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Whole transcriptome analysis reveals dysregulated oncogenic lncRNAs in natural killer/T-cell lymphoma and establishes MIR155HG as a target of PRDM1

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Abstract

Natural killer/T-cell lymphoma is a rare but aggressive neoplasm with poor prognosis. Despite previous reports that showed potential tumor suppressors, such as PRDM1 or oncogenes associated with the etiology of this malignancy, the role of long non-coding RNAs in natural killer/T-cell lymphoma pathobiology has not been addressed to date. Here, we aim to identify cancer-associated dysregulated long non-coding RNAs and signaling pathways or biological processes associated with these long non-coding RNAs in natural killer/T-cell lymphoma cases and to identify the long non-coding RNAs transcriptionally regulated by PRDM1. RNA-Seq analysis revealed 166 and 66 long non-coding RNAs to be significantly overexpressed or underexpressed, respectively, in natural killer/T-cell lymphoma cases compared with resting or activated normal natural killer cells. Novel long non-coding RNAs as well as the cancer-associated ones such as SNHG5, ZFAS1, or MIR155HG were dysregulated. Interestingly, antisense transcripts of many growth-regulating genes appeared to be transcriptionally deregulated. Expression of ZFAS1, which is upregulated in natural killer/T-cell lymphoma cases, showed association with growth-regulating pathways such as stabilization of P53, regulation of apoptosis, cell cycle, or nuclear factor-kappa B signaling in normal and neoplastic natural killer cell samples. Consistent with the tumor suppressive role of PRDM1, we identified MIR155HG and TERC to be transcriptionally downregulated by PRDM1 in two PRDM1-null NK-cell lines when it is ectopically expressed. In conclusion, this is the first study that identified long non-coding RNAs whose expression is dysregulated in natural killer/T-cell lymphoma cases. These findings suggest that ZFAS1 and other dysregulated long non-coding RNAs may be involved in natural killer/T-cell lymphoma pathobiology through regulation of cancer-related genes, and loss-of-PRDM1 expression in natural killer/T-cell lymphomas may contribute to overexpression of MIR155HG; thereby promoting tumorigenesis.

Keywords

Natural killer/T-cell lymphoma, long non-coding RNA, ZFAS1, PRDM1, MIR155HG, TERC

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Introduction

Natural killer/T-cell lymphoma (NKTCL) is a rare malignancy with aggressive clinical course.[1] The incidence of NKTCL is remarkably higher in East Asian and South-Central American countries compared to the rest of the world,[2] which may be related to the presence of single-nucleotide polymorphisms (SNPs) in *HLA-DPB1* locus based on a recent epidemiological study.[3] The cell of origin of NKTCLs is mostly natural killer (NK) cells, but they may also derive from αβ or γδ-T cells.[4] NKTCLs may

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be subdivided into two subtypes based on the primary site of the tumors: NKTCL of nasal type and extra-nasal NKTCL with notable differences in clinical features.[5] NKTCLs invariably display Epstein–Barr virus (EBV) positivity; however, its role on NKTCL pathobiology is not clear yet.[6] Compared with B and T-cell malignancies, diagnosis of NKTCL patients may be more challenging due to lack of rearrangement in NK-cell receptor genes; however, a recent study suggested abnormal KIR expression profiles, including selective expression of KIR2DL4 as a potential diagnostic biomarker for NKTCL.[7]

Earlier studies reported mutations leading to loss-of-function of *FAS*[8] and *P53*[9] in NKTCL cases. Genomic investigations on NKTCL cases coupled with in vitro assays on NK-cell lines showed several candidate tumor suppressor genes (e.g. PRDM1, HACE1, DAPK1, BCOR, SOCS6, PTPN6, DDX3X)[10–15] or oncogenes (e.g. janus kinase 3 (JAK3), signal transducers and activators of transcription 3 (STAT3), STAT5B)[16,17] whose expression or activity is dysregulated through genomic copy number variations, oncogenic mutations, or promoter hypermethylation. Among these genes, the role of tumor suppressor PRDM1 has been investigated more comprehensively in NKTCL as well as other lymphomas. Three studies showed that PRDM1 is located in frequently deleted 6q21 locus, and its ectopic expression inhibits cell growth through cell cycle arrest and/or apoptosis.[10,11,18] Promoter-associated CpG island hypermethylation–mediated silencing of PRDM1 is also frequently observed both in NKTCL cases and malignant NK-cell lines.[10,18,19] In addition, a previous report showed that miR-223 can inhibit PRDM1 in NKTCLs.[20] Altogether, these studies suggest that PRDM1 is a frequently inactivated tumor suppressor gene in NKTCL. PRDM1 acts as the master regulator of plasma cell differentiation by turning off the transcriptional program associated with germinal center B cells.[21] Consequently, PRDM1 inactivation is common in B-cell malignancies.[22,23] However, its transcriptional targets whose deregulation is associated with NKTCL pathobiology are currently unknown.

There is very little information on the role of transcripts of the non-coding part of the genome in NKTCL pathogenesis. Among these few reports, mir146a was implicated as a candidate tumor suppressor with prognostic value in NKTCLs.[24] However, no study has been reported to date regarding the diagnostic or therapeutic potential of dysregulated long non-coding RNAs (lncRNAs) on NKTCL etiology.

lncRNAs are long transcripts (>200 bp) with no protein-coding potential.[25] It has been postulated that there are more than 30,000 lncRNAs present in the human genome.[26] lncRNAs have been shown to regulate expression or activity of genes involved in cancer[27] through a variety of different mechanisms such as regulation of alternative splicing[28] or enhancement of transcription.[29] Several reports showed that lncRNAs regulate processes critical to cancer such as cell proliferation,[30,31] metastasis;[32,33] thereby affect patient survival.[34,35]

Here, we analyzed the whole-transcriptome sequencing data of 17 NKTCL cases and 3 normal NK-cells reported earlier,[17] and identified novel and cancer-associated lncRNAs overexpressed or underexpressed in NKTCLs potentially associated with disease pathobiology. Furthermore, we identified MIR155HG, TERC, and other cancer-associated lncRNAs transcriptionally regulated by tumor suppressor PRDM1[18] in two malignant NK-cell lines.

Materials and methods

Patient samples, cell lines, and normal NK-cells

The characteristics of all NKTCL cases (n = 17), normal NK-cells (resting NK, PBNK48h, NKCOD12), and NK-cell lines (i.e. NK92 and KHYG1) used in this study were described previously,[17] and they are also available in Table S1. NK92 and KHYG1 cell lines were cultured, respectively, in 20% or 10% fetal bovine serum RPMI medium supplemented with 100 units/mL penicillin G (Sigma-Aldrich, St. Louis, MO, USA), 100 g/mL streptomycin (Sigma-Aldrich), and 5–7 ng/mL rhIL2 (recombinant human interleukin-2; R&D Systems, Minneapolis, MN, USA) at 37°C in a humidified incubator with 5% CO_2. KHYG1 and NK92 cell lines were obtained from the Health Science Research Resource (Osaka, Japan) and the German Collection of Microorganism and Cell Culture (DSMZ, Braunschweig, Germany), respectively.

The isolation and activation procedures for normal human NK-cells used in this study were described in detail previously.[14,17] NK-cell isolation kit (Miltenyi Biotech Inc., Bergisch Gladbach, Germany) was used to isolate resting NK-cells from peripheral blood of healthy people. Isolated cells were stained with CD56-allophycocyanin (CD56-APC) (Miltenyi Biotec Inc., Bergisch Gladbach, Germany) and CD3-phycoerythrin (CD3-PE) (Miltenyi Biotech Inc.) to evaluate NK-cell purity with a FACSCalibur flow cytometer (BD Biosciences, Bedford, MA, USA). Cells with >95% $CD56^+/CD3^-$ purity were considered as human NK-cells and used for future experiments (Figure S1). Activated NK-cells were obtained by either culturing resting NK-cells for 2 days in interleukin 2 (IL2)-supplemented normal NK-cell growth medium (i.e. PBNK48h) or by enrichment and expansion of primary NK-cells co-cultured with K562 Clone 9.mbIL21 cell line, which is an NK-cell target engineered to display additional activation molecules on the cell surface, for 12 days (i.e. NKCOD12).[36]

Whole transcriptome sequence analysis

All RNA-Seq raw data files (17 NKTCL cases, 3 normal NK samples, empty vector (i.e. PMIG) or PRDM1α-transduced NK92 cell line) used in this study are available as FASTQ files of 100 bp paired-end reads generated through Illumina Genome Analyzer IIx or Hiseq2000 platform in the National

Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database with the following study number: SRP049695. FASTQ raw data files were aligned with TopHat[38] to the hg38 human genome assembly. Binary alignment map (BAM) files generated were transferred to the Galaxy main instance[37] and all subsequent analyses of RNA-Seq files were performed using bioinformatics tools available in the Galaxy platform. For each RNA-Seq file, transcript assembly, discovery, and abundancy estimates were performed by Cufflinks program v2.2.1[38] using the following input parameters: Max Intron Length: 300,000; Min Isoform Fraction: 0.1; Pre mRNA Fraction: 0.15; Use Reference Annotation: Yes.; Perform Bias Correction: Yes; Use multi read correct: Yes; Apply length correction: Cufflinks Effective Length Correction (http://cole-trapnell-lab.github.io/cufflinks). The "gencode.v24.long_noncoding_RNAs.gtf" file from GENCODE[39] was used for annotation of lncRNA transcripts with Cufflinks. The same Cufflinks parameters used to measure lncRNA expression were used for quantification of transcript expression of protein-coding genes, and the "gencode.v24.annotation.gtf" file was used for annotation of these genes.

lncRNAs upregulated or downregulated in NKTCL cases compared to three normal NK-cells were identified by applying the following criteria: (1) Median fragments per kilobase of exon per million fragments mapped (FPKM) >1, (2) $p < 0.05$ (Student's t test), and (3) 1.5-fold increase or 1.5-fold decrease in median transcript expression in NKTCL cases (n = 17) compared to that of three normal NK-cells for upregulated or downregulated lncRNAs, respectively.

BAM alignment files were used for quantification of lncRNAs in PRDM1 or empty vector–transduced NK92 cells using the Cuffdiff program[38] within the Galaxy platform (http://cole-trapnell-lab.github.io/cufflinks/cuffdiff/). The Cuffdiff parameters used are as follows: Library normalization method: Geometric; Dispersion estimation method: Pooled; Min alignment count:10; Include Read Group Datasets: No; Apply length correction: Cufflinks effective length correction.

Pathway and biological process analysis

Reactome software was used to identify enrichment of pathways or biological processes for genes whose transcription shows strong positive ($R > 0.7$) or negative ($R < -0.7$) correlation with that of ZFAS1 in NKTCL cases (n = 17) and normal NK-cells (n = 3) Reactome (http://www.reactome.org/PathwayBrowser). A binomial test was applied to calculate the probability of enrichment of ZFAS1-correlated genes. The corrected p values after multiple testing using the Benjamini–Hochberg procedure were considered for evaluating statistical significance.

LncRNA2Function[40] was applied to identify the functional role of lncRNAs dysregulated in NKTCL cases. lncRNAs upregulated or downregulated in NKTCL cases (n = 17) compared with those of normal NK-cells (n = 3) were used as input to identify significant biological processes or pathways they are involved in. In this analysis, LncRNA2Function software identified each gene co-expressed with at least five lncRNA, and then annotated the lncRNAs with biological processes or signaling pathways based on the set of genes co-expressed with these lncRNAs. The p value for each pathway/biological process is calculated by the hypergeometric test and corrected by the Benjamini–Hochberg method. The cutoff for corrected p value is set as 0.05.

Ectopic expression of PRDM1α in NK92 and KHYG1 cells

NK92 and KHYG1 cells were retrovirally transduced with PMIG (i.e. empty vector) or PRDM1α-FL construct[18] as follows: Retroviruses were generated by co-transfection of 4 μg of empty plasmid vector or vector containing PRDM1α-FL (Figure S2(a)) with 4 μg of the packaging plasmid pCL-Ampho using TurboFect transfection reagent (Thermo Fisher Scientific, San Jose, CA, USA) into 60%–70% confluent 293T cells seeded 24 h earlier for generation of the retrovirus. After 24 h of transfection, the medium was replaced with 3 mL fresh cell culture medium. At 48 h post-transfection, the supernatants were collected, spun down at 2095*g* for 5 min at 4°C to remove the cellular debris and filtered with a 0.45-μm filter. A volume of 500,000 cells were mixed with 1 mL retroviral supernatant in a well of 12-well plate in the presence of 10 μg/mL polybrene (Chemicon-Millipore, Billerica, MA, USA). After that, spin-occulation was performed by centrifuging cells at 524*g* for 90 min at 4°C. After centrifugation, the cells were incubated in a humidified 5% CO_2 incubator at 37°C for 7 h. After 7 h, the cells were spun down and the supernatants including the virus were replaced with fresh NK-cell line growth medium.

After 48 h of transduction, green fluorescent protein positive (GFP^+) cells were sorted from empty vector or PRDM1α-transduced NK92 or KHYG1 cells using fluorescence-activated cell sorting (FACS) at the University of Nebraska Medical Center (UNMC) cell analysis facility. Sorted cells were then spun down at 300*g* for 5 min, and the cell pellet was resuspended in TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative reverse transcription polymerase chain reaction

RNA isolation, reverse transcription, and real time polymerase chain reaction (PCR) were performed as described previously.[12] Briefly, RNA was isolated with RNeasy Kit (Qiagen, Gaithersburg, MD, USA), and then reverse transcribed with Superscript II reverse transcriptase (Life Technologies, Grand Island, NY, USA). Dynamo HS SyBr Green q-PCR kit (Thermo Fisher Scientific) was used for the amplifications. Ectopic PRDM1α messenger RNA

(mRNA) levels were detected using PRDM1α and flag tag specific primers. ΔΔCt method was used for quantification of relative transcript levels of MIR155HG or TERC. RPL13A was used as the housekeeping gene for calibration of transcript expression. Melting curves were evaluated to ensure specificity during PCR amplifications. The sequences of primers used for quantitative reverse transcription PCR (qRT-PCR) are as follows: PRDM1α-FL forward: 5′-TCA TGAAGTTGCCTCCCAGCAA-3′, PRDM1α-FL reverse: 5′-TCATCGTCGTCCTTGTAATCAGCG-3′; MIR155HG forward: 5′-ACGGTTGTGCGAGCAGAGAATCTA-3′, MIR155HG reverse: 5′-CTCATCTAAGCCTCACAACA ACCT-3′; TERC forward: 5′-TCTAACCCTAACTGAGA AGGGCGT-3′; TERC reverse: 5′-TGCTCTAGAATGAAC GGTGGAAGG-3′; and RPL13A forward: 5′-ACCGTCTC AAGGTGTTTGACG-3′, RPL13A reverse: 5′-GTACTT CCAGCCAACCTCGTG-3′.

MIR155HG expression analysis in PRDM1α-transduced NK-cell lines by DNA microarray

DNA microarray was performed on empty vector or PRDM1α-transduced KHYG1 or NK92 cells 48 h post-transduction using GeneChip® Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA, USA). Overall, we observed more than 1.5-fold downregulation of 219 transcripts in NK92 cell line and 466 transcripts in KHYG1 cell line. We also observed more than 1.5-fold upregulation of 188 transcripts in NK92 and 243 transcripts in KHYG1 cell lines transduced with PRDM1α. There were 118 transcripts downregulated or upregulated more than 1.5-fold in both NK92 and KHYG1 cell lines. In this study, expression of MIR155HG, TERC, and six other cancer-associated lncRNAs (i.e. USP30-AS1, HOXB-AS1, ITGB2-AS1, CRNDE, GMDS-AS1 and PAXIP-AS1) was evaluated, and the full profile will be reported later in an independent study.

Evaluation of PRDM1 occupancy on MIR155HG and TERC promoters by chromatin immunoprecipitation sequencing

PRDM1 protein occupation on MIR155HG promoter was evaluated with chromatin immunoprecipitation sequencing (ChIP-Seq) in normal NK-cells obtained by activating NK-cells with IL2 for 3 days or through co-culturing peripheral blood lymphocytes for 14 days with the K562-Cl9-mb21, an NK-cell target cell line engineered to express cell surface molecules on K562 cell line.[36] A ChIP-grade PRDM1 antibody (Cell Signaling Technology, MA, USA) was used to immunoprecipitate chromatin associated with PRDM1 in these two samples. Immunoprecipitated fragments of these activated NK samples as wells as their input samples (i.e. negative control) were then sequenced with Illumina Genome Analyzer IIx (NKCOD14-PRDM1 sample) or Illumina HiSeq 2000 platforms (NKCOD14-Input, PBNKD3-PRDM1, and PBNKD3-Input samples) at UNMC or Tufts University next-generation sequencing (NGS) core facilities. We identified the ChIP-Seq binding sites of PRDM1 using CisGenome[41] and MACS[42] programs and determined the statistically significant peaks identified to be common by both the programs for each sample. These analyses revealed 21,206 and 14,765 PRDM1 binding sites in NKCOD14 and PBNKD3 cells, respectively, when both the programs were run with default parameters and with the following statistical cutoffs: Cisgenome (false discovery rate [FDR] < 0.01) and Model-based Analysis for ChIP-Seq (MACS) ($p < 10^{-5}$). Predicted peaks were observed on promoters of genes and on intergenic or intragenic regions. Consistent with a previous report,[43] the ChIP-Seq analyses revealed PRDM1 occupancy on LTA (tumor necrosis factor (TNF)-β) promoter, showing the feasibility of the procedure. In this study, only MIR155HG and TERC were evaluated and the full ChIP-Seq profile will be reported in the future in a separate study.

DNA microarray analysis of public datasets

Previously reported HG-U133 Plus 2.0 DNA Microarray data of 19 NKTCL cases deposited in the NCBI Gene Expression Omnibus (GEO) database (accession number: GSE19067) were reanalyzed using GEO2R bioinformatics tool[44] to evaluate the relationship between MIR155HG or ZFAS1 expression with gender and age of these patients.

Results

ZFAS1 and several other cancer-associated lncRNAs are significantly overexpressed in NKTCL cases

We investigated lncRNAs significantly dysregulated in NKTCL cases (n = 17) compared with those of normal NK-cells (n = 3) using Cufflinks software. The top overexpressed or underexpressed lncRNAs observed in our study were in general poorly characterized with no known biological function or dysregulation in any cancer type (Table 1). Overall, we observed more than 1.5-fold overexpression of 166 lncRNAs in NKTCL cases (Table S2). SNGH5, a lncRNA suggested as a biomarker in malignant melanoma,[45] was also overexpressed in NKTCLs (Figure 1(a)). Interestingly, antisense transcripts of genes regulating cell growth such as RAB30, ARAP1, PRMT5, IL21R, or PAX8 showed significant upregulation in NKTCL cases compared with normal NK-cells (Figure 1(b)–(f)).

ZFAS1 is associated with many growth-related or oncogenic pathways in NKTCLs

In NKTCL cases, we observed significant upregulation of ZFAS1 (Figure 2(a)) that has been implicated in different

Table 1. The most significantly upregulated or downregulated lncRNAs in NKTCL cases.

lncRNA name	Normal NK-cell median expression value (FPKM)	NKTCL median expression value (FPKM)	The ratio of median expression levels (NKTCL/normal NK)	t test
Top five lncRNAs significantly overexpressed in NKTCL cases compared to those in normal NK-cells				
CTD-2540B15.11	1.33	13.31	10.02	0.0003
RP11-291B21.2	8.02	79.61	9.93	0.0041
CTD-2369P2.8	1.43	10.15	7.12	0.0008
SNHG5	41.56	229.74	5.53	0.0066
RP11-626G11.3	2.00	10.81	5.42	0.0206
Top five lncRNAs significantly underexpressed in NKTCL cases compared to those in normal NK-cells				
RP11-932O9.9	73.98	1.04	0.01	0.041154
RP11-612B6.2	204.47	7.26	0.04	0.023023
AC006129.2	55.60	8.08	0.15	0.039223
RP11-229E13.4	483.52	71.61	0.15	0.033174
CTD-3252C9.4	15.24	2.60	0.17	8.11E-08

lncRNA: long non-coding RNA; NKTCL: natural killer/T-cell lymphoma; NK: natural killer; FPKM: fragments per kilobase of exon per million fragments mapped.

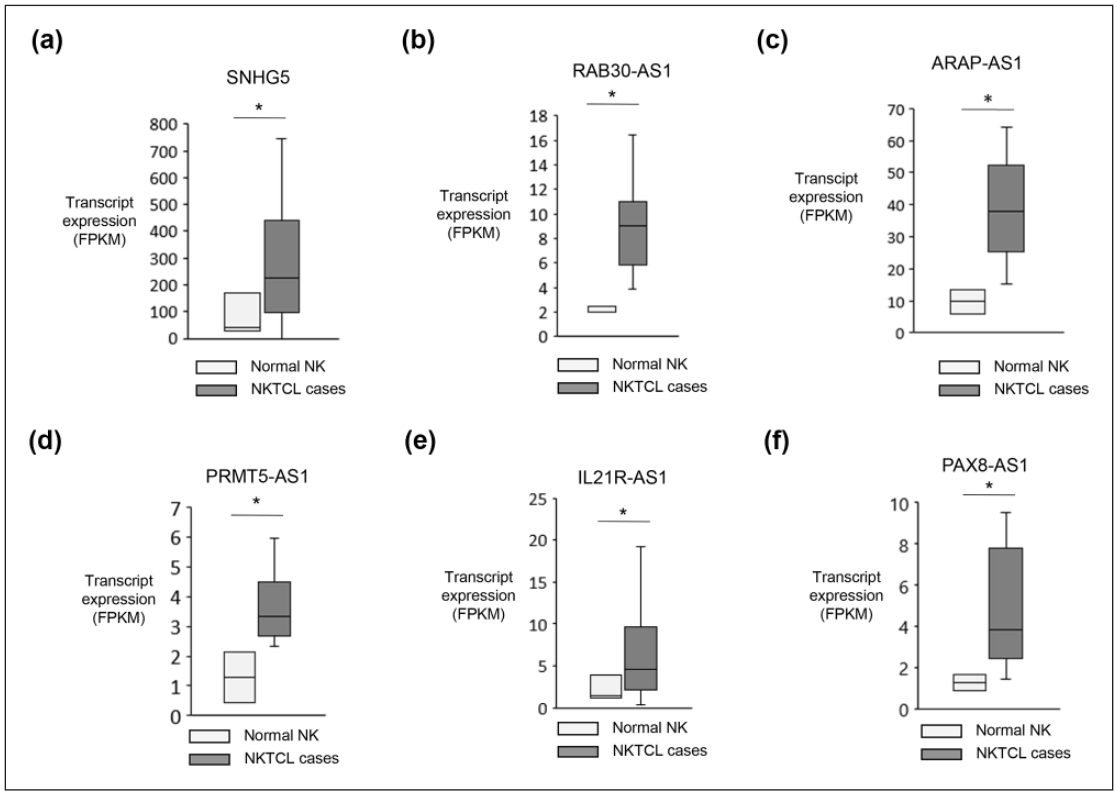


Figure 1. Representation of overexpressed cancer-associated lncRNAs in NKTCL cases. (a–f) lncRNAs including antisense transcripts of growth-regulating genes identified to be overexpressed in NKTCL cases (n = 17) compared to those of normal NK-cells (resting, 2 day IL2 or co-culture activated NK-cells; n = 3) were shown as box-whisker plots (*$p < 0.05$; t test).

cancer types[46–48] and whose function is relatively better characterized compared with other overexpressed lncRNAs identified in our analysis; therefore, we evaluated the signaling pathways or biological processes associated with this lncRNA. First, we identified genes that show positive or negative correlation of transcription expression among 20 NK samples (i.e. 17 NKTCL cases and 3 normal NK samples). We observed strong positive correlation ($R > 0.7$) of expression between ZFAS1 lncRNA and 70 genes. We then determined the list of genes showing negative

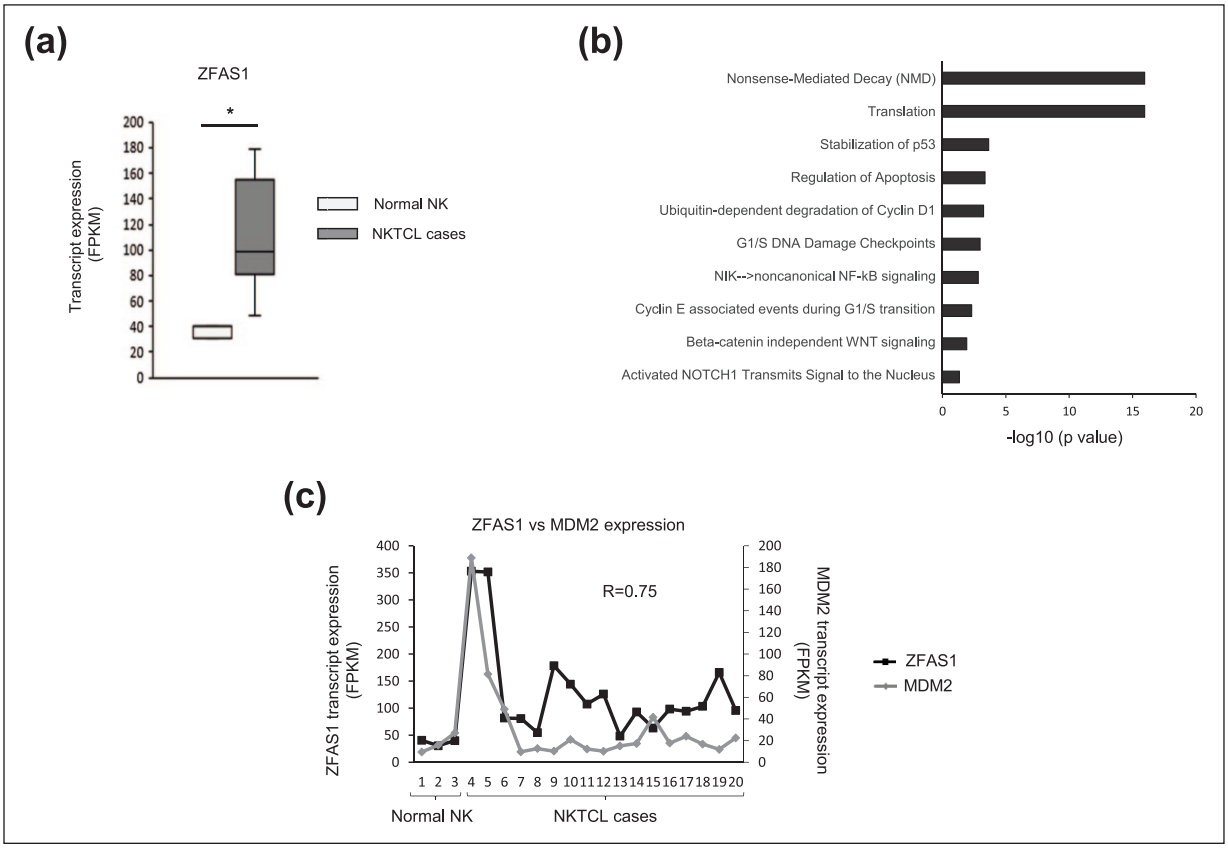


Figure 2. ZFAS1 is overexpressed in NKTCL cases and ZFAS1-associated genes are enriched in pathways regulating proliferation and survival. (a) Median expression levels of ZFAS1 transcript in normal NK-cells (n = 3) and NKTCL cases (n = 17) are shown with a box-whisker plot (*p = 0.0003). (b) Significantly enriched signaling pathways determined with the Reactome software based on genes whose expression positively (R > 0.7) or negatively (R < −0.7) correlated with ZFAS1 expression in NKTCL cases (n = 17) and normal NK-cells (n = 3). (c) The expression levels of ZFAS1 and MDM2 among normal NK-cells (n = 3) and NKTCL cases (n = 17) are shown with a dual-axis graph. R: Pearson product–moment correlation.

correlation with ZFAS1 expression and observed 413 genes whose expression showed strong negative correlation (R < −0.7) with ZFAS1. After that, we used all these 483 ZFAS1-correlated genes as input to identify overrepresented pathways associated with these genes using the Reactome software. Overall, the most deregulated biological processes centered around DNA replication/cell cycle, DNA repair, metabolism, and immune system (Figure S3). Interestingly, we observed very high overrepresentation of non-sense-mediated decay (69 of 123 pathway genes), non-canonical nuclear factor-kappa B (NF-κB) signaling (9 of 61 pathway genes), β-catenin independent WNT signaling (14 of 163 pathway genes), and pathways regulating cell cycle and apoptosis mainly through p53-dependent G1/S checkpoint (Figure 2(b)). These associations altogether suggest that ZFAS1 may promote tumorigenesis through deregulation of the biological processes critical for cellular growth and neoplastic transformation. Of note, expression of MDM2, an E3 ubiquitin protein ligase with critical functions in the regulation of P53 protein stability,[49] was positively correlated with ZFAS1 (R = 0.75) suggesting that ZFAS1 may regulate P53 pathway (Figure 2(c)).

Several cancer-associated lncRNAs are downregulated in NKTCL cases

We observed transcriptional downregulation of 66 lncRNAs in NKTCL cases compared to those in normal NK-cells (Table S3). Antisense transcripts of PRKCQ1 (Figure 3(a)), FGD5 (Figure 3(b)), SVIL (Figure 3(d)), EDRF1 (Figure 3(e)), MDC1 (Figure 3(f)), and VIM (Figure 3(g)) genes were among the significantly underexpressed lncRNAs. We also observed downregulation of C5orf66 (Figure 3(c)) that has recently been reported to be dysregulated in lung cancer.[50] Taurine up-regulated 1 (TUG1), another critical lncRNA with tumor suppressor function regulated by P53[51] was also downregulated in NKTCL cases (Figure 3(h)).

Pathway analysis of lncRNAs dysregulated in NKTCL cases

To identify the signaling pathways and biological processes related to lncRNAs dysregulated in NKTCL cases, we determined whether there is enrichment of pathways

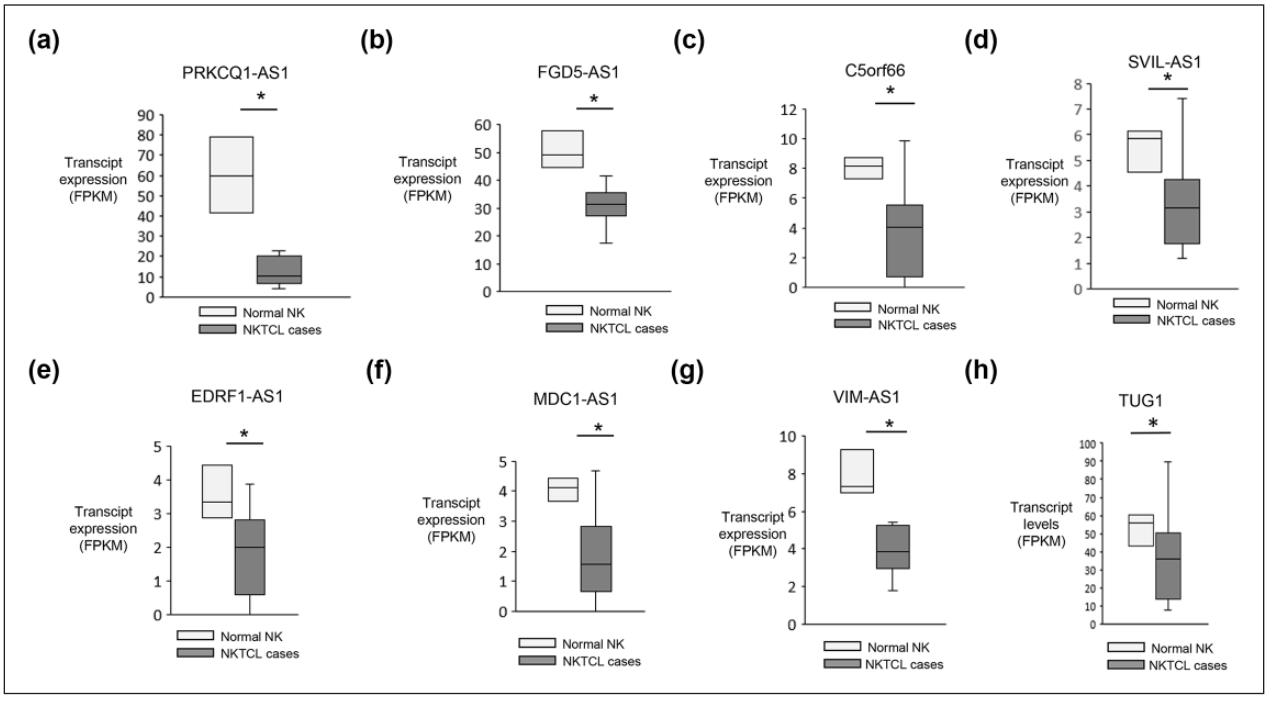


Figure 3. Representation of cancer-related lncRNAs significantly downregulated in NKTCL cases. (a–h) Eight representative lncRNAs, including antisense transcripts of cancer-related genes that are downregulated in NKTCL cases (n = 17) compared to those of normal resting or activated NK-cells (n = 3) are shown with box-whisker plots (*$p < 0.05$).

for lncRNAs overexpressed (>1.5-fold compared to normal NK-cells) and underexpressed (<1.5-fold compared to normal NK-cells) in these patient samples. Intriguingly, several biological processes related to NK-cell activation (i.e. DAP12 signaling, NK-cell-mediated cytotoxicity) or cell growth and survival (i.e. caspase cascade in apoptosis, phosphoinositide 3-kinase (PI3K) signaling, JAK-STAT pathway) were remarkably enriched among deregulated lncRNAs (Figure 4(a)). Similarly, dysregulated lncRNAs were significantly overrepresented among NK-cell activation, cell migration, or cell growth-related biological processes (Figure 4(b)).

Ectopic expression of PRDM1α is associated with transcriptional regulation of cancer-associated lncRNAs in PRDM1-null NK92 cell line determined with RNA-Seq

As the transcription factor PRDM1 was reported by two independent groups[11,18] as a tumor suppressor gene silenced in NKTCLs, we aimed to determine lncRNAs transcriptionally regulated by PRDM1 in PRDM1-null NK92 cell line[10] by comparing the lncRNA expression pattern of PRDM1α or empty vector–transduced NK92 cell line 48 h post-transduction. We observed more than 1.5-fold upregulation of 169 and downregulation of 103 lncRNAs by PRDM1α, respectively. Intriguingly, two well-characterized, oncogenic lncRNAs (i.e. MIR155HG and TERC) were downregulated by PRDM1α in NK92 cells (Figure 5(a)). In addition, we observed transcriptionally upregulated lncRNAs such as CRNDE or LUCAT1 (Figure 5(b)) whose overexpression was reported to promote carcinogenesis.[52,53] We then evaluated the expression level of seven of these PRDM1-regulated lncRNAs by DNA microarray, which have the annotated probe sets available, to cross-validate the RNA-Seq data. We observed that six of these seven lncRNAs (i.e. USP30-AS1, HOXB-AS1, ITGB2-AS1, CRNDE, GMDS-AS1, and PAXIP-AS1) showed transcriptional downregulation or upregulation in the same direction as RNA-Seq results, thereby cross-validating RNA-Seq data (Figure S4). The only exception was the expression of MFI2-AS1 which did not show transcriptional upregulation in PRDM1α-transduced NK-cell lines (data not shown).

MIR155HG and TERC are downregulated by PRDM1 in two PRDM1-null NK-cell lines

Next we performed qRT-PCR to cross-validate the findings of RNA-Seq and observed that MIR155HG is significantly downregulated in PRDM1α-transduced KHYG1 or NK92 cell line compared to empty vector–transduced ones (Figure 6(a)). Similarly, qRT-PCR revealed significant downregulation of TERC expression in these two cell lines PRDM1 post-transduction (Figure 6(b)). We also observed significant downregulation of MIR155HG by DNA microarray (Figure 6(c)). In addition, we observed significant upregulation of MIR155HG expression in NKTCL cases compared to that in normal NK-cells by RNA-Seq (Figure 6(d)), whereas TERC

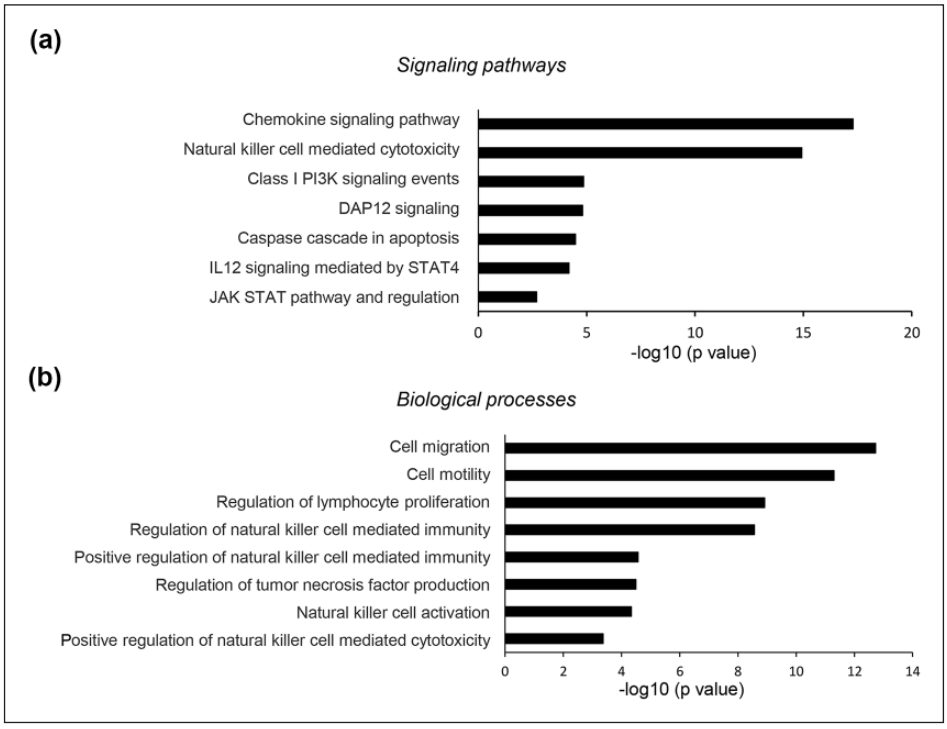


Figure 4. lncRNAs dysregulated in NKTCL cases are associated with NK-cell activation–related signaling pathways and biological processes. The genes whose expression correlates with lncRNAs overexpressed or underexpressed in NKTCL cases in comparison to normal NK-cells were evaluated using the lncRNA2 function program as described in the section "Materials and methods." (a) Signaling pathways (b) and biological processes significantly associated with lncRNAs dysregulated in NKTCL cases are shown.

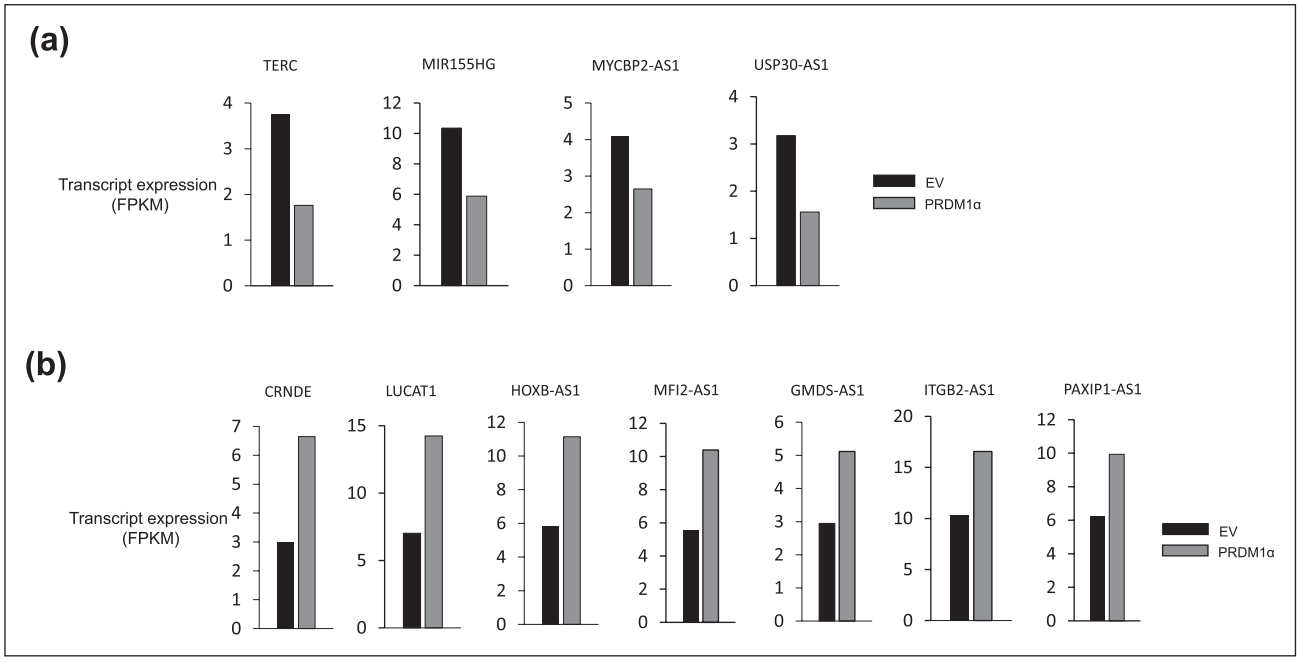


Figure 5. lncRNAs transcriptionally regulated by PRDM1α in NK92 cell line. NK92 cell line was transduced with the empty vector or PRDM1α construct. At 48 h post-transduction, RNA-Seq was applied on RNA isolated from GFP^+ cells sorted by FACS. FPKM transcript levels were shown for representative cancer-associated lncRNAs (a) downregulated or (b) upregulated by PRDM1 in NK92 cells.

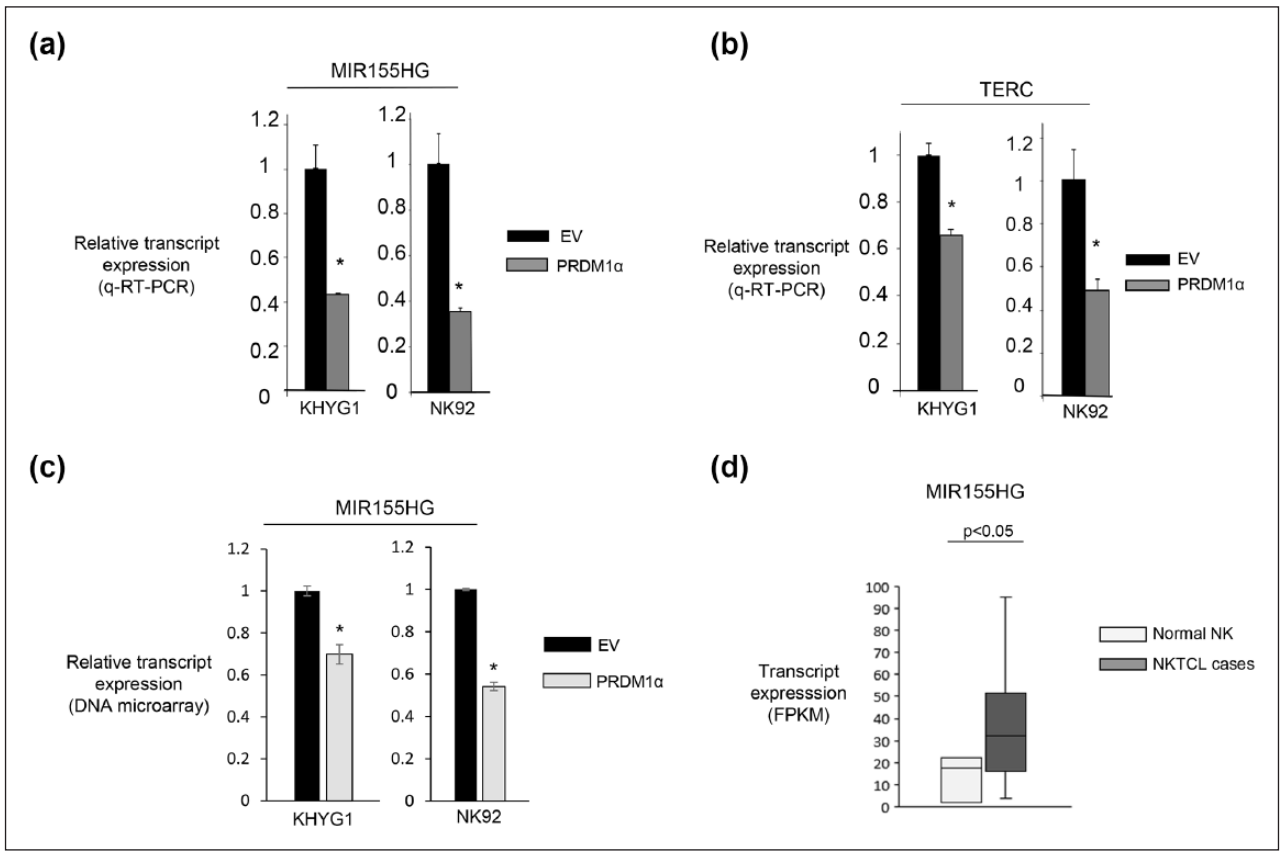


Figure 6. MIR155HG and TERC are transcriptionally downregulated in PRDM1α-transduced NK92 and KHYG1 cell lines. Relative transcript expression levels by qRT-PCR for (a) MIR155HG (b) and TERC on GFP-sorted KHYG1 or NK92 cell lines transduced with the empty vector (EV) or PRDM1α are shown. qRT-PCR was performed 48 h post-transduction of GFP-sorted cells. (c) Relative MIR155HG expression was determined by HG-U133 Plus 2.0 for empty vector or PRDM1α-transduced KHYG1 or NK92 cell lines as described for (a) and (b). For all panels, the expression level of each gene is shown as the relative fold difference of PRDM1α versus empty vector–transduced cells. Data are mean ± SD of two biological replicates for each comparison. (d) MIR155HG expression in NKTCL cases (n = 17) and three normal NK-cell samples by RNA-Seq (*$p < 0.05$; t test).

expression generally was very low (FPKM < 1) in both the normal NK-cells and NKTCL cases with no significant difference (data not shown). Ectopic expression of PRDM1α was detected with qRT-PCR in these two PRDM1α-transduced NK-cell lines (Figure S1(c)).

Evaluation of PRDM1 occupation on MIR155HG and TERC promoters by ChIP-Seq

Visual investigation of aligned ChIP-Seq files of PRDM1–immunoprecipitated co-cultured NK-cells (i.e. NKCOD14) or 3 day IL2 activated NK-cells (i.e. PBNKD3) in comparison to their corresponding input ChIP-Seq files did not reveal any PRDM1 binding on or around the MIR155HG promoter, suggesting that MIR155HG may be an indirect target of PRDM1 in NK-cells. Similarly, no peak was visually observed on or in the vicinity of TERC promoter in these two files, suggesting that this lncRNA may also be an indirect transcriptional target of PRDM1 in NK-cells.

The relationship of ZFAS1 and MIR155HG expression to gender and age of NKTCL patients

We then evaluated whether MIR155HG and ZFAS1 expression correlates with the gender or age variables using normalized DNA microarray data available in GSE19067. There were no significant differences in transcript expression for these two lncRNAs with respect to age or gender (Figure S5(a)–(d)).

Discussion

No study, to date, concentrated on the role of lncRNAs in NKTCL etiology, and in the current study, whole transcriptome sequencing (WTS) analysis revealed that several dysregulated lncRNAs potentially associated with NKTCL pathobiology. Interestingly, expression of antisense lncRNAs of several cancer-associated genes was dysregulated implying

the possibility of regulation of these genes by their antisense lncRNA transcripts. Consistent with this possibility, VIM-AS1, a lncRNA downregulated in colon cancers, was reported to regulate VIM expression.[54] Whether MDC1-AS1 regulates MDC1, an E3-ubiutin ligase that mediates DNA damage checkpoint by causing degradation of P53;[55] or whether PRMT5-AS1 regulates PRMT5, which has been shown to promote the NF-κB pathway,[56] requires further investigation.

ZFAS1 overexpression in NKTCL cases may potentially promote NK-cell growth through distinct mechanisms: First, ZFAS1 may contribute to neoplastic transformation of NK-cells through deregulation of P53-mediated pathways. In support of this, ZFAS1 was reported to be remarkably overexpressed in colorectal cancer tissues, and it has been shown to regulate cell cycle and apoptosis in a P53-dependent manner.[48] Given that ZFAS1 expression shows strong correlation with MDM2 raises the possibility that ZFAS1 may regulate P53 indirectly through regulating MDM2; however, future mechanistic studies are needed to address this possibility. Of note, inactivating mutations of P53 was reported in NKTCLs previously,[9] and p53-mediated pathways, including genotoxic–stress signaling pathways were already shown by gene expression profiling to be dysregulated in NKTCLs.[57] Altogether, these observations suggest deregulation of p53-mediated pathways by multiple genetic and epigenetic mechanisms in NKTCLs. In addition, ZFAS1 may promote growth by deregulating several P53-independent pathways since overactivation of them such as NF-κB, WNT, or NOTCH1 pathways (Figure 2(b)) was already reported previously in NKTCL cases.[57,58] Intriguingly, our observation that some lncRNAs transcriptionally dysregulated in NKTCL cases are enriched in pathways related to NK-cell activity such as NK-cell-mediated cytotoxicity or DAP12 signaling[59] suggests that lncRNAs may have important roles in regulating NK-cell activation during immune reactions.

PRDM1 acts as a tumor suppressor gene silenced through genetic and epigenetic mechanisms in NKTCLs;[11,18] consequently, it may be possible to speculate that the tumor suppressive effect of PRDM1 may, in part, be through transcriptional regulation of lncRNAs. Importantly, mir155 was reported to activate the PI3K pathway through P85α downregulation in diffuse large B-cell lymphoma (DLBCL),[60] and SH2 domain-containing inositol 5'-phosphatase 1 (SHIP1), a negative regulator of PI3K/AKT signaling pathway, was reported to be a target of mir155.[61] Of note, mir155 is a positive regulator of NK-cell activation, including interferon gamma (IFNγ) production.[62] In addition, mir155 overexpression led to the expansion and activation of NK-cells in vivo.[63] Intriguingly, mir155 has recently been reported to be overexpressed in NKTCL,[64] and its overexpression may account for the activation of AKT pathway observed previously by DNA microarray in NKTCL cases.[58] These observations suggest that loss-of-function of PRDM1 may, at least in part, be responsible for mir155 overexpression in NKTCLs. TERC, a lncRNA downregulated by PRDM1α in NK92 cells, is the RNA component of human telomerase that is the gene responsible for maintaining telomere length,[65] and thereby it promotes proliferation. TERC overexpression is associated with many cancer types, including lung cancer[66] and cervical neoplasia.[67] However, TERC did not show upregulation in NKTCL, suggesting that it may not have a notable role in pathobiology of this neoplasm. Biological functions of most lncRNAs transcriptionally regulated by PRDM1 are currently unknown, and further studies involving in vitro functional characterization may be needed. Of note, it is possible that antisense transcripts may contribute to transformation by regulating their corresponding genes (e.g. MYCBP2 or PAXIP1). Altogether these observations suggest the possibility that PRDM1 silencing in NKTCL may be associated with deregulation of lncRNAs important in NK-cell proliferation and survival.

In conclusion, we report that ZFAS1 and several other cancer-associated lncRNAs and oncogenic pathways significantly deregulated in NKTCLs by these lncRNAs for the first time, which may be used as therapeutic targets or diagnostic/predictive biomarkers in the future. These lncRNAs may have roles in NK-cell activation as well as NKTCL pathobiology. Moreover, we provide a possible connection between tumor suppressor PRDM1 and cancer-related lncRNAs (e.g. MIR155HG) in terms of their potential roles in NKTCL pathobiology.

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E.B., Q.G., B.A., and H.Y. performed in vitro experiments and analyzed WTS data. W.C.C supervised in vitro experiments and edited the manuscript. C.K. conceived, designed, and supervised the project, analyzed the data, and wrote the manuscript.

Declaration of conflicting interests

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