MicroRNA Expression Profiling in Classical Hodgkin Lymphoma

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Running title: MicroRNAs in Hodgkin lymphoma
ABSTRACT

MicroRNAs (miRNAs) are negative regulators of gene expression that play an important role in hematopoiesis and tumorigenesis. We analyzed miRNA expression in classical Hodgkin lymphoma (cHL) and the influence of Epstein Barr virus (EBV) infection on the miRNA expression profiles. The expression of 157 miRNAs in lymph nodes from 49 cHL patients and 10 reactive lymph nodes (RLNs) was analyzed by real time PCR. Hierarchical clustering revealed three well-defined groups: nodular sclerosis cHL, mixed cellularity cHL, and RLNs. A distinctive signature of 25 miRNAs differentiated cHL from RLNs, and 36 miRNAs were differentially expressed in the nodular sclerosis and mixed cellularity subtypes. These results were validated in a set of 30 cHLs and five RLNs and in three cHL cell lines. mir-96, mir-128a and mir-128b were selectively downregulated in cHL with EBV. Our findings suggest that miRNAs play an important role in the biology of cHL and may be useful in developing therapies targeting miRNAs.
INTRODUCTION

Mature microRNAs (miRNAs) are naturally occurring small non-coding RNAs that act as negative regulators of gene expression through messenger RNA interference. These molecules were described for the first time in 1993 by Victor Ambros and colleagues in Caenorhabditis elegans, and to date, hundreds of miRNAs have been identified in other species, including viruses. miRNAs are encoded by intronic or intergenic DNA regions, primarily as large molecules that can exceed 1 Kb, and are cleaved by an RNase complex into fragments with characteristic stem-loop structures. In the cytoplasm, a RNase called Dicer further cleaves miRNA to generate a duplex molecule of 21-25 nucleotides in length. One of the two chains is the mature miRNA that binds a protein complex called the RNA-induced silencing complex (RISC). When a miRNA and a messenger RNA exhibit total complementarities, RISC is capable of degrading target messenger RNA, whereas if an incomplete base pairing complementarity takes place, translational silencing of the target occurs. Through these mechanisms, miRNAs decrease translation of human genes.

miRNAs play an important role in cellular proliferation and differentiation and embryonic development, and they also act as oncogenes or tumor suppressor genes. Notably, the majority of miRNAs are found in cancer-associated genomic regions or in chromosome–fragile sites, suggesting an important role for miRNAs in human tumorigenesis. There is also evidence that the influence of miRNAs in oncogenesis might be indirectly driven. For example, the presence of some viruses in a cell may change the host miRNA pattern. Viruses may participate in the origin of some tumors, like the Epstein Barr virus (EBV) in Hodgkin lymphoma (HL).
HL is a neoplasm characterized by the presence of relatively few tumoral cells (Hodgkin and Reed-Sternberg cells) in a non-neoplastic microenvironment. Hodgkin and Reed-Sternberg cells arise from germinal center B-cells. Classical HL (cHL) is subclassified according to the morphology of Reed-Sternberg cells and the composition of the cellular background into nodular sclerosis, mixed cellularity, lymphocyte-rich, and lymphocyte depletion. The two former subtypes are the most frequent forms of cHL and contain a variable proportion of neoplastic cells.

EBV is present in the malignant cells of 40-60% of cHL patients. However, the precise role of the EBV in the pathogenesis of cHL is unknown. It has been reported that viruses have their own miRNA set, and that there is an interaction between the host miRNAs and virus miRNAs. The interaction between the virus and the malignant cells in cHL might be mediated in part by miRNAs.

To investigate whether a specific expression signature of miRNAs is associated with cHL, we assessed the expression of 156 miRNAs, the majority of which are related to hematopoiesis or tumorigenesis, in lymph nodes from patients with nodular sclerosis and mixed cellularity cHL and compared the expression patterns with those in reactive lymph nodes (RLNs). We also examined the influence of EBV on the expression pattern of miRNAs in cHL patients.

METHODS

Approval for these studies was obtained from the Institutional Review Board of Hospital Clinic, Barcelona. Informed consent was provided according to the declaration of Helsinki.
TISSUE SAMPLES AND CELL LINES

Forty-nine specimens of formalin-fixed paraffin-embedded lymph nodes from patients diagnosed with cHL (37 nodular sclerosis and 12 mixed cellularity) between January 1996 and June 2005 were assessed. All patients were diagnosed and followed up in a single institution. Table 1 shows the clinical and biological characteristics of the patients. Ten RLNs were used as controls. In addition, a validation set of 30 formalin-fixed paraffin-embedded lymph nodes from cHL patients (22 nodular sclerosis and 8 mixed cellularity) and 5 RLNs was analyzed. Finally, human HL cell lines L-428 and HD-MY-Z (nodular sclerosis) and L-1236 (mixed cellularity) were analyzed to discriminate between miRNAs expressed in the cell and those present only in the micro-environment. The cell lines were cultured in RPMI 1640 containing 20% fetal calf serum (Invitrogen, Paisley, UK).

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections, as previously described.19 Briefly, paraffin sections on silane-coated slides were dewaxed and subjected to antigen retrieval (Target Retrieval Solution, Dako, Denmark) in a microwavable pressure cooker. Primary antibodies against CD20 (L26, Dako), CD5 (NCL-CD5, Novocastra, Germany), CD30 (BerH2, Dako), and CD15 (Leu M1, Novocastra) were incubated, and the slides were counterstained in Gill's hematoxylin and mounted in Pertex (Histolab GmbH, Germany).
RNA EXTRACTION, REVERSE TRANSCRIPTION AND REAL TIME PCR QUANTIFICATION

Total RNA was extracted from 49 formalin-fixed paraffin-embedded cHL lymph nodes and from 10 RLNs, using RecoverAll™ Total Nucleic Acid Isolation (Ambion, Applied Biosystems, Foster City, CA) as per the manufacturer’s protocol. The same methods were used for RNA extraction in the validation data set of 30 cHL lymph nodes and 5 RLNs. RNA was extracted from the three cell lines using Trizol® total RNA isolation reagent (Gibco BRL, Life Technologies, Gaithersburg, MD) as per the manufacturer’s protocol.

cDNA was synthesized from total RNA using gene-specific primers of 156 different mature miRNAs (TaqMan MicroRNA Assay Protocol, Early Access Kit, Applied Biosystems) (Supplementary Table 1). Real time PCR was performed using an Applied Biosystems 7500 Sequence detection system.

miRNA ANALYSIS BY CHROMOGENIC IN SITU HYBRIDIZATION

Fluorescein (FITC) 5’ labelled locked-nuclei-acid-incorporated (LNA) miRNA ribo probes for mir-21, 134, 138, and 155 (miRCURY™ LNA detection, Exiqon) were used in formalin-fixed, paraffin-embedded tissue sections on silane coated slides (Vision BioSystem, Mount Beberly, AU). Chromogenic in situ hybridization was performed in an automated platform Bond Max (Vision Biosystems). Slides were pretreated with Protease 1 for 10 min at 37°C. A total amount of 300 microliter of 25nM probe was hybridized in 1x sodium chloride-sodium citrate hybridization buffer (SSC) (Innogenetics) up to 50° C for 2 hours. We used a pre-diluted mouse anti-FITC antibody (Vision BioSystems) for 20-60 minutes followed by a biotin-free, polymeric horseradish peroxidase (HRP)-linker antibody conjugate system.
DAB was used as a chromogen reacting for 10 minutes and hematoxilyn was used as a counterstain.

**EBV ANALYSIS**

The presence of EBV in cHL lymph nodes was examined by in situ hybridization for EBV RNA in an automated platform BenchMark XT (EBER 1 and 2, Inform EBER, Ventana Medical Systems, Tucson, AZ), and real time PCR using specific primers and probe for the highly conserved segment BamH1W of EBV.²⁰

**STATISTICAL ANALYSIS**

miRNA expression data was normalized by two different approaches: global-median normalization and let7-a miRNA as previously described.²¹ Data was analyzed using BRB Array Tools version 3.5.0 software and TIGR Multiexperiment viewer version 4.0 software. Hierarchical clustering was performed using average linkage and Euclidean distance. To identify miRNAs with significant differential expression between the two histological subgroups and those that might be influenced by the presence of EBV, two multivariate permutation tests were performed: Significance Analysis of Microarrays (SAM) and Student’s t-test based on multivariate permutation (with random variance model). Differences between miRNAs were considered statistically significant if the P-value was less than 0.001. The Prediction Analysis of Microarrays (PAM) and Class Prediction Methods (BRB Array tools) were used to determine a set of miRNAs able to classify the samples into cHL and RLNs and to differentiate between the two histological subgroups. To identify functional interactions of putative target genes of miRNAs, obtained from Mirbase,²²
TargetScan\textsuperscript{23} and Tarbase\textsuperscript{24} databases, we used DAVID database\textsuperscript{25} and GENIG (http://alggen.lsi.upc.edu).
RESULTS

miRNA PATTERNS IN cHL PATIENTS AND IN RLNs

Unsupervised hierarchical clustering of the cHL and RLN samples was performed on the entire set of unfiltered data. The heat map of miRNA expression categorized three well-defined clusters corresponding to nodular sclerosis cHL, mixed cellularity cHL, and RLNs (Fig. 1). Thirty-eight miRNAs were differentially expressed in cHL versus RLNs (Fig. 2; Supplementary Table 2), and a set of 55 miRNAs was able to further classify all samples into nodular sclerosis cHL, mixed cellularity cHL or RLNs. (Fig. 1). A specific miRNA expression signature consisting of 25 of these 55 miRNAs precisely differentiated between cHLs and RLNs (Fig. 1; Table 2). In addition, 36 miRNAs were differentially expressed in nodular sclerosis and mixed cellularity subtypes (Fig. 2). These findings were confirmed in the validation set of 30 cHLs and 5 RLNs (Supplementary Figure 1).

ANALYSIS OF miRNA EXPRESSION IN HUMAN HL CELL LINES

cHL tumors are composed of different reactive cell types and tumor cells represent a minority. To elucidate whether different signatures might discover different tumor compositions rather than be specific for tumor cells, we analyzed the 25-miRNA signature that discriminated between cHLs and RLNs in three different human HL cell lines: L-428, HD-MY-Z and L-1236 (Fig. 3). We found that 20 of the 25 miRNAs analyzed were expressed by the human HL cell lines and were therefore likely to have been expressed by the tumor cells rather than the microenvironment. The five miRNAs (miR-220, miR-302a, miR-302b, miR-302c and miR-325) that were not expressed in the cell lines yet had been overexpressed in the cHL cases may have been expressed by the reactive microenvironment.
MiR-21, miR-27a, miR-147, miR-182, miR-183 and miR-216 were the most strongly upregulated miRNAs in the HL cell lines (Fig. 3a).

Moreover, we analyzed the 36 miRNAs that were differentially expressed in the nodular sclerosis and mixed cellularity subtypes and found that 32 were expressed in the cell lines; miR-122a, miR-154, miR-302d and miR-371 were not expressed in the cell lines suggesting differences in the reactive microenvironment. MiR-34a, miR-128b, miR-129 and miR-200a were the most strongly differentially expressed miRNAs between L-428 and HD-MY-Z nodular sclerosis HL cell lines versus L-1236 mixed cellularity HL cell line.

**miRNA ANALYSIS BY CHROMOGENIC IN SITU HYBRIDIZATION**

In order to examine whether miRNAs that were more highly expressed in cHLs than in RLNs were detected preferentially in tumor or reactive cells, we analyzed 20 cHL lymph nodes using highly sensitive chromogenic in situ hybridization. We first analyzed miR-155 as a positive control, as previously described in cHL. We observed a cytoplasmic signal in Hodgkin and Reed-Sternberg cells, as well as in scattered reactive lymphocytes and activated histiocytes, as recently reported (Fig. 4). Based on functional and target analyses and on the chromosomal locations of the miRNAs (Table 2), we selected three miRNAs with a potential role in tumorigenesis and analyzed them by chromogenic in situ hybridization in the 20 cases: miR-21 (validated target PTEN, encoded in 17q32.2); miR-134 (putative target J-chain, encoded in 14q32.3); and miR-138 (putative target PU.1, encoded in 7q32.2). In all 20 cases, a cytoplasmic signal was observed in Hodgkin and Reed-Sternberg cells, and we also observed a certain degree of miRNA expression in surrounding lymphocytes. Moreover, a nuclear signal was identified in some reactive tumor-infiltrating lymphocytes for miR-21 and miR-138 (Fig. 4).
EFFECT OF EBV INFECTION ON miRNA EXPRESSION IN cHL

Ten miRNAs were differentially expressed in EBV+ cHL compared to EBV- cHL. In EBV+ cases, miR-96, 128a, 128b, 129 and 205 were underexpressed and miR-28, 130b, 132, 140, 330 were overexpressed (Fig. 5). In the subgroup of nodular sclerosis cHL, three miRNAs (miR-96, 128a and 128b) were significantly underexpressed in EBV+ cHL compared to EBV- cHL. All but one of the mixed cellularity cHLs were EBV+.

ASSOCIATION BETWEEN miRNA EXPRESSION AND CLINICAL PARAMETERS

We analyzed a possible association of miRNA expression with clinical and biological patient characteristics: age, gender, B symptoms, bulky mass, anemia, leukocytosis, lymphocytopenia, hypoalbuminemia, ESR, LDH, β-2-microglobulin, extranodal involvement, stage, Hasenclever index, positivity for CD15, positivity for CD20, and density of tumoral cells and T lymphocytes in the tumor microenvironment. We found that miR-138 was overexpressed in Ann-Arbor stage I-II disease (P=0.003), while miR-328 was overexpressed in Ann-Arbor stage III-IV disease (P=0.004). In order to further investigate this possible association between miRNA expression and disease stage, we analyzed the expression of miR-138 and miR-328 in 30 additional patients from the validation data set (18 stage I-II; 12 stage III-IV). When results for both the original set and the validation set were combined, we found that miR-138 was overexpressed in stage I-II disease but not in stage III-IV (P=0.001) (Supplementary Figure 2), but no association was observed between miR-328 expression and disease stage.
DISCUSSION

Prior studies have shown that a small subset of miRNAs may define tumor entities better than microarray expression data from thousands of messenger RNAs.30 In the present study, we have characterized for the first time a 25-miRNA signature that can differentiate between cHLs and RLNs. In addition, a small number of miRNAs were differentially expressed in mixed cellularity and nodular sclerosis subtypes. Finally, overexpression of one miRNA (miR-138) was related with Ann Arbor stage I-II cHL. miR-138 has been reported to be overexpressed in other tumors31, where it seems to be associated with an undifferentiated state32.

The differential miRNA expression observed between cHLs and RLNs may be explained by cytogenetic changes in the Hodgkin and Reed-Sternberg cells. The genomic region 17q has previously been associated with frequent gains in cHL28, and miR-21 is encoded in this chromosomal region (Table 2). Other previously described chromosomal gains in cHL28 include 2p, where mirR-216 is encoded, 22q, where miR-185 is encoded, and 14q, where miR-134 is encoded. One of the most frequent losses involves 4q, where miR-302a, miR-302b, and miR-302c are encoded28,33, and 3p, where miR-135a is encoded34.

The analysis of the 25-miRNAs cHL signature in cell lines showed a set of strongly upregulated miRNAs (miR-21, miR-27a, miR-147, miR-182 and miR-216) and a set of downregulated miRNAs (miR-126, miR-135a and miR-204) supporting our observations in patient samples. However, other miRNAs showed a different expression pattern in the patient samples compared to the cell lines. Those differences could be due to the expression
of miRNAs by the reactive microenvironment in patient samples or to the molecular and chromosomal alterations of cell lines produced by the immortalization process\textsuperscript{35}. Chromogenic in situ hybridization showed a preferential expression of miR-21, miR-134 and miR-138 in the cytoplasm of Hodgkin and Reed-Sternberg cells, suggesting that miRNA silencing may be biologically relevant in cHL tumor cells; moreover, the miRNA expression observed in lymphocytes provides some evidence that miRNA expression in the tumor microenvironment is also important to the biology and clinical behavior of cHL.\textsuperscript{13,36,37} The cHL tumor microenvironment differs among the histological subtypes, and in the present study, a differential pattern of miRNA expression was observed in mixed cellularity and nodular sclerosis subtypes. The subsequent analysis of cell lines revealed that some miRNAs were also differentially expressed in the Hodgkin and Reed-Sternberg cells in the two histological subtypes. Although prior studies had shown that miR-155 is overexpressed in cHL,\textsuperscript{26} the molecular signature in the present study does not include miR-155. However, chromogenic in situ hybridization showed that miR-155 was constantly expressed in atypical cells in all the cHL cases analyzed as well as in reactive lymphocytes and activated macrophages.\textsuperscript{26,27}

Since miRNAs differentially expressed in cHL cases target putative and validated genes involved in survival, apoptosis and B-cell functions, our findings can provide the basis for a better understanding of the complex mechanisms of transformation of a normal B-cell into a tumor cell. One of the most striking features of cHL tumor cells is their acquisition of survival advantages while largely lacking expression of most of the B-cell associated genes.\textsuperscript{14,38,39} miR-21, which was overexpressed both in cHL lymph nodes and in the human HL cell lines, has been reported to favor cell survival by indirect upregulation of antiapoptotic genes.\textsuperscript{40-44} cHL is a neoplasm of B-cells characterized by an incomplete B-cell
phenotype, due to downregulation of some transcription factors crucial to the full development of a mature B-cell program.\textsuperscript{14} The mechanisms causing downregulation of the transcriptional program in cHL cells are not totally understood, but some epigenetic events are proving to be important in the silencing of B-cell genes.\textsuperscript{45} miRNAs may be a new regulatory epigenetic event, with an important role regulating the translation of a number of different genes in the complicated regulatory network that leads normal B cells from the germinal center\textsuperscript{46} to Hodgkin and Reed-Sternberg cells.

Prior reports had suggested that virus miRNAs can play an important role in the host-pathogen interaction networks. Moreover, viruses might trigger changes in the host miRNA expression pattern, thus favoring cancer development.\textsuperscript{12,47,48} We identified a subset of 10 host miRNAs whose expression was influenced by the presence of EBV. The effect of EBV on host miRNAs might explain the reported association of EBV with the clinical course of cHL patients.\textsuperscript{49} Interestingly, only one of the miRNAs differentially expressed in EBV+ cases was included in the 25-miRNA expression signature differentiating cHL from RLN, leading us to speculate that EBV is not a primary transforming event in cHL, a concept that is also supported by the fact that the majority of the cHL cases were EBV-.\textsuperscript{49}

In summary, cHLs express a characteristic miRNA signature different from that of normal RLNs, with a small number of miRNAs differentially expressed in mixed cellularity and nodular sclerosis and one differentially expressed in early and advanced stage disease. Some of these miRNAs were expressed in Hodgkin and Reed-Sternberg cells but not in reactive cells. In addition, EBV influences host miRNA expression in cHL. These findings suggest that miRNAs may play an important role in the biology of cHL, and they may be useful in the development of therapies targeting miRNAs in tumor cells in cHL patients.\textsuperscript{40,50}
Acknowledgements

Supported in part by RETICS 07 from Instituto de Salud Carlos III, by Program Project grants (BM-05/219) from “La Caixa” and by FISS 050209 and FISS 060087 from the Spanish Ministry of Public Health. O. Balague is a fellow supported by the Instituto de Salud Carlos III.

The authors are grateful to Dr. Isabel Sanchez, CNIO, Madrid, for providing the three cell lines, to Ingrid Lopez and Dolors Fuster for their excellent histology work, to Silvia Pairet for technical assistance, to Dr. Beatriz Zafra from the Spanish Division of Menarini Diagnostics for her support in the situ hybridization development, to Dr. Eva Bandres for her support in the data analysis, and to Renee O’Brate for her assistance in writing the manuscript.

Authors' Contributions

Alfons Navarro designed and performed the research, analyzed the data and wrote the paper; Anna Gaya designed the research, selected cases, analyzed the clinical data and wrote the paper; Antonio Martinez designed and performed the research, analyzed the data and wrote the paper; Alvaro Urbano-Ispizua designed the research, analyzed the data and wrote the paper; Aina Pons performed the research and analyzed the data; Olga Balagué performed the research and analyzed the data; Bernat Gel analyzed the data and performed the statistical analysis; Pau Abrisqueta analyzed the clinical data; Armando Lopez-Guillermo analyzed the clinical data; Rosa Artells analyzed the data; Emili Montserrat wrote the paper; Mariano Monzo designed the research, analyzed the data and wrote the paper.

The authors declare no competing financial interest.
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Ref Type: Generic


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FIGURE LEGENDS

**Figure 1.** miRNA expression pattern in cHL

Panel A: Unsupervised hierarchical cluster analysis categorized three clusters corresponding to nodular sclerosis (NS), mixed cellularity (MC) and reactive lymph nodes (RLN). A corresponding typical histology is shown. The data was presented as log10 of relative quantification normalized in regard to global-median and relative to the 10 reactive lymph nodes median as calibration method.

Panel B: A set of 55 miRNA classified all samples into NS, MC or RLN (PAM analysis). In blue, the 25 miRNAs comprising the miRNA expression signature capable of distinguishing between cHLs and RLNs.

**Figure 2.** Differential expression of miRNAs in cHL subtypes and in reactive lymph nodes

Panel A: nodular sclerosis (NS) vs reactive lymph nodes (RLN)

Panel B: mixed cellularity (MC) vs reactive lymph nodes

Panel C: NS vs MC

Panel D: all cHL cases (NS and MC) vs RLN. miRNAs overexpressed (in red) or underexpressed (in green) are shown in boxes (SAM analysis)

**Figure 3.** Analysis in human Hodgkin lymphoma (HL) cell lines: L-428 and HD-MY-Z (nodular sclerosis) and L-1236 (mixed cellularity)

Panel A: 25-miRNA signature. This figure shows the log2 of fold change (-ΔΔCt) of the 13 miRNAs most differently expressed in the cell lines. The dotted line indicates a fourfold difference in expression compared to the mean of expression of reactive lymph nodes.
Panel B: 32 of the 36 miRNAs differentially expressed in lymph nodes of nodular sclerosis and mixed cellularity subtypes were also differentially expressed in the cell lines. Panel B depicts only the 11 miRNAs with at least a twofold difference in expression between the two subtypes.

**Figure 4.** Chromogenic in situ hybridization of cHL cases

Cytoplasmatic expression in Hodgkin and Reed-Sternberg cells of miR-21 (A), miR-134 (B), miR-138 (C) and miR-155 (D). miR-155 was used as positive control of the hybridization technique. (Microscope, Olympus BX51 [Olympus, Center Valley, PA, USA]; camera, Olympus DP70; lens, UPlanFI 40x/0.75; software, Olympus DP Controller)

**Figure 5.** MiRNAs differentially expressed in EBV+ versus EBV- cases

### TABLES

<table>
<thead>
<tr>
<th>Table 1. Main clinical characteristics of cHL patients</th>
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<tr>
<td>Characteristic</td>
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<td>Median age (range), years</td>
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<tr>
<td>Gender (M/F)</td>
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<tr>
<td>Histology</td>
</tr>
<tr>
<td>Nodular sclerosis</td>
</tr>
<tr>
<td>Mixed cellularity</td>
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<tr>
<td>EBV+*</td>
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<tr>
<td>B symptoms</td>
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<td>Bulky mass</td>
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<tr>
<td>Anemia (Hb &lt;105 g/L)</td>
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<tr>
<td>Leukocytosis (&gt;15 x 10⁹/L)</td>
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<td>Stage III-IV</td>
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<td>Hasenclever index†</td>
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* 8/35 (26%) nodular sclerosis vs 7/10 (70%) mixed cellularity (P=0.01).

† calculated only in 36 patients with advanced disease (stages III-IV, B symptoms or bulky mass).
Table 2. Composition of the miRNA signature of cHL. The putative targets shown were selected based on functional aspects (David Database) and on described gene associations (GENIG).

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Chromosomal Location</th>
<th>Expression Level</th>
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<tr>
<td>miR-21</td>
<td>17q23.2</td>
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<td>PTEN‡, TPM1‡, TRAIL-3,</td>
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<td>NFkB-REPRESOR FACTOR</td>
</tr>
<tr>
<td>miR-335</td>
<td>7q32.2</td>
<td>Low</td>
<td>ANGPT1</td>
</tr>
</tbody>
</table>

† Putative target genes identified from Mirbase and TargetScan by using DAVID database and GENIG software.

‡ Target genes experimentally validated identified from Tarbase.
Figure 2.
Figure 3.
Figure 4.
MicroRNA expression profiling in classical Hodgkin lymphoma

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