ORIGINAL ARTICLE

Expressions of MicroRNA 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 non-coding RNAs that play important roles in the post-transcriptional regulation of gene expression. This study was aimed to evaluate the relevance of microRNA in classical mixed cellularity Hodgkin lymphoma (MCHL) pathogenesis.*

Methods: *The expressions of 157 microRNAs in lymph nodes from 20 paediatric and 20 adult patients with MCHL and 20 normal lymph nodes (controls) were analysed.*

Results: *The patients' mean age was 7.4 years for the children and 47.4 years for the adults. Most of the patients were male (n = 14, 70%) in both groups. Stage III disease (n = 9, 45%) was common in the paediatric 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 paediatric tumours.*

Conclusion: *These results suggest that microRNAs may play an important role in the biology of paediatric MCHL and may be useful in developing therapies targeting microRNAs.*

Keywords: Children and adults, microRNA, mixed cellularity Hodgkin lymphoma

INTRODUCTION

MicroRNAs (miRNAs) are small (20–22 nucleotides), non-coding RNA molecules, which do not code a protein, involved in the post-transcriptional and translational regulation of gene expression (1, 2). The first microRNA (miRNA), lin-4, was identified in *Caenorhabditis 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 non-functional. Over the past decade, the field of RNA research had rapidly expanded, with a concomitant increase in the number of miRNAs identified in human 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 is subdivided into two main types as nodular lymphocyte-predominant HL (NLPHL) and classical HL (cHL) according to their histology and tumour cell characteristics (7–10). The main tumour cells of cHL, Hodgkin and Reed-Sternberg

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(HRS) cells are derived from pre-apoptotic germinal centre B cells and are characterized by a loss of B-cell phenotype. The cure rates approach 80%–90% of patients and it is one of the greatest successes of multidisciplinary oncological treatment. However, 15%–20% of patients are resistant to therapy or relapse after treatment (9). Few studies had been conducted until now concerning the role of miRNA in HL and these studies had shown that miRNA expression analyses might help to understand the physiopathology of HLs (11, 12).

Studying the differences in age-related miRNA expression profiles in MCHL may enhance our understanding of the pathophysiology of the condition and thereby improve the quality of patient treatment. Childhood MCHL have better prognoses and chemotherapy responses than adult cases. Understanding the epigenetic and other factors among these two groups may provide the opportunities to develop new miRNA activating or slicing-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 statistical associations between microRNA levels and MCH. 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 the 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 regarding the children. Demographic information was obtained from the patients' records and registries. Forty tumour specimens were selected from 40 cases of MCHL. These samples consisted of 20 cases of adult MCHL and 20 cases of paediatric MCHL. In addition, 20 normal lymph nodes were collected for an independent sample test to confirm the previous microRNA profiling results. In the power analysis, the 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 accept patients from all over the country.

Samples and RNA isolation

Hematoxylin and eosin (HE) staining were used to select appropriate paraffin blocks and to identify the viable tumour areas. For the assessment of MicroRNA, the selected tumour tissue parts were gently punched out of the paraffin blocks from the 40 patients with MCHL. All of the tissues were obtained before any chemotherapy or radiotherapy was done on them. These 40 patients' samples were deparaffinized with 1-ml xylene and washed two times with 1-ml absolute ethanol. The deparaffinized tissue samples were placed in tubes containing ceramic beads and were homogenized using a Magna Lyser device (Roche) at 7500 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, USA), and the integrity of the RNA was checked on Agilent 2100 Bioanalyzer Eukaryote Total RNA Nano assay. Good-quality RNA samples were used for the subsequent analysis. RNA concentration more than 100 ng/µl and RNA integrity number more than 10 were used as the criteria for good quality. For miRNA expression microarray analysis, we used human miRNA microarray, Release 21.0, 8x60K microarray slides (Agilent Technologies, Santa Clara, CA, USA).

MicroRNA analysis

Microarray analysis was performed by Agilent using the Agilent human miRNA microarray, Release 21.0, 8x60K microarray slides (Agilent Technologies, Santa Clara, CA, USA) 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, 100 ng of total RNA was labelled using the Agilent miRNA Complete Labelling and Hyb Kit (P/N 5190-0456), that was treated with calf intestine alkaline phosphatase for 30 minutes at 37°C before labelling. Samples (4 µl) were diluted with 2.8 µl of dimethyl sulfoxide (DMSO) denatured for seven minutes at 100°C and labelled 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 Technologies, Santa Clara, CA, USA). After labelled miRNA were kept on ice, and completely dried for 3 hours using vacuum concentrator with heater SpeedVac instrument (Thermo). The samples were hybridized

at 55°C for 20 hours in an Agilent SureHyb chamber (G2534A; Agilent Technologies, Santa Clara, CA, USA) rotated at 20 rpm. The arrays were washed with Gene Expression Wash Buffer (Agilent Technologies, Santa Clara, CA, USA) at 37°C under the required ozone conditions before scanning with an Nimblegene Ms200 microarray scanner (Roche).

The microarray data analysis was done, 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, the extracted data were analysed using GeneSpring GX (Version 10.0) Software (Agilent Technologies, Santa Clara, CA, USA). After the 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 paediatric tumours. 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 5× 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 minutes at 42˚C and 5 minutes at 95˚C, cDNA was diluted in 80× 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 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds, and annealing/extension at 60°C for 60 seconds, in a LightCycler 480II Real-Time PCR System (Roche).

Bioinformatic and statistical analyses

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). The differences in the relative quantification of the target miRNAs in Hodgkin lymphomas tissue RNA or control sample's RNA were analysed by two-sided Mann Whitney's *U* test. Statistical analyses were done using SPSS 15.00. p-Value < 0.05 was considered statistically significant.

RESULTS

The patients' mean age was 7.4 ± 3.8 years (2–13 years) in the paediatric cases and 47.4 ± 16.5 years (19–75 years) in the adult cases. Most of the cases were males (70%, n = 14) in both groups (M:F ratio is 2.33) reflecting the gender distribution of cases in Turkey. Chemotherapy and radiotherapy were the treatment modalities that were applied to a total of 40 patients according to their features. All the paediatric patients had treatment according to the German Society of Paediatric Oncology and Hematology Hodgkin Lymphoma Trial 95. The 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 the adult cases were treated with doxorubucin, bleomycin, vinblastine, dacarbazine (ABVD), bleomycine, etoposide, doxorubucine, cyclophosphamide, vincristine, procarbazine, prednisone (BEACOPP). Moreover, according to the clinical stage and bulky lesions, involved-site radiation therapy was recommended.

Most tumours in both groups were located in the cervical lymph nodes (14 children/16 adults). Thirty-six cases (90%) were alive, while four cases (10%) were deceased. The rate of the relapses was slightly higher in the adult cases (15%, n = 3) than in the paediatric cases (10%, n = 2). In the paediatric group, five cases (25%) were stage I, five cases (25%) were stage II, nine cases (45%) were stage III and a case (5%) was stage IV. In the adult group, 18 cases (90%) were stage II and two cases (10%) were stage III (Figure). Although the presence of Ebstein–Barr virus (EBV) latent membrane proteins in the RSCs was slightly higher than in paediatric patients, there was no statistically significant difference between the two groups (p = 0.262). Similarly, the rate of positive staining with CD15 antibody was higher in adult cases. But it was not statistically significant (p = 0.150). The features of the patients are demonstrated in Table 1.

In this study, we used a commercially available MiRNA assay for the evaluation of 2005 miRNAs. In the bioinformatic analyses, without correction, 36 miRNAs were found to be different among the groups.

Table 1: The clinical features of the cases

	Paediatric patients	Adult patients	*p*
Age (mean of groups)	7.45 ± 3.8 (2–13)	47.4 ± 16.7 (19–75)	**< 0.001***
Gender (male/female)	M = 14, 70%/ F = 6, 30%	M = 14, 70%/ F = 6, 30%	0.634
Location (cervical/other regions)	n = 16, 80% Cervical	n = 14, 70% Cervical	0.358
	n = 4, 20% other regions	n = 6, 300% other regions	
Stage (early/ advanced)	n = 10, 50% early stage	n = 18, 90% early stage	**0.007**
	n = 10, 50% advanced stage	n = 2, 10% advanced stage	
Presence of B symptoms)	n = 8, 40%	n = 10, 50%	0.376
Prognosis (alive/ died)	n = 18, 90% alive	n = 18, 90% alive	0.698
Presence of EMV-LMP	n = 13, 65%	n = 10, 50%	0.262
Presence of CD15	n = 12, 60%	n = 16, 80%	0.150

*Statistically significant *p* values are in bold.

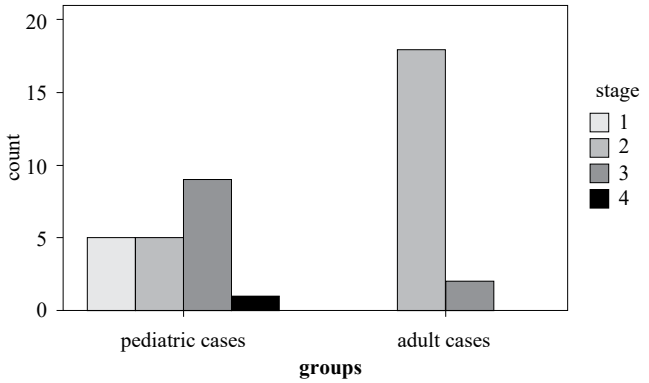

Figure: Distributions of the paediatric and adult cases according to the stage ($p = 0.014$).

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 the paediatric cases to be statistically important. None of the non-tumoural samples and adulthood HLs showed alteration in any of the miRNA tested. With miRNA sequencing, paediatric 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 with the control lymph node samples and adult cases. The properties of these miRNAs are shown in Table 2. MiR-1273e, miR-4322, miR-5008-5p, miR-6511b-5p and miR-508-5p expressions were not found to be related with any stage ($p > 0.05$) in the Kruskal–Wallis test. Interestingly, none of these miRNAs were 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 paediatric MCHL.

DISCUSSION

In this study, we evaluated the distribution of 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 tumour tissue of patients. The potential differences in the distribution of these miRNAs were investigated by age and also on the basis of tissue type, patient survival, and the presence of EBV-latent membrane proteins. The paediatric 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.

The differences in the miRNA expressions had been implicated in various malignancies and numerous miRNAs have been found to be associated with the pathogenesis of some cancers (13–19). But there is relatively a few studies that reported on the 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 the functional

Table 2: The properties of the miRNAs that are statistically different in paediatric mixed cellular Hodgkin lymphoma cases

	Regulation	*p*-Value	Log FC	Active sequence	Chr	miRbase accession no
miR-1273e	Up	0.0011	4.61	TCCACTTCCTGGGTTC	Chr17	MIMAT0018079
miR-4322	Up	0.0021	4.39	CCCCACGCGCTG	Chr19	MIMAT0016873
miR-5008-5p	Up	0.0021	4.53	CCACTGTGCCCCA	Chr1	MIMAT0021039
miR-6511b-5p	Up	0.01	5.299	TGTCAGCCCCACTTC	Chr16	MIMAT0025847
miR-508-5p	Down	0.0022	−4.66	CATGAGTGACGCCCTC	ChrX	MIMAT0004778

silencing of MIR21 and MIR30-d in L428 cells showed an increased sensitivity to doxorubicin-induced apoptosis, due to mitochondrial dysfunction and the activation of TP53-CDKN1A pathways (21). In this way, both miRNA21 and miRNA30D play a role in cHL tumourigenesis and therapy response. Similarly, low miRNA 135 levels were found to be associated with higher relapse rates and shorter diseases-free survival (24). miRNA 16, miRNA 24, miRNA 155, miRNA 124-a and miRNA 328 were also commonly reported miRNAs that were associated with HL pathogenesis (11, 20, 25). In the present study, we evaluated 157 common miRNAs and we did not determine any differentiation in the adult cases and the non-tumoural samples. In contrast, the paediatric 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 with the control lymph node samples and the adult cases. Interestingly, none of these miRNAs were 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 to a standard nomenclature system (2, 3). When we made a search according to the main numbers of these five miRNAs, there were relatively more reports of miRNA 1273 in the 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 played an important role in the cell cycles by many genes. They found 449 miR-1273e binding sites on the mRNAs of 413 target genes. Similarly, Zhang *et al* (14) reported that flourouroacil altered the microRNA expression profile and acted as a negative regulatory on miRNA 1273e (14).

In Turkey, Hodgkin Lymphoma was observed in 1.6/100 000 in women and 2.2/ 100 000 in men according to the Turkish Cancer Statistics Report 2015 of the 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 the Turkish Paediatric Oncology group cancer statistics records, between 2009 and 2016, 947 of the 12 671 recorded paediatric malignancy cases are HLs. The MSHL cases contributed approximately 56% of all the HL cases. In the paediatric cases, the male/female ratio was 2:8.

The malignant cells of cHL are derived from pre-apoptotic germinal centre B cells that have lost their normal B cell phenotype. The alterations in the cell cycle and the apoptosis pathways contribute to their resistance to apoptosis and sustained cell cycle progression. CDKN1A, encoding p21 plays a basic role in this process (11). The 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 numerous studies, it was shown that special miRNAs such as miR-17 and miR-106a upregulated cell cycle progression and contributed to a dysfunctional p53 pathway. Jones *et al* (26) also reported that the plasma levels of miR-494, miR-1973, and miR-21, were higher in patients with HL than in the control's plasmas. They also claimed that in patients with cHL, circulating cell-free miRNAs could reflect disease response once therapy has commenced (11, 21, 26).

The 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) analysed miRNA expression in cHL and the influence of EBV infection on the miRNA expression profiles (26). The distinctive signatures of miR-96, miR-128a and miR-128b were selectively down-regulated in cHL with EBV. Our findings suggested that the presence of EBV–LMP was more common in the paediatric group and EBV might also play an important role in the biology of paediatric MCHL (8–10, 21).

Previous studies showed that one miRNA could 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, the origin of miR-1273e was not established. Ivashchenko *et al* (13) did not find the miR1273E's origin, either. In this study, we also discovered that coding gene of MiR-4322 was located on chromosome 19, MiR-5008-5p on chromosome 1, MiR-508-5p on chromosome X, and MiR-6511b on chromosome 16. There were only a few studies about these five MiRNAs in the literature. Therefore, we could not make a comprehensive evaluation about the target genes of these 5 miRNAs (13, 14).

CONCLUSION

In summary, the expressions of five specific miRNAs (miR1273e, miR4322, miR5008-5p, miR508-5p and miR6511b) were found to be different between the paediatric and adult MSHL cases. These preliminary findings suggest a potential role of miRNAs in the paediatric HLs. But further studies are needed to corroborate and extend our results. The data of this study might warrant further more detailed investigations using larger sample sizes.

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AUTHORS' NOTE

GD and SA conceived the paper, oversaw the data collection, conducted the data analyses, wrote the manuscript and approved the final version. MC and HTY participated in the study design, data analyses and interpretations, critically revised the manuscript and approved the final version. CC, YO, DSK, BD and HO participated in the study design, data analyses, and the interpretations of the data and revision of the manuscript and approved the final version. NO participated in the interpretations of the data and the revision of the manuscript and approved the final version.

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