Imaging, Diagnosis, Prognosis

MicroRNA-146a Downregulates NFκB Activity via Targeting TRAF6 and Functions as a Tumor Suppressor Having Strong Prognostic Implications in NK/T Cell Lymphoma

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Abstract

Purpose: We investigated prognostic implications of microRNAs in extranodal NK/T cell lymphoma (NKTL).

Experimental Design: We measured miRNA expression in NKTL tissues and cell lines, using real-time PCR, and analyzed its role in NKTL, using cell lines.

Results: Multivariate analysis showed low miR-146a expression ($P < 0.001$; HR = 13.110), primary non–upper aerodigestive tract lesion (non-UAT; $P = 0.008$; HR = 5.376) and high International Prognostic Index (IPI; ≥ 3; $P = 0.013$; HR = 3.584) to be independent poor prognostic factors. miR-146a expression could subdivide UAT-NKTL into 2 prognostic groups, resulting in the following prognostic groups: (i) UAT$^{Low-146a}$, (ii) UAT$^{High-146a}$, and (iii) non-UAT. Compared with UAT$^{High-146a}$, UAT$^{Low-146a}$ showed distinctively poor prognosis ($P < 0.001$; HR = 15.620), similar to the non-UAT group. *In vitro*, miR-146a overexpression in NKTL cell lines, SNK6 and YT, inhibited nuclear factor κB (NFκB) activity, suppressed cell proliferation, induced apoptosis, and enhanced chemosensitivity. TNF receptor–associated factor 6, a target of miR-146a and a known NFκB activator, was downregulated by miR-146a in SNK6 and YT cells. Promoter methylation of *miR-146a* gene was observed in SNK6 and YT cells, as well as in NKTL tissues with low miR-146a expression, and miR-146a expression was induced by the conversion of methylation status with a demethylating agent in SNK6 and YT cells.

Conclusions: These results suggest that miR-146a might function as a potent tumor suppressor in NKTL and be useful for patient assessment and therapeutic targeting. *Clin Cancer Res; 17(14); 4761–71. ©2011 AACR.*

Introduction

microRNAs (miRNA) are small, noncoding RNAs that are known to negatively regulate target genes at the post-transcriptional level. They play important biologic roles by regulating cells in growth, development, apoptosis, and hematopoiesis (1). miRNA analysis might be important in clinical practice, because of its small size allowing good preservation in formalin-fixed, paraffin-embedded (FFPE) tissue and good quantitative correlation in the amounts of miRNAs between FFPE and fresh-frozen tissues (2).

To date, work on miRNAs in tumors has focused on understanding miRNAs as (i) tools for diagnosis or classi-fication of tumors (3), (ii) tumor-specific biomarkers or prognostic factors predicting clinical outcome or therapeu-tic response (4), and (iii) regulators of oncogenes or tumor suppressor genes (5). Differential expressions of miRNAs have been reported in many solid tumors, showing the clinical significance (4–6). In hematolymphoid malignan-cies, miRNAs and their clinical implications have primarily been investigated in B-cell neoplasms (7–9). However, few studies have focused on T or NK cell neoplasms, such as extranodal NK/T cell lymphoma (NKTL), which was strongly associated with Epstein–Barr virus (EBV) infection (10).

Some miRNAs played an important role in EBV-asso-ciated tumors, including Hodgkin lymphoma, Burkitt lym-phoma, and nasopharyngeal carcinoma (8, 11, 12). Recently, the miRNA expression has been investigated as regulators of *EBV* oncogenes, that is, latent membrane protein 1 (*LMP1*; ref. 13–15). Furthermore, miR-21 was shown to play an important oncogenic role via down-

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Translational Relevance

miRNA functions as an important regulator of gene expression by inhibiting translation of mRNA. Because of its small size and stability, the level of miRNA can be readily measured by real-time PCR in clinical samples including formalin-fixed, paraffin-embedded (FFPE) tissues. In this study, we showed that miR-146a played an important role as a tumor suppressor with clinico-pathologic significance in extranodal NK/T cell lymphoma (NKTL). miR-146a suppressed TNF receptor–associated factor 6 (TRAF6)/nuclear factor κB (NFκB) pathway in NKTL cell lines, which resulted in reduced cell proliferation, increased apoptosis, and enhanced chemosensitivity. These in vitro data were consistent with those observed in NKTL patients whose prognosis and responsiveness to chemotherapy were significantly dependent on the miR-146a level measured from FFPE tumor tissues. These results provide clinical applicability of miR-146a as a biomarker predicting patient prognosis, as well as a potential therapeutic target for modulation of tumor cell behavior in NKTL.

regulation of tumor suppressors in NKTL cell lines (10). Therefore, we hypothesized that other specific miRNAs might also participate in a crucial pathway in NKTL.

NKTL is a malignant lymphoma with a predominant NK immunophenotype with generally aggressive behavior despite its heterogeneous outcome (16, 17). Unfortunately, therapeutic options are limited to conventional chemotherapy and radiation therapy (16). Therefore, more practical risk stratification has been needed to help make clinical decisions. We investigated the role of miRNAs in NKTL in prognosis or therapeutic response for the use as additional biomarkers, valid prognostic factors, and potential therapeutic targets.

Materials and Methods

Patients and samples

Fifty NKTL cases diagnosed in Seoul National University Hospital (SNUH) from 1990 to 2007 were included by the following criteria: (i) available paraffin blocks, (ii) sufficient amounts of tissues, (iii) available medical records, and (iv) confirmed diagnosis by 3 hematopathologists (J.H.P., Y.K.J. and C.W.K.) by World Health Organization criteria (16). All cases were included in a previous study, and histology review, immunohistochemistry, and EBV–in situ hybridization were conducted as previously described (18), with the additional immunostaining of LMP1 (DakoCytomation). All cases showed EBV positivity in most tumor cells. The cases harboring more than 70% large cells were classified as large/anaplastic cell type and the rest small/medium cell type. Clinical information was reviewed by hemato-oncologists (T.M.K. and D.S.H.). The primary site was classified as upper aerodigestive tract (UAT) or non-UAT (17). The age of patients ranged from 10 to 79 years (mean, 49.2 years). The Institutional Review Board in SNUH approved this study.

Cell lines and reagents

SNK6, established from EBV-positive NKTL, was kindly provided by Prof. Shimizu and cultured in RPMI-1640 media supplemented with 10% heat-inactivated human plasma and 700 U/mL of recombinant interleukin-2 (IL-2). YT, an EBV-positive human NK cell line, was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and cultured in Iscove's modified Dulbecco's medium supplemented with 20% heat-inactivated FBS. All cells were tested for Mycoplasma. Etoposide was purchased from Boryung Pharm and 5-aza-2′-deoxycytidine (5-azadC) from Sigma.

Selection of miRNAs

miRNAs were selected by previously reported biologic relevance in NKTL. miR-9* and miR-189 were selected as representative miRNAs with expected high and low expression in NKTL in a study comparing YT cells and normal NK cells (19). miR-146a and miR-155 had shown close relationship with LMP1/nuclear factor κB (NFκB) in nonhematologic and hematologic cells (13, 14, 20–22). miR-106a had been involved in T-cell lymphoma and leukemia (23).

RNA extraction, reverse transcription, and real-time PCR for miRNA quantification

Total RNA was extracted from cell lines using TRIzol reagent (Invitrogen) or from 10-μm-thick FFPE tissue sections, using RecoverAll Total Nucleic Acid Isolation Kit for FFPE samples (Applied Biosystems), and stored at $-80°C$ until the time of use after measuring the concentration with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc.).

For reverse transcription and real-time PCR, 10 ng of total RNA per sample was used with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and the RT primers included in the TaqMan MicroRNA Assay [Applied Biosystems; catalogue nos. 4395459 (miR-155), 4378067 (miR-189), 4395342 (miR-9*), 4395280 (miR-106a), 4373132 (miR-146a), and 4373381 (RNU6B)], according to the manufacturer's instruction except for the use of an IQ5 thermal cycler (Bio-Rad), and related products. The signal from FAM dye (490 nm) was collected during 50 cycles of amplification. The threshold cycle (C_t) was normalized to U6 snRNA (Applied Biosystems; catalogue no. 4373381), producing differences of C_t [(ΔC_t); $C_{t(U6)} - C_{t(miR)}$] as relative amounts of miRNAs.

Synthesis of miR-146a mimics and inhibitors and preparation of TRAF6 siRNA

miR-146a mimics and inhibitors, as well as a negative control of miRNA mimics (negative mimics) or inhibitors (negative inhibitors), were synthesized by GenePharma (www.genepharma.com) with the following sequences: miR-146a mimics (5′-UGAGAACUGAAUUCCAUGGGUU

-3'), miR-146a inhibitors (5'-AACCCAUGGAAUUCA-GUUCUCA-3': 2'Ome modification), and negative mimics/inhibitors (5'-UUGUACUACACAAAAGUACUG-3'). TNF receptor (TNFR)–associated factor 6 (TRAF6) siRNA was purchased from Santa Cruz Biotech (TRAF6 siRNA: sc-36717; control siRNA: sc-37007).

Transfection of miR-146a mimics and inhibitors, and TRAF6 siRNA

miR-146a mimics/inhibitors or negative mimics/inhibitors, as well as TRAF6 siRNA or scrambled siRNA, were transfected into the cells by using Lipofectamine 2000 (Invitrogen) with the transfection efficacy of 25% (SNK6) and 30% (YT). Details are described in the Supplementary Methods.

MTT assay

Cell viability was monitored by the MTT (Sigma) colorimetric assay. Details are described in the Supplementary Methods.

Reverse transcriptase-PCR

At 24 hours posttransfection, total RNA of harvested cells was extracted using TRIzol reagent (Invitrogen). For reverse transcriptase-PCR (RT-PCR), 5 μg total RNA was reverse transcribed using RT-PCR kits (Promega) and PCR was conducted with the following conditions: 35 cycles of 94°C (1 minute), 55°C (1 minute), and 72°C (2 minutes). Details are described in the Supplementary Methods.

Western blot analysis

Western blot was conducted with anti-IκB, anti-β-actin, anti-TRAF6, anti-Bcl-2 antibodies (all from Santa Cruz Biotechnology), and anticleaved caspase-3 antibody (Cell Signaling Technology), and cells harvested and lysed for 24 hours posttransfection, as previously described (24).

Reporter gene assay

A NFκB-luciferase-reporter construct (pGL2 vector), containing many NFκB signal binding motifs, was cotransfected with miR-146a mimics/inhibitors or negative mimics/inhibitors into SNK6 or YT cells by using Lipofectamine 2000 and cultured for 24 hours. At 24 hours posttransfection, the cells were lysed and centrifuged (12,000 × g, 4 minutes, 4°C). Supernatant fractions were recovered, and luciferase activities were determined using a single sample luminometer (FB12 luminometer; Berthold Detection Systems). Details are described in the Supplementary Methods.

Methylation-specific PCR

SNK6 and YT cells were treated with 1 μmol/L of 5-azadC for 72 hours, and genomic DNA was extracted and purified using QUIAamp DNA mini kits (Qiagen). Unmethylated cytosines in 500 ng DNA aliquots were converted to uracil by using MethylCode Bisulfite Conversion kits (Invitrogen). Methylation-specific PCR (MSP) for *miR-146a* promoter was conducted using the following primers: 5'-

TAGATATTATTTAAGGTAAAGAGAGGAATG-3' (unmethylated sense), 5'-CATAATAACCTATAATAAAAAAATCACT-3' (unmethylated antisense), 5'-TTAGATATTATTTAAGG-TAAAGAGAGGAAC-3' (methylated sense), and 5'-AATAA CCTATAAAAAAATCGCT-3' (methylated antisense). Details are described in the Supplementary Methods.

Statistical analysis

miRNA expression levels and clinicopathologic parameters were analyzed using SPSS 12.0 (SPSS Inc.) to conduct the χ^2 test, the Fisher's exact test, the Student t test, the Mann–Whitney test, Kaplan–Meier survival analysis, and multivariate Cox proportional hazard analysis for overall survival, with the P value less than 0.05 as statistically significant. The cases in which the patient was alive or lost to follow-up at the time of analysis were marked as censored.

miRNA expression was divided into high or low expression by the cutoff point showing maximum χ^2 (minimum P value) as a prognostic factor (25), which is a systematic search of almost all observed values as the candidate cutoff point and choosing the one associated with a maximum χ^2 (or minimum P value) as the final cutoff point (25), resulting in the cutoff points of −1.15 (miR-146a), 1.8 (miR-155), 0.5 (miR-106a), −7 (miR-9*), and −11.2 (miR-189). miR-146a expression levels of SNK6 and YT cells were −0.57 and −2.97.

Results

Clinical characteristics of NKTL patients and the relationship between miRNAs and clinicopathologic variables

Supplementary Table S1 summarizes clinical characteristics of NKTL patients. Supplementary Table S2 shows the relationship between miRNAs and clinicopathologic variables in NKTL, showing frequent correlation of clinical parameters with miR-155, or less frequently, with miR-146a.

Prognostic implications of miRNAs in NKTL

In univariate survival analysis, several well-known clinical parameters were observed to have prognostic significance, including International Prognostic Index (IPI) group, NKTL Prognostic Index (NKPI) group, Eastern Cooperative Oncology Group (ECOG) PS, primary site, lactate dehydrogenase (LDH), stage, number of extranodal sites, and B symptoms. Moreover, low expression of both miR-155 and miR-146a showed significantly poor prognosis (Table 1 and Fig. 1A; $P = 0.0016$ and 0.0057, respectively).

Multivariate analysis was conducted with IPI, primary site, B symptoms, and expression level of miR-146a and miR-155, because other significant clinical parameters were closely associated with IPI. Multivariate analysis with IPI, primary site, B symptoms, and miR-146a revealed that low miR-146a expression ($P < 0.001$; HR = 13.110), non-UAT ($P = 0.008$; HR = 5.376), and high IPI (≥ 3; $P = 0.013$; HR = 3.584) were independent poor prognostic factors (Table 1). In contrast, multivariate analysis with IPI,

Table 1. Significant prognostic factors in NKTL by univariate and multivariate analysis

Variables	Univariate analysis				Multivariate analysis			
	HR	95% CI		P	HR	95% CI		P
IPI group (0, 1, 2 vs. 3, 4, 5)	5.860	1.716	20.016	<0.0001[a]	3.584	1.304	9.901	0.013[b]
NKPI group (1, 2 vs. 3, 4)	7.973	2.515	25.274	<0.0001[a]				
ECOG performance status (0, 1 vs. higher)	5.218	1.583	17.202	<0.0001[a]				
Primary site (UAT vs. non-UAT)	4.168	1.052	16.516	0.0008[a]	5.367	1.567	18.519	0.008[b]
LDH (normal vs. elevated)	5.115	1.997	13.103	0.0013[a]				
miR-146a (high vs. low)	4.045	0.912	17.931	0.0016[a]	13.110	3.304	52.018	<0.001[b]
Stage (1, 2 vs. 3, 4)	3.341	1.030	10.841	0.0043[a]				
Number of extranodal sites (0, 1 vs. higher)	3.341	1.030	10.841	0.0043[a]				
miR-155 (high vs. low)	3.187	1.197	8.482	0.0057[a]				
B symptoms (absent vs. present)	2.788	1.112	6.989	0.0185[a]	0.067	0.931	4.762	0.067

[a]P <0.05 by univariate analysis.
[b]P <0.05 by multivariate analysis.

primary site, B symptoms, and miR-155 showed that only high IPI ($\geq$3; P = 0.045; HR = 3.003) and non-UAT (P = 0.049; HR = 2.874) were independent poor prognostic factors whereas miR-155 was not (P > 0.05).

Prognostic implications of miR-146a in NKTL stratified by primary site and IPI group

Because low miR-146a expression was observed only in the UAT-NKTL, we investigated the prognostic value of miR-146a in the UAT-NKTL by Kaplan–Meier survival analysis. Figure 1B shows 2 prognostically distinct groups, namely, (i) UAT with low miR-146a expression (UAT$^{Low-146a}$) and (ii) UAT with high miR-146a expression (UAT$^{High-146a}$) groups. Compared with UAT$^{High-146a}$ group, UAT$^{Low-146a}$ group showed distinctively poor prognosis. Among low miR-146a expression, high IPI ($\geq$3), and presence of B symptoms, low miR-146a expression was observed as the only independent prognostic factor in UAT-NKTL by multivariate analysis (N = 40; P < 0.001; HR = 15.620).

Three distinct clinicopathologic groups in NKTL: UAT$^{Low-146a}$, UAT$^{High-146a}$, and non-UAT

On the basis of the aforementioned findings, we constructed 3 clinicopathologic prognostic groups, that is, UAT$^{Low-146a}$, UAT$^{High-146a}$, and non-UAT, and compared the survival curves using Kaplan–Meier analysis (Fig. 1C). Compared with the UAT$^{High-146a}$, the UAT$^{Low-146a}$ group showed distinctively poor prognosis, which was similar to the non-UAT group.

We also analyzed and compared clinicopathologic characteristics among these 3 groups (Table 2). Interestingly, the UAT$^{Low-146a}$ showed more similarities to the non-UAT than to the UAT$^{High-146a}$ group. Compared with the UAT$^{Low-146a}$, the non-UAT showed higher expression of miR-146a, similar to that of the UAT$^{High-146a}$ group (Fig. 1D). Taken together, these results indicate that UAT$^{Low-146a}$-NKTL is a distinct poor prognostic subgroup

of UAT-NKTL, which is clinicopathologically similar to non-UAT than to UAT$^{High-146a}$ (Fig. 1D and Table 2).
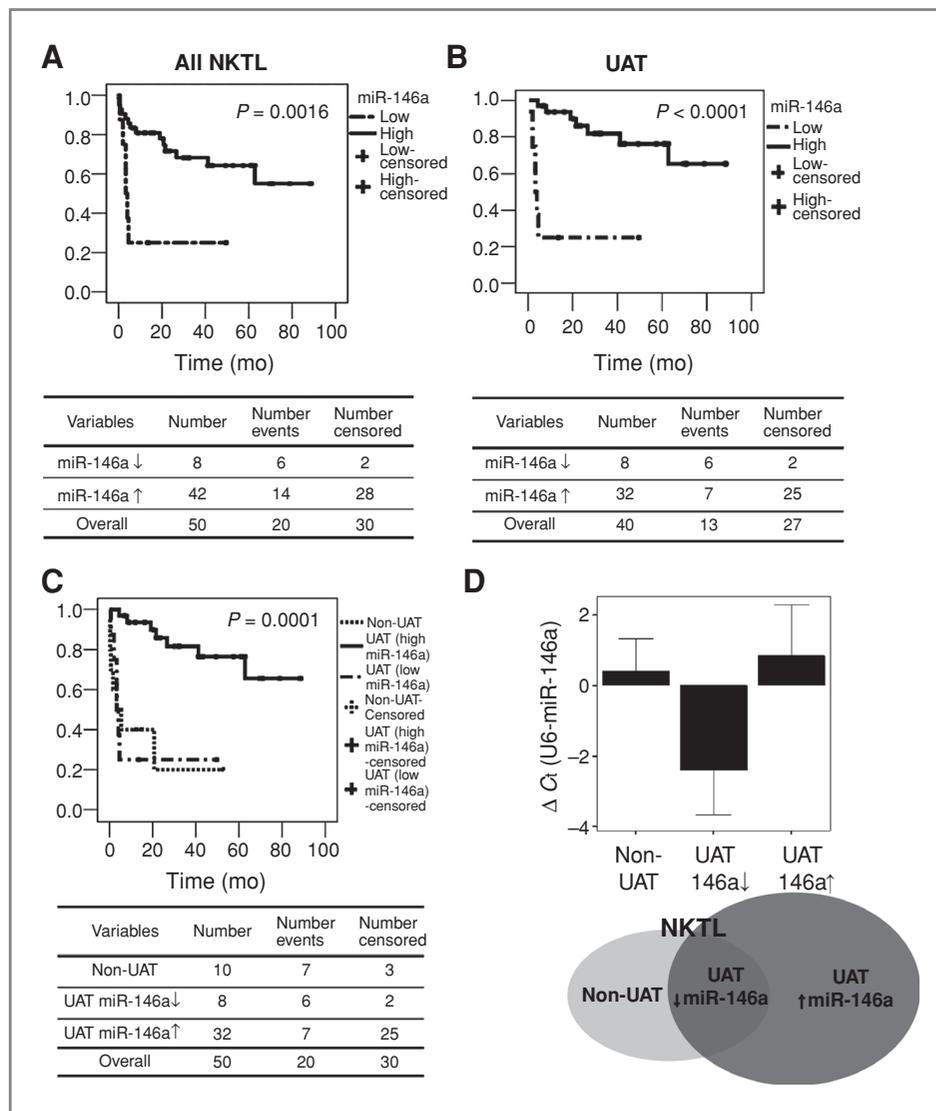
Inhibition of cell proliferation and induction of apoptosis with suppression of the NFκB pathway by miR-146a in SNK6 and YT cells

To clarify the biological mechanism for the poor prognosis in the low miR-146a expression NKTL group, we investigated the cellular responses to upregulation or downregulation of miR-146a in 2 EBV-positive NKTL cell lines (SNK6 and YT). We hypothesized that (i) miR-146a levels might regulate cell proliferation or survival in NKTL cells and (ii) because most of the patients in this study were primarily treated with chemotherapy, miR-146a levels might have an effect on the chemosensitivity of NKTL cells.

To investigate the effects of miR-146a on cell proliferation, we transfected the cells with miR-146a mimics to upregulate miR-146a activity or miR-146a inhibitors for downregulation of miR-146a activity to SNK6 and YT cells. After transfection, changes of miR-146a levels were checked by real-time PCR in all related experiments and the effective suppression or enhancement of miR-146a expression was observed (data not shown). Compared with the negative controls, cell proliferation was inhibited by the miR-146a mimics and enhanced by the miR-146a inhibitors in both the SNK6 and YT cells by the MTT assay (Fig. 2A). These results indicated that miR-146a negatively regulated cell proliferation in the SNK6 and YT cells.

We investigated the expression levels of Bcl-2, an important antiapoptotic molecule. Transfection with the miR-146a mimics resulted in a decrease in Bcl-2 mRNA expression and protein levels, whereas transfection with the miR-146a inhibitors induced an increase in Bcl-2 expression (Fig. 2B). On Western blot analysis, cleavage of caspase-3 was observed by the transfection of miR-146a mimics, concomitantly with Bcl-2 downregulation (Fig. 2B). These data indicate that miR-146a induced apoptosis along with

Figure 1. Three prognostic groups driven by miR-146a in NKTL. A–D, Kaplan–Meier overall survival curve shows a poor prognostic group of low miR-146a expression within total NKTL ($N = 50$; A), or within UAT ($N = 40$; B), showing 3 prognostic groups (UAT$^{Low-146a}$, UAT$^{High-146a}$, and non-UAT; C) with the comparison of miR-146a expression levels and their relationship (D).

suppression of Bcl-2 expression in NKTL cell lines. To determine how miR-146a was decreasing cell proliferation and inducing apoptosis, we focused on the NFκB, as a known upstream molecule of Bcl-2. NKTL patient outcome has been associated with NFκB pathway involving chemoresistance, which has also been mediated by NFκB/Bcl-2 in various other tumors (26–28). Thus, we investigated whether miR-146a might be involved in NFκB regulation in NKTL cell lines. Cotransfection of a NFκB-luciferase-reporter construct (pGL2 vector) containing various NFκB signal binding motifs and miR-146a mimics showed a decreased luciferase activity compared with cotransfection of pGL2 vector and negative mimics in the SNK6 and YT cells (Fig. 2C). By Western blotting, IκB (a negative regulator of NFκB) accumulated upon transfection of miR-146a mimics whereas IκB expression decreased with transfection of miR-146a inhibitors (Fig. 2C). These data suggest that miR-146a inhibits the NFκB pathway in the SNK6 and

YT cells and therefore might function as a tumor suppressor inhibiting cell proliferation and inducing apoptosis with the suppression of Bcl-2 expression.

Downregulation of TRAF6 by miR-146a in SNK6 and YT cells

To clarify the miR-146a target mRNA involved in the pathogenesis of NKTL, we used a Web-based miRNA database (microRNA.org; www.targetscan.org; and pictar.mdc-berlin.de) to search the computationally predicted candidate mRNAs. Among multiple candidate mRNAs with simulated binding capacity in its 3′ untranslated region (UTR), we hypothesized that TRAF6 might be a possible important target in NKTL, considering its biological significance in NK cells. TRAF6 was observed as a common and powerful predicted target of miR-146a in multiple miRNA databases and was known to be physiologically important in innate immune cells functioning as a signal transducer in

Table 2. Comparison of clinicopathologic characteristics between non-UAT ($N = 10$), UAT$^{Low-146a}$ ($N = 8$), and UAT$^{High-146a}$ ($N = 32$) groups in NKTL ($N = 50$)

Clinicopathologic parameters	Non-UAT No. (%)	UAT$^{Low-146a}$ No. (%)	UAT$^{High-146a}$ No. (%)	P
High IPI ($\geq$3)	7/10 (70)	4/8 (50)	3/31 (9.7)	<0.001[a]
High stage (3, 4)	6/10 (60)	3/8 (37.5)	4/32 (12.5)	0.008[a]
NKPI group (3, 4)	7/10 (70)	5/7 (71.4)	3/32 (9.4)	<0.001[a]
Presence of regional LN involvement	0/10 (0)	1/8 (12.8)	6/32 (18.8)	0.326
Presence of BM involvement	1/10 (10)	1/8 (12.8)	1/32 (3.1)	0.509
Number of extranodal sites ($\geq$2)	5/10 (50)	3/8 (37.5)	5/32 (15.6)	0.069
ECOG performance status ($\geq$2)	6/10 (60)	4/8 (50)	3/32 (9.4)	0.002[a]
Elevated LDH level	7/10 (70)	6/7 (85.7)	10/30 (33.3)	0.014[a]
Presence of B symptoms	8/10 (80)	3/8 (37.5)	10/32 (31.2)	0.023[a]
Cell size (large/anaplastic)	3/10 (30)	2/8 (25)	5/32 (15.6)	0.325
LMP1 expression in IHC	5/10 (50)	2/8 (25)	6/32 (18.8)	0.144

Abbreviations: LN, lymph node; BM, bone marrow; IHC, immunohistochemistry.
[a]$P < 0.05$ between the 3 groups.

the NFκB pathway (20). Therefore, we measured TRAF6 expression level after transfection with the miR-146a mimics and found that the miR-146a mimics inhibited TRAF6 expression at the mRNA and protein levels (Fig. 3A). Moreover, to determine whether TRAF6 was involved in the activation of the NFκB pathway in NKTL cell lines, we directly silenced TRAF6 by siRNA, which resulted in upregulation of IκB and downregulation of Bcl-2 in SNK6 and YT cells (Fig. 3A). These data indicate that miR-146a might inhibit NFκB pathway via degradation of TRAF6, thereby playing a tumor-suppressive role in NKTL.

Enhancement of chemosensitivity to etoposide by miR-146a in SNK6 and YT cells and the relationship between miR-146a expression and response to chemotherapy in NKTL patients

To clarify the effects of miR-146a on the chemosensitivity of SNK6 and YT cells, we investigated the response to etoposide in SNK6 and YT cells because (i) etoposide-induced cell death was closely associated with NFκB activity (29) and (ii) etoposide was a main component of the ifosfamide, methotrexate, etoposide, and prednisone (IMEP) regimen, which was applied to 64% (32 of 50) of the total patients in this study, which consisted of 75% (6 of 8) of patients with low miR-146a expression and 62% (26 of 42) of patients with high miR-146a expression.

Etoposide was added at 24 hours after transfection with the miR-146a mimics/inhibitors, and the relative cell viability was measured by the MTT assay at 48 hours after transfection. As shown in Figure 3B, transfection with the miR-146a mimic itself caused cell death in 31% of SNK6 and 36% of YT cells. The etoposide concentration inhibiting 50% of SNK6 or YT cells was substantially reduced with the miR-146a mimics by 10- to 100-fold. Therefore, miR-146a was found to enhance chemosensitivity to etoposide in SNK6 and YT cells.

To investigate the clinical significance of miR-146a–induced chemosensitivity observed in the SNK6 and YT cells, we analyzed the relationship between miR-146a expression and the response to initial chemotherapy in NKTL patients, who received chemotherapy alone or with radiation therapy ($N = 48$). We divided the patients into "responder" (complete remission and partial remission) and "no responder" (stable disease and progressive disease). NKTL patients with low miR-146a expression showed a higher frequency of no responders (7 of 8, 87.5%), whereas those with high miR-146a were more frequently responders (26 of 40, 65%), and miR-146a expression significantly correlated with chemotherapy response in NKTL ($N = 48$; $P = 0.006$; Fig. 3C).

Upregulation of miR-146a expression by a demethylating agent (5-azadC) treatment in SNK6 and YT cells

As a mechanism of low miR-146a expression, we hypothesized that the promoter methylation of miR-146a would be able to prevent the NFκB-induced miR-146a expression. Treatment of SNK6 and YT cells with a demethylating agent, 5-azadC, markedly upregulated miR-146a expression (Fig. 4A). Concomitant with this, the conversion of miR-146a methylation status from methylated into unmethylated by 5-azadC was observed in the both cell lines (Fig. 4B). These data suggest that miR-146a promoter methylation might be a mechanism of miR-146a suppression in NKTL cell lines.

Correlation between promoter methylation status and expression level of *miR-146a* in individual NKTL patient tissues

To confirm that promoter methylation is an important mechanism of miR-146a suppression in NKTL patients, we also investigated methylation status of miR-146a by

Figure 2. Reduction of cell proliferation and induction of apoptosis by miR-146a via NFκB pathway in NKTL cell lines. A, cell proliferation was inhibited by miR-146a mimics but enhanced by miR-146a inhibitors compared with negative controls by the MTT assay in SNK6 and YT cells. B, Bcl-2 was downregulated by miR-146a mimics but increased by miR-146a inhibitors at mRNA level in SNK6 (left) and YT cells (middle) and at protein level in SNK6 cells (right). Cleavage of caspase-3 was observed by miR-146a mimics, concomitantly with Bcl-2 downregulation (right). C, cotransfection of an NFκB-luciferase-reporter construct (pGL2 vector) containing several NFκB signal binding motifs and miR-146a mimics caused reduction of luciferase activity, compared with transfection of the pGL2 vector only in SNK6 and YT cells (left). IκB expression was increased by transfection with the miR-146a mimics but markedly decreased by transfection with the miR-146a inhibitors, compared with negative controls as determined by Western blot analysis (right).
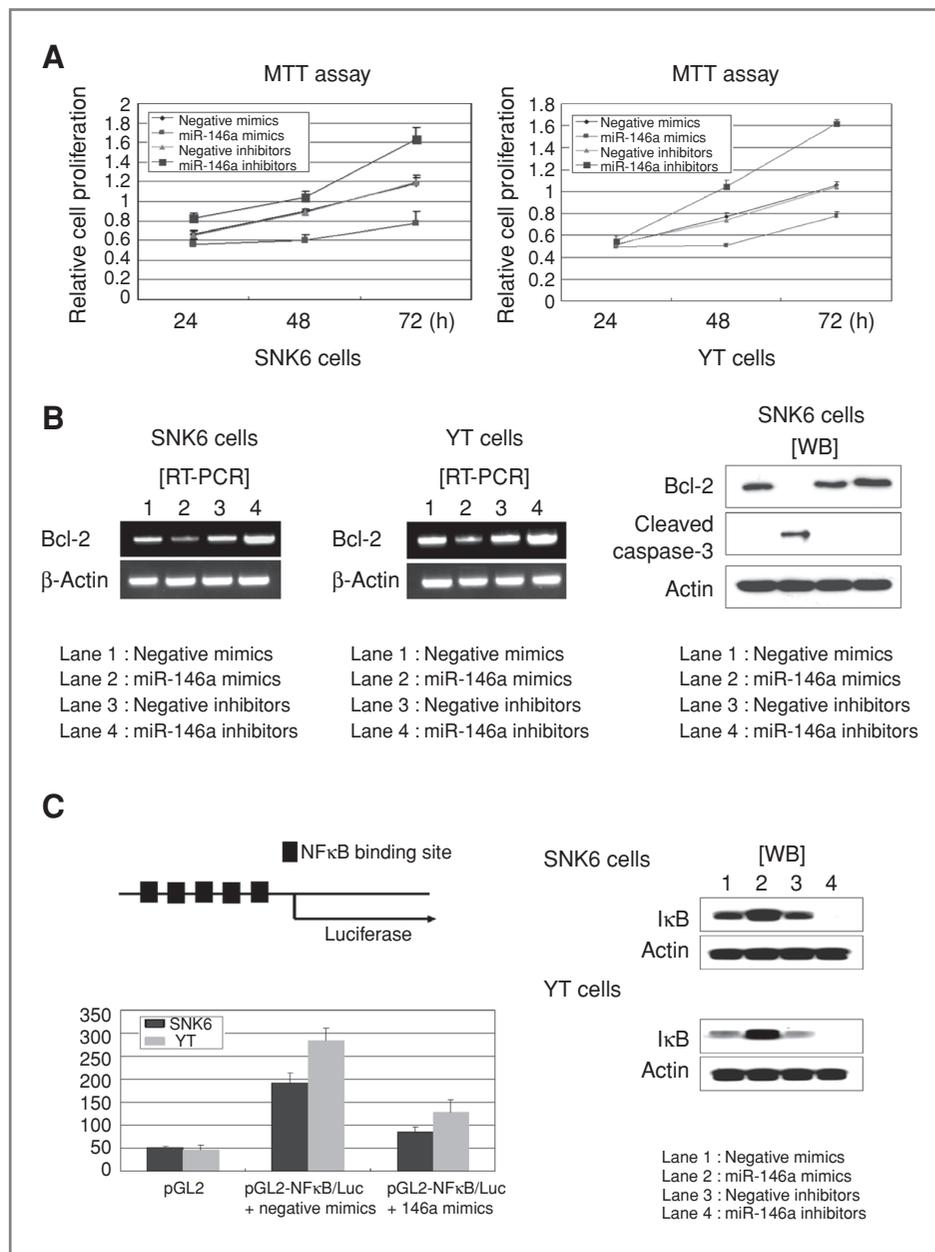
MSP in representative individual cases containing all 8 cases of low miR-146a expression and randomly selected 6 cases of high miR-146a expression from 50 cases of NKTL and compared the results with the relative expression levels. All 8 cases of low miR-146a expression ($\Delta C_t < -1.15$) showed methylated promoter status, whereas high miR-146a ($\Delta C_t > -1.15$) cases showed mainly unmethylated promoter status (Fig. 4C). These results indicate that promoter methylation might be an important mechanism for different expression level of miR-146a in NKTL patients. Overall, the possible pathogenesis of NKTL involving miR-146a suppression was illustrated (Fig. 4D).

Discussion

In this study, the prognosis of NKTL could be predicted by miR-146a, which functioned as a tumor suppressor *in vitro*, and correlated with chemotherapy response. Previous reports showed that miR-146a played a tumor-suppressive or oncogenic role, depending on tumor types (30–32). Our *in vivo* and *in vitro* results showed that miR-146a might function as a potent tumor suppressor in NKTL by suppressing cell proliferation, as well as enhancing apoptosis and chemosensitivity by inhibition of NFκB activity. The NFκB pathway has been observed as an important chemoresistance mechanism in various tumors including
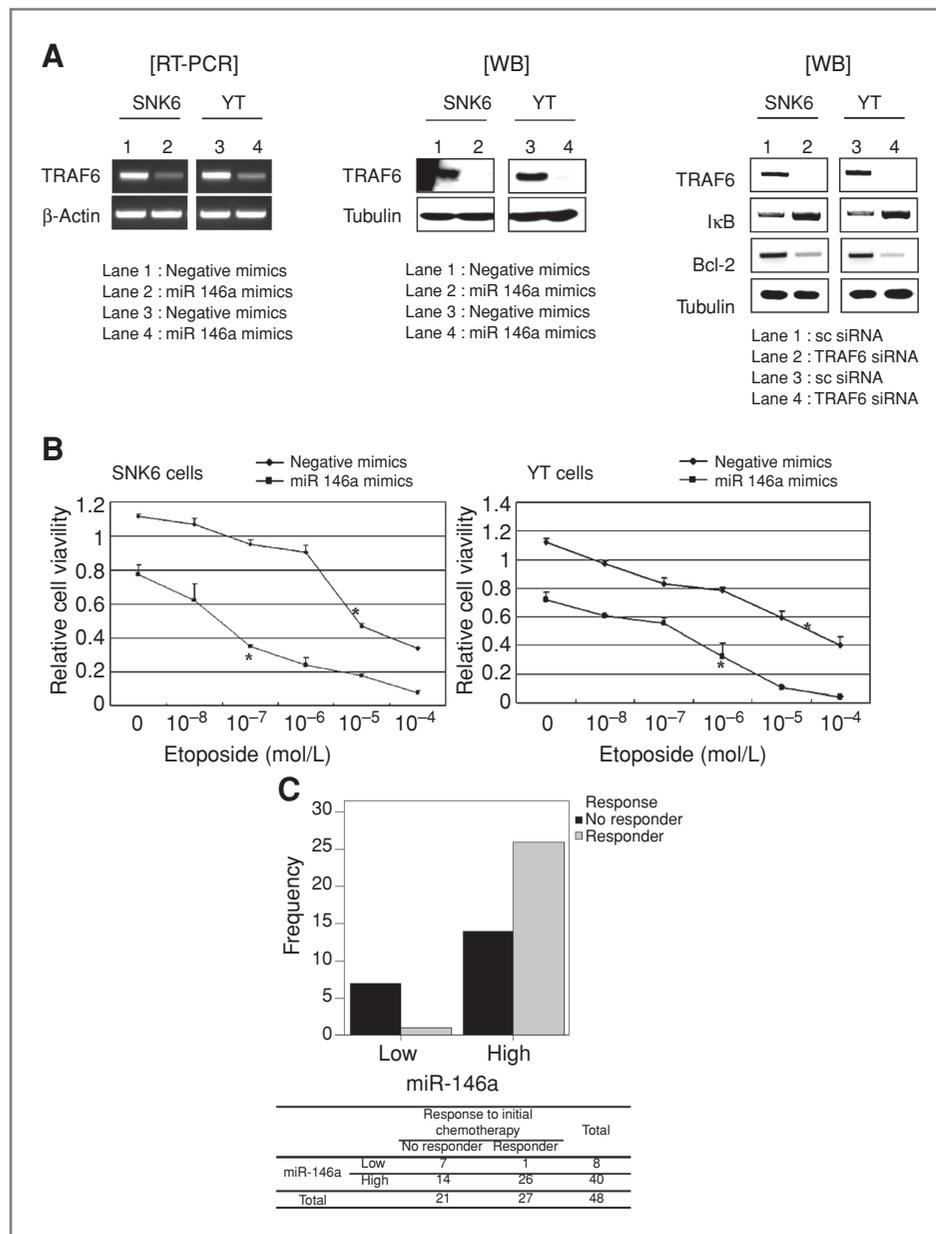
Figure 3. TRAF6 as a target of miR-146a, and chemosensitivity-enhancing role of miR-146a in NKTL. A, TRAF6 expression was decreased by miR-146a mimics in SNK6 and YT cells at mRNA level (left) and protein level (middle). Direct inhibition of TRAF6 by siRNA targeting TRAF6 caused IκB accumulation and Bcl-2 downregulation in SNK6 and YT cells, which was similar to the action of the miR-146a mimics (right). B, the chemosensitivity-enhancing role of miR-146a was shown in NKTL cell lines and NKTL patients. The transfection with the miR-146a mimics themselves caused cell death of about 31% of SNK6 and 36% of YT cells. The etoposide concentration inhibiting 50% of SNK6 or YT cells (marked as *) was substantially reduced by transfection with miR-146a mimics by 10- to 100-fold in SNK6 (left) and YT cells (right) compared with negative controls. C, in NKTL patients, miR-146a expression was well correlated with response to initial chemotherapy in NKTL patients ($N = 48$), revealing frequent "responders" in the high miR-146a group while frequent "no responders" in the low miR-146a expression group.

NKTL (26, 33, 34). NFκB-induced chemoresistance is mediated in part via the *mdr-1* gene and its protein product, P-glycoprotein, a known poor prognostic indicator in NKTL (35, 36). Bcl-2 expression is also induced by NFκB and can inhibit chemotherapy-induced apoptosis in cancer (27, 37, 38), which is consistent with our results showing that Bcl-2 upregulation induced by suppression of miR-146a is associated with chemoresistance in SNK6 and YT cells.

As a putative target gene of *miR-146a*, we focused on *TRAF6*, which has a miR-146a binding site in the 3′ UTR. TRAF6 is a member of the TRAF protein family and is known to transduce activating signals from the TNFR or Toll-like receptor (TLR)/IL-1 receptor family to NFκB. At the molecular level, TRAF6 functions as an E3 ubiquitin ligase (39) that activates IκB kinase (IKK), resulting in degradation of IκB, and nuclear translocation and activation of NFκB as a physiologic response. The role of TRAF6 has primarily been studied in innate immunity in response to harmful stimuli (40, 41). TRAF6 has also been intermittently investigated in several cancer cells (38, 39, 42, 43). Activation of some signaling mechanisms, including Akt pathway, have been suggested to be involved in TRAF6-mediated oncogenesis (39). In malignant lymphoma, TRAF6 is known to mediate oncogenesis of marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue, which is associated with *Heliobacter pylori*–induced antigenic stimulation and accompanying

Figure 4. Promoter methylation as a mechanism of *miR-146a* downregulation and possible pathogenesis in NKTL with low miR-146a expression. A, after treatment with 5-azadC, miR-146a expression distinctively increased in SNK6 and YT cells. B, concomitant with this, MSP showed a change of methylation status of *miR-146a* from methylated to unmethylated by 5-azadC. C, miR-146a expression was well correlated with its promoter methylation status in NKTL tissues. The NKTL cases with low *miR-146a* expression showed methylated promoter status, whereas those with high *miR-146a* expression harbored unmethylated promoters. D, in immune cells, stimulation of TRAF6 via TNFR, TLR/IL-1R, or EBV infection leads to enhancement of NFκB activity, which upregulates miR-146a expression. The increased miR-146a level, in turn, suppresses TRAF6 and NFκB activity, which properly terminates the immune response (negative feedback loop). In NKTL with low miR-146a expression, enhanced TRAF6 and NFκB activity fail to induce miR-146a expression due to methylation of *miR-146a* gene or other factors, which allows uncontrolled TRAF6 and NFκB activity, leading to uncontrolled cell proliferation, inhibition of apoptosis, and induction of chemoresistance (disruption of negative feedback loop).
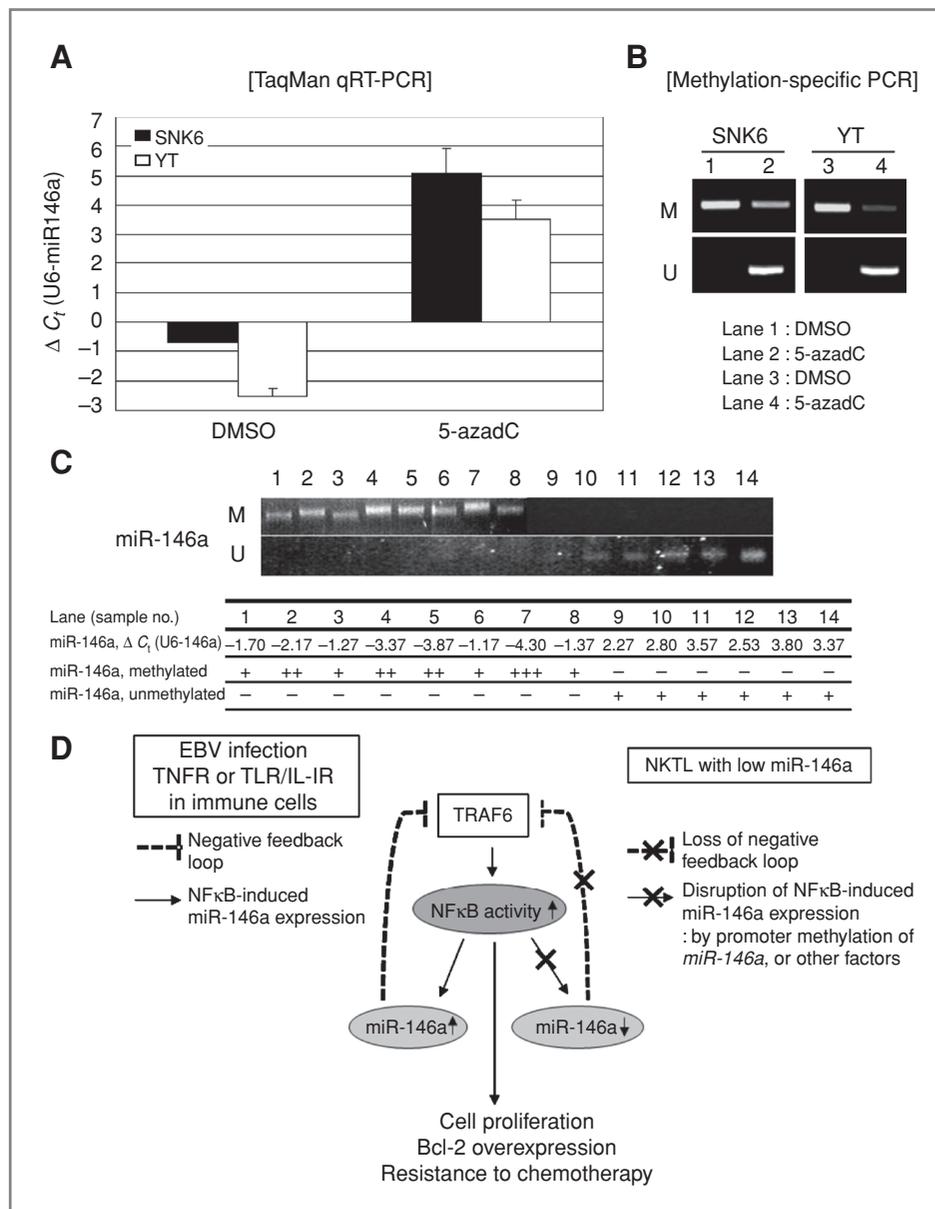
inflammation in tumorigenesis (42). By direct knockdown of TRAF6 using siRNA (Fig. 3A), we observed the inhibition of NFκB activity, which suggests that TRAF6/NFκB pathway is also an important mechanism in NKTL.

During the inflammatory or antiviral response, the TRAF6/NFκB pathway has been reported to be tightly regulated by a negative feedback loop with miR-146a as a key component (Fig. 4D), serving to prevent excessive reaction and to protect host tissues (13, 20). EBV infection in hematologic cells leads to LMP1-mediated activation of the NFκB pathway, which then induces miR-146a expression. miR-146a, in turn, inhibits NFκB by downregulating TRAF6 or other targets (13, 20). From this physiologic regulation, we hypothesized that the miR-146a/TRAF6/NFκB pathway might also be important in

NKTL, and in a subset of NKTL, the integrity of this negative feedback loop might be disrupted, unlike physiologic response. We observed that UAT^Low-146a tissue harbored unrecovered low levels of miR-146a with poor prognosis and NKTL cell lines with downregulated miR-146a harbored activated TRAF6/NFκB pathway, which might be a critical event determining biological behavior of NKTL (Fig. 4D). As a mechanism of miR-146a downregulation causing disruption of the negative feedback regulation of miR-146a/TRAF6/NFκB, we showed that promoter methylation of *miR-146a* gene might be important by *in vitro* study (Fig. 4A and B). The close correlation between the promoter methylation status and expression level of miR-146a in NKTL tissues might support this mechanism (Fig. 4C) and might also explain the biolo-

gical relevance of the cutoff value of miR-146a expression originally determined by clinical significance. Alternatively, deletion of *miR-146a* gene might be considered as an additional possible mechanism (44), which remains to be clarified. It also is yet to be elucidated whether miR-146a downregulation is associated with neoplastic transformation (i.e., "initiation"), or acquisition of a more aggressive phenotype (i.e., "progression"), in NKTL.

NKTL is a disease of clinicopathologic heterogeneity (17), showing different prognosis by several clinicopathologic parameters (17, 18, 45, 46). Despite the relatively good prognosis of UAT-NKTL (17), the combination of miR-146a could provide additional prognostic information associated with different chemoresistance and clinicopathologic features in this study. This subgroup analysis reflects the heterogeneity of NKTL even within the UAT-NKTL. UAT$^{Low-146a}$-NKTL was observed as poor prognostic group requiring more aggressive treatment and more careful follow-up. Moreover, our miRNA-based prognostication and subclassification might be important for its potential therapeutic application (47).

Although the patients of this study did not receive homogeneous treatment, the treatment modality or chemotherapy regimen did not affect the prognosis and showed no correlation with miR-146a level (data not shown). These facts suggest that treatment heterogeneity had little influence on the conclusion of the present study.

Recently, miR-21 was reported to play an important role in oncogenesis of NKTL cells via Akt pathway (10). Akt has been reported to interact with NFκB as a downstream or upstream molecule of NFκB (48, 49) Therefore, these 2 miRNA-regulated pathways, that is, miR-21–regulated Akt pathway and miR-146a–regulated NFκB pathway, might interact with each other to enhance their roles, which need to be clarified further in NKTL cells.

In summary, low expression of miR-146a could successfully define 3 clinicopathologic subgroups, that is, UAT$^{Low-146a}$, UAT$^{High-146a}$, and non–UAT-NKTL. *In vitro* studies with SNK6 and YT cells showed that miR-146a overexpression suppressed cell proliferation, induced apoptosis, and enhanced chemosensitivity by inhibiting NFκB pathway by targeted downregulation of TRAF6, which was consistent with the strong association between low miR-146a expression and frequent chemoresistance in NKTL patients. Practically, miR-146a would be used for prediction of prognosis, even with small amounts of FFPE tissue archived for several years. Therefore, a novel miRNA-based prognostic marker, along with conventional prognostic factors, would facilitate collective assessment of NKTL, providing the possibility of putative therapeutic targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

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