately 80% [2,4]. At the present time, the standard treatment for HL is a chemotherapy scheme consisting of Adriblastin, Bleomycin, Vinblastine and Dacarbazine (ABVD), associated or not with radiotherapy. This combination has been used for over 20 years and has high efficiency and a low toxicity profile [2,4].

Even though most patients respond well to the treatment, some show no effective changes in their clinical condition or relapse after complete remission of the disease [5,6]. In the early stages of treatment, a robust tool that stratifies distinct types of patients called the International Prognostic Score (IPS) [4] has been widely used in clinical practice to identify patients that are non-responsive to

treatment. However, this system does not consider genetic and epigenetic changes as factors involved in the development of the disease or in the response to treatment.

MicroRNAs (miRNAs) are endogenous small non coding RNAs (approximately 23 nt) that regulate genic expression at the post-transcriptional level by pairing with the 3' UTR region of the messenger RNA and repressing its translation [7,8]. Because miRNAs regulate thousands of genes involved in cellular processes, such as proliferation, differentiation and apoptosis, aberrant expression or alteration of the characteristic miRNA profile of a tissue contributes to the development of various human diseases, including cancer [9-12]. MiRNAs are considered promising biomarkers of cancer, mainly because (I) they circulate in biofluids (including blood, serum and plasma); (II) they can easily be sampled and (III) they are highly stable in plasma [8,13,14].

The aim of this study was to evaluate the expression profile of five miRNAs (hsa-miR-9, hsa-miR-20a, hsa-miR-21, hsa-miR-26a and hsa-miR-155) described in the literature [15-17] in peripheral blood samples from untreated HL patients; HL patients treated with ABVD; and healthy volunteers without HL to see if these miRNAs are blood biomarkers of HL and if ABVD treatment modifies the expression profiles of these miRNAs.

## Methods

### Sample collection and ethical aspects

The present study comprised three sample groups: group 1-patients diagnosed with HL who had not received any radiologic or chemotherapeutic treatment (n=4); group 2-HL patients treated with

the ABVD chemotherapeutic scheme, including patients from group 1 (n=7); and group 3-Healthy volunteers without HL or any other blood disease (n=14). The characteristics of the included patients are summarized in Table 1. The study followed the Declaration of Helsinki, and all patients and volunteers signed a consent form for participation in the study that had been approved by the Pará State University ethics committee CCBS-UEPA 08/2011).

Characteristics	Patients	
	Before Treatment (n = 4)	After Treatment (n = 7)
Median Age (range)	35 (21 - 54)	29 (13 - 54)
Gender		
Male	2	4
Female	2	3
Histologic Subtype		
Nodular Sclerosis	2	5
Mixed Cellularity	2	2
Stage		
I - II	3	5
III - IV	1	2
IPS		
< 3	1	2
Does not apply	3	5
Average Time of Treatment (range)	-	6.7 months (6 - 8)

**Table 1:** Clinical characteristics of the Hodgkin Lymphoma patients, before and after the treatment with ABVD

Samples were obtained from patients at Ofir Loyola Hospital (HOL-Belém/Brazil) in two moments: Before starting the ABVD scheme and 3 months after the sixth cycle of ABVD. Blood was collected in PAXgene Blood RNA tubes (Qiagen, Germany) and stored at -80°C until total RNA extraction.

### Total RNA extraction, reverse transcription and quantitative real time PCR (qRT-PCR)

Total RNA was extracted from the 25 blood samples using a PAXgene Blood RNA kit (Qiagen, Germany) according to the manufacturer's instructions. RNA was quantified using a Qubit® 2.0 fluorometer (Life Technologies, Carlsbad, CA) and was then stored at -80°C until performing miRNA profiling.

TaqMan MicroRNA Assays (Thermo Fisher Scientific, Waltham, MA) were used to measure miRNA expression levels. Ten nanograms of total RNA was reversed transcribed using a TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. qRT-PCR was performed in a ABI Prism 7500 thermal cycler using TaqMan Universal Master Mix and TaqMan MicroRNA Assays according to the manufacturer's instructions.

To evaluate differences in the expression levels between each group, the comparative Ct method was used [18], and the endogenous control RNU24 was used to normalize the expression values. The NormFinder algorithm [19] was also used to measure the existing variation in the studied miRNAs in all studied groups, including the endogenous control.

### Statistical analysis

-ΔCt values were used to verify differences in the miRNA expression levels between the (1) untreated HL patients, (2) patients with HL treated with ABVD and (3) Healthy volunteers and 2-ΔCt values were used to present relative expression in graphic form.

The Shapiro-Wilk test was used to determine if the data followed a Gaussian distribution, and One Way ANOVA or Kruskal-Wallis were used to compare the miRNA expression levels between the groups if the data followed parametric or non-parametric distribution, respectively. Differences were considered statistically significant differences at P<0.05, and False Discovery Rate (FDR) correction was applied for multiple pairwise comparisons. All tests and graphics were done using the R statistical package (www.R-project.org).

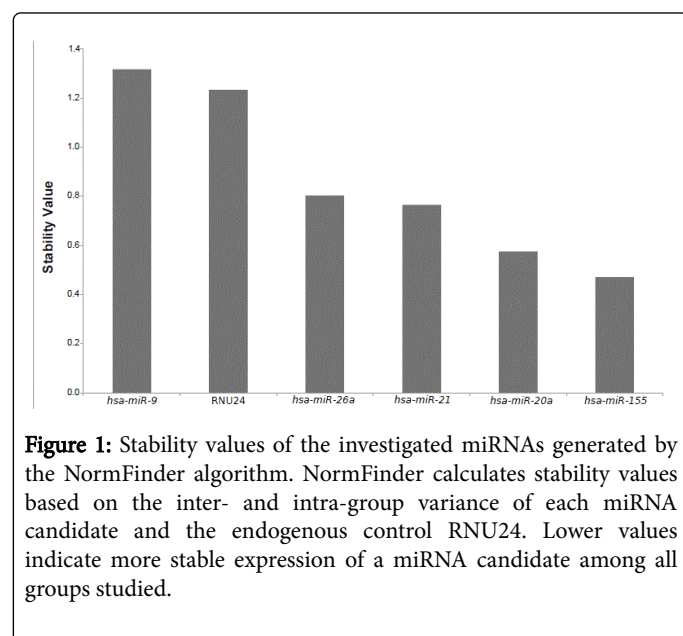
## Prediction of target genes

Target genes regulated by the studied miRNAs were predicted using two online tools: TargetCompare [20] (lghm.ufpa.br/targetcompare), which is able to determine the target genes of miRNAs and the disease that is related to those target genes and to identify simultaneous targets of two or more miRNAs; and miRTarBase [21] (<http://mirtarbase.mbc.nctu.edu.tw>), which predicts possible target genes of a miRNA in addition to indicating which molecular biology technique was used to validate the miRNA-gene interaction. Only genes validated with strong evidence (by a Reporter assay, Western Blot or qRT-PCR) were considered. Targets predicted by both tools that had any relation with hematological diseases or malignant neoplasias were considered in this study.

## Results

### RNU24 does not act as a good endogenous control

The NormFinder algorithm was used to determine stability values (lower values indicate a more stable expression) for all miRNAs investigated, including the endogenous control RNU24 (Figure 1). This endogenous control had one of the highest stability values of all of the examined miRNAs, indicating that it is not a reliable control for the normalization of expression data. The NormFinder analysis also suggested that a better strategy for normalizing the data would be to use hsa-miR-155 as the endogenous control, as it presented the most stable value. To normalize hsa-miR-155 expression we used the mean values of hsa-miR-21 and hsa-miR-155 expression, also suggested by Normfinder.

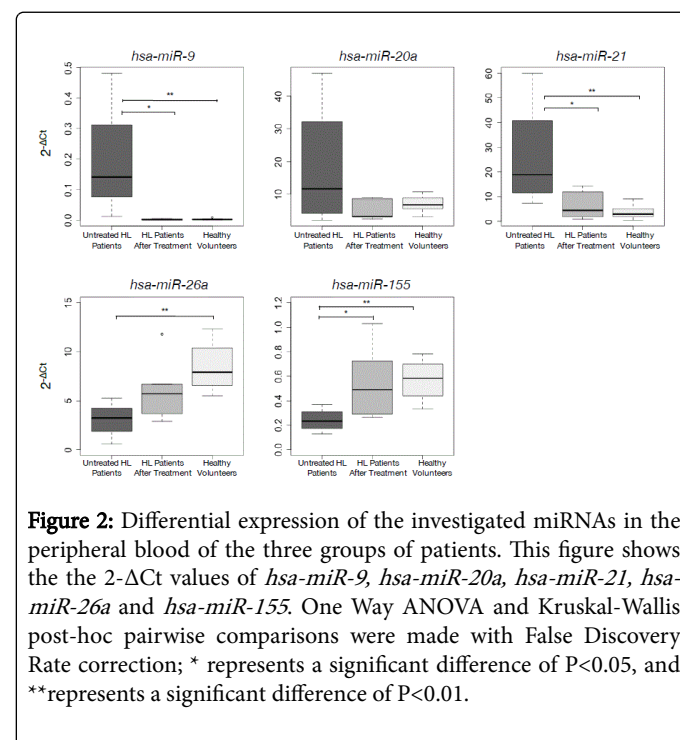


**Figure 1:** Stability values of the investigated miRNAs generated by the NormFinder algorithm. NormFinder calculates stability values based on the inter- and intra-group variance of each miRNA candidate and the endogenous control RNU24. Lower values indicate more stable expression of a miRNA candidate among all groups studied.

### hsa-miR-9, hsa-miR-21, hsa-miR-26a and hsa-miR-155 are differentially expressed in patients with Hodgkin lymphoma

After normalization of the expression data, we observed that the data followed both parametric and non-parametric distribution. ANOVA post-hoc pairwise comparisons showed statistically significant differences in the expression levels of hsa-miR-21 ( $P=0.0043$ ) and hsa-miR-155 ( $P=0.0184$ ) in patients from group 1

compared to patients from group 3 (Figure 2). Hsa-miR-20a was the only miRNA whose expression profile was not different between the studied groups ( $P=0.428$ ). Kruskal-Wallis post-hoc pairwise comparisons showed statistically significant differences in the expression levels of hsa-miR-9 ( $P=0.025$ ) and hsa-miR-26a ( $P=0.011$ ).



**Figure 2:** Differential expression of the investigated miRNAs in the peripheral blood of the three groups of patients. This figure shows the the 2- $\Delta$ Ct values of *hsa-miR-9*, *hsa-miR-20a*, *hsa-miR-21*, *hsa-miR-26a* and *hsa-miR-155*. One Way ANOVA and Kruskal-Wallis post-hoc pairwise comparisons were made with False Discovery Rate correction; \* represents a significant difference of  $P<0.05$ , and \*\* represents a significant difference of  $P<0.01$ .

Compared to group 3, hsa-miR-9 and hsa-miR-21 were up-regulated by 55- and 5.23-fold, while hsa-miR-26a and hsa-miR-155 were down-regulated by 1.35- and 2.36-fold in group 1, respectively.

### ABVD treatment altered the expression of *hsa-miR-9*, *hsa-miR-21* and *hsa-miR-155*

For *hsa-miR-9*, *hsa-miR-21* and *hsa-miR-155* expression, in addition to being different between untreated HL patients and the control group, it was also significantly different ( $P=0.025$ ,  $P=0.019$  and  $P=0.019$ , respectively) between untreated HL patients (group 1) and HL patients treated with ABVD (group 2). The treatment reduced the expression of this miRNA in 62-, 2.48- and 2.05-fold, respectively, not differing from the expressions observed in volunteers without HL (group 3;  $P=0.5353$ ,  $P=0.4654$ ,  $P=0.4654$ , respectively; Figure 2).

### Predicted target genes of *hsa-miR-9*, *hsa-miR-21*, *hsa-miR-26a* and *hsa-miR-155*

Based on the differential expressions of these four miRNAs, we used the online tools TargetCompare and miRTarBase to predict which genes those four miRNAs targeted.

Both platforms predicted the SIRT1 gene as a target gene of *hsa-miR-9*, and RASGRP1, TIMP3 and MTAP genes were predicted as target genes of hsa-miR-21. Because these two miRNAs are up-regulated in untreated HL patients (group 1), we searched for target genes that were shared by these two miRNAs, and the only such gene that was identified was the TGFBR2 gene.

The platforms predicted *HMGA1*, *HMGA2*, *RB1* and *MTDH* genes as possible targets of *hsa-miR-26a*, and *SOCS1* as a possible target of *hsa-miR-155*. The validated common targets of these two down-regulated miRNAs were *JARID2*, *SMAD1*, *SOCS6* and *EZH2*.

## Discussion

There is still no consensus in the literature regarding the best method of normalizing data obtained by relative quantification of miRNAs in whole blood and plasma/serum samples. The most common endogenous controls used to normalize miRNA expression in tissues are small nucleolar RNAs (snoRNAs). Because nucleic acids were extracted from whole blood samples collected in PAXgene tubes, we expected that the chosen endogenous control (RNU24) would be stable between samples. However, the results of the NormFinder algorithm showed that RNU24 was among the miRNAs whose expressions varied the most, indicating that it is not a reliable endogenous control for the normalization of miRNA expression data. Other snoRNAs, such as RNU43, RNU44, RNU48 and RNU6B, have been shown to have highly variable expression in cancer and to introduce bias in miRNA analyses when they are used for normalization [22]. The NormFinder analysis suggested that a better strategy for normalizing the data would be to use *hsa-miR-155* and the mean values of *hsa-miR-21* and *hsa-miR-155* expressions as endogenous controls; therefore, we decided to adopt this strategy.

We analyzed the expression profiles of *hsa-miR-9*, *hsa-miR-20a*, *hsa-miR-21*, *hsa-miR-26a* and *hsa-miR-155* (identified in distinct studies in cell lines and lymph nodes to be specially altered in HL [15-17]) in peripheral blood samples in patients diagnosed with HL (before and after treatment with ABVD) and in healthy volunteers without the disease.

Our results showed that the expression profiles of *hsa-miR-9* and *hsa-miR-21* were up-regulated ( $P=0.0083$  and  $P=0.0043$ , respectively) and the expression profile of *hsa-miR-26a* ( $P=0.011$ ) was down-regulated in untreated patients with HL compared to controls. These results were similar to those observed in HRS cells found in lymph nodes of patients with HL and in cell lines of the disease [16,17]. We observed a down-regulation of *hsa-miR-155* (2.36-fold;  $P=0.0043$ ) in the peripheral blood of untreated patients with HL and an absence of differences in the expression of *hsa-miR-20a* in all groups studied ( $P=0.428$ ).

Of all the studied miRNAs, *hsa-miR-9*, *hsa-miR-21* and *hsa-miR-155* were the ones whose expression profile was altered by ABVD treatment (Figure 2;  $P=0.025$ ,  $P=0.019$  and  $P=0.019$ , respectively). In addition to being a characteristic biomarker of HL, *hsa-miR-9*, *hsa-miR-21* and *hsa-miR-155* may be promising new markers of ABVD treatment response as a reduction in *hsa-miR-9* and *hsa-miR-21* expression and an elevation in *hsa-miR-155* expression were observed in treated HL patients and their expression profiles were similar and had no significant difference ( $P=0.79$ ) to the controls without HL.

Up-regulation of *hsa-miR-9* has been observed in HL [15] and other types of cancer [23,24]. In ovarian cancer [25], this miRNA was found to be closely related to tumorigenesis and elevated tumor sensitivity to treatment with cisplatin and other chemotherapeutic drugs. Although the positive correlations between the up-regulation of *hsa-miR-9* expression and treatment response in ovarian cancer and HL may not involve the same mechanisms, this observed correlation could explain why HL is a cancer type that has one of the highest cure rates and

lower treatment toxicity rates [26] and supports the hypothesis that this miRNA may be a good biomarker of ABVD treatment response.

The *SIRT1* gene was identified as a possible target of *hsa-miR-9*, and its expression in HL would be reduced by the high regulatory activity of this miRNA. Yang *et al.* [27] made a similar observation in gastric cancer: decreased expression of this gene was associated with an elevated proliferation rate and high clonal potential.

Up-regulation of the expression of *hsa-miR-21* in the blood circulation and in solid tumors has been previously observed in various studies and in several types of cancer, including HL [28-32]. A recent meta-analysis of 36 studies regarding circulating *hsa-miR-21* expression [33] revealed that up-regulation of this miRNA was able to distinguish patients with cancer from patients without cancer, with 78% specificity and 82% sensitivity. We observed similar results to those of Jones *et al.* [34]: we found the circulating level of *hsa-miR-21* to be up-regulated in untreated patients with HL, indicating the importance of this miRNA in the general mechanism of carcinogenesis. As elevated expression of *hsa-miR-21* in the blood/plasma has also been observed at the site of origin of the tumor, this miRNA is a promising diagnostic biomarker for the development of malignant neoplasias.

Elevated levels of *hsa-miR-21* and *hsa-miR-30D* were associated with chemoresistance in L428 Hodgkin Lymphoma cell line [6]. When transfected with *hsa-miR-21* inhibitors, L428 cells presented a slight increase in apoptosis rates and when treated with the *hsa-miR-21* inhibitors in combination with doxorubicin the cells presented a major increase in apoptosis. This also suggests that *hsa-miR-21* has an important role in the dynamics of treatment response to chemotherapeutics.

One of the predicted target genes of *hsa-miR-21* was the *TIMP3* gene (a tumor suppressor that induces programmed cell death), and low expression of this gene as well as the up-regulation of *hsa-miR-21* have already been observed in gastric cancer [35,36]. These data suggest that the changes in the expressions of these two genes might be related and reinforces the role of *hsa-miR-21* in cancer development.

The *TGFBR2* gene, a common target of *hsa-miR-9* and *hsa-miR-21*, was previously shown to be down-regulated in B cell lymphoma [37], and this gene is involved in growth suppression. Reduction in the level of TBR1 protein allows the cell to grow without limitation because it compromises growth suppression signaling.

This is the first study to evaluate down-regulated circulating miRNAs in the blood of patients with HL, and *hsa-miR-26a* expression was found to be reduced by 1.35-fold compared with the control group. This down-regulated expression profile has also been observed in HRS cells in lymph nodes of patients with HL [16], in non-Hodgkin lymphomas, such as natural killer/T cell lymphoma [38] and in solid tumors, such as breast cancer [39]. In breast cancer cell lines, the down-regulation of *hsa-miR-26a* was found to be related to the up-regulation of the *HMGA1* gene, which consequently caused increased proliferation.

The other miRNA that was down-regulated in the blood of untreated patients with HL was *hsa-miR-155*, and this is not consistent with the findings of Jones *et al.* [34]; however, this discrepancy can be explained by the differences in the strategies used for nucleic acid extraction from the blood between these two studies. We extracted RNA from whole blood, which included the RNA present in circulating T cells. Huffacker *et al.* [40] observed that *hsa-miR-155*



expression in T cells was closely related to antitumoral activity: mice that had *hsa-miR-155* deficiency in their T CD4+ and CD8+ cells had limited antitumoral activity. Because the *hsa-miR-155* expression profile in untreated HL patients was reduced compared to healthy volunteers without the disease, we can speculate that a similar mechanism to that observed in the mouse study occurs during the development of HL, as immune system evasion is one of the hallmarks of cancer [41].

Analysis of miRNA expression profiles in the peripheral blood of patients with HL has potential for use in clinical practice when the expression levels of most of the miRNAs examined are altered in the presence of the disease. Of all five studied miRNAs, *hsa-miR-9*, *hsa-miR-21*, *hsa-miR-26a* and *hsa-miR-155* were differentially expressed in HL patients that had not received any radiologic or chemotherapeutic treatment compared to the healthy control group without the disease. This difference is important because the differential expression of these miRNAs can separate these two groups.

In addition to the potential of the miRNAs *hsa-miR-9*, *hsa-miR-21* and *hsa-miR-155* as biomarkers of HL, they were also found to be promising biomarkers of ABVD treatment response. Thus, these data suggest that it should have a tracking on the expression levels of these miRNAs in the patients considered remitted after the treatment to verify its efficiency and risks in the relapse of the disease.

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## Authorship

Contribution: AVVDB and ARS conceived and designed the study; AVVDB collected samples from the patients; LM, AMPC and AV realized the experiments; LM analysed the data; LM and ARS wrote the first draft of the manuscript; AVVDB and AV contributed to the final version of the manuscript.

Conflict of interest disclosure: The authors declare that no competing interests exist.

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