TUMORIGENESIS AND NEOPLASTIC PROGRESSION

The PTEN-AKT-mTOR/RICTOR Pathway in Nasal Natural Killer Cell Lymphoma Is Activated by miR-494-3p via PTEN But Inhibited by miR-142-3p via RICTOR

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Nasal natural killer (NK) cell lymphoma (NNL) is an Epstein-Barr virus—associated lymphoma of cytotoxic NK cell origin. The Epstein-Barr virus—encoded miR-BART20-5p inhibits T-bet (TBX21), the master transcription factor of cytotoxic NK cells. To further explore the roles of miRNAs in NNLs, we measured the miRNA expression profiles of 36 NNLs. miR-21, miR-142-3p, miR-126, miR-451, and miR-494-3p were the top five miRNAs with the highest expression levels. By using pathway analysis, we identified associations between all of the five miRNAs with the PTEN-AKT-mTOR pathway, in which PTEN suppresses the oncogenic AKT, and mTOR mediates the oncogenic effects of AKT. YT and NK92 cells derived from NK cell lymphomas were used. miR-494-3p inhibited PTEN with secondary activation of AKT in NK92 cells, and miR-142-3p inhibited RICTOR, a key component of the mTOR complex, with secondary suppression of AKT in YT cells. Significantly, T-bet inhibited the PTEN-AKT-mTOR/RICTOR pathway through induction of PTEN and suppression of RICTOR. Therefore, a molecular circuit of T-bet, PTEN, AKT, and RICTOR is regulated by miR-BART20-5p, miR-494-3p, and miR-142-3p. This circuit is involved in the pathogenesis of NNL. Hence, antagonists to miR-BART20-5p or miR-494-3p, miR-142-3p mimics, or AKT inhibitors may be useful in NNL therapy. (Am J Pathol 2015, 185: 1487–1499; http://dx.doi.org/10.1016/j.ajpath.2015.01.025)

Nasal natural killer (NK) cell lymphoma (NNL) is an Epstein-Barr virus—associated lymphoma of cytotoxic NK cell origin. Because cytotoxic NK cells normally eradicate viral infections, it is interesting to investigate how the EBV may evade and transform NK cells in NNL. Interferon (IFN)-γ is the primary cytotoxic cytokine of NK cells, and T-bet (T-box 21/T-box expressed in T cells; official name TBX21) is the master transcription factor for IFN-γ. The EBV-encoded miR-BART20-5p inhibits both Tbet and IFNG in NNL. This inhibition allows the survival of EBV inside NK cells and possibly also the malignant transformation of NK cells. The EBV encodes at least 44 miRNAs; the functions of most EBV-encoded miRNAs in the pathogenesis of NNL remain to be explored. In addition, it is unclear how miRNAs encoded in the human genome contribute to the pathogenesis of NNL through interactions with the EBV-encoded miRNAs.

The miRNA expression profiles of normal NK cells are well known. Although the human genome encodes >1000 miRNAs, human CD56+ NK cells express only 48 miRNAs. Similarly, murine NK cells express only a limited set of miRNAs. In murine NK cells, the top 10 miRNAs constitute 65% of all miRNA transcripts, and miR-21 alone, the most abundantly expressed miRNA, accounts for approximately 30% of all miRNA transcripts.

In contrast to normal NK cells, data on the miRNA expression profiles of malignant NK cells are limited. Both NNLs and EBV-negative peripheral T-cell lymphomas

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TP53 induces transcription of regulate the PTEN-AKT-mTOR/RICTOR pathway in NNLs. The master transcription factor of cytotoxic NK cells, may pathway, and inhibition of RICTOR was proposed, in which inhibition of plentifully expressed in NNLs. A PTEN-AKT-mTOR/PI3K pathway was regulated by miRNAs in NNL. 

AKT1 is a well-characterized oncogene, whose oncogenic activities are modulated by the tumor suppressors TP53 and PTEN, which are regarded as the guardian of the genome. There are multiple interactions among the oncogenic AKT and the tumor suppressors TP53 and PTEN. For example, TP53 increases transcription of PTEN. Conversely, PTEN induces TP53 via phosphatase-dependent and phosphatase-independent mechanisms. In the phosphatase-dependent pathway, cytokines and antigen receptor activation promote phosphorylated p38, 3,4,5 triphosphates to produce phosphorylated AKT (pAKT), which may phosphorylate mouse double minute 2 homolog to accelerate TP53 degradation. PTEN removes the phosphate from phosphatidylinositol 3,4,5 triphosphates, prevents formation of pAKT, and blocks degradation of TP53. In the phosphatase-independent pathway, interactions between PTEN and TP53 maintain high acetylation of TP53 and enhance the DNA binding ability of TP53. Through these mutual interactions, increased TP53 and PTEN may cause degradation of pAKT.

The oncogenic effects of AKT are mediated through the mammalian target of rapamycin (mTOR), including mTOR complexes (mTORCs) 1 and 2. The mTORCs are activated by AKT. Conversely, a key component of mTORC2, RICTOR, the independent companion of mTORC2 (RICTOR), may phosphorylate and activate AKT.

Both mTORC1 and mTORC2 have been implicated in the development of thymocytes and lymphoblastic lymphoma of immature T-cell origin. Furthermore, in diffuse large B-cell lymphoma, the activity of mTORC2 is important in the development of drug resistance to the mTOR inhibitor rapamycin. These data support an oncogenic AKT-mTOR pathway. However, regulation of the AKT-mTOR pathway by miRNAs in NNLs is not well documented.

In this study, genome-wide miRNA expression profiles in 36 NNLs showed that miR-149-3p and miR-142-3p were abundantly expressed in NNLs. A PTEN-AKT-mTOR/RICTOR pathway was proposed, in which inhibition of RICTOR by miR-142-3p inactivates the oncogenic AKT pathway, and inhibition of PTEN by miR-494-3p activates the oncogenic AKT pathway. Finally, we showed that T-bet, the master transcription factor of cytotoxic NK cells, may regulate the PTEN-AKT-mTOR/RICTOR pathway in NNLs.

Materials and Methods

Tissue Samples

From the archives at the Pathology Department of National Taiwan University Hospital (Taipei), a series of 36 NNLs were selected. Diagnoses were made on pretreatment nasal biopsy specimens by a combination of histopathology, diffuse positive immunostaining for CD3, focal to diffuse positive immunostaining for CD56, and in situ hybridization for EBV-encoded small RNA. The ethics committee of the National Taiwan University Hospital approved the study.

Genome-Wide miRNA Profiles in 36 NNLs

A series of 36 NNLs were used. The nCounter miRNA Expression Analysis System (NanoString Technologies, Seattle, WA) was used to obtain the expression profiles of 651 human miRNAs. A panel of six synthetic miRNAs was used as internal positive controls. Approximately 0.1 μg of total RNAs was extracted from formalin-fixed, paraffin-embedded tissue blocks. Mature miRNAs were ligated to sequence-specific miRTags, and the miRTagged mature miRNAs were hybridized to fluorescent reporter probes and captured probes. After hybridization, the samples were immobilized to a solid-phase cartridge, and a charge-coupled device digital analyzer was used for data acquisition. The data were normalized to the internal positive controls (Supplemental Table S1).

Cell Lines and Transfection

YT and NK92 cells are EBV-infected lymphoma cell lines of NK cell origin. They were obtained from the German Collection of Microorganisms and Cell Culture (Braunschweig, Germany). YT cells were grown in 80% Iscove’s Modified Dulbecco’s Medium plus 20% fetal bovine serum. NK92 cells were grown in 75% α–minimal essential medium, 12.5% fetal bovine serum, 12.5% horse serum, 2 mmol/L l-glutamate, and 10 ng/mL IL-2.

For transfection experiments, 20 to 40 μg of enhanced green fluorescent protein—expressing plasmids was electroporated into the cells, followed by selection with G418 until >90% were positive for enhanced green fluorescent protein.

Luciferase Activity Assay for Inhibition of PTEN by miR-494-3p or RICTOR by miR-142-3p

Luciferase assays were performed in YT or NK92 cells with a plasmid miRCS-GLO-targetCS (Supplemental Figure S1 and Table 1) derived from the pmirGLO Dual-Luciferase miRNA target expression vector (Promega, Madison, WI). The plasmid, miRCS-GLO-targetCS, includes cloning sites for both miRNA and 3’ untranslated region (UTR) of the target. The firefly luciferase was the primary reporter to monitor inhibition of the targets by miRNAs. A Renilla luciferase was used as an internal control for normalization.

For inhibition of PTEN by miR-494-3p, the 3’UTR of PTEN, approximately 3.3 K, was inserted downstream of the firefly luciferase coding region, and the wild-type (WT) or mutant (MT) miR-494 stem-loop precursor was inserted into the miRNA expression cassette. The resultant vector,
WT-miR-494-GLO-PTEN-3'UTR, MT-miR-494-GLO-PTEN-3'UTR, or miRCS-GLO-PTEN-3'UTR, was transfected into NK92 cells. For inhibition of RICTOR by miR-142-3p, the 3'UTR of RICTOR, approximately 4.4 K, was inserted downstream the firefly luciferase coding region, and the WT or MT miR-142 stem-loop precursor was inserted into the miRNA expression cassette. The resultant vector, WT-miR-142-GLO-RICTOR-3'UTR, MT-miR-142-GLO-RICTOR-3'UTR, or miR-CS-GLO-RICTOR-3'UTR, was transfected into YT cells.

Luciferase activities were measured according to the manufacturer’s protocol. The ratio of firefly luciferase activity/Renilla luciferase activity was obtained and was normalized to the ratio obtained when an empty vector expressing no miRNAs was used as a control.

Plasmids for Localization of Target Sites with Luciferase Assay

Two plasmids were derived from the plasmid, WT-miR-494-GLO-PTEN-3'UTR, to localize the miR-494-3p binding on the PTEN-3'UTR (Supplemental Figure S1 and Table 1). In the first plasmid, WT-miR-494-GLO-MT1-PTEN-3'UTR, the seed region of the first predicted binding site at PTEN-3'UTR, was replaced by GAATTc, the EcoRI site. In the second plasmid, WT-miR-494-GLO-MT2-PTEN-3'UTR, the seed region of the second predicted binding site at PTEN-3'UTR, was replaced by GAATTc, the EcoRI site. Luciferase assays were done in NK92 cells transfected with plasmids with WT, MT1, or MT2 PTEN-3'UTR.

A plasmid was derived from the plasmid WT-miR-142-GLO-RICTOR-3'UTR to localize the miR-142-3p binding on the RICTOR-3'UTR (Supplemental Figure S1 and Table 1). In the plasmid, WT-miR-142-GLO-MT-RICTOR-3'UTR, the predicted binding site at RICTOR-3'UTR, was replaced by CCGCGG, the SacII site. Luciferase assays were done in YT cells transfected with plasmids with WT or MT RICTOR-3'UTR.

Plasmid for Antagomirs to miR-494-3p or miR-142-3p

Antagomir to miR-494-3p or miR-142-3p was inserted into the miRNA cloning site in the miRCS-GLO-targetCS vector (Supplemental Figure S1 and Table 1). The target cloning site was left empty.

The vector expressing antagomir to miR-494-3p was transfected into YT cells, and Western blot analysis for PTEN was performed. The vector expressing antagomir to miR-142-3p was transfected into NK92 cells, and Western blot analysis for RICTOR was performed.

Western Blot Analysis

Western blot analysis was performed with antibodies against luciferase (rabbit polyclonal) from GeneTex (San Antonio, TX); TP53 (rabbit polyclonal) from Abcam (Cambridge, MA); PTEN (number 9552, rabbit polyclonal), RICTOR (number 2140, rabbit polyclonal), and pAKT (Ser473) (D9E, rabbit monoclonal) from Cell Signaling (Danvers, MA); and T-bet (4B10, mouse monoclonal) and glyceraldehyde-3-phosphate dehydrogenase (FL-355, rabbit polyclonal) from Santa Cruz Biotechnology (Santa Cruz, CA).

Chromatin IP for Binding of T-bet on the PTEN Promoter or Binding of TP53 on the miR-142-3p Promoter

Chromatin immunoprecipitation (IP) was done with the SimpleChIP assay on YT or YT–T-bet cells. Briefly, YT or YT–T-bet cells were fixed in 1% formaldehyde for cross-linking. After lysis of the cells, the nuclei were pelleted, and the chromatin was digested with micrococcal nuclease.

For binding of T-bet to the PTEN promoter, the chromatin was reacted with a T-bet antibody (mouse monoclonal, clone 4B10; Santa Cruz Biotechnology) or mouse IgG as negative control, or a mouse antibody against histone 3 (MABI 0301; Active Motif, Carlsbad, CA) as a positive control. Protein G magnetic beads were used for isolation of the chromatin, and protein-DNA cross-linking was reversed with proteinase K. The DNAs were amplified with the following primers: 5'-AGCTGAGTCCAGACATGTGTA-3' (forward) and 5'-AGCTGGACAGTCGGCTCCCCAAAT-3' (reverse).

For binding of TP53 on the miR-142-3p promoter, the chromatin was reacted with a TP53 antibody (rabbit polyclonal; Abcam) or rabbit IgG as negative control, or a rabbit antibody against histone 3 (D2B12, rabbit monoclonal; Cell Signaling) as positive control. The DNAs were purified and amplified with the following primers: 5'-GGGGATGGGGTGGAGC-CTTAT-3' (forward) and 5'-CTCCTTGTGGGCTTCCATTAG-3' (reverse).

Quantitative RT-PCR for miR-494-3p, Anti–miR-494-3p, miR-142-3p, and Anti–miR-142-3p

Total RNAs were extracted from cells with the TRIZol method (Sigma-Aldrich, St. Louis, MO). A stem-loop RT-PCR was performed according to a published protocol.12 For RT, a specific reverse primer, 5'-N44-AGAGTT-3' for miR-494-3p, 5'-N44-TGAAAC-3' for anti–miR-494-3p, 5'-N44-TCCATA-3' for miR-142-3p, or 5'-N44-TGTAAGT-3' for anti–miR-142-3p, was used, where N44 is the stem-loop 5'-GTGGTATCCATGGTGCGGTTGGTTAATG-3'. For PCR, a specific forward PCR primer, 5'-GGGGATGGGGTGGAGCATGTTCTAATAATG-3', was used, where GGATGGGGTGGAGCAGTTCTAATAATG is the full PCR fragment, and a universal reverse PCR primer, 5'-TAGCAGCCACATGGTGAGTTTCTCTG-3', was used. The difference between the threshold cycles of miR-494-3p, anti–miR-494-3p, miR-142-3p, and anti–miR-142-3p, and U6 snRNA [dTCT(CTmiRNA – CTU6)] was
calculated from the mean values of duplicate measurements. This definition of dCT implies an inverse correlation between dCTs and miRNA levels.

Caspase Activities in YT and NK92 Cell Lines Treated with AKT Activator SC79 or AKT Inhibitor GSK690693

NK92, NK92 transfected with miR-494-3p, or NK92 transfected with anti-miR-142-3p was treated with an AKT activator SC79 (Sigma-Aldrich, St. Louis, MO). YT, YT transfected with miR-142-3p, or YT transfected with anti-miR-494-3p was treated with an AKT inhibitor GSK690693 (Sigma-Aldrich). Caspase activities were measured with a luminescent assay Caspase-Glo 3/7 (Promega, St. Louis, MO), in which the luciferase substrate aminoluciferin is generated from Z-DEVD-aminoluciferin after caspase cleavage.

Briefly, 10,000 cells were grown in 200 μL medium containing 25 μmol/L SC79 or GSK690693 for 24 hours. An equal volume of reagent containing reagents for lysis, the substrate Z-DEVD-aminoluciferin, and luciferase were added. The mixture was incubated at 25°C for 30 minutes, and the luminescence was measured.

Immunohistochemistry for PTEN, RICTOR, and pAKT

Immunoperoxidase stains on formalin-fixed, paraffin-embedded tissue sections with antibodies against PTEN (number 31392, rabbit polyclonal; Abcam), RICTOR (number 56578, mouse monoclonal; Abcam), and pAKT (Ser473) (D9E, rabbit monoclonal; Cell Signaling). Antigen retrieval was performed in Tris buffer at pH 8. The primary antibody was applied to the slides at 37°C for 45 minutes. Biotin-conjugated secondary antibodies, peroxidase-conjugated streptavidin, and 3,3′-diaminobenzidine were used sequentially for completing the reactions. The percentages of positive cells, as averages of 10 randomly selected high-power fields, were determined.
miRNAs in NNLs Regulate the AKT Pathway

Pathway analysis for the top five miRNAs in NNLs, miR-21, miR-142-3p, miR-126, miR-451, and miR-494-3p, was performed on the DIANA-miRExTra web server (http://diana.cslab.ece.ntua.gr/hexamers, last accessed January 23, 2015). Except for miR-142-3p, four of the five top miRNAs have been shown experimentally to regulate the AKT pathway (Figure 1B and Table 2). In addition, a target site for miR-142-3p on the 3’ UTR of RICTOR was identified with the RNAhyb program.25

These miRNAs may either activate or inactivate the AKT pathway (Table 2). For example, both miR-126-3p and miR-142-3p inhibit the AKT pathway, whereas miR-494-3p, miR-21-5p, and miR-451-5p all activate the AKT pathway. We are interested in the inhibition of PTEN by miR-494-3p, because of the importance of the AKT pathway in nasal lymphoma, and in miR-142-3p, because the regulation of RICTOR by miR-142-3p is a novel finding.

miR-494-3p/PTEN/pAKT Pathway

Overexpression of miR-494-3p Inhibits PTEN with Secondary Up-Regulation of pAKT in NK92 Cells

Two binding sites for miR-494-3p at the 3’ UTR of PTEN mRNA were identified with the RNAhyb online program (Figure 2A). The 5′ end of the first binding site is located at the 2297th nucleotide after the stop codon. This is a strong model, because of the perfect matches at the seed region (ie, between the second and eighth nucleotides at the 5′ end of miR-494-3p and the 3′ end of the target, and also because of the presence of only small internal mismatches of at most dinucleotides but not large internal bulges). The second binding site is located at the 2776th nucleotide after the stop codon. This is a weak model, because of significant mismatches at the 5′ end and the internal bulges.

In NK92 cells, overexpression of miR-494-3p, but not mutant miR-494-3p, inhibited the expression of luciferase with wild-type PTEN-3’UTR. In addition, miR-494-3p inhibited PTEN-3’UTR with a mutated second binding site but not PTEN-3’UTR with a mutated first binding site (Figure 2B). These data confirmed the first binding site for miR-494-3p in addition, Western blot analysis showed miR-494-3p, but not a mutant, inhibited expression of luciferase-PTEN-3’UTR in NK92 cells (Figure 2C).

For further confirmation, a plasmid expressing miR-494-3p was transfected into NK92 cells, and a plasmid expressing anti-miR-494-3p was transfected into YT cells. NK92 cells and YT cells are lymphoma cells of NK origin. NK92 cells have higher expression of PTEN and lower expression of pAKT than YT cells. As predicted, miR-494-3p inhibited PTEN with secondary induction of pAKT in NK92 cells, whereas anti-miR-494-3p induced PTEN with secondary inhibition of pAKT in YT cells (Figure 2D).

Functionally, pAKT\textsuperscript{weak} NK92 cells are more sensitive to the AKT activator SC79 than pAKT\textsuperscript{strong} NK92-miR-494-3p cells. In contrast, PAKT\textsuperscript{strong} YT cells are more sensitive to the
AKT inhibitor GSK690693 than pAKT\textsuperscript{weak} YT-anti-miR-494-3p cells (Figure 2E).

Overexpression of miR-142-3p Inhibits \textit{RICTOR} with Secondary Down-Regulation of pAKT in YT Cells

A binding site for miR-142-3p at the 3′UTR of \textit{RICTOR} mRNA was identified with the RNAhyb online program (Figure 3A).\textsuperscript{25} The 5′ end of the binding site is located at the 864\textsuperscript{th} nucleotide after the stop codon. Luciferase assay showed that miR-142-3p, but not a mutant, inhibited expression of luciferase-\textit{RICTOR}-3′UTR in YT cells. In addition, miR-142-3p inhibited luciferase-\textit{RICTOR}-3′UTR, but not luciferase-MT-\textit{RICTOR}-3′UTR (Figure 3B).

For further confirmation, a plasmid expressing miR-142-3p was transfected into YT cells, and a plasmid expressing anti-miR-142-3p was transfected into NK92 cells. YT cells have higher pAKT than NK92 cells, although the levels of RICTOR are approximately equal. As predicted, miR-142-3p inhibited \textit{RICTOR} with secondary suppression of pAKT in YT cells, whereas anti-miR-142-3p induced \textit{RICTOR} with secondary induction of AKT in NK92 cells (Figure 3C).

In the AKT-mTOR pathway, pAKT not only mediates the oncogenic effects of pAKT, but RICTOR in the mTOR complex 2 may phosphorylate and activate AKT.\textsuperscript{16} Therefore, there is possibly a miR-142-3p-\textit{RICTOR}-AKT pathway in NNL.\textsuperscript{1,2} Because a genome-wide screen identified the promoter of \textit{PTEN} as one of the T-bet binding sites,\textsuperscript{34,35} there might be interactions between the miR-BART20-5p-T-bet-TP53 pathway and the miR-494-3p-PTEN-AKT pathway.

A model for the interactions between the transcription factor T-bet and the promoter of \textit{PTEN} is shown in Figure 4A. The 5′ end of the binding site is at position -6101 before the transcription start site. YT cells are lymphoma cells of NK cell origin. These cells express no or weak T-bet. Chromatin IP was done in YT cells and YT cells transfected with a plasmid expressing T-bet (YT-T-bet cells). Consistent with the model, there was stronger binding of T-bet to the \textit{PTEN} promoter in YT-T-bet cells than in YT cells (dCT: 7.8 in YT−T-bet versus 13.6 in YT) (Figure 4B).

Furthermore, Western blot analysis showed that T-bet induces \textit{PTEN} and TP53 with inhibition of pAKT in YT−T-bet cells (Figure 4C). Because inhibition of AKT by \textit{PTEN} and TP53 is well established,\textsuperscript{12,13,15} we conclude that T-bet induces both \textit{PTEN} and \textit{TP53} to inhibit AKT in YT−T-bet cells.

Tumor Suppressor TP53 Induces miR-142-3p

\textbf{Figure 4,} A−C, demonstrates a connection between the T-bet−TP53 pathway and the miR-494-3p-PTEN-AKT pathway. Because of the down-regulation of RICTOR in YT−T-bet cells (Figure 4C), TP53 might directly regulate miR-142-3p\textsuperscript{36} and secondarily the RICTOR-pAKT pathway.

The expression level of miR-142-3p was higher in YT−T-bet cells than in YT cells (dCT: 0.45 in YT−T-bet versus 2.72 in YT) (Figure 4D). This finding supported indirect induction of miR-142-3p by T-bet through TP53.

A binding site for TP53 on the promoter of miR-142-3p was noted.\textsuperscript{16} The 5′ end of the binding site was at position -166 before the transcription start site (Figure 4E). The

\textbf{Table 2} Regulation of the AKT Pathway by miRNAs

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Target</th>
<th>Name</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>126-3p\textsuperscript{26}</td>
<td>IRS1</td>
<td>Insulin receptor substrate 1</td>
<td>A protein that is phosphorylated by insulin receptor tyrosine kinase</td>
</tr>
<tr>
<td>142-3p\textsuperscript{*}</td>
<td>\textit{RICTOR}</td>
<td>RPTOR-independent companion of mTOR complex 2</td>
<td>The mTOR is a Ser/Thr kinase that regulates cell growth and proliferation; mTOR may exist as mTOR complex 1 or mTOR complex 2; RICTOR is a component of mTORC2 that may phosphorylate and activate AKT</td>
</tr>
<tr>
<td>494-3p\textsuperscript{27,28}</td>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
<td>A phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase that suppresses tumor by negatively regulating the AKT signaling pathway</td>
</tr>
<tr>
<td>21-5p\textsuperscript{*}</td>
<td>\textit{PIK3R1}</td>
<td>Phosphoinositide-3-kinase, regulatory subunit 1</td>
<td>A regulatory component of PI3K, which is a lipid lipase important for cell growth</td>
</tr>
<tr>
<td>21-5p\textsuperscript{30−32}</td>
<td>SPRY</td>
<td>Sprout homolog</td>
<td>An inhibitor of receptor tyrosine kinase</td>
</tr>
<tr>
<td>451-5p\textsuperscript{33}</td>
<td>M025</td>
<td>Calcium-binding protein 39, CAB39</td>
<td>A calcium-binding protein</td>
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\textsuperscript{*} A predicted target by RNAhyb or http://www.microrna.org.

\textbf{Figure 3A} demonstrates the interaction between the T-bet-TP53 pathway and the miR-494-3p-PTEN-AKT pathway. Inhibition of \textit{PIK3R1} was demonstrated with the RNAhyb online program. These cells express no or weak T-bet. Chromatin IP was done in YT cells and YT cells transfected with a plasmid expressing T-bet (YT-T-bet cells). Consistent with the model, there was stronger binding of T-bet to the \textit{PTEN} promoter in YT−T-bet cells than in YT cells (dCT: 7.8 in YT−T-bet versus 13.6 in YT) (Figure 4B).

Furthermore, Western blot analysis showed that T-bet induces \textit{PTEN} and TP53 with inhibition of pAKT in YT−T-bet cells (Figure 4C). Because inhibition of AKT by \textit{PTEN} and TP53 is well established,\textsuperscript{12,13,15} we conclude that T-bet induces both \textit{PTEN} and \textit{TP53} to inhibit AKT in YT−T-bet cells.
Figure 2  A miR-494-3p/PTEN/pAKT pathway: Overexpression of miR-494-3p inhibits PTEN with secondary up-regulation of pAKT in NK92 cells. A: Models for the interactions between miR-494-3p and the 3′ untranslated region (UTR) of PTEN mRNA. The 5′ end of the target site is predicted to be located at the 2297th or 2776th nucleotide after the stop codon. B: Confirmation of the first model with a luciferase assay in NK92 cells. The activity of luciferase is inhibited in NK92 cells with transfections of both a wild-type (WT) miR-494-3p and a wild-type first binding site at position 2297 (lanes 1 and 2). The inhibition is not removed by a mutated second binding site at position 2776 (lane 3), but is removed by mutations at either the first binding at position 2297 or miR-494-3p (lanes 4 and 5). C: Confirmation of the model with Western blot analysis for luciferase in NK92 cells. Wild-type miR-494-3p, but not mutant (MT) miR-494-3p, inhibits the expression of luciferase-PTEN-3′UTR in NK92 cells. D: Overexpression of miR-494-3p inhibits PTEN with secondary induction of pAKT in NK92 cells, whereas overexpression of anti-miR-494-3p induces PTEN with secondary suppression of pAKT in YT cells. Western blot analysis in NK92 overexpressing miR-494-3p or YT cells overexpressing anti-miR-494-3p supports miR-494-3p/PTEN/pAKT pathway, in which miR-494-3p inhibits PTEN and PTEN inhibits pAKT (left column). Quantitative RT-PCR for miR-494-3p or anti-miR-494-3p. For x axis, cell lines; y axis, dCT for miR-494-3p (5.8 in NK versus 0.89 in NK-miR-494-3p) and dCT for anti-miR-494-3p (17.6 in YT versus 9.7 in YT-anti-miR-494-3p) (right column). E: NK92 cells are more sensitive to the AKT activator SC79 than NK92-miR-494-3p cells (median caspase activity, 1.04 versus 0.85; P = 0.005). In contrast, YT cells are more sensitive to the AKT inhibitor GSK690693 than YT-anti-miR-494-3p cells (median caspase activity, 1.18 versus 1.00; P = 0.005). For y axis, caspase activity in cells treated with the drug relative to untreated cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
model was confirmed with chromatin IP that showed stronger binding of TP53 to the miR-142-3p promoter in YT–T-bet cells than in YT cells (dCT: 7.9 in YT–T-bet versus 12.7 in YT) (Figure 4F).

These data provide additional evidence that TP53 may regulate miR-142-3p. However, this finding does not exclude additional regulators on the promoter of miR-142-3p.
miR-494 Correlates Inversely with PTEN, and miR-142-3p Correlates Inversely with RICTOR in NNLs

Immunohistochemical stains for PTEN, RICTOR, and pAKT were performed on 24 NNLs. Representative immunostains are shown in Figure 5A–F.

The levels of miRNAs were measured with the NanoString technology. There is an inverse correlation between miR-494-3p and miR-142-3p in 36 NNLs ($r = -0.47$, $P = 0.003$). NNLs can be classified into 18 miR-494-low/miR-142-high NNLs and 18 miR-494-high/miR-142-low NNLs (Figure 5A).
Immunohistochemistry for PTEN, RICTOR, and pAKT was performed on 12 miR-494low/miR-142high NNLs and 12 miR-494high/miR-142low NNLs (Figure 5, B–G). The 12 miR-494low/miR-142high NNLs had higher PTEN and lower RICTOR and pAKT than the 12 miR-494high/miR-142low NNLs. The median percentages of positive cells were 62% versus 43% for PTEN at \( P < 0.01 \), 40% versus 68% for RICTOR at \( P < 0.01 \), and 40% versus 55% for pAKT at \( P < 0.03 \) (Figure 5H).

There is an inverse correlation between RICTOR and PTEN in the 24 cases (Figure 5I), between miR-494-3p and PTEN (\( r = -0.46, P = 0.02 \) for all 24 cases). For x axis, percentages of PTEN+ cells; y axis, percentages of RICTOR+ cells. Red circles, seven cases outside the 95% CI; black circles, 17 cases inside the 95% CI.

Discussion

NNL is an EBV-associated lymphoma that arises from the nasal mucosa. Our data show that miR-494-3p inhibits PTEN with secondary induction of AKT, whereas miR-142-3p inhibits RICTOR with secondary suppression of AKT (Figure 5J). In addition, T-bet induces both PTEN and TP53 to inhibit pAKT. Modulation of the molecular loop by miRNAs is probably critical in the development of NNL (Figure 5J).
growth through induction of apoptosis, this model agrees with the general conclusion that the EBV rescues tumor cells from apoptosis.37

The finding that miR-494-3p inhibits PTEN to activate AKT in NNL echoes reports that miR-494-3p inhibits PTEN in myeloid cells.27,28 Other than miR-494-3p, miR-2129,30 one of the top 10 highly expressed miRNAs in NNLs (average level, 369 in NNLs), and miR-155,6 with an average expression level of 31 in our series, may also inhibit PTEN to activate AKT. These data support the recent conclusion on the combinatorial regulation of miRNAs: a given miRNA may have hundreds of different mRNA targets, and a given target might be regulated by multiple miRNAs.38 Because the binding for miR-21 on the 3′UTR of PTEN has not been reported and is not predicted by online programs (http://www.microrna.org, last accessed January 23, 2015), and the expression of miR-155 is weak in our series, we decided to focus on the role of miR-494 in the AKT pathway.

miR-142-3p has both oncogenic and tumor-suppressing effects in various cell lines.39,40 We found that miR-142-3p may be regulated by TP53, and miR-142-3p may inhibit RICTOR with secondary suppression of the oncogene pAKT. This suggests a tumor-suppressing effect of miR-142-3p; however, miR-142-3p might still be oncogenic through alternative pathways.

Our studies are based mainly on two EBV-infected lymphoma cell lines of NK cell origin, YT and NK92. These studies demonstrate the existence of a miR-494-PTEN-AKT pathway (Figure 2) and a miR-142-3p-AKT pathway (Figure 3), but do not capture the whole complexities of NNLs, as demonstrated by the finding of an inverse correlation between miR-494-3p and miR-142-3p (Figure 5H). The data from the biopsy specimens thus compensate for the inadequacy of the cell line data.

The mechanism and the biological significance of the inverse correlation are unclear. Because all NNLs are infected with EBV, miR-494-4p-high/miR-142-3p-low NNLs and miR-494-3p-low/miR-142-3p-high NNLs might express different virus-encoded genes or miRNAs. Additional studies, such as genome-wide screens, will be necessary to identify these differences.

Despite these uncertainties, miR-494-4p-high/miR-142-3p-low NNLs have higher levels of pAKT than miR-494-3p-low/miR-142-3p-high NNLs. The former group might benefit from AKT inhibitor therapy, whereas the latter group might require AKT activator therapy,41–43 Therefore, a lower AKT level does not necessarily imply a less important role when therapeutic interventions are considered.

These considerations are further supported by cell line studies. NK92 cells, which express weaker AKT than NK92-494-3p or NK92-anti-142-3p cells, are more sensitive to the AKT activator SC79. YT cells, which express stronger AKT than YT-anti-494-3p or YT-142-3p cells, are more sensitive to the AKT inhibitor GSK690693 (Figures 2E and 3D).

In addition to NNL, loss of PTEN is also present in a subset of AKT pathway-dependent diffuse large B-cell lymphomas of the germinal center type.44,45 Because miR-21 inhibits PTEN, antisense miR-21 has been used to increase the cytotoxic effects of traditional doxorubicin, cyclophosphamide, vincristine, and prednisone therapy in cell lines of B-cell origin.46,47 These reports also suggest that adding antisense miR-494-3p to the standard radiochemotherapy of NNL could potentially improve the outcome of NNL. Finally, miR-494 is up-regulated by the extracellular signal–regulated kinase 1/2 pathway.48 Therefore, a pharmacological inhibitor of the extracellular signal–regulated kinase pathway might be therapeutically effective too.

The miRNA expression profiles in normal human or murine NK cells are well documented.3,4 When the most abundant 20 miRNAs are compared, only nine are common to malignant NK cells and normal NK cells (Supplemental Figure S2). For example, both normal and malignant NK cells express high levels of miR-21 and miR-142-3p, but miR-494 is found only in malignant NK cells. This finding demonstrates significant alterations during malignant transformation, in part due to the presence of EBV-encoded miRNAs, such as miR-BART20-5p, in malignant NK cells only.

T-bet induces PTEN, TP53, and IFN-γ, consistent with CHIP-on-CHIP data that T-bet regulates the promoters of PTEN, TP53, and IFNG (Figure 5J).34,35 This model does not consider inhibition of TBX21 and IFNG by miR-29,49,50 because of the low expression of miR-29 in NNLs (average level, 50) (Supplemental Table S1).

In conclusion, in NNLs, miR-494-3p inhibits the tumor suppressor PTEN with secondary induction of AKT, whereas miR-142-3p inhibits RICTOR with secondary suppression of AKT. We propose a model in which miR-BART20-5p, miR-494-3p, and miR-142-3p cooperate to inhibit a loop, where T-bet induces both PTEN and TP53 to inhibit AKT. This model is consistent with recent molecular data that the AKT pathway is critical in the pathogenesis of NNL,6,8,51 suggesting potential targets of therapy through antagonists to miR-494-3p, mimics of miR-142-3p, or pharmacological manipulations of the phosphatidylinositol 3-kinase/AKT/mTOR pathway.52

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Supplemental Data

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