The American Journal of Pathology, Vol. ■, No. ■, ■ 2017

ELSEVIER

The American Journal of PATHOLOGY

ajp.amjpathol.org

miR-24-3p Is Overexpressed in Hodgkin Lymphoma and Protects Hodgkin and Reed-Sternberg Cells from Apoptosis

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Accepted for publication February 16, 2017.

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miRNAs play important roles in biological processes, such as proliferation, metabolism, differentiation, and apoptosis, whereas altered expression levels contribute to diseases, such as cancers. We identified miRNAs with aberrant expression in Hodgkin lymphoma (HL) and investigated their role in pathogenesis. Small RNA sequencing revealed 84 significantly differentially expressed miRNAs in HL cell lines as compared to germinal center B cells. Three up-regulated miRNAs—miR-23a-3p, miR-24-3p, and miR-27a-3p—were derived from one primary miRNA transcript. Loss-of-function analyses for these miRNAs and their seed family members resulted in decreased growth on miR-24-3p inhibition in three and of miR-27a/b-3p inhibition in one HL cell line. Apoptosis analysis indicated that the effect of miR-24-3p on cell growth is at least in part caused by an increase of apoptotic cells. Argonaute 2 immunoprecipitation revealed 1142 genes consistently targeted by miRNAs in at least three of four HL cell lines. Furthermore, 52 of the 1142 genes were predicted targets of miR-24-3p. Functional annotation analysis revealed a function related to cell growth, cell death, and/or apoptosis for 15 of the 52 genes. Western blotting of the top five genes showed increased protein levels on miR-24-3p inhibition for CDKN1B/P27^{kip1} and MYC. In summary, we showed that miR-24-3p is up-regulated in HL and its inhibition impairs cell growth possibly via targeting CDKN1B/P27^{kip1} and MYC. *(Am J Pathol 2017, ■: 1–13; http://dx.doi.org/10.1016/j.ajpath.2017.02.016)*

Q5 Hodgkin lymphoma (HL) is a B-cell–derived lymphoma classified into classic HL (cHL) and nodular lymphocyte–predominant HL.[1] Nodular lymphocyte–predominant HL is a more rare subtype of HL, accounting for approximately 5% of all cases.[2] cHL accounts for 95% of all HL cases and is characterized by a minority of Hodgkin and Reed-Sternberg (HRS) tumor cells,[3] which have lost their normal B-cell phenotype.[4] Furthermore, cHL is subclassified according to the morphology of HRS cells and the composition of the cellular background into nodular sclerosis, mixed cellularity, lymphocyte-rich, and lymphocyte-depleted cases.[5]

miRNAs are short noncoding RNA molecules with unique expression patterns in different tissue and cell types.[6,7] They inhibit gene expression by binding to complementary sequences at the 3′ untranslated region of their target gene transcripts.[8] One single miRNA can interact with multiple targets.[9] The first human cancer type reported to be associated with miRNAs was chronic lymphocytic leukemia.[10] After that, many aberrant miRNA expression patterns have been linked to specific types of cancer.[11] Depending on their set of target genes, miRNAs can act as oncogenes or tumor suppressor genes.[12–14]

So far, multiple miRNAs are deregulated in B-cell lymphoma and for a subset of them pivotal functions have been shown in the pathogenesis.[10,15,16] Using small RNA

Y.Y. received a fellowship of the Graduate School of Medical Sciences of the University of Groningen. Q3

Disclosures: None declared.

http://dx.doi.org/10.1016/j.ajpath.2017.02.016

sequencing, Landgraf et al[17] generated among others miRNA expression profiles of four Epstein-Barr virus−cHL cell lines. Van Vlierberghe et al[18] identified 12 up-regulated and three down-regulated miRNAs in microdissected HRS cells from nine cHL patients and HL cell lines compared to $CD77^+$ germinal center (GC)-B cells. Gibcus et al[19] determined the miRNA profile of HL cell lines in comparison to GC-B cell−derived lymphoblastoid cell lines and other B-cell lymphoma cell lines and showed increased expression of the miR-17 to miR-92 cluster, miR-16, miR-21, miR-24, and miR-155 in HL. Functional studies in HL are limited, but for some of the miRNAs their putative role has been established. miR-135a targets JAK2, which leads to reduced Bcl-xL levels in HL.[20] The miR-17/106b seed family targets CDKN1A encoding for the P21 protein and inhibition of this seed family results in a G_1-phase cell cycle arrest.[21] HuR and Dicer were shown to be targets of the oncogenic miR-9 and inhibition of miR-9 resulted in higher cytokine production levels.[22] A significant correlation between miR-124a methylation status and a high-risk international prognostic score was found in HL.[23]

Herein, we established an HL-specific miRNA expression profile using small RNA sequencing and validated differential expression of selected miRNAs. Furthermore, we determined the effects of miR-23a/b-3p, miR-24-3p, and miR-27a/b-3p inhibition on cell growth. To identify target genes regulated by these miRNAs, Ago2 RNA immunoprecipitation (Ago2-RIP) followed by a microarray analysis was performed on four HL cell lines. Targeting of selected Ago2-IP−enriched miR-24-3p−target genes was confirmed using Western blotting.

Materials and Methods

Culturing of HL Cell Lines and Sorting of GC B Cells

L540 (nodular sclerosis, T-cell derived), KM-H2 (mixed cellularity), L1236 (mixed cellularity), L428 (nodular sclerosis), and HDLM2 (nodular sclerosis) HL cell lines were cultured in RPMI 1640 medium (Cambrex Biosciences, Walkersville, MD), and the SUPHD1 (lymphocyte depleted) HL cell line was cultured in McCoy 5A medium (Cambrex Biosciences) at 37°C in an atmosphere containing 5% CO_2. Culture medium was supplemented with 2 mmol/L ultra-glutamine 1 (Cambrex Biosciences), 100 U/mL penicillin/streptomycin, and 5% L428, 10% L1236, KM-H2, and HDLM2, or 20% L540 and SUPHD1 fetal bovine serum (Cambrex Biosciences).

GC-B cells were sorted from tonsil tissue samples of three HL donors aged between 2 and 6 years. Two of the three GC-B cells were purified >98% from human tonsils based on expression of $CD20^+IgD^-CD38^+$ as previously described.[24] The third sample was magnetic-activated cell sorting purified >95% based on expression of $IgD^-CD138^-CD3^-CD10^+$. Briefly, a freshly prepared tonsillar cell suspension was prepared and depleted from IgD^+ (naïve), $CD138^+$ (plasma cells), and $CD3^+$ (T cells) using LD columns and $IgD^-Biotin^+$ anti-Biotin beads, CD138-beads, and CD3-beads (Miltenyi Biotec, Leiden, the Netherlands). Next, we positively enriched the flow-through fraction for $CD10^+$ cells using CD10 beads and LS columns (Miltenyi Biotec). Purity of the GC-B cell population was confirmed by fluorescence-activated cell sorting using antibodies against CD20, IgD, and CD38 as indicated above. All cells of $CD20^+IgD^-CD38^+$ were considered to be GC-B cells. The procedures were according to the guidelines of the medical ethics board of the University Medical Center Groningen. Written informed consent was obtained for the use of the tonsil samples from the parents of the children.

RNA Isolation

RNA was isolated from the total cell lysate fractions and the Ago2-IP fractions of HL cells using miRNeasy mini kit (Qiagen, Hiden, Germany), according to manufacturer's protocol. The RNA concentration was measured by a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA), and the integrity was evaluated on a 1% agarose gel.

Small RNA Library Preparation, Sequencing, and Data Analysis

Small RNA libraries were generated from 2000 ng total RNA using TruSeq Small RNA Sample Preparation Kit and TruSeq small RNA indices (Illumina, San Diego, CA). All RNA samples were analyzed on an Illumina 2000 HiSeq high-throughput sequencing platform. Briefly, 3′- and 5′-adaptor sequences were removed using the CLC Genomics Workbench (CLC Bio, Cambridge, MA). RNA reads were analyzed with miRDeep 2.0 and annotated against miRBASE (miRBASEv21) allowing one mismatch. Novel miRNAs were identified using miRDeep. Total read counts were normalized to read counts per million. Read counts for miRNAs with the same mature sequence were merged. For statistical analysis, we included all unique miRNAs with at least 50 read counts in the sum of all seven samples that is the four samples of HL cell lines and the three samples of GC-B cells. The list with both known and novel miRNAs was further analyzed by GeneSpring GX 12.5 PA software (Agilent Technologies, Santa Clara, CA). Significantly differentially expressed miRNAs were identified using a moderated *t*-test with Benjamini-Hochberg multiple testing correction and a fold change >4. The small RNA sequencing data were deposited in the Gene Expression Omnibus database (*http://www.ncbi.nlm.nih.gov/geo*; accession number GSE92616).

Quantitative Real-Time PCR

miRNA expression levels were measured using the TaqMan miRNA quantitative PCR assay (Thermo Fisher Scientific

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