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A microRNA-mediated regulatory loop modulates NOTCH and MYC oncogenic signals in B and T cell malignancies

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Abstract

Growing evidence suggests that microRNAs facilitate the cross-talk between transcriptional modules and signal transduction pathways. MYC and NOTCH1 contribute to the pathogenesis of lymphoid malignancies. NOTCH induces MYC, connecting two signaling programs that enhance oncogenicity. Here we show that this relationship is bidirectional and that MYC, via a microRNA intermediary, modulates NOTCH. MicroRNA-30a, a member of family of microRNAs that are transcriptionally suppressed by MYC, directly binds to and inhibits NOTCH1 and NOTCH2 expression. Using a murine model and genetically modified human cell lines, we confirmed that microRNA-30a influences NOTCH expression in a MYC-dependent fashion. In turn, through genetic modulation, we demonstrated that intracellular NOTCH1 and NOTCH2, by inducing MYC, suppressed microRNA-30a. Conversely, pharmacological inhibition of NOTCH decreased MYC expression, and ultimately de-repressed microRNA-30a. Examination of genetic models of gain and loss of microRNA-30a in diffuse large B-cell lymphoma (DLBCL) and T-acute lymphoblastic leukemia (T-ALL) cells suggested a tumor suppressive role for this microRNA. Finally, the activity of the microRNA-30a-NOTCH-MYC loop was validated in primary DLBCL.

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and T-ALL samples. These data define the presence of a microRNA-mediated regulatory circuitry that may modulate the oncogenic signals originating from NOTCH and MYC.

**Introduction**

The transcriptional factors MYC and NOTCH1 play critical role in the pathogenesis of B and T cell malignancies\(^1,^2\). Gain of function mutations in NOTCH1 are predominantly found in T-ALL (acutelymphoblastic leukemia)\(^3\), but recent evidence implicate NOTCH1 and NOTCH2 also in subsets of mature B-cell malignancies\(^4^–^7\). Likewise, despite the fact that MYC is more prominently linked to B cell lymphoma biology, its relevance to T-ALL pathogenesis is well established, at least in part due to NOTCH1’s ability to induce MYC expression\(^8^–^10\). This interplay extends to other members of the NOTCH pathway, and NOTCH2 also transcriptionally induce MYC\(^11\). Conversely, it remains unclear whether MYC also positively regulates NOTCH expression/activity, a potentially beneficial mechanism to sustain the signals originating from these oncogenes. However, as concrete evidence for direct transcriptional regulation of NOTCH1 and NOTCH2 by MYC is lacking\(^12^–^14\), if such regulatory node exists, it would probably involve intermediaries.

MicroRNAs (miRNAs) are ideal candidates to mediate the potential effect of MYC activity on NOTCH expression. Indeed, growing evidence suggests that these non-coding RNAs are often the key elements in facilitating the cross-talk between transcriptional modules and pathways\(^15,^16\). Furthermore, while data from multiple cell models suggested that MYC functions as a master regulator of miRNA expression\(^1,^17\), the full scope of miRNA dysfunction in lymphoid malignancies remains to be defined\(^18\). Thus, identification of a miRNA that may bridge the oncogenic MYC and NOTCH nodes could improve our understanding of the pathogenesis of these disorders.

To investigate this concept, we considered miRNAs that we had earlier reported to display aberrant copy number/expression in DLBCL\(^19\), and that were independently shown to be directly regulated by MYC\(^17\). This approach identified the microRNA-30 family as a candidate for putative MYC-dependent regulation of NOTCH1 and NOTCH2 expression. We focused on microRNA-30a (miR-30a) to validate this interplay, since among the members of the miR-30 family it had the less well-characterized interaction with MYC and the NOTCH pathway\(^17,^20,^21\), thus assuring that our findings would add new knowledge to the field.

Herein, we confirmed that MYC negatively influences miR-30a expression, and discovered that this miRNA directly targets NOTCH1 and NOTCH2. Using genetic and pharmacological models, we characterized a regulatory loop, where by the MYC-mediated inhibition of miR-30a de-represses NOTCH, eventually modulating its own expression. Further, we showed that miR-30a altered the fitness of B and T-cell malignancies, consistent with a tumor suppressive role. Finally, to determine the relevance of this finding beyond genetically engineered cell models, we examined primary human tumors and found a significant correlation between the expression of miR-30a and NOTCH2 in diffuse large B-cell lymphomas (DLBCL) and between NOTCH1 mutational status and miR-30a expression in T-ALLs.

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Material and Methods

Cell Lines and Primary Tumors

Diffuse large B cell lymphoma (DLBCL) cell lines (SU-DHL6, SU-DHL7 and OCI-Ly18) and T-ALL cell lines (DND-41 and KOPT-K1) were cultured at 37 °C in 5% CO2 in RPMI-1640 medium containing 10% (vol/vol) fetal bovine serum (FBS), penicillin (100 units/mL), streptomycin (100μg/mL), Hepes buffer (10 mmol/L), and L-glutamine (600 μg/mL). HEK-293 cells were maintained in Dulbecco’s modified Eagle medium with 10% FBS. Sixteen primary DLBCL specimens were obtained from our local tumor bank, as reported earlier 19. Five primary T-ALL samples obtained from the Division of Hematology, Medical University of Graz, Austria. The clinical and pathological features of these tumors are summarized in Supplementary Tables 1 and 2. The use of primary tumor samples was approved by the Review Boards of the UTHSCSA, USA, and Medical University of Graz, Austria, and conforms to the Declaration of Helsinki.

Mice and isolation of murine mature splenic B lymphocytes

Eμ-Myc transgenic male mice (MycTg+/−) on a C57BL/6 background (B6.Cg-Tg[IgHMyc]22Bri/J) were purchased from The Jackson Laboratory and maintained by in house breeding with C57BL/6 female mice to generate MycTg+/− and C57BL/6 (WT) littermates. Five to fourteen weeks old sex matched WT and MycTg+/− mice littermates (all without clinical evidence of lymphoma) were euthanized and their splenic B cells purified using the mouse B-Cell Enrichment kit (Stem Cell Technologies), as we describe earlier22. The purity and degree of enrichment was determined by FACS-based measurement of CD19-positive cells, in pre- and post-separation aliquots - the fraction of CD19-positive cells was above 95%. All animal procedures were approved by the Institute Animal Care and Use Committee of the University of Texas Health Science Center San Antonio.

Luciferase reporter assays

Three PCR-generated fragments containing the miR-30a binding sites (one in NOTCH1, two in NOTCH2) (Supplementary Figure 1) were individually cloned in the 3’UTR of the luciferase gene (pMIR vector, Ambion). Site directed mutagenesis was used to modify three of the six “seed sequence” nucleotides in each construct, as we described23. After sequencing verification, these constructs were cotransfected in HEK-293 cells with the pCMVβ-gal plasmid (5ng) and 100 nM each of chemically synthesized pre–miR-30a or pre–miR negative control oligonucleotides (Sigma). Cells were harvested 48 h after transfection, and luciferase and β-galactosidase activities measured, as we described24. The primers sequences are listed in Supplementary Table 3.

Generation of stable genetic models of miR-30a and NOTCH

A fragment of ~ 600bp encompassing the precursor miR-30a sequence was PCR amplified, sequence verified and cloned into the MSCV–eGFP retrovirus vector, as we described24. Intracellular NOTCH1 and NOTCH 2 (ICN1 and ICN2) were subcloned from pCDNA3 into MSCV-eGFP to generate MSCV-ICN1 and MSCV-ICN2, respectively. To produce a miR-30a specific sponge construct, sense and antisense oligonucleotides containing two
mature miRNA-30a sequences separated by a short “spacer” were synthesized and annealed, as reported earlier\textsuperscript{25}, and cloned in tandem in the 3’UTR of the eGFP gene (pEGFP-C3 plasmid, Clontech). The sponge sequences included bulged sites (Supplementary Table 3) located just after the seed sequence (nucleotides 9–12) promoting a mispairing and thus forming more stable interactions with the miRNA. A construct containing six mature miR-30a complementary repeats arranged in tandem in the 3’UTR of eGFP was used for downstream studies. A control sponge, scrambled sequence that does not match any human microRNA, was similarly designed and cloned. It should be noted that due to the similarity between the members of the miR-30 family, we cannot exclude the possibility that some of the effects derived from expression of the miR-30a-sponge construct, derive from the functional sequestration of other members of this miRNA family. Cell models stably expressing MSCV-miR-30a, MSCV-ICN1, MSCV-ICN2 or an empty MSCV vector, were created by retrovirus transduction and sorting of eGFP positive cells to high purity (>95%), as we described\textsuperscript{26}. The cell lines stably expressing the sponge plasmids (miR-30a or scrambled) were generated by electroporation, followed antibiotic (G418) selection and eGFP-based cell sorting. The direct interaction between miR-30a and NOTCH1 was examined by electroporating the T-ALL cells with a 125nM of an antisense oligonucleotide (5’TGTAAACTACACTCTATT 3’) (Exiqon) that binds to the miR-30a site on NOTCH1 3’UTR, thus preventing this miRNA from gaining access to the mRNA.

**Immunoblotting**

Whole cell lysates were extracted, separated by SDS-PAGE electrophoresis, transferred to PVDF membrane, as we described\textsuperscript{27}. For detection of relevant proteins the following antibodies were used: c-MYC (Santa Cruz, sc-40), NOTCH1 (Santa Cruz, sc-32745), NOTCH2 (Cell Signaling, #5732S), cleaved NOTCH1 (val1744) (Cell Signaling, #2421S), cleaved Caspase-3 (Asp175) and Caspase 3 (Cell Signaling, #9661 and #9662). The membranes were stripped with One Minute Western Blot Stripping Buffer (GM Biosciences) and re-probed with anti-β-actin antibody (Sigma-Aldrich, #A2228).

**In Vitro Drug Treatments**

DND-41 and KOPT-K1 cells were exposed to 100nM or 200nM of the gamma-secretase inhibitor Compound E (Enzo Life Sciences, Inc.) or DMSO control for 24, 48 and 72 hrs. Subsequently, cells were harvested for RNA and protein based analyses. All experiments were validated in two to three biological replicates.

**Cell growth, cell cycle and apoptosis analyses**

The growth pattern of the genetically modified T-ALL cell lines and isogenic controls was defined by plating the cells at equal density (0.6 to 1 × 10\textsuperscript{5} cell/ml) in fresh media (Day 0), in triplicate. Cell number and viability were determined daily by an automated blue (TB) exclusion assay (Cellometer Vision CBA system, Nexcelom) and side-by-side manual counting (Neubauer chamber). At Day 5, these cells were also analyzed for cell cycle distribution and apoptosis, using propidium iodide (PI) and Annexin V staining, respectively, followed by FACS analyses, as we described\textsuperscript{24}. The growth pattern of electroporated T-ALL cell lines was defined by plating viable cells 24h after electroporation.
at equal density (1 × 10^5 cell/ml) in fresh media (Day 0), in triplicate. Cell number and viability were determined at 48h and 72h electroporation by an automated blue (TB) exclusion assay (Cellometer Vision CBA system, Nexcelom) and side-by-side manual counting (Neubauer chamber). In the DLBCL cell lines expressing scramble or miR-30a specific sponges, apoptosis was measured in response to oxidative or starvation stresses. In brief, cells were grown in serum free media or in the presence of 25μM of H2O2 (Sigma) for 48h (10% serum-containing media and DMSO as controls, respectively) and FACS-based Annexin V staining (PE Annexin V Apoptosis Detection Kit I; BD Pharmingen) used to determine apoptosis rate. All experiments were validated in two to three biological replicates.

**Quantification of miR-30 expression**

Relative expression of miR-30a, -30b, -30c, -30d, -30e was defined by stem-loop quantitative TaqMan real-time reverse transcription polymerase chain reaction (RT-PCR) assay (MicroRNA assays; Applied Biosystems Inc.), as we reported. Small Nucleolar (Sno) RNA-202 and Sno RNA U18 were used for normalizing the expression in murine and human cells respectively.

**Sanger sequencing**

High molecular weight genomic DNA was isolated from five primary T-ALL samples using the GentraPuregene DNA purification kit (Qiagen). 50ng of DNA was PCR amplified for 40 cycles for regions spanning mutational hot-spots in NOTCH1, exons 26, 27 and 34. Amplicons were verified on agarose gel, bi-directionally sequenced and results analyzed using Mutation Surveyor software.

**Statistics**

The Mann–Whitney test was used to determine the significance of the difference in NOTCH2 expression in primary DLBCL dichotomized by low and high miR-30a expression. Significance of the differences in miR-30a expression in in primary T-ALL and T-ALL cell lines was tested with one-way ANOVA. For all other assays, the statistical analyses were performed with two-tailed Student’s t test. P <0.05 was considered significant. Data analyses were performed in the Prism software (version 5.0; GraphPad) and Excel software (Microsoft).

**Results**

**Functional binding sites for miR-30a are present in the 3’UTRs of NOTCH1 and NOTCH2**

Earlier, we reported on miRNAs that displayed aberrant copy number and expression in DLBCL. Independently, a subset of these miRNAs, including the miR-30 family, was shown to be directly regulated by MYC. Here, we centered our investigations on miR-30a as the prediction algorithm Target Scan (Release 6.2) identified NOTCH1 and NOTCH2 as high confidence candidate targets for this miRNA. Further, among the members of the miR-30 family, miR-30a had the less well-characterized interaction with MYC and the NOTCH pathway, therefore assuring that our findings would add new knowledge to the field. A single highly conserved miR-30a binding site was predicted in the 3’UTR of...
**NOTCH1** and two non-conserved sites in the 3′ UTR of **NOTCH2** (Supplementary Figure 1). To test their functionality, we used prototypical reporter assays, whereby DNA fragments containing each of three sites in the 3′ UTR of the luciferase gene are cloned downstream to a reporter gene, as described. To ascertain the specificity of these miRNA/target gene interactions, for each site we also created constructs containing three interposed point mutations in the seed-sequence (miRNA’s nucleotides 2 to 7) disrupting the putative binding sites. Expression of miR-30a resulted in significant suppression of the luciferase activity in all three seed-sequence wild-type constructs (corresponding to one **NOTCH1** and both **NOTCH2** sites, p<0.05, Student’s t-test), whereas miR-30a had no effect towards the mutant constructs (Figure 1A). These data indicate that miR-30a directly binds to **NOTCH1** and **NOTCH2**’s 3′UTRs and may inhibit their expression.

**Genetic modulation of miR-30a impinges on NOTCH1 and NOTCH2 expression**

To define if the binding of miR-30a to the 3′UTRs of **NOTCH1** and **NOTCH2** inhibited their expression, we generated a panel of four cell lines – two DLBCL (SU-DH-6 and SU-DHL7) and two T-ALL (KOPT-K1 and DND-41) – stably expressing miR-30a from a retrovirus system (MSCV-eGFP), as described (Supplementary Figure 2). Note that we purposely selected to use NOTCH1-mutant T-ALL cell lines in this study, for we suggest that it is in this context that the relevance of miR-30a suppression to the pathogenesis of this disorder may be better demonstrated. In both disease models, expression of miR-30a suppressed NOTCH1 and NOTCH2 expression (Figure 1B). In the **NOTCH1** mutant T-ALL cell lines, the miR-30a effects were also readily detected upon examination of the **NOTCH1** intracellular domain (NICD) with an antibody that recognizes the Val1744 position (cleaved NOTCH1). To expand this observation to loss of function models, we used a miR-30a-specific sponge construct, an elaborate model of functional depletion of miRNAs that provides stable competitive titration of these small non-coding RNA from their targeting sites. The activity and specificity of the “sponge constructs” was confirmed by its ability to abrogate the effects of miR-30a in the **NOTCH1** binding-site luciferase reporter assay (Supplementary Figure 3). Again, a panel of four cell lines – two DLBCL (SU-DHL7 and OCI-Ly18) and two T-ALL (KOPT-K1 and DND-41) – were generated with stable expression of sponge constructs directed at miR-30a or a scrambled sequence. Of note, repeated attempts to create SU-DHL6 stably expressing miR-30a-sponge were unsuccessful. Thus, we used a related GCB-like DLBCL cell line (OCI-Ly18) to create the fourth loss of function model, guaranteeing the representative nature of our observations. In agreement with the overexpression model, sequestering miR-30a away from its target genes with “sponges” resulted in an increase in NOTCH1 and NOTCH2 expression (Figure 1C). As expected, in the DLBCL cell lines NOTCH2 was more consistently expressed than NOTCH1, suggesting that the former is the principal biological target of miR-30a in mature B cell tumors. Finally, as we indicated earlier, the use of NOTCH-1 mutant T-ALL cell lines was important for it allowed us to show that a miRNA can modulate the expression (Figure 1B–C), and function (see below), of constitutively active **NOTCH1**, a finding that may eventually have therapeutic implications. Indirectly, our data also suggest that the suppression of miR-30a may be an important component of mutant-**NOTCH1**’s transcriptional activity.
**MYC regulates and it is regulated by the miR-30a-NOTCH1/2 axis**

The data shown above confirm that miR-30a directly suppress NOTCH1 and NOTCH2 expression. As MYC has been reported to suppress the expression of the miR-30 family by directly binding to their promoters (defined by ChIP-PCR)\(^1\), we tested whether modulating MYC levels/activity altered the levels of miR-30a in our models and, consequently, NOTCH1 and NOTCH2. To that end, we first used the Myc transgenic (MycTg) mouse. Mature splenic B cells were isolated from nine mice (4 WT and 5 MycTg littermates) and the expression of miR-30a was determined by stem-loop real-time RT-PCR. In agreement with a role for Myc in the transcriptional regulation of miR-30 family\(^1\), the expression of miR-30a was significantly lower in B cells from the Myc Tg animals than from their WT littermate controls (mean relative expression 0.78±0.1 vs. 1.38±0.3, an approximately 50% reduction, \(p<0.05\) Student’s t-test) (Figure 2A). Next, we used western blotting to examine the expression of NOTCH1 and NOTCH2 in these cells, and found that NOTCH1 levels were higher in B cells from the MYC Tg mice (Figure 2A) than from the WT mice, presumably, at least in part, due to the lower expression levels of miR-30a. Supporting the role of miR-30a in regulating this process, no change in the expression of NOTCH2 was found in this model, which we attribute to the lack of conservation of the NOTCH2 miR-30a binding sites in the mouse genome (Supplementary Figure 1). Thus, MYC appears to suppress miR-30a expression and consequently impinge on NOTCH levels.

We next considered the reverse, i.e., the role of miR-30a in the regulation of MYC; we reasoned that if the regulatory circuitry that we are describing is a “closed loop”, then not only will MYC suppress miR-30a, but miR-30a by modulating NOTCH expression should eventually affect MYC levels. Indeed, examining the miR-30a gain or loss of function models generated in DLBCL and T-ALL cell lines, we found that suppression of NOTCH1 and NOTCH2 by miR-30a (Figure 1B) was associated with inhibition of MYC expression (Figure 2B - top). Conversely, the functional depletion of miR-30a by the “sponge” construct increased MYC expression (Figure 2B - bottom), probably as a consequence of the NOTCH1 and/or NOTCH2 de-repression in these cells (Figure 1B); at present, we cannot fully exclude the possibility that other, still uncharacterized, miR-30a targets also contribute to up-regulation of MYC in our cell models.

**Genetic and pharmacological modulation of NOTCH1 and NOTCH2 modifies miR-30a expression**

We showed above that the modulation of the MYC/miR-30a axis interferes with NOTCH1 and NOTCH2 expression. Next, we attempted to isolate the role of NOTCH in this circuitry. To achieve this goal, we first stably expressed constitutively active NOTCH1 and NOTCH2 constructs (ICN1 and ICN2 - intra-cellular NOTCH1 and NOTCH2, respectively) in the DLBCL cell line SU-DHL7. Using western blot, we confirmed the expression of ICN1 or ICN2 in these cells and demonstrated the consequent induction of MYC (Figure 3A). Then, we measured miR-30a expression in these cells, and found it to be significantly lower in ICN1- and ICN2-positive than in the SU-DHL7-MSCV control cells (\(p<0.05\), two-sided Student’s t-test) (Figure 3A). In complementary assays, we exposed NOTCH1-mutant T-ALL cell lines to a gamma-secretase inhibitor (GSI), Compound E, and determined the temporal relationship between ICN1 inhibition, MYC suppression, miR-30a modulation. In
these assays, ICN1 showed early and sustained inhibition, which was followed by MYC
downregulation and lastly (as expected) miR-30a de-repression (a ~ 50% increase in its
expression at 72h)(Figure 3B). It is important to note that with these genetic and
pharmacological models (Figure 3A and 3B, respectively) we did not expect to find a
massive modulation (suppression or induction, respectively) of miR-30a. Surely, as any
other gene, miR-30a’s transcriptional regulation is controlled by other mechanisms beyond
the NOTCH-MYC interplay, which remain intact in these models. The central purpose of
these assays was to demonstrate that positive and negative perturbations of NOTCH1/2
activity eventually leads to miR-30a down and upregulation, respectively, which our data
suggest occurs via MYC. Finally, since MYC has been suggested to repress the expression
of other members of the miR-30 family, we tested whether the genetic and/or
pharmacological modulation of NOTCH1 would broadly impact on miR-30 expression.
Indeed, ectopic expression of ICN1 suppressed, whereas exposure to Compound E de-
repressed the expression of multiple members of this miRNA family (Supplementary Figure
4). These data suggest that the network of genes impinged upon by the NOTCH-MYC-
miR-30 circuitry expands beyond the miR-30a targets.

**MiR-30a has tumor suppressive properties towards T-ALL and DLBCL cell lines**

The data above, established the presence of a regulatory loop that when engaged by
activation of either NOTCH or MYC could result in modulation of their own and each
other’s expression, at least in part via the suppression of miR-30a. It follows that miR-30a
expression could disrupt the growth advantage provided by oncogenic NOTCH and MYC.
To test this possibility, we first examined the effects of stably expressing miR-30a in
NOTCH1-mutant T-ALL cell lines. In both models, we found a significant decrease in the
growth rate of miR-30a expressing cells when compared to their isogenic controls
expressing an empty MSCV vector – a ~ 32% to 50% suppression in DND-41 and KOPT-
K1 growth (p<0.01two-sided Student’s t-test, Figure4A and Supplementary Figure 5).
MicroRNAs target multiple mRNAs. Thus, to confirm that the observed growth suppression
resulted from the direct effects of miR-30a towards NOTCH1, we used an antisense
oligonucleotide (Target Site Blocker - TSB) that blocked the access of this miRNA to its
binding site on NOTCH1’s 3’UTR. In these assays, the TSB oligonucleotide was
electroporated into a TALL cell line stably expressing MSCV-miR-30a, alongside mock-
electroporated empty MSCV and miR-30a cells. Subsequently, viable cells were plated at
equal density and growth pattern defined at 48 and 72h post-transfection, accompanied by
western blot analysis of NOTCH1 expression. As shown in Figure 4B, blocking miR-30a
targeting of NOTCH1 restored the growth rate of the miR-30a-expressing cells to the levels
of their isogenic MSCV-only controls, in association with depression of NOTCH1
expression. In agreement with these observations, and to demonstrate that these are not
exclusive features of an overexpression model, we found that the functional depletion of
miR-30a with the sponge construct enhanced the fitness of the cells and they grew at a
significantly faster pace than isogenic cells expressing a scrambled sponge - a ~ 40%
increase in DND-41 and KOPT-K1 cell numbers (p<0.01, two-sided Student’s t-test, Figure
4B and Supplementary Figure 5). Cell cycle arrest and/or apoptosis probably accounts for
the significantly slower growth pattern found in miR-30a overexpressing cells
(Supplementary Figure 5), similarly to the reported effects of GSI in NOTCH1 mutant cell
The heightened apoptosis rate of the miR-30a-expressing cells was confirmed by Annexin V staining (Supplementary Figure 6), as well as cleaved caspase-3 western blotting (Supplementary Figure 7). Contrary to the NOTCH1-mutant T-ALL cell lines, ectopic expression of miR-30a or its sponge-mediated depletion did not immediately alter the growth pattern of genetically modified DLBCL cell lines, perhaps suggesting that miR-30a play a more dominant role in the context of NOTCH1 activation. Nonetheless, upon challenging these cells with serum starvation and H202-induced oxidative stress, which attempts to reflect the actual tumor microenvironment and recapitulate the effects of chemotherapy, we consistently found that depletion of miR-30a rendered DLBCLs significantly more resistant to apoptosis induced by these signals (a ~ 25% to 100% change, depending on the cell type and stimulus) (Figure 4C). The lower apoptosis rate of the cells expressing miR-30a sponge was confirmed by Annexin V staining (Figure 4C), as well as cleaved caspase-3 western blotting (Supplementary Figure 7). Of note, microRNAs are primarily fine tuners of gene expression, and many other programs regulate NOTCH and MYC expression. In agreement with this concept, in all genetic models of miR-30a gain or loss of function that we generated, residual expression of NOTCH1, NOTCH2 and MYC was present. Thus, as we showed in Figure 4, a marked and significant but not a complete disruption of cell proliferation and/or apoptosis is the expected outcome of modulating miR-30a in DLBCL and T-ALL cell lines.

**MiR-30a expression correlates with NOTCH1 and NOTCH2 status in primary T-ALL and DLBCL biopsies**

To verify if the regulatory circuitry involving miR-30a and NOTCH1 or NOTCH2 was active in primary samples, we studied cohorts of T-ALL and DLBCL, respectively. First, we obtained high quality DNA and RNA from five T-ALL samples not previously characterized for their NOTCH1 mutational status. Sequencing of exons 26, 27 and 34 identified two samples as NOTCH1-WT, and three with typical missense mutations targeting the heterodimerization mutation, alone or combined with truncating variants in the PEST domain (Supplementary Table 1). Next, we used a stem-loop real-time RT-PCR assay to quantify miR-30a in these samples, alongside with the NOTCH1 mutant T-ALL cell lines. In agreement with the NOTCH-MYC-miR-30a axis that we are reporting on, we hypothesized that miR-30a expression would be lower in NOTCH1 mutant T-ALLs. Indeed, in this small cohort, we found that in primary T-ALLs NOTCH1 mutational status correlated well with miR-30a expression (Figure 5A), and that as a group the NOTCH1-mutant T-ALL cell lines expressed the lowest levels of this miRNA, perhaps owing to their more homogenous cell population. We also examined the relationship between miR-30a and NOTCH in primary DLBCL biopsies, focusing on NOTCH2 given its more prominent role in mature B cell biology. Here, we collected sixteen primary DLBCL biopsies for which good quality RNA and protein were available; this cohort was also enriched for samples that displayed only limited (less than 10%) T cell infiltrates, thus favoring a more precise analysis of the tumor cells with minor influence of the infiltrating non-malignant cells. Quantification of miR-30a in these DLBCLs revealed a broad continuum of relative expression (mean 3.11, range 0.2 to 26.5). Next, we investigated NOTCH2 expression in all 16 samples, and grouped the eight DLBCLs with highest and the eight with lowest miR-30a levels (median relative expression 2.9 vs. 0.7, respectively) for analysis. As shown in Figure 5B, expression of...
NOTCH2 significantly correlated with miR-30a levels, and it was markedly higher in tumors expressing low levels of this miRNA (p<0.01, Mann Whitney test). We also sequenced NOTCH2 in eleven of the sixteen DLBCLs analyzed, as part of a larger next generation sequencing project (not shown) and found it to be in WT configuration (mean coverage for the NOTCH2 locus in the 11 samples = 35×, range 23× to 58×). These data suggest that the regulatory loop that includes NOTCH and miR-30a is active in the two distinct primary neoplasms of interest, and that this circuitry may contribute to the oncogenic thrust of these malignancies. Of note, our ability to identify a significant correlation between NOTCH and miR-30a, despite the fact that these transcription factors are regulated by multiple inputs, highlights the relevance of this miR-30-dependent axis in B and T cell biology.

Discussion

In this report, we identified and characterized a regulatory loop that includes miR-30a, NOTCH1 or NOTCH2 and MYC. In particular, our findings suggest that the earlier picture, i.e. that NOTCH induces MYC\textsuperscript{8–10}, was incomplete and that the relationship between these transcriptional programs is bidirectional, i.e., MYC, by suppressing miR-30a, also impinges on NOTCH expression. Certainly, a full grasp of the relative contribution of this circuitry to NOTCH function, in normal and malignant settings, awaits the development of genetically engineered mice (GEM) with specific disruption of the miR-30a binding site in NOTCH1 and NOTCH2.

Nonetheless, our cell models and initial analysis of primary samples suggest that miR-30a may play a part in regulating NOTCH and MYC signals. Conceivably, in T-ALLs with mutant NOTCH1, MYC up-regulation could lead to miR-30a suppression and, secondarily, NOTCH1 de-repression. This regulatory loop could also start at the “MYC position” leading first to miR-30a suppression, followed by NOTCH1 and NOTCH2 de-repression, which upon NOTCH ligand exposure could further induce MYC expression and activity. This concept is supported by our in vitro data, however, we fully recognize that, as it is the case with any other complex transcriptional program, multiple factors influence the expression and activity of all players identified herein (MYC, miR-30a and NOTCH). Thus, the most robust messages from our findings are that a MYC can indirectly modulate NOTCH1/2 levels via miR-30a, that in the examined in vitro models miR-30a has tumor suppressive properties, and that in primary tumors a correlation is found between miR-30a levels and NOTCH1/2 mutational status/expression. To determine whether miR-30a is a central modulator of NOTCH and MYC oncogenicity, it will require the development and characterization of new GEM models; in this regard, we suggest that with all its limitations\textsuperscript{33}, examination of a xenograft model would add limited, if any, information beyond the data presented here.

Recent evidence suggests that NOTCH1 and NOTCH2 are mutated in various mature B cell tumors, most notably in chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL) and marginal zone lymphoma (MZL), but also in DLBCL\textsuperscript{4–6, 34}. Characteristically, these mutations target the protein’s PEST domains resulting primarily in their overexpression. Our data, in genetically modified cell lines and primary DLBCL biopsies,
suggest that miR-30a downregulation could also up-regulate NOTCH1 and NOTCH2 in B cell malignancies. Thus, as aberrant MYC activation is a frequent event in the pathogenesis of various B and T cell malignancies, and MYC suppresses miR-30a, abnormally high levels of NOTCH1 and NOTCH2 may be a more common component in the pathogenesis of these tumors than previously appreciated.

In NOTCH1 mutant T-ALLs, the MYC-miR-30a-NOTCH axis that we described may primarily function to maintain an uninterrupted flux of the oncogenic signals between MYC and NOTCH, via downregulation of miR-30a. Approximately 20% of T-ALLs also have mutations in the ubiquitin ligase FBXW7\textsuperscript{35, 36}. Interestingly, recent evidence suggests that the central consequence of a common FBXW7 mutation (R465C) is MYC stabilization\textsuperscript{35}. Thus, a scenario could be envisioned whereby in T-ALLs with FBXW7 mutations the higher levels of NOTCH derive both from its deficient degradation (i.e., stabilization) and its derepression secondary to the MYC-mediated miR-30a suppression.

Interestingly, a broader interaction between the miR-30 family and the NOTCH pathway may exist. Recent reports demonstrated that miR-30a, -30b and -30c directly inhibit the NOTCH ligand Delta-like 4 (DLL4) in endothelial cells\textsuperscript{37, 38}. Furthermore, miR-30b was shown to target NOTCH1 in hematopoietic stem cells, while miR-30c regulates NOTCH1 expression in the human and murine myeloid cell compartment\textsuperscript{20, 21}. Thus, it is possible that other members of this miRNA family also play a role in the circuitry that we identified herein, as suggested by the preliminary quantification of miR-30b-e in the ICN1-expressing and GSI-treated models. More importantly, however, by ectopically expressing miR-30a, we confirmed that this miRNA can independently influence the NOTCH-MYC regulatory loop. Our data add knowledge to the emerging relationship between miR-30 family and the NOTCH pathway in two ways: we demonstrated that miR-30a targets NOTCH1 in human and murine lymphocytes, and showed that NOTCH2 is also a target of miR-30a.

In summary, in this report we showed that the relationship between NOTCH and MYC is bidirectional and that in addition to the earlier evidence that NOTCH induces MYC\textsuperscript{8–10}, MYC itself, by suppressing miR-30a, also impinges on NOTCH expression. These observations may prove relevant to the pathogenesis of multiple neoplasms that rely on MYC and NOTCH signals for initiation and maintenance, and future examination of large and well-characterized cohorts of patients with mature follow up should help define if this interplay has important clinical implications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. NOTCH1 AND NOTCH2 are direct targets of miR-30a

A) Luciferase constructs containing the NOTCH1 and NOTCH2 miR-30a binding sites in wild-type (WT) or mutant (Mut) configuration were cotransfected with miR-30a, or control oligonucleotides. MiR-30a inhibited luciferase activity in the NOTCH1 and NOTCH2 WT but had no significant effect in the mutant construct (P < 0.05, Student’s t-test). Data shown are mean ± SEM of the ratio of luciferase activity (miR-30a vs. control oligonucleotide); experiments were performed in triplicate and repeated two to three times. The two NOTCH2 binding sites are labeled as NOTCH2.1 and 2.2.

B) Western blot analysis of the DLBCL cell lines (SU-DHL6 and SU-DHL7) or T-ALL cell lines (DND-41 and KOPT-K1) depicts NOTCH1 and NOTCH2 inhibition upon stable expression of miR-30a. In the T-ALL model, the effects of miR-30a are also detected with antibody that specifically recognizes the intracellular domain of NOTCH1 (ICN1) released by cleavage. The SU-DHL6 cell line does not express NOTCH1.

C) Western blot analysis of the DLBCL cell lines (SUDHL7 and OCI-Ly18) or T-ALL cell lines (DND-41 and KOPT-K1) stably expressing of miR-30a-specific sponge construct (30a-spg), or a control construct containing a scrambled sequence (ctrl-spg), shows that functional sequestering of this miRNA markedly de-represses NOTCH1 and NOTCH2 expression. The OCI-Ly18 cell line does not express NOTCH1.
Figure 2. MYC regulates and it is regulated by the miR-30-NOTCH1/2 axis

A) Genetic gain of MYC impinges on miR-30a and Notch1 expression. Mature B cells were isolated from the spleens of five Myc Tg mice and four WT littermate controls. MiR-30a levels were significantly lower in B cells expressing the Myc transgene (mean relative expression 0.78±0.1 vs. 1.38±0.3, p<0.05, two-tailed Student’s t-test) and correlated with higher Notch1 expression. Notch2 levels were not distinct among these mice, in agreement with the lack of conservation of the miR-30a binding in murine Notch2.

B) Genetic modulation of miR-30a modifies MYC expression in multiple DLBCL and T-ALL cell line models. Western blot analysis depicts suppression or upregulation of MYC upon stable expression of miR-30a (upper panel) or a miR-30a-specific sponge construct (lower panel), respectively.
Figure 3. Genetic and pharmacological modulation of NOTCH1 and NOTCH2 modifies miR-30 expression

A) Stable expression of intracellular NOTCH1 and NOTCH2 (ICN1 and ICN2) in the DLBCL cell line SU-DHL7 led to upregulation of MYC expression, which was accompanied by downregulation of miR-30a (bottom panel) (p<0.05, two-tailed Student’s t-test). MiR-30a relative expression data are mean ± SEM of three independent biological replicates performed in duplicate (six data points).

B) Exposure of two T-ALL cell lines to the gamma-secretase inhibitor Compound E (100–200nM), resulted in a temporally related ICN1 suppression (detected as early at 24h), followed by MYC inhibition and subsequently by a significant de-repression of miR-30a (a ~ 50% increase in expression at 72h). (A) MiR-30a relative expression data are mean ± SEM of two independent biological replicates performed in duplicate or triplicate (five data points) *(p<0.05, two-tailed Student’s t-test).
Figure 4. MiR-30a has tumor suppressive properties towards T-ALL and DLBCL cell lines

A) The growth pattern of two NOTCH1-mutant T-ALL cell lines stably expressing miR-30a or an empty vector (MSCV) was monitored using an automated trypan blue (TB) exclusion assay (Cellometer Vision CBA system, Nexcelom). In both models, miR-30a expression significantly reduced cell proliferation (32% and 50% decrease in DND-41 and KOPT-K1, respectively; **p<0.01, two-tailed Student’s t-test).

B) DND-41 cells stably expressing MSCV or miR-30a were mock-transfected or transfected with a target site blocker (TSB) oligonucleotide. In mock-transfected cells, miR-30a expression significantly repressed cell growth (**p<0.01, two-tailed Student’s t-test), whereas these effects were lost in cells transfected with the NOTCH1-miR-30a TSB (ns = non-significant). TSB-transfected miR-30a-expressing cells also showed significantly higher growth rate than their isogenic mock-transfected miR-30a counterparts. Western blotting confirms the NOTCH1 derepression in cells exposed to the TSB oligonucleotide.

C) Functional inactivation of miR-30a with stable expression of a specific sponge construct significantly enhanced the growth rate of the T-ALL cells (a ~ 40% increase in DND-41 and KOPT-K1 at day 5; **p<0.01, two-tailed Student’s t-test).

D) In the DLBCL model, sequestering miR-30a from its targets with a sponge construct, rendered these cells significantly more resistant to apoptosis induced by broadly genotoxic stresses, starvation (0% serum) or oxidation (H2O2, 25μM) for 24 or 48h (**p<0.01, two-tailed Student’s t-test). Control and 10% serum indicate the cells exposed to vehicle or grown in full media and basal apoptosis rate for comparison to H2O2 treatment and serum deprivation, respectively. All data shown are mean...
± SD of an assay performed in triplicate. Two to three biological replicates of each assay were completed, each time in triplicate.
Figure 5. The miR-30a/NOTCH regulatory axis is active primary tumors

A) Quantification of miR-30a in a cohort of five primary T-ALLs revealed lower miR-30a expression in NOTCH1 mutant than WT samples. Data shown relative expression of miR-30a for each sample (and mean for the group), measured in triplicate and normalized by the levels of the small nucleolar RNA U18 (p=0.56, one way ANOVA, p<0.05 Dunnett’s multiple comparison test, WT samples vs. mutant cell lines).

B) NOTCH2 levels defined by western blotting were significantly higher in primary DLBCL biopsies with low miR-30a expression (p<0.05, two-sided Mann-Whitney test for the densitometric values – shown in lower left panel). Expression of miR-30a in these samples is shown on the lower right panel (p<0.05, two-sided Mann-Whitney test), and depicts the relative expression for each sample (and mean of the group) quantified in triplicate and normalized by the levels of the small nucleolar RNA U18.