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Notch-mediated repression of miR-223 contributes to IGF1R regulation in T-ALL

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ABSTRACT

To identify microRNAs regulated by oncogenic Notch signaling, we performed microarray-based miRNA profiling of T-cell acute lymphoblastic leukemia (T-ALL) cells before and after treatment with γ-secretase inhibitor (GSI) to block Notch signaling. We show miR-223 levels increase after GSI treatment suggesting that active Notch signaling represses miR-223 expression. We also demonstrate that insulin-like growth factor-1 receptor (IGF1R) is regulated by miR-223 in this context, but observe no apparent effects on cell growth by overexpression or knock-down of miR-223 alone. We conclude that miR-223 contributes to IGF1R regulation, but may act in concert with other genes and/or microRNAs to alter T-ALL biology.

1. Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive cancer of immature T cells that affects both children and adults alike. While current chemotherapy regimens achieve cure in ~80% of pediatric patients, adults fare much more poorly with only 40% 5-year overall survival [1]. Further improvements, especially for patients with relapsed/refractory disease, will require introduction of novel approaches and more specific, targeted therapies aimed at underlying pathogenetic mechanisms [2]. Several major oncogenes have been identified in T-ALL including NOTCH1, HOX11/11L2, TAL1/SCL, LMO1/2, LYL1, and CALM-AF10 [3].

MicroRNA (miRNAs) are a class of endogenously encoded small non-coding RNAs (~22 nucleotides) that recognize partially complementary sequences in the 3′ UTR of target mRNAs and repress their expression via translation inhibition and/or transcript decay [4,5]. miRNAs are becoming increasingly appreciated for their ability to regulate a wide range of physiological and pathological processes including human leukemia [6,7]. Given that Notch1 is an important oncogene in T-ALL, and that the Notch receptor functions ultimately as a nuclear transcriptional factor, we hypothesized that Notch1 might regulate a set of miRNAs that contribute to its oncogenic activity in this context. We report here an unbiased screen for Notch1-regulated miRNAs in T-ALL using expression profiling by miRNA microarrays. We find that limited set of miRNAs to be regulated by Notch1 in human T-ALL, and that one in particular, miR-223, contributes to regulation of insulin-like growth factor-1 receptor (IGF1R) expression at the protein level. This result is of interest given that Notch-regulated IGF1R signaling was recently shown to be important for T-ALL cell growth and leukemia-initiating activity in vivo [8].

2. Materials and methods

2.1. Cell culture

Human T-ALL cell lines were grown in RPMI 1640 medium supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, and antibiotics. All cell lines used in this study with NOTCH1/FBW7/PTEN mutational status and GSI response are summarized in Table S1.

2.2. miRNA expression profiling

Human T-ALL cell lines Jurkat and P12 Ichikawa were treated with compound E (Calbiochem), a potent γ-secretase inhibitor (GSI), for 4 days to block Notch signaling. Cells were then harvested and total RNA extracted using TRIzol reagent (Invitrogen). Sample RNAs were profiled by Exigon using their miRCURY LNA™ microRNA array system (v11.0). Briefly, total RNA for each sample was labeled with Hy3, mixed with Hy5-labeled RNA from a common reference pool, and hybridized to miRCURY LNA™ microRNA arrays containing capture probes for 1264 different miRNA species. The common reference pool was generated by mixing all sample RNAs from the study. Arrays were scanned to obtain both Hy3 and Hy5 signals. Capture probes with both Hy3 and Hy5 signals greater than 1.5x of the median signal intensity received "present" calls, while those failing this detection threshold were excluded from further analysis. Expression level for each miRNA species deemed "present" is reported as $\log_2$(Hy3/Hy5) ratio in Table S2. Microarray data are also available from the Gene Expression Omnibus (accession no. GSE35993).

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2.3. Real-time quantitative PCR

Initial real-time qPCR studies were performed by "Exiqon Services", as contract work. Briefly, 10 ng total RNA was reverse transcribed in 10 μl reactions using the miRCURY LNA™ Universal RT microRNA PCR, polyadenylation and cDNA synthesis kit (Exiqon); each sample was processed in triplicates. cDNA was diluted 80× and 4 μl was used in 10 μl PCR reactions according to the protocol for miRCURY LNA™ Universal RT microRNA PCR; each microRNA was assayed once by qPCR in triplicate cDNA. The amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche) in 384 well plates. The following assays were performed: miR-223, miR-223*, and as reference miR: miR-103, miR-191, and miR-423-5p. LightCycler® 480 software was used to determine the Cp value, and to generate amplification and melting curves. LinRegPCR (ver 11.5) software was used to determine the amplification efficiency. The average amplification efficiency was used to correct the Raw Cp values. Reference miRNAs were analyzed for stability using SLqPCR algorithim (similar to geNorm) and miR-191 selected as the most stable reference miRNA and subsequently used to normalize all measurements on a well-to-well basis.

Subsequent real-time qPCR studies were performed by generating first-strand cDNA from total RNA by reverse transcription with SuperScript III (Invitrogen) using stem-loop primers specific for miR-223 (5′-GTT GGC TCT GGT GCA GGG TCC GAG GTA TTC GCA CCA GAG CCA ACG GGG TA-3′ and a normalization control miRNA, miR-191 (5′-GTT GGC TCT GGT GCA GGG TCC GAG GTA TTC GCA CCA GAG CCA ACC AGC TG-3′). qPCR amplification was then carried out using primers specific for miR-223 (5′-CCG CCC GTG TCA GTT TGT CA-3′) and miR-191 (5′-CCC GCA ACG GAA TCC CAA AAG-3′) in combination with a universal stem-loop primer (5′-GTG CAG GGT CCG AGG T-3′). Each sample was assayed in triplicate using a Dyad Disciple thermal cycler equipped with Chromo4 optical head (Bio-Rad). miR-223 expression levels were calculated by the ΔΔCt method with normalization to miR-191.

2.4. Luciferase reporter assay

A portion of the IGF1R 3′ UTR containing putative miR-223 target sites was cloned downstream of the luciferase gene in the pGL3 reporter plasmid (Promega). Luciferase reporter and miRNA expressing plasmids were transfected at a 2:1 ratio into HeLa cells using Lipofectamine Plus (Invitrogen) along with Renilla luciferase transfection control plasmid. Luciferase reporter activity was measured 42 h later using the Dual Luciferase Reporter (DLR) assay kit (Promega).

2.5. Viral vectors and transduction

High titer, replication defective retrovirus was produced by transient transfection of 293T producer cells as described [9]. Lentivirus was produced in a similar manner, but utilizing pCMVdR8.74, pCMV-VSV-G, and pRSV-Rev packaging vectors. HES1 and c-Myc were overexpressed from the MSCV-IRES-GFP (Mig) retroviral vector. miR-223 was overexpressed from the MSCV-PGK-GFP retroviral vector [10]. miR-223 knockdown was achieved using lentivirus overexpressing miR-223 target sequences from a polymerase II promoter, whereas the scrambled target control lacks sequence complementarity to any of the known microRNAs [11]. Doxycycline-inducible expression of dominant negative Mastermind-like 1-GFP fusion protein (DnMAML)[12] was achieved using pLVX-Tet-On Advanced and pLVX-Tight-puro lentivectors (Clontech), the latter containing DnMAML cDNA insert. Viral transduction was performed by spinoculation with 4 μg/ml polybrene as described [9]. Transduced cells were selected with G418 and/or puromycin as appropriate, or FACS sorted by GFP expression. Doxycycline-inducible expression was achieved by treatment of cells with 500 ng/mL doxycycline (Sigma) for 4 days.

2.6. Western blot

Whole cell extracts were prepared, separated by SDS-PAGE, and transferred to nylon filters. Blots were probed with antibodies directed against IGF1Rα (sc-712, Santa Cruz) and ERK2 (sc-154, Santa Cruz), followed by HRP-conjugated secondary antibody and detected by chemiluminescence. Band intensities were quantitated using ImageJ software.

2.7. Flow cytometry

Fresh cells were stained with antibodies directed against IGF1R (aIR3; Calbiochem) followed by APC-conjugated donkey anti-mouse IgG secondary antibody and analyzed by flow cytometry using a FACSCalibur cytometer (BD Biosciences) and FlowJo software (Treestar). Virally transduced cells were sorted by GFP expression using an Influx sorter (Cytopeia).

2.8. Statistics

Prism 5 software (GraphPad) was used for all statistical analyses.

3. Results

3.1. Microarray profiling of Notch1-regulated miRNAs in T-ALL

In order to identify miRNAs that are regulated by Notch signaling, we performed miRNA expression profiling of Jurkat and P12 Ichikawa T-ALL cell lines treated with GSI to block Notch signaling vs. DMSO vehicle control. For this initial discovery/screening step, we utilized the Exigon miRCURY LNA™ microRNA array platform which features locked nucleic acid capture probes for 1264 different miRNA species. Overall, 33–37% of miRNA species were detected in the samples (Table S2). Unexpectedly, only 3 miRNAs were identified to be significantly altered upon inhibition of Notch signaling in Jurkat cells, whereas no miRNAs appeared significantly changed in P12 Ichikawa cells (Fig. 1A). miRPlus-A1065 was found to be decreased by approximately 1.5-fold when Notch signaling was inhibited by GSI treatment, whereas miR-223, along with its complementary "star" strand (miR-223*), were found to be increased approximately 1.5-fold (Fig. 1B).

miRPlus-A1065 is predicted by TargetScan 5.1 software to target lysophospholipase II (LYPLA2) and collagen, type XXII, α1 (COL22A1), but is an Exigon proprietary miRNA species and not otherwise reported in the literature. In contrast, miR-223 has previously been described to be involved in several aspects of normal and malignant hematopoiesis [13–15] and thus we elected to focus further studies on miR-223.

3.2. Notch1 regulates miR-223 in human T-ALL cells

To validate the microarray results, we performed real-time quantitative PCR (qPCR) analysis for miR-223 and miR-223* in Jurkat and P12 Ichikawa cells, as well as an additional 6 human T-ALL cells lines. The qPCR assay for miR-223* yielded expression results at or below the assay detection limit and thus further validation could not be pursued (data not shown). In contrast, miR-223 was readily detected, and expression levels increased significantly with GSI treatment in Jurkat, but not P12 Ichikawa cells, thus confirming the microarray findings (Fig. 2A). As well, 4 of 6 additional human T-ALL cell lines (PF382, RPMI 8402, DND41, KOPTK1) showed significantly increased miR-223 with GSI treatment, while the remaining 2 cell lines (ALLSIL and HPBALL) exhibited miR-223 expression near the limit of detection (data not shown). Of note, both GSI "sensitive" and "resistant" cell lines, the former of which undergo G0/G1 cell cycle arrest upon treatment with GSI [16–18], showed regulation of miR-223 by Notch, suggesting that changes in miR-223 occur regardless of effects on cell cycle progression.

Subsequent time course analysis showed miR-223 expression to increase gradually, reaching a maximum after 3–4 days of GSI treatment (Fig. 2B). To exclude that miR-223 upregulation was due to effects of GSI on targets other than Notch signaling, T-ALL cells were transduced with lentiviral vectors to achieve doxycycline-inducible dominant negative MAML1 (DnMAML) expression to block Notch signaling [12,18], which also confirmed miR-223 upregulation (Fig. 2C). Taken together, these results support the conclusion that Notch1 signaling negatively regulates the expression of miR-223 in human T-ALL cells. Of note, mouse acute T-cell leukemias generated experimentally by retroviral transduction of bone marrow with activated Notch1 [19] showed no detectable miR-223 expression even after GSI treatment suggesting Notch1 may only regulate miR-223 in human cells (data not shown).

3.3. Notch1-mediated repression of miR-223 does not involve HES1

Because canonical Notch signaling results in transcriptional activation, our observation that inhibition of Notch signaling leads