Expressions of Micro RNA of Mixed Cellularity Hodgkin Lymphomas are Different in Paediatric and Adult Patients

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ABSTRACT

Objective: MicroRNAs represent an emerging class of small noncoding RNAs that play important roles in the posttranscriptional regulation of gene expression. The aim of this study is evaluation the relevance for microRNA in classical mixed cellularity Hodgkin lymphoma (MCHL) pathogenesis.

Methods: Expressions of 157 microRNAs in lymph nodes from 20 pediatric and 20 adult patients with MCHL were analyzed.

Results: The mean age was 7.4 years in children and 47.4 years in adults. Most patients were male (70%, n=14) in both groups. Stage III disease (N = 9.45%) was common in pediatric group, while stage II disease (N = 18.90%) in others. Thirty-six cases (90%) were alive, while four (10%) were deceased. With microRNA sequencing it was determined that miR-1273e, miR-4322, miR-5008-5p, and miR-6511b-5p were upregulated, while miR-508-5p was down regulated in only pediatric tumors.

Conclusion: These results suggest that microRNAs may play an important role in the biology of pediatric Mixed Cellularity Hodgkin lymphomas and may be useful in developing therapies targeting microRNAs.

Keywords: Children and adults, children and adults mixed cellularity Hodgkin Lymphoma, Micro RNA

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INTRODUCTION

MicroRNAs (miRNAs) are small (20-22 nucleotides), non-protein-coding RNA molecules involved in the post-transcriptional and translational regulation of gene expression (1, 2). The first microRNA (miRNA), lin-4, was identified in C. elegans in 1993, but the term 'microRNA' was not introduced until 2001 (3). Now their names are assigned under a standard nomenclature system (2, 3). Previously, the majority of the human genome was thought to be nonfunctional purpose. Over the past decade, the field of RNA research has rapidly expanded, with a concomitant increase in the number of miRNAs identified in genome (3). Currently, more than 1000 miRNAs have been described for the human genome (4). Although most of them do not have established exact function, the association between their expression and tumorigenesis is widely accepted (4-6). Most miRNAs are encoded by highly conserved DNA regions and found in cancer-associated regions of the genome or in fragile sites (1-6).

Hodgkin's lymphoma (HL) is one of the most frequently occurring lymphomas and subdivided into two main types as nodular lymphocyte predominant HL (NLPHL) and classical HL (cHL) according to their histology and tumor cell characteristics (7-10). The main tumor cells of cHL, Hodgkin and Reed-Sternberg (HRS) cells are derived from preapoptotic germinal center B cells and are characterized by a loss of B-cell phenotype. Cure rates approach 80-90% of patients and it is one of the greatest success of multidisciplinary oncological treatment. However 15-20% of patients are resistant to therapy or relapse after treatment (9). Few studies have been conducted until now concerning the role of miRNA in HL and these studies have shown that miRNA expression analyses may help to understand the physiopathology of HLs (11-12).
Studying differences in age-related miRNA expression profiles in MCHL might enhance our understanding of the pathophysiology of the condition and thereby improve the quality of patient treatment. Childhood MCHL have better prognosis and chemotherapy response than adult cases. Understanding the epigenetic and other factors among these two groups might give us opportunities to develop new miRNA activating or silencing based targeted therapy agents.

In this study, we aimed to evaluate the expression patterns of miRNAs, to correlate their expression levels with age and other clinicopathological features of patients and to investigate the role of microRNAs in tumorigenesis of MCHL. In this cross-sectional study from Izmir Turkey, we aimed to look for whether the pattern of miRNA expression from MCHL tissue is different between paediatric and adult patients. And to identify epigenetic factors that might influence the pathophysiology of MCHL in paediatric and adult patients.

SUBJECTS AND METHODS
The Local Ethics Committee of the Izmir Dr. Behcet Uz Children’s Hospital approved the research protocols, and written informed consent was provided by the participants or their parents for children. Demographic information was obtained from the patient records and registries. Forty tumor specimens were selected from 40 cases of MCHL. These samples consisted of 20 cases of adult MCHL and 20 cases of pediatric MCHL. In addition, 20 normal lymph nodes were collected for an independent sample test to confirm the previous microRNA profiling results. In power analysis, minimum sample size calculated was 16 cases with 25% precision (http://sampsize.sourceforge.net/iface/). We think that our cases with
n=20 for each group would represent our country, because these two hospitals from which the cases were collected accepts patients from all over the country.

Samples and RNA isolation

Hematoxylin and eosin (HE) staining was used to select appropriate paraffin blocks and to identify the viable tumor areas. For the assessment of MicroRNA, the selected tumor tissue parts gently were punched out of the paraffin blocks from 40 patients with MCHL. All of the tissues were obtained before any chemotherapy or radiotherapy. These 40 patients’ samples were deparaffinized with in 1ml xylene and washed two times with in 1 ml absolute ethanol. Deparaffinized tissue samples were placed in tubes containing ceramic beads and were homogenized using a Magna Lyser device (Roche) at 7,500 rpm for 45 seconds. Total RNA was extracted from these tissues using an RNeasy Mini Kit (Qiagen), in accordance with the manufacturer's instructions. Each sample was eluted in 100 μL of RNase-free water. The quantity was measured on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the integrity of the RNA was checked on Agilent 2100 Bioanalyzer Eukaryote Total RNA Nano assay. Good-quality RNA samples were used for subsequent analysis. RNA concentration more than 100 ng/µl and RNA integrity number more than 10 were used as criteria for good- quality. For miRNA expression microarray analysis, we used Human miRNA Microarray, Release 21.0, 8x60K microarray slides (Agillent).

MicroRNA analysis

Microarray analysis was performed by Agilent using the Agilent Human miRNA Microarray, Release 21.0, 8x60K microarray slides (Agilent, Santa Clara, CA) and the protocol provided by the company. The array included 2005 (2026 with controls) microRNAs. Briefly, 100 ng of total RNA, also used for qRT-PCR, 100ng of total RNA was labeled using the Agilent
miRNA Complete Labeling and Hyb Kit (P/N 5190-0456), that was treated with calf intestine alkaline phosphatase for 30 minutes at 37°C before labeling. Samples (4 µl) were diluted with 2.8 µl of DMSO, denatured for 7 minutes at 100°C and labeled in a total volume of 11,3 µl at 16°C for 2 hours using pCp0-Cy3 in T4 RNA ligation buffer supplied in the company kit (5190-0408; Agilent). After labeled miRNA were kept on ice, and completely dried 3 hours with using vacuum concentrator with heater SpeedVac instrument (Thermo). Samples were hybridized at 55°C for 20 hours in an Agilent SureHyb chamber (G2534A; Agilent) rotated at 20 rpm. The arrays were washed with Gene Expression Wash Buffer (Agilent) at 37°C under required ozone conditions before scanning with an Nimblegene Ms200 microarray scanner (Roche).

Microarray data analysis was performed using Agilent Feature Extraction Software (www.agilent.com/chem/fe) with the protocol available for miRNA expression analysis at www.agilent.com/chem/feprotocols. Also extracted data was analyzed with using GeneSpring GX (Version 10.0) Software (Agilent). After analysis, we found that miR-1273e, miR-4322, miR-5008-5p, and miR-6511b-5p were upregulated, while miR-508-5p was down regulated in only pediatric tumors. For all of these 5 miRNAs, we confirmed with Real time PCR using the commercially available LNA™ PCR primer set,UniRT (Exiqon). cDNA was synthesized using the Universal cDNA Synthesis Kit II (Exiqon), in accordance with the manufacturer's instructions. The reaction mixture consisted of 2 µL of total RNA (5 ng/µL), 2 µL of 5x Reaction buffer, 1 µL of Enzyme mix, 0.5 µL of Synthetic RNA spike ins), 4.5 µL of Nuclease-free water in a final reaction volume of 10 µL. After Incubated for 60 min at 42°C and 5 min at 95°C, cDNA diluted in 80x in nuclease free water. The Real Time PCR reaction mixture for the analysis consisted of 4 µL of a diluted template cDNA, 5 µL of ExiLENT SYBR® Green master mix (Exiqon), and 1 µL of microRNA primer mix in a total reaction volume of 10 µL. Real-time PCR was done with precycling heat activation at 95°C
for 10 mins, followed by 45 cycles of denaturation at 95°C for 10 seconds, and annealing/extension at 60°C for 60 seconds, in an LightCycler 480II Real-Time PCR System (Roche).

Bioinformatic and statistical analysis

The miRNA expression analysis was conducted using the Relative Quantitation method. In this analysis, the formulae for the relative quantification of each of the genes were as follows: (dCt of each miRNA) = (Ct of each miRNA) − (Ct of U6 and snord48 snRNA), and (relative quantification of each miRNA) = 2−(dCt of each miRNA). Differences in relative quantification of the target miRNAs in Hodgkin lymphomas tissue RNA or control sample’s RNA were analyzed by two-sided Mann Whitney's U test. Statistical analyses were done using SPSS 15.00. P < 0.05 was considered statistically significant.

RESULTS

The mean age was 7.4± 3.8 years (2- 13 years) in pediatric cases and 47.4± 16.5 years (19-75 years) in adult cases. Most cases were male (70%, n=14) in both groups (M:F ratio is 2.33) reflecting the sex distribution of cases in Turkey. The chemotherapy and radiotherapy were the treatment modalities which were applied to the total of 40 of patients according to their individual features. All pediatric patients had treatment according to German Society of Pediatric Oncology and Hematology Hodgkin Lymphoma Trial 95. Patients in treatment group 1 (TG1; early stages) received two cycles of vincristine, prednisone, procarbazine, and doxorubicin or vincristine, prednisone, etoposide, and doxorubicin chemotherapy; additional two or four cycles of cyclophosphamide, vincristine, prednisone, and procarbazine were added in TG2 (intermediate stages) or TG3 (advanced stages), respectively. Radiotherapy was given to each patient after chemotherapy. All adult cases were treated with ABVD
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(doxorubicin, bleomycin, vinblastine, dacarbazine), BEACOPP (bleomycin, etoposide, doxorubicine, cyclophosphamide, vincristine, procarbazine, prednisone). And according to the clinical stage and bulky lesions, involved-site radiation therapy was recommended.

Most tumors in both groups were located in cervical lymph nodes (14 children/16 adults). Thirty-six cases (90%) were alive, while four cases (10%) were deceased. Rate of relapses was slightly higher in adult cases (15%, N=3) than pediatric cases (10%, N=2). In pediatric group, five cases (25%) were stage I, five cases (25%) were stage II, 9 cases (45%) were stage III and a case (5%) was stage IV. In adult group; 18 cases (90%) were stage II and two cases (10%) were stage III (Figure). Although the presence rate of Ebstein Barr virus latent membrane proteins in RSCs was slightly higher in pediatric patient, there was no statistical importance between two groups (p=0.262). Similarly, the rate of positive staining with CD15 antibody was higher in adult cases. But there was no statistical significance (p=0.150). Features of patients were demonstrated in Table I.

In this study, we used a commercially available MiRNA assay for evaluation 2005 miRNAs. In bioinformatic analysis, without correction 36 miRNAs was found to be different among groups. Benjamini-hochberg, Storey with Bootstrapping and Westfall-Young correction methods combination showed miR-1273e, miR-4322, miR-5008-5p, and miR-6511b-5p upregulation, and miR-508-5p downregulation in pediatric cases to be statistically important. None of the nontumoral samples and adulthood HLs showed alteration in any of the miRNA tested. With miRNA sequencing, pediatric cases showed a lower expression of miR-508-5p and a higher expression of miR-1273e, miR-4322, miR-5008-5p, and miR-6511b-5p compared to control lymph node samples and adult cases. Properties of these miRNAs are shown on Table 2. MiR-1273e, miR-4322, miR-5008-5p, miR-6511b-5p and miR-508-5p expression was not found to be related with stage (p>0.05) in Kruskal Vallis test. Interestingly; none of these miRNAs was not reported before in HLs. These results suggest
that childhood and adulthood MCHLs may have different genetic background and miRNAs may play an important role in the biology of pediatric MCHL.

DISCUSSION

In this study, we evaluated the importance of a specific set of miRNAs in the clinical course of MCHL (especially the age). To explore this hypothesis, we monitored the expression of a panel of 157 miRNAs extracted from the tumor tissue of patients. Potential differences in the distribution of these miRNAs were investigated by age and also on the basis of tissue-type, patient survival, presence of EBV- latent membrane proteins. Pediatric MCHL cases showed a lower expression of miR-508-5p and a higher expression of miR-1273e, miR-4322, miR-5008-5p, and miR-6511b-5p.

Difference of the miRNA expressions has been implicated in various malignancies and several miRNAs have been determined associated with pathogenesis of some cancers (13-19). But there is relatively a few studies reported miRNA expression status in HLs (20-24). In a recent study, Sánchez-Espiridión et al (21) reported an association between survival and a miRNA signature with MIR21, MIR30-e, MIR30-d, MIR92-b. In addition, they suggested that functional silencing of MIR21 and MIR30-d in L428 cells showed increased sensitivity to doxorubicin-induced apoptosis, due to mitochondrial dysfunction and activation of TP53-CDKN1A pathways (21). By this way, both miRNA21 and miRNA30D play a role in cHL tumorigenesis and therapy response. Similarly, low miRNA 135 levels are found associated with higher relapse rates and shorter diseases-free survival (24). MiRNA 16, miRNA 24, miRNA 155, miRNA 124-a and miRNA 328 are also commonly reported miRNAs associated with HL pathogenesis (11, 20, 25). In the present study, we evaluated of 157 common miRNAs and we didn’t determine any differentiation in adult cases and
nontumoral samples. Contrary, pediatric cases showed a lower expression of miR-508-5p and a higher expression of miR-1273e, miR-4322, miR-5008-5p, and miR-6511b-5p compared to control lymph node samples and adult cases. Interestingly none of these miRNAs were not reported before in HLs.

Currently, sequential numerical identifiers are assigned to each novel miRNAs. If the mature miRNAs differ by only one or two nucleotides, they are allocated letter suffixes according a standard nomenclature system (2, 3). When we made a search according to the main numbers of these 5 miRNAs, there are relatively more report of miRNA 1273 in literature (13, 14). Ivashchenko et al (13), searched the binding sites of miR-1273 family on the mRNA of target genes and they reported that miRNA 1273 family acts an imported role on the cell cycles by several genes. They found 449 miR-1273e binding sites on the mRNAs of 413 target genes. Similarly Zhang et al (14) reported that flourouroacil alters the microRNA expression profile and acts as negative regulatory on miRNA 1273e (14).

In Turkey, Hodgkin Lymphoma is observed in 1.6/100.000 in women and 2.2/100000 in men according to Turkish Cancer Statistics Report 2015 of Ministry of Health. It is the second most common malignancy among 15-24 years of age after testis cancer for men and thyroid cancer for women. In Turkish Pediatric Ongology group cancer statistics records, between 2009-2016; 947 of 12671 recorded pediatric malignancy cases are Hodgkin lymphomas. MSHL cases contributes approximately 56% of all Hodgkin Lymphoma cases. In pediatric cases male/female ratio is 2.8.

Malignant cells of cHL are derived from preapoptotic germinal center B cells that have lost their normal B cell phenotype. Alterations in the cell cycle and apoptosis pathways contribute to their resistance to apoptosis and sustained cell cycle progression. CDKN1A, encoding p21 plays a basic role in this process (11). P21 is regulated by p53 and can function as a cell cycle inhibitor when in the nucleus or as an apoptosis inhibitor when localized in the
cytoplasm. In several studies, it was shown that special MiRNAs such as miR-17 and miR-106a, upregulate to cell cycle progression and contribute to a dysfunctional p53 pathway. Jones et al (26) also reported that plasma levels of miR-494, miR-1973, and miR-21 were higher in patients with HL than control plasmas. They also claimed that in patients with cHL, circulating cell-free miRNAs can reflect disease response once therapy has commenced (11, 21, 26).

The Epstein–Barr virus (EBV), also called human herpesvirus 4 (HHV-4), is one of eight viruses in the herpes family, and is one of the most common viruses in humans. It is also associated with particular forms of malignancy including the HL. Navorra et al (27) analyzed miRNA expression in cHL and the influence of EBV infection on the miRNA expression profiles (26). A distinctive signatures of miR-96, miR-128a, and miR-128b were selectively down-regulated in cHL with EBV. Our findings suggest that the presence of EBV-LMP is more common in pediatric group and EBV may also play an important role in the biology of pediatric MCHL (8-10, 21).

Previous studies show that one miRNA can bind to one or more mRNAs, and some mRNAs have multiple binding sites for different miRNAs that are within the same family. In the present study, origin of miR-1273e was not established. Ivashchenko et al (13) didn’t found miR1273E’s origin, either. In this study, we also determined that coding gene of MiR-4322 is located chromosome 19, MiR-5008-5p on chromosome 1, MiR-508-5p on chromosome X and MiR-6511b on chromosome 16. There are only a few studies about these 5 MiRNAs in the literature. Therefore we couldn’t make a comprehensive evaluation about the target genes of these 5 MiRNAs (13, 14).
CONCLUSION
In summary; the expressions of 5 specific MiRNAs (MiR1273e, MiR4322, MiR5008-5p, MiR508-5p and MiR6511b), were found to be different between pediatric and adult MSHL cases. These preliminary findings suggest a potential role of MiRNAs in pediatric HLs. But further studies are needed to corroborate and extend our results. The data of this study might warrant further more detailed investigation using a larger sample size.

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AUTHORS’ NOTE
G Diniz and S Aktas, conceived paper, oversaw data collection, conducted data analysis, wrote manuscript and approved final version. M Ceyhan and H Tosun Yıldırım participated in study design, data analysis and interpretation, critically revised manuscript and approved final version. C Ceylan, Y Oymak, Dudu Solakoglu Kahraman, B Demirag and H Oniz participated in study design, data analysis, and interpretation of data and revision of manuscript and approved final version. N Olgun interpretation of data and revision of manuscript and approved final version.
REFERENCES


Table 1: The clinical features of the cases

<table>
<thead>
<tr>
<th></th>
<th>Pediatric Patients</th>
<th>Adult Patients</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (mean of groups)</strong></td>
<td>7.45±3.8 (2-13)</td>
<td>47.4±16.7 (19-75)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Gender (male/female)</strong></td>
<td>M=14.70%/ F=6.30%</td>
<td>M=14.70%/ F=6.30%</td>
<td>0.634</td>
</tr>
<tr>
<td><strong>Location (Cervical/other regions)</strong></td>
<td>N=16, 80% Cervical</td>
<td>N=14, 70% Cervical</td>
<td>0.358</td>
</tr>
<tr>
<td></td>
<td>N=4, 20% other regions</td>
<td>N=6, 300% other regions</td>
<td></td>
</tr>
<tr>
<td><strong>Stage (early/advanced)</strong></td>
<td>N=10, 50% early stage</td>
<td>N=18, 90% early stage</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>N=10, 50% advanced stage</td>
<td>N=2, 10% advanced stage</td>
<td></td>
</tr>
<tr>
<td><strong>Presence of B symptoms</strong></td>
<td>N=8, 40%</td>
<td>N=10, 50%</td>
<td>0.376</td>
</tr>
<tr>
<td><strong>Prognosis (alive/died)</strong></td>
<td>N=18, 90% alive</td>
<td>N=18, 90% alive</td>
<td>0.698</td>
</tr>
<tr>
<td><strong>Presence of EMV-LMP</strong></td>
<td>N=13, 65%</td>
<td>N=10, 50%</td>
<td>0.262</td>
</tr>
<tr>
<td><strong>Presence of CD15</strong></td>
<td>N=12, 60%</td>
<td>N=16, 80%</td>
<td>0.150</td>
</tr>
</tbody>
</table>

Table 2. The properties of the miRNAs that are statistically different in Pediatric mixed cellular Hodgkin Lymphoma cases.

<table>
<thead>
<tr>
<th>miR</th>
<th>Regulation</th>
<th>p value</th>
<th>Log FC</th>
<th>Active sequence</th>
<th>Chr</th>
<th>mirbase accession No</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-1273e</td>
<td>up</td>
<td>0.0011</td>
<td>4.61</td>
<td>TCCACTTCCTGGG TTC</td>
<td>Chr17</td>
<td>MIMAT0018079</td>
</tr>
<tr>
<td>miR-4322</td>
<td>up</td>
<td>0.0021</td>
<td>4.39</td>
<td>CCCACGCAGCTG</td>
<td>Chr19</td>
<td>MIMAT0016873</td>
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<tr>
<td>miR-5008-5p</td>
<td>up</td>
<td>0.0021</td>
<td>4.53</td>
<td>CCACTGTGCCCCA</td>
<td>Chr1</td>
<td>MIMAT0021039</td>
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<tr>
<td>miR-6511b-5p</td>
<td>up</td>
<td>0.01</td>
<td>5.299</td>
<td>TGTCAGCAGCCACTTC</td>
<td>Chr16</td>
<td>MIMAT0025847</td>
</tr>
<tr>
<td>miR-508-5p</td>
<td>down</td>
<td>0.0022</td>
<td>-4.66</td>
<td>CATGAGTGACGCCCTC</td>
<td>ChrX</td>
<td>MIMAT0004778</td>
</tr>
</tbody>
</table>
Figure: Distributions of the pediatric and adult cases according to the stage ($p = 0.014$).