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Research paper

Expression of the miR-150 tumor suppressor is restored by and synergizes with rapamycin in a human leukemia T-cell line

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ABSTRACT

miR-150 functions as a tumor suppressor in malignancies of the lymphocyte lineage and its expression is significantly reduced in these cells. However, the mechanism of miR-150 repression is unknown and so are pharmacological interventions that can reverse it. Here, we report that reduced expression of miR-150 in human Jurkat T-cell acute lymphoblastic leukemia (T-ALL) cells is mediated by constitutive mTOR signaling, a common characteristic of T-ALL cell lines and clinical isolates. Activating mTOR signaling in non-malignant T cells also resulted in a significant miR-150 down-regulation. Conversely, treatment with a pharmacological mTOR inhibitor, rapamycin, increased miR-150 expression in a dose-dependent manner in Jurkat cells, as well as in other leukemia cells. Interestingly, ectopic over-expression of miR-150 acted in a feed-forward loop and further sensitized Jurkat cells to a rapamycin-induced cell cycle arrest by targeting a large network of cell cycle genes. These findings suggest that miR-150 is normally expressed in quiescent T lymphocytes to reinforce an anti-proliferative state, and that mTOR signaling promotes cell proliferation in part by inhibiting miR-150 expression. Restoration of the miR-150-dependent anti-proliferative loop constitutes a novel mechanism underlying the efficacy of rapamycin in a T-ALL cell line. Further investigation of this mechanism in clinical isolates of T-ALL and other hematopoietic malignancies could help better guide development of targeted therapies.

1. Introduction

The mechanistic/mammalian target of rapamycin (mTOR) is a conserved protein kinase that induces cell growth, proliferation and changes in metabolism in response to mitogenic signals [1]. Unsurprisingly, pathogenesis and progression of numerous cancers, including T-cell acute lymphoblastic leukemia (T-ALL), is associated with constitutive activation of mTOR, caused primarily by mutations in its upstream regulators, such as AKT and PTEN [2,3]. In the majority of T-ALL patients, constitutive mTOR activation negatively affects outcomes [4]. Conversely, inhibition of mTOR signaling slows down T-ALL cell growth *in vitro* [5,6] and improves survival in T-ALL mouse models [7]. Clinical studies of the mTOR inhibitor everolimus, alone or in combination with chemotherapy, showed promising levels of toxicity and efficacy in non-Hodgkin lymphoma [8] and relapsed/refractory T-ALL [9] patients. Although several downstream targets of mTOR are known, the molecular basis for the efficacy of mTOR inhibition against T-ALL remains incompletely understood.

microRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate gene expression by inducing translational repression and/or degradation of mRNA molecules with partial sequence complementarity [10]. Key pathways involved in oncogenesis are targeted by miRNAs [11,12] and profound alterations in miRNA expression are well documented in malignantly transformed cells [13–15]. It was reported recently that mTOR signaling can interfere with miRNA biogenesis [16].

miR-150 is a highly abundant miRNA in cells of the lymphoid lineage [17–19], but its expression is strongly reduced in hematopoietic malignancies, such as acute myeloid leukemias [20], NK/T cell lymphoma [21], advanced cutaneous T-cell lymphoma [22] and multiple T-ALL cell lines [23]. Ectopic expression of miR-150 negatively affects survival of these cells [20,21,23] and reduces their metastatic potential [22], suggesting that it acts as a tumor suppressor. Indeed, restoring miR-150 expression in chronic myeloid leukemia cells significantly slows tumor growth in a xenograft model [20]. However, the mechanism for miR-150 down-regulation in any type of hematopoietic malignancy is unknown. Given the high levels of constitutive mTOR activation in T-ALL cells, we set out to test a hypothesis that mTOR signaling induces oncogenic down-regulation of miR-150 in these cells. We show that two different classes of mTOR inhibitors, rapamycin and

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PP242, rescue miR-150 expression in Jurkat T-ALL cells. This result connects mTOR signaling to the regulation of miRNA expression in a T-ALL cell line, specifically to the down-regulation of a tumor suppressor miRNA. We also report a novel feed-forward regulatory loop, whereby miR-150 reinforces a rapamycin-induced cell cycle arrest by targeting a functional gene network linked to the cell cycle. We propose that restoration of this novel miRNA-dependent regulatory loop by rapamycin is a novel mechanism underlying the efficacy of mTOR inhibitors against T-ALL and suggest that it should be studied further in primary cells.

2. Methods

2.1. Ethics statement

All experiments were covered by Human Subjects Research Protocols approved by the Institutional Review Board of The Scripps Research Institute.

2.2. Cells

Jurkat (ATCC, Cat # TIB-152) cells were grown in RPMI-1640 with 2 mM L-glutamine, 10 mM HEPES and 1 mM sodium pyruvate; primary T cells were grown in RPMI-1640 with 2 mM L-glutamine. All media contained 10% heat-inactivated FBS, penicillin and streptomycin. Primary naïve ($CD4^+CD45RA^+CD45RO^-$) and memory ($CD4^+CD45RA^-CD45RO^+$) T cells were isolated from peripheral blood collected from healthy donors ages 18–30 years. Briefly, peripheral blood mononuclear cells were enriched by Ficoll-histopaque (Sigma-Aldrich) density gradient centrifugation, and T cells were purified from these cells by negative selection using magnetic beads (Miltenyi Biotec). Cells were stimulated with anti-CD3/CD28 beads (Life Technologies) using manufacturer's instructions for 48 h. All other cell lines were obtained from ATCC and grown following the recommended culture methods. All cells were grown at 37 °C in a 5% CO2 humidified chamber. Rapamycin (Sigma-Aldrich) and PP242 (Tocris) were reconstituted in DMSO. Rapamycin was used at 100 nM unless otherwise noted. Anti-CD95 antibody (Millipore) was used at 0.1ug/ml.

2.3. qRT-PCR

Total RNA was extracted using Trizol (Life Technologies), cleaned up using the miRNeasy kit (Qiagen) and subjected to on-column DNase digestion (Qiagen). Mature miRNAs were detected with the qScript system and PerfeCTa miRNA assays (Quanta BioSciences). Quantification was done using the delta Cq method relative to RNU6. For pri-miRNA quantification, cDNA was synthesized using high capacity cDNA reverse transcriptase kit (Life Technologies) and used to template qPCR reactions with Taqman assays (Life Technologies) specific for pri-miR-150. Cq values were normalized to 18S RNA. For pre-miRNA quantification, primers were designed to the pre-miRNA region directly upstream of the mature sequence (pre-miR-150: TCCCCATGG CCCTGTCTC). Total RNA was fractionated (miRNeasy kit, Qiagen) to only contain molecules < 200 nt, converted to cDNA using qScript microRNA cDNA Synthesis Kit (Quanta BioSciences) and used to template SYBR green qPCR reactions with a specific 5′ primer and the universal Quanta 3′ primer. Cq values were normalized to RNU6. Because pri-miRNAs can be detected by pre-miRNA primers, we confirmed that pri-miRNAs were absent in the < 200 nt RNA fraction.

2.4. microRNA-seq

Small RNA sequencing libraries were prepared using the TruSeq kit (Illumina) and sequenced on an Illumina HiSeq instrument with 100-base single-end reads. QC and read filtering was done using the FASTX toolkit (v.0.0.13; http://hannonlab.cshl.edu/fastx_toolkit/) and sequencing adapters were trimmed off using a custom python script. Reads that did not contain any adapter sequence were discarded and trimmed reads were aligned to mature miRNA sequences (miRbase v.17 [24]) using bowtie (v.0.12.9 [25]) with -v 0 –a options. On average, 1.6 million reads were mapped to mature miRNAs. miRNA read counts were normalized to the median tRNA expression [26]. miRNAs with less than 10 reads were considered not expressed and removed from further analysis.

2.5. Microarrays

Jurkat cells were grown in 4 independent cultures for several passages, transiently transfected with pre-miR-150 or control (scrambled) pre-miRNA and then treated with rapamycin or DMSO for 72 h. Total RNA was analyzed on the Agilent Bioanalyzer to confirm integrity. cDNA was synthesized and labeled using the Ambion whole transcript (WT) expression kit (Life Technologies), hybridized to 24-array HuGene 1.1ST plates and run on the GeneTitan Multi-Channel Instrument (Affymetrix). Data analysis was done in Genomics Suite (Partek). Probe intensities were normalized using the RMA method. Expression changes were calculated using 1-way ANOVA with the p-value < 0.05 as a cutoff for significance. Network and pathway enrichment analyses were performed using Ingenuity Pathway Analysis software and GO term enrichment was performed using GOrilla: http://cbl-gorilla.cs.technion.ac.il [27].

2.6. Western blotting

All antibodies were obtained from Cell Signaling and were used with the ODYSSEY CLx infrared imaging system (LI-COR): phospho-Ser473-Akt (D9E), polyclonal Akt, phospho-Thr389-p70S6K (108D2), p70S6K (49D7), alpha-tubulin (DM1 A), and LC3B (D11).

2.7. miR-150 over-expression and knockdown

Pre-miR-150 or control pre-miRNA vectors (PMIR150-PA-1 and PMIRH000-PA-1, System Biosciences) were transfected into Jurkat cells at 18 nM using Amaxa 4-D Nucleofector. MiR-150 LNA or control LNA (426828-00 and 199020-00, Exiqon) were transfected at 200 nM. Transfection efficiencies were monitored using copGFP co-expressed on the pre-miRNA vector or by transfection with FITC-labeled LNA probes. Transfection efficiencies ranged between 94–96%. Cells were recovered in complete media for 3–4 h prior to drug treatment.

2.8. Proliferation and cell cycle analysis

Cells were plated at 80,000 cells per well in 96-well plates and BrdU was added to the media for the final 18 h of culture at 0.01 mM. Cells were lysed and BrdU incorporation quantified using Ziva Cell Proliferation Assay kit (Jaden BioScience Inc). For cell cycle analysis, 0.01 mM BrdU was added to culture medium for 24 h, cells were stained with anti-BrdU antibodies and 7-AAD (BD Biosciences) and analyzed by flow cytometry.

2.9. Luciferase assays

3′UTRs were amplified from Jurkat genomic DNA using the following primers – ARRB1: CACATGGGCGATCGCGAAGTGAGGATGGG TGTC and GAGGCGGCCGCAAGCTTGGAAACATGACCTGC; FADS1: CAACAGCGATCGCGCCCAGTCTGGAAGAAGAGGAGG and CACAGCG GCCGCGGTGCTTTGAGGACTTGGTCTTGG; CDK2: TAGCGCGATCGCA GCCCCCAGCCCTAATCTCACCC and TCCGCGGCCGCAGGAGGTGGAC GTCAGAGGAAAATGGG and cloned into the psiCheck2 Dual-luciferase reporter vector (Promega). To make the positive control reporter, oligonucleotides bearing 2 complementary miR-150 sequences separated by an EcoRI site were annealed and inserted into the psiCheck2 vector (