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ORIGINAL ARTICLE

Natural killer cell lymphoma shares strikingly similar molecular features with a group of non-hepatosplenic γδ T-cell lymphoma and is highly sensitive to a novel aurora kinase A inhibitor *in vitro*

J Iqbal[1], DD Weisenburger[1], A Chowdhury[2], MY Tsai[2], G Srivastava[3], TC Greiner[1], C Kucuk[1], K Deffenbacher[1], J Vose[4], L Smith[5], WY Au[3], S Nakamura[6], M Seto[6], J Delabie[7], F Berger[8], F Loong[3], Y-H Ko[9], I Sng[10], X Liu[11], TP Loughran[11], J Armitage[4] and WC Chan[1], for the International Peripheral T-cell Lymphoma Project

[1]Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE, USA; [2]Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE, USA; [3]Departments of Pathology and Medicine, University of Hong Kong, Queen Mary Hospital, Hong Kong, China; [4]Division of Hematology and Oncology, Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE, USA; [5]College of Public Health, University of Nebraska Medical Center, Omaha, NE, USA; [6]Departments of Pathology and Cancer Genetics, Aichi Cancer Center Research Institute, Nagoya University, Nagoya, Japan; [7]Department of Pathology, University of Oslo, Norwegian Radium Hospital, Oslo, Norway; [8]Department of Pathology, Centre Hospitalier Lyon-Sud, Lyon, France; [9]Department of Pathology, Samsung Medical Center, Sungkyunkwan University, Seoul, Korea; [10]Department of Pathology, Singapore General Hospital, Singapore and [11]Penn State Hershey Cancer Institute, Pennsylvania State University College of Medicine, Hershey, PA, USA

Natural killer (NK) cell lymphomas/leukemias are rare neoplasms with an aggressive clinical behavior. The majority of the cases belong to extranodal NK/T-cell lymphoma, nasal type (ENKTL) in the current WHO classification scheme. Gene-expression profiling (GEP) of 21 ENKTL and NK-cell lymphoma/leukemia patients, 17 NK- and T-cell lines and 5 indolent NK-cell large-granular-lymphocytic proliferation was performed and compared with 125 peripheral T-cell lymphoma (PTCL) patients previously studied. The molecular classifier derived for ENKTL patients was comprised of 84 transcripts with the majority of them contributed by the neoplastic NK cells. The classifier also identified a set of γδ-PTCLs both in the ENKTL cases as well as in cases initially classified as PTCL-not otherwise specified. These γδ-PTCLs expressed transcripts associated with the T-cell receptor (TCR)/CD3 complex, suggesting T cell rather than NK-cell lineage. They were very similar to NK-cell tumors by GEP, but were distinct from cytotoxic (αβ)-PTCL and hepatosplenic T-cell lymphoma, indicating derivation from an ontogenically and functionally distinct subset of γδ T cells. They showed distinct expression of Vγ9, Vδ2 transcripts and were positive for TCRγ, but negative for TCRβ by immunohistochemistry. Targeted inhibition of two oncogenic pathways (AURKA and NOTCH-1) by small-molecular inhibitors induced significant growth arrest in NK-cell lines, thus providing a rationale for clinical trials of these inhibitors in NK-cell malignancies.
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Introduction

Natural killer (NK)-cell malignancies are rare and present mainly as a lymphoma and less commonly as leukemia termed aggressive NK-cell leukemia (ANKL).[1] Extranodal NK-cell lymphoma typically occurs in the nasal/paranasal areas with prominent angioinvasion and angiodestruction and accompanying necrosis. Other extranodal sites may also be involved and are frequently associated with hemophagocytic syndrome at the advanced stage.[2] There have also been reports of rare T-cell lymphomas in these locations with very similar clinicopathologic features, and these lesions are grouped under the heading of extranodal NK/T-cell lymphoma, nasal type (ENKTL).[3] ENKTL shows strong geographic predilection, with much higher frequencies in East Asia and Central and South America.[4] ANKL is a related disorder with highly aggressive clinical course in contrast to the chronic lymphoproliferative disorder of NK cells.[5] Although ENKTL localized to the nasal region is responsive to radiation therapy, patients with disseminated disease have a very poor outcome.[6,7]

There is a spectrum of lymphomas with cytotoxic (CT) molecules belonging to αβ or γδ T-cell lineage[8] including hepatosplenic γδ T-cell lymphomas (HSTCL),[9] enteropathy-associated T-cell lymphoma (EATCL)[10] and CT T-cell lymphoma of the skin and subcutaneous tissue.[11,12] These tumors express the surface T-cell receptor (TCR)/CD3 complex, exhibit clonal TCR rearrangement and are negative for Epstein–Barr virus (EBV) genome in the tumor cells. It is unclear if these entities have distinct gene expression profiles (GEPs) that can distinguish them from each other and from ENKTL.

Owing to the rarity of the disease and the difficulty in obtaining adequate biopsy specimens, the molecular mechanisms underlining ENKTL are largely unknown. Only a few genome-wide profiling studies using NK-cell lines[13,14] and a limited number of NK-cell[15] and γδ T-cell lymphoma cases have been performed.[16] In this study, we have defined molecular signatures for ENKTL and related malignancies. We have also identified a number of oncogenic pathways in NK-cell tumors and validated the potential significance of the Notch-1 and aurora kinase A pathways by inhibitor studies *in vitro*.

Correspondence: Professor WC Chan, Department of Pathology and Microbiology, Center for Research in Lymphoma and Leukemia, 983135 Nebraska Medical Center, Omaha, NE 68198-3135, USA.
E-mail: jchan@unmc.edu
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Materials and methods

Patient source and cell lines

A series of ENKTL ($n=18$), ANKL cases ($n=2$) and an EBV (−) aggressive NK-cell lymphoma ($n=1$) diagnosed pathologically were studied[4,7] for their GEP. We compared their GEP with a series of peripheral T-cell lymphoma (PTCL) cases from our recent study[17] including 44 PTCL-not otherwise specified (NOS) cases, 4 HSTCL, 2 EATCL cases, 11 CT (αβ)-PTCL[17] and five indolent NK-cell large-granular-lymphocytic proliferation cases. The pathology review, diagnostic criteria and clinical data for these cases have been described.[4,7] The Institutional Review Board of the University of Nebraska Medical Center approved this study.

The characteristics of malignant NK-cell lines, γδ T-cell lines[18] and other T-cell lines are summarized in Supplementary Table 1a. The cell lines were cultured as previously described.[17] Normal resting and activated NK cells,[19] and T-cell subsets were used for comparative analysis.

RNA isolation and GEP

We used HG-U133 plus 2 arrays (Affymetrix Inc., Santa Clara, CA, USA) for GEP as described previously.[17] The raw data was uploaded in BRB-ArrayTools (version 3.7.0)[20] for analysis. The classifier for ENKTL was constructed using the Bayesian algorithm as described earlier.[21] We selected genes at a significance level ($P<0.001$) and a mean fold difference ($\geqslant$4-*fold*) between the ENKTL and PTCL groups for the Bayesian algorithm. Classification precisions were evaluated using leave-one-out cross-validation.[20] Differential gene-expression and pathway analysis was performed using random-variance *T*-test ($P<0.005$), significance analysis of microarrays (with false discover rate <0.1 and $\geqslant$3-*fold* change)[22] and gene-set-enrichment-analysis computational programs.[23] The microarray data is available in the Gene Expression Omnibus database of NCBI through the accession numbers GSE19067 and GSE8059.

Evaluation of re-classified cases

The re-classified cases by GEP were re-reviewed by DD Weisenburger. Additional immunostains and TCRγ gene rearrangement analyses were performed when feasible to evaluate the diagnoses of these cases. The antibody for TCRγ (clone γ3.20) (Thermo Scientific/Pierce Biotechnology/Endogen, Rockford, IL, USA) was incubated with the slide for 2 h at 1:80 dilution. Antigen retrieval was performed at 115 °C in 1 mM EDTA (pH 8.0) for 15 min followed by 15 min cooling at room temperature. Immunostaining was performed on the Dako Autostainer using a Dako EnVision Dual Link Peroxidase/DAB detection kit (Dako North America Inc., Carpinteria, CA, USA) and reactive tonsil was used as control.

Clinical correlation

The Kaplan–Meier method was used to estimate overall survival and event-free survival of the patients, and the log-rank test was used to compare the survival distributions.[4]

Treatment of NK-cell lines with aurora kinase A (AURKA) and Notch-1 inhibitors

NK-cell lines with (SNK6, NKYS and KAI3) or without (KHYG1) EBV were treated with an AURKA inhibitor (MK-8745) (Merck & Co., Inc., Whitehouse Station, NJ, USA). Cell viability was determined using CellTiter-GloLuminescent-Cell Viability Assay (Promega Inc, Madison, WI, USA). Notch-1 inhibitors (Compound-E and Compound-34, ENZO Life-Sciences, Inc, Plymouth Meeting, PA, USA) were similarly tested in NK-cell lines. B-cell line (DHL16) was used as negative and HL (L428) and T-cell line (Jurkat) as positive controls for Notch-1 experiments. AURKA phosphorylation status and co-activators and substrates (TPX2, TP53 and Survivin) were evaluated by western blots (ECL Plus kit; GE-Healthcare Bio-Science, Piscataway, NJ, USA). Apoptosis and cell cycle analysis was performed with flow cytometry[24] (BD FACScalibur, BD-Bioscience, San Jose, CA, USA). Analysis of the list mode data was performed using ModFit software (VeritySoftwarehouse, Topsham, ME, USA).

Results

Patient and cell line characteristics

We have identified four distinct groups of CT lymphomas: NK-cell lineage (NKCL), HSTCL, CT (αβ)-PTCL and γδ-PTCL (non-hepatosplenic, NHS) according to their lineage and gene-expression profile as detailed in subsequent sections, and their characteristics are summarized in Table 1a. NKCL refers to NK-cell lineage malignancies of ENKTL and ANKL entities. The

Table 1a Clinical characteristics of the different groups of cytotoxic lymphomas[a]

	NKCL	*γδ-PTCL (NHS)*	*HSTCL*	*CT (αβ)-PTCL*
Number of cases	17	5	4	11
Age (years)				
Median	49	55.5	33	58
Range	(24–79)	(18–87)	(20–46)	(41–80)
Percentage (%)				
Gender				
Male	53	60	75	90
Female	47	40	25	10
IPI				
Low (0–2)	63	100	50	50
High (3–5)	37	0	50	50
Chemo				
CHOP like	19	80	25	75
Other	56	20	75	25
None	25	0	0	0
Radiation				
No	56	100	100	100
Yes	44	0	0	0
Response				
Complete	50	60	50	50
Partial	0	0	0	12
No	44	40	50	38
Not determined	6	0	0	0
Median survival (years)				
OS	2.55	2.42	1.17	1.53
EFS	2.5	2.4	0.8	0.82

Abbreviations: CT, cytotoxic; EFS, event-free survival; GEP, gene-expression profiling; HSTCL, hepatosplenic γδ T-cell lymphomas; OS, overall survival; NHS, non-hepatosplenic; NKCL, NK-cell lineage malignancies; PTCL, peripheral T-cell lymphoma.
[a]*Note*: CT-(αβ) PTCL subtypes were identified in a previous study (Iqbal *et al*.[17]) and γδ-PTCL (NHS) subtypes were identified in this study by GEP. Categories with two or fewer cases are not included.

γδ-PTCL, non hepatosplenic (NHS) refers to cases originally classified as PTCL, but reclassified as γδ T-cell lineage lymphoma in this study (In GEP analysis, two cases of ENKTL were observed to belong to the γδ T-cell lineage as mentioned in molecular analysis, see below). The complete clinical data was available in 17 (of 19) NKCL and 2 ENKTL patients of γδ T-cell lineage. All these patients exhibited an aggressive clinical course with a median overall survival of only 2.5 years (Supplementary Figure 1). The two cases diagnosed with stage-1 disease showed overall survival exceeding 18 years, indicating that the disease may be curable at this stage. The majority of the patients included in this study were from East Asia (~70%) with a male to female ratio of ~1:1 and median age at diagnosis of 49 years (range, 24–79 years). The majority of NKCL cases were examined for pan T- and NK-cell markers and showed expression of the NK-cell marker CD56 and cytoplasmic CD3ε, but were negative for surface CD3ε (*Leu4*) and TCRγ rearrangement (7 of 7, 100%) and other T-cell makers including CD5, CD4 and CD8 (Table 1b). As expected, these cases also showed positive staining for the CT molecules TIA1 and/or granzyme B (Table 1b). The major characteristics of the cell lines are summarized in Supplementary Table 1a. The presence of EBV genome was detected in the majority of NKCL cases (18 of 19, 95%), NK (8 of 10, 80%)- and γδ T-cell lines (3 of 3, 100%). The clinicopathological findings of the EBV (−) NK-cell lymphoma case have been reported previously.[25]

Table 1b NKCL: immunophenotype, TCR rearrangement and EBV status

Immune markers	*Status*
Cytoplasmic CD3ε	18/19 (+)
CD56	16/16 (+)
TIA1	13/14 (+)
Granzyme B	4/4 (+)
EBER-1	18/19 (+)[a]
CD2	6/7 (+)
Surface CD3ε (leu4)	6/6 (−)
TCRγ rearrangement	7/7 (−)
TCRβ	5/5 (−)
CD5	18/18 (−)
CD8	8/10 (−)
CD4	13/13 (−)

Abbreviations: EBV, Epstein–Barr virus; NKCL, NK-cell lineage malignancies; TCR, T-cell receptor.
[a]The pathological characteristics of an EBV-negative NK-cell lymphoma case has been reported previously by Martin *et al.*[25]

Molecular classifier for ENKTL and the identification of a group of γδ-PTCL

Unsupervised hierarchical clustering showed that ENKTL and ANKL cases formed a distinct cluster from the other PTCL entities, with a few interspersed PTCL-NOS cases. The majority of the ENKTL cases showed a very uniform gene-expression profile and the molecular classifier consisted of 84 transcripts. There were similar number of up- and down-regulated transcripts (41 vs 43) (Figure 1) and most of the up-regulated genes were essentially contributed by NK cells, as they showed an expression pattern similar to normal NK cells (resting and IL-2 activated) and NK-cell lines (Figure 2c). These genes included killer cell immunoglobulin- or lectin-like receptor (*KIR or KLR*) family members, NK-cell-associated markers, CT molecules and a group of distinct chemokines primarily expressed by NK cells or CT T cells. Some of the transcripts were also noted in normal $CD8^+$ T cells (resting and IL12 activated). *TCRδ* mRNA, but not *CD16 (FCGR3A)* was represented in the classifier. Certain up-regulated transcripts, encoding *KRT19* (mainly expressed in endothelial cells) and *VSIG4* (macrophages) were absent from the NK-cell lines and normal NK cells, implying their derivation from stromal cells (Table 2). The down-regulated genes were largely associated with T-cell biology and stromal components including T-cell markers (*CD3* subunits) and genes involved in T-cell differentiation, activation and chemotaxis, transcripts from B cells and macrophages/dendritic cells.

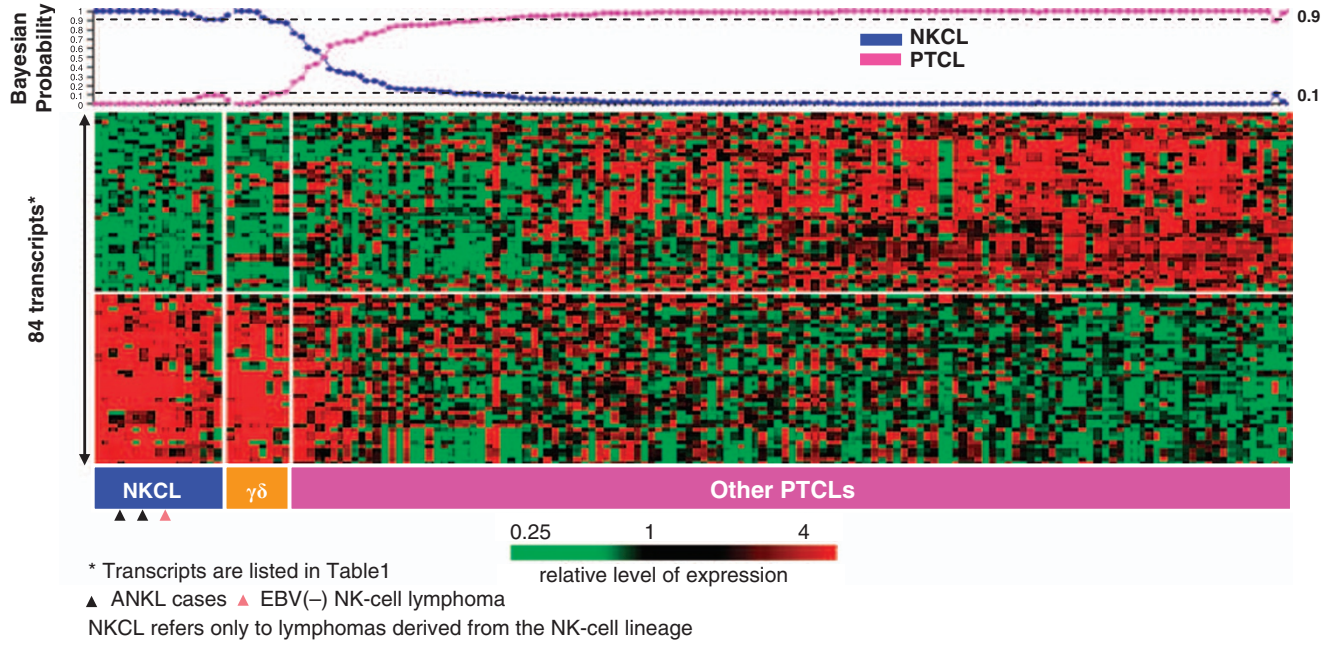


Figure 1 Gene-expression-based molecular predictors for ENKTL. The probability that a case is classified as ENKTL vs PTCL is shown at the top. A small subset of PTCL-NOS cases was re-classified as ENKTL, which, on further examination, showed γδ T-cell differentiation. Each column represents a case and each row represents the expression level of a gene. Gene-expression levels are depicted according to the color scale shown.

The molecular classifier was initially constructed using all pathologically diagnosed ENKTL cases and later evaluated in PTCL cases as well as two ANKL, one EBV(−) NK-cell lymphoma case and five indolent NKLGL. The accuracy of the classifier was evaluated by leave-one-out cross validation (LOOCV) and was robust in re-classifying 15 of 18 pathological diagnosed ENKTL. The two ANKL cases, the EBV(−) NK-cell lymphoma and NK-cell lines were included by the ENKTL classifier. However, 3 of 3 γδ T-cell lines derived from ENKTL,[18] 1 EATCL and 4 (*5 biopsies*) of 44 PTCL-NOS cases were also classified as ENKTL with >90% probability. A small number of PTCL cases ($n=5$) showed greater similarity (>60–85% probability) to ENKTL, when evaluated with the classifier and included four HSTCL (72–85% probability) and one CT (αβ) PTCL (~60% probability). These cases did not show any association with EBV, and when evaluated with significance analysis of microarrays analysis, showed significant gene-expression differences with the cases classified as ENKTL with >90% probability (see below). The three molecularly unclassified cases of ENKTL had low expression of NK-cell markers (*CD56* and *KIR molecules*), high expression of endothelial cell-related genes and did not express other PTCL subtype signatures, suggesting that they had a low number of neoplastic cells.

The four PTCL-NOS cases and one EATCL predicted to be ENKTL with >90% probability were further examined. Interestingly, two transcripts (*CD3γ* and *CD3δ*) were expressed by the re-classified EATCL, PTCL-NOS and two ENKTL cases. Differential gene-expression analysis of the latter group of cases against the remaining cases showed significantly ($P<0.005$) higher expression of transcripts encoding the TCR complex including *CD3γ*, *CD3δ*, *TCRγC2*, *TCRVγ9* and *TARP* (>10-*fold*), the activation and differentiation molecules (*CD69* and *LAT2*) consistent with T-cell lineage differentiation (Figure 2a; Supplementary Table 2). There was high expression of *TCRγV9* and *Vδ2* that correlates with the high incidence of Vγ9 and Vδ2 usage reported previously in nasal T-cell lymphoma and chronic EBV infection.[26] We also observed higher expression of *IKZF2* ($P=0.0001$), a T-cell-restricted IKAROS family member,[27] and T-cell adaptor protein *SH2B3* (*LNK*, $P=0.008$)[28] and a repertoire of transcripts encoding *KIR* molecules (Supplementary Table 2). The analysis indicated that this group of cases was T-cell lymphomas expressing the γδ TCR. The two ENKTL cases of γδ T-cell lineage were positive for EBER, and showed a very similar GEP to three γδ T-cell lymphoma lines,[18] as well as the remaining group of 15 cases derived from NK-cell lineage, thus clearly showing the close relationship among the ENKTL cases not only morphologically and clinically, but also in gene expression profiles despite their separate lineage derivation. The ENKTL cases of γδ T-cell lineage were also remarkably similar by GEP to the other five re-classified cases (4 PTCL-NOS, 1 EATCL) that are designated as γδ-PTCL, (NHS) (see below).

Gene-set-enrichment-analysis identified gene signatures associated with IL12 signaling, proliferation, and chromosome 6q arm in γδ T-cell lineage lymphomas compared with NKCL tumors. The analysis of overall survival showed no significant difference between these tumors similar to a previous report.[29]

We performed hierarchical clustering of the ENKTL/ANKL, the re-classified γδ-PTCL (NHS), CT (αβ)-PTCL[17] and four HTSCL cases using the ENKTL classifier. The ENKTL molecular classifier clustered the re-classified γδ-PTCL with ENKTL cases in one cluster as they showed very similar expression profile (Figure 2b and c), whereas the CT (αβ)-PTCL and HSTCL clustered separately.

The re-classified γδ-PTCL (NHS) from PTCL-NOS cases showed extranodal involvement including skin ($n=2$), lung ($n=1$) or neck LN ($n=1$) and with a CD3+ and TCRβ (βF1) negative immunohistochemical profile, when examined, consistent with γδ T-cell lineage (Supplementary Table 3). CD56 protein expression was not consistent (1 of 2 cases positive), and two of the four cases showed *CD56* mRNA expression at a similar range as in NK-cell malignancies. Two of the four cases were double negative for CD4 and CD8, and one case was double positive. Interestingly, 3 of 3 cases were negative for EBV by EBER *in situ* hybridization. A representative re-classified case is shown to be positive for TCRγ by immunohistochemistry (Figure 2d). The γδ-PTCL (NHS) originally classified as EATCL

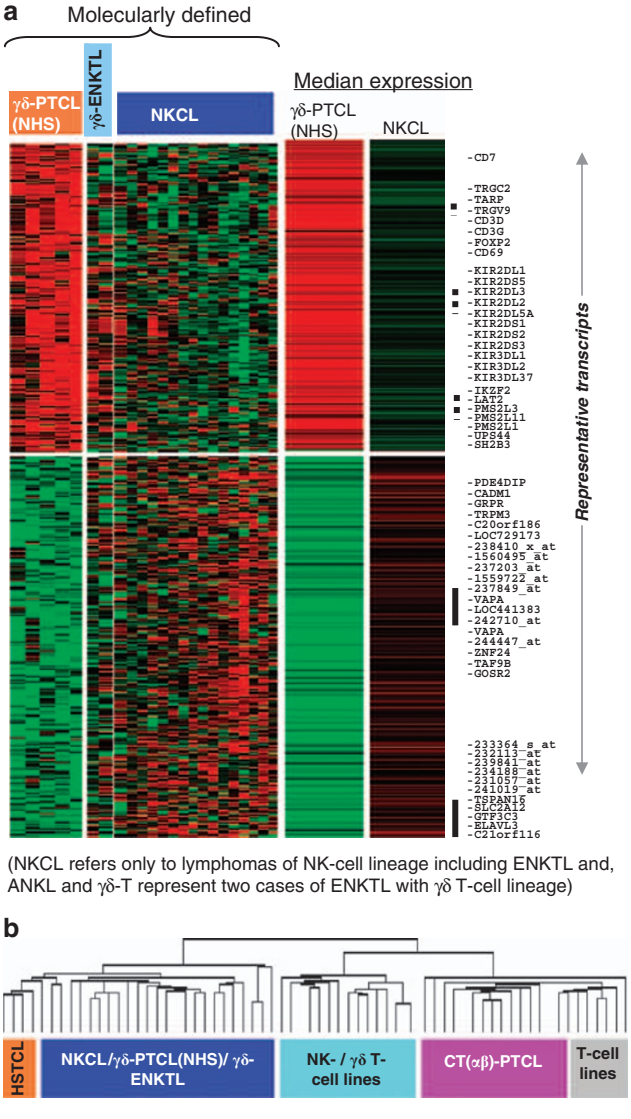


Figure 2 (**a**) Differentially expressed genes between γδ-PTCL and NKCL. A unique subgroup within PTCL-NOS with features of γδ T cells (γδ-PTCL) was identified to have a very similar GEP to ENKTL with only a small subset showing differential expression ($P<0.005$, 448 transcripts). Two cases of ENKTL with γδ T-cell phenotype are also evaluated with this set of differential gene-expression signature. (**b**) Hierarchical clustering according to the ENKTL classifier showed a distinct cluster of CT (αβ)-PTCL, HSTCL and ENKTL with γδ-PTCL interspersed among the ENKTL cases. (**c**) Expression of ENKTL classifier genes in different cytotoxic PTCL entities, cell lines and normal cells. List of the genes on right indicate that they are differentially expressed in cytotoxic entities. (**d**) A representative γδ-PTCL case identified by GEP immunostained for TCRγ (clone γ3.20, Thermo Fisher Scientific Inc., Rockford, IL, USA) (original magnification ×20).

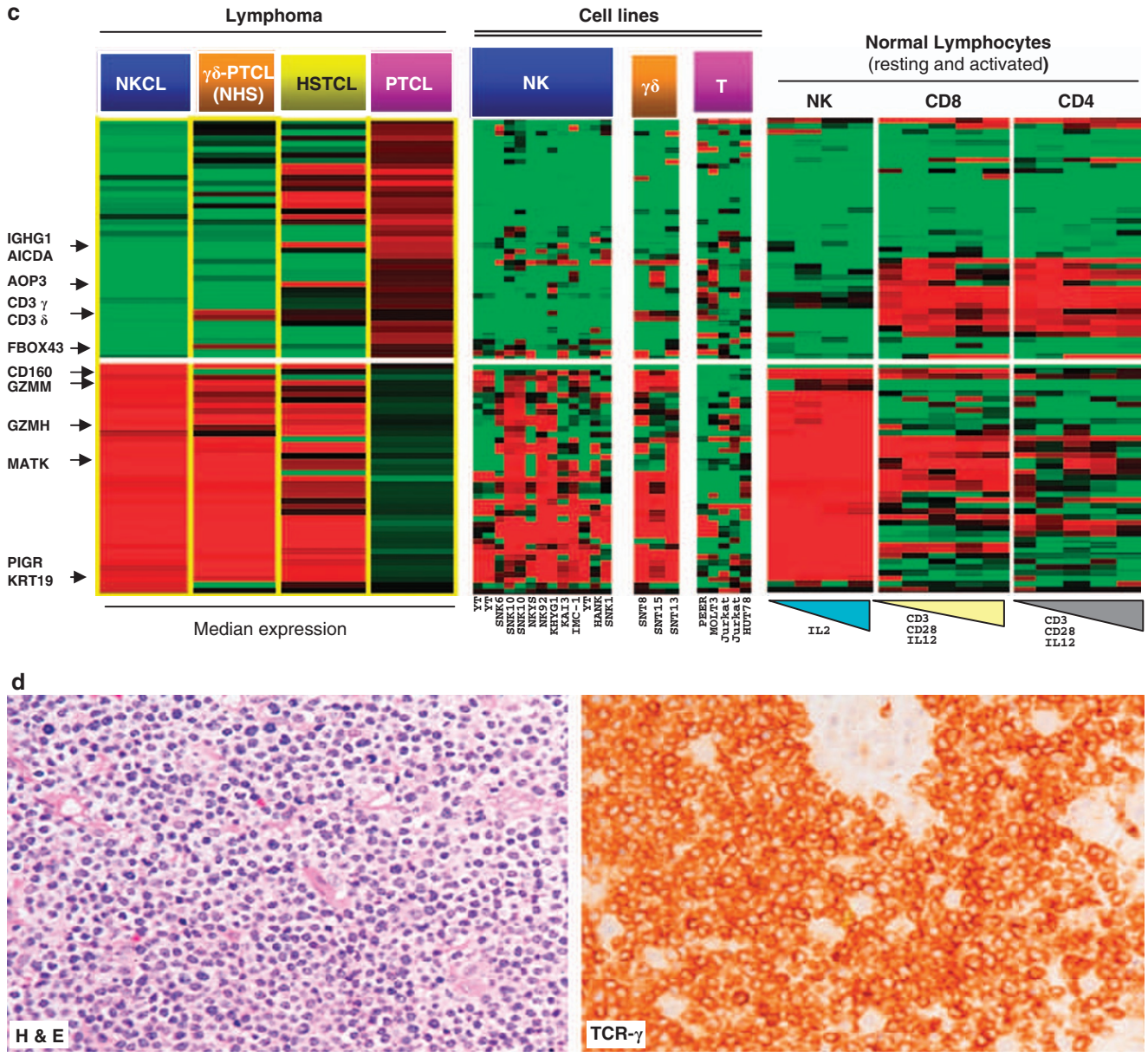


Figure 2 Continued.

presented with terminal ileum involvement and showed no enteropathic changes. The immunohistochemical profile was characterized by CD3+, CD56+, CD8+, CD5−, CD2−, CD4−, TCRβ(βF1) negative with expression of CT molecules TIA1 and Granzyme B. This case was also negative for EBV. There was no significant difference in expression of *CD103 (ITGAE)* mRNA compared with other γδ-PTCL (NHS) or NKCL.

Comparison of the NKCL and NHS γδ-PTCL with PTCLs, HSTCL and CT(αβ)-PTCL

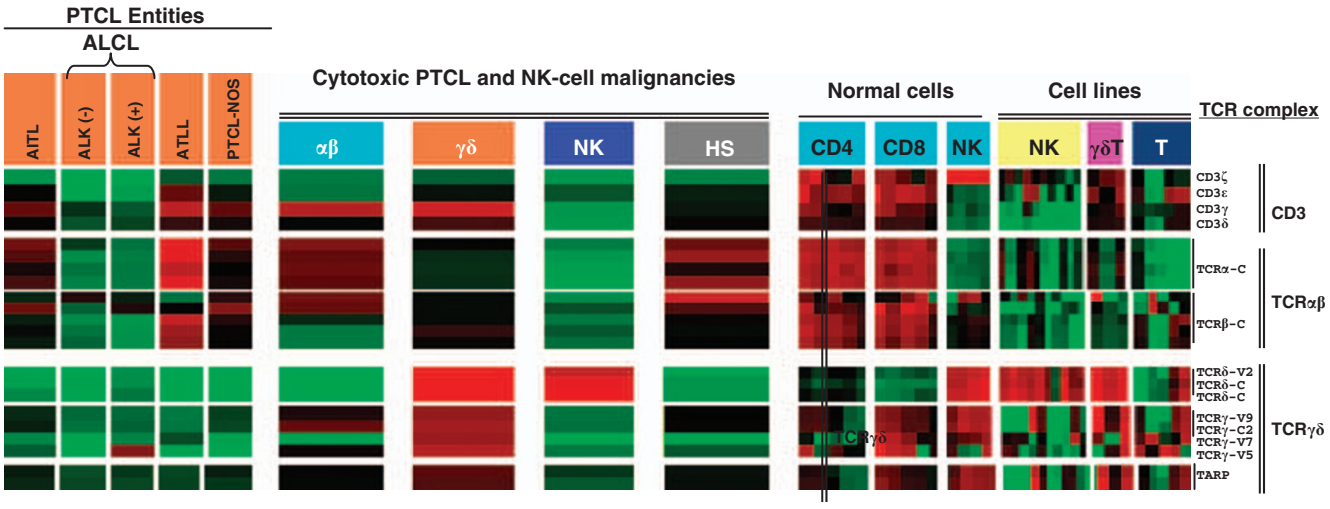
PTCLs. Supervised analysis of NKCL vs PTCLs for differential gene expression using the significance analysis of microarrays algorithm included many members of KIR glycoproteins, leukocyte Ig-like receptors (LIR) and sialic acid-binding immunoglobulin-like lectin (SIGLEC) in NKCL (Table 2). A few transcripts encoding KLR transmembrane-calcium-dependent (C-type) lectin proteins including *NRC1 (NKp46), NRC3 (NKp30)* and *KLRK1(NKG2D),* NK-cell activation markers and genes critical for CT function were highly expressed in NKCL (Table 2). NKCL showed a limited cytokine profile, with cytokines mainly involved in angiogenesis (*IL8* and *CXCL17*), migration of monocytes/macrophages (*CCL-8,-7,-4, CXCR3)* and the pro-inflammatory response (INFγ). There were many down-regulated genes relevant to T-cell biology as mentioned above.

Hepatosplenic γδ T-cell lymphomas. The four cases of this rare lymphoma formed a distinct hierarchical cluster, but showed similarity to ENKTL (70–85% probability with the classifier). The differentially expressed transcripts showed the characteristic anatomical (liver/spleen) distribution of the neoplastic cells, with high expression of metabolism and B-cell-related genes. Even without the liver/splenic signature, HSTCL has a distinct GEP reflecting T-cell biology and its γδ T-cell derivation. It has lower expression of genes involved with cytotoxicity including *GZM-A,-M,* and

Table 2 Differentially expressed genes[a] between molecularly defined NKCL malignancies and PTCLs

	Genes in classifier[b]	*Differentially expressed genes determined by SAM analysis*[c]
Up-regulated genes		
KIR or KLR family members and SIGLEC	KLRD1, KLRC3, KLRC2, KIR2DL4	KLRK1, KLRC4, KIR3DS1, KIR2DS5, KIR2DS3, KIR2DS2, KIR2DS1, KIR3DL3, KIR2DL5A, KIR2DL4, KIR2DL3, SIGLECP3, SIGLEC9, SIGLEC16, SIGLEC11, SIGLEC1,
Cytokines/receptors	XCL2, XCL1, CCR1, CCL5	IL8, IL11, IFNγ, CXCR3, CXCL17, CCL4, CCL3, CCL7, CCL8
Cytotoxic molecules	GZMM, GZMH, GZMA, GNLY, CTSW	GZMB, GZMK, FASLG, PRF1
NK-cell markers and NK-cell-activation-related molecules	NCAM1, CD244, CD160, SH2DIB, NKG7	NCR1, NCR3, KLRK1, FCGR3B/CD16, CD96, CD300A, CD163, SLAMF7, EOMES, TBX21, NKTR
Immunoregulatory mediators or receptors	VSIG4, PTGDR	LILRP2, LILRB3, LILRB2, LILRA3, LILRA2, PTGER2
Fc receptor family/miscellaneous genes	PIGR, MCTP2, MATK, HOPX, TCRδ	FCRLB, FCGR2C, FCGR1B, FCGR1A, FCER1G, CD47
Down-regulated genes		
Cytokines/receptors	CXCL13, CCL21, TNFRSF25, TNFRSF17	TNFSF11, TNFRSF9, TNFRSF8, TNFRSF11B, LTA, IL7, IL6R, IL2RA, IL28RA, IL22RA2, IL21R, IL21, IL1RAP, IL12B, CXCL14, CCL22, CCL20, CCL17, CCR7, CCR6, CCR4
B-cell-related genes	FCRL5, IgM and G1, AICD, POU2AF1 (BOB1)	BLK, BTLA, CD24, CD22, CD79A,-B,
T-cell-related genes	CD27, CD3G, CD3D, ICOS, MAL, TCF7, PKIA	TCRα, TCF4, MALT1, IKZF2, CTLA4, CD8-A and -B, CD28, CD6, CD5, CD200
Macrophages/dendritic cells-related genes	CR1, CR2(CD21), C7, ZC3H12D	CD1E, CD1C

Abbreviations: ENKTL, extranodal NK/T-cell lymphoma, nasal type; NHS, non-hepatosplenic; NKCL, NK-cell lineage; SAM, significance analysis of microarrays.
[a]$P<0.005$ and >fourfold.
[b]Gene in classifier were identified using ENKTL cases vs PTCL.
[c]SAM was performed using NKCL vs PTCL (excluding δγ-PTCL (NHS) and only representative genes (FDR<0.1 and threefold) are included).



Abbreviation :ATLL: Angioimmunoblastic T-cell lymphoma, ALCL-ALK(-) :Anaplastic large cell lymphoma, anaplastic lymphoma kinase (negative); ALCL-ALK(+) Anaplastic large cell lymphoma, anaplastic lymphoma kinase (positive); ATLL: Adult T-cell leukemia/lymphoma, PTCL-NOS: Peripheral T-cell lymphoma-not otherwise specified

Figure 3 The expression of different TCR and CD3 subunits is different in cytotoxic PTCL subtypes and NKCL.

several adhesion molecules compared with NKCL (Supplementary Table 4). When γδ-PTCL (NHS) subtype was compared with HSTCL, similar differential expression as noted with NKCL was observed aside from the TCR complex-related transcripts. Notably, the CT molecules *GNLY (33-fold), GZMB (11-fold), GZMM (4-fold)* and transcription factor *TWIST1 (19-fold)* previously shown to be highly expressed in Sézary syndrome[30] and pan T-cell marker *CD7 (8-fold)* were significantly up-regulated in γδ-PTCL (NHS). Gene-set-enrichment-analysis also showed significant enrichment of proliferation-related gene signatures with NHS γδ-PTCL, whereas gene signatures associated with angiogenesis, liver metabolism and B-cell-related gene signature were significantly enriched in HSTCL. A notable difference in contrast to the former (*NKCL* vs *HSTCL*) comparison was that no significant differential expression of *KIR* or *KLR* family members was observed between HSTCL vs γδ-PTCL (NHS).

CT (αβ)-PTCL. The CT (αβ)-PTCL[17] subgroup was readily separable from NKCL by the classifier and showed higher expression of genes associated with proliferation, anti-apoptosis and T-cell activation and differentiation (Supplementary Figure 2a; Table 5). The γδ-PTCL (NHS) cases and the two ENKTL of γδ T-cell lineage were also molecularly distinct from CT(αβ)-PTCL subtype. Aside from the expression of transcripts encoding different TCR chains, the γδ-PTCL (NHS) cases exhibited GEP very similar to NK-cell lymphoma including distinct expression of *KIR* or *KLR* family members, also observed previously[16] (Figure 3; Supplementary Table 6). On the contrary, CT(αβ)-PTCL showed up-regulated expression of the full repertoire of T-cell markers including co-stimulatory genes (*CD27,CD28* and *CD82*), genes involved in T-cell activation (*IKZF1, TCF4*), a characteristic group of cytokines/receptors and a higher expression of genes associated with proliferation (Supplementary Figure 3b).

Identification of activated pathways in NKCL

Several gene signatures (for example angiogenesis, genotoxic stress and proliferation) and signaling pathway (for example TGFβ, Notch and Wnt) were significantly enriched in NKCL, when compared with IL2-activated normal NK cells and indolent NK-large-granular-lymphocytic proliferation cases (Table 3). Surprisingly, the signatures of NF-κB pathway/target genes were not enriched in NKCL.

NK-cell lines are susceptible to Notch and AURKA inhibitor

We investigated the significance of selected up-regulated pathways by observing the effect of inhibiting the relevant pathways on NK-cell lines. The two Notch inhibitors tested are potent inhibitors of γ-secretase and Notch processing. Both

Table 3 Enriched pathways and gene signatures in NK-cell lineage malignancies

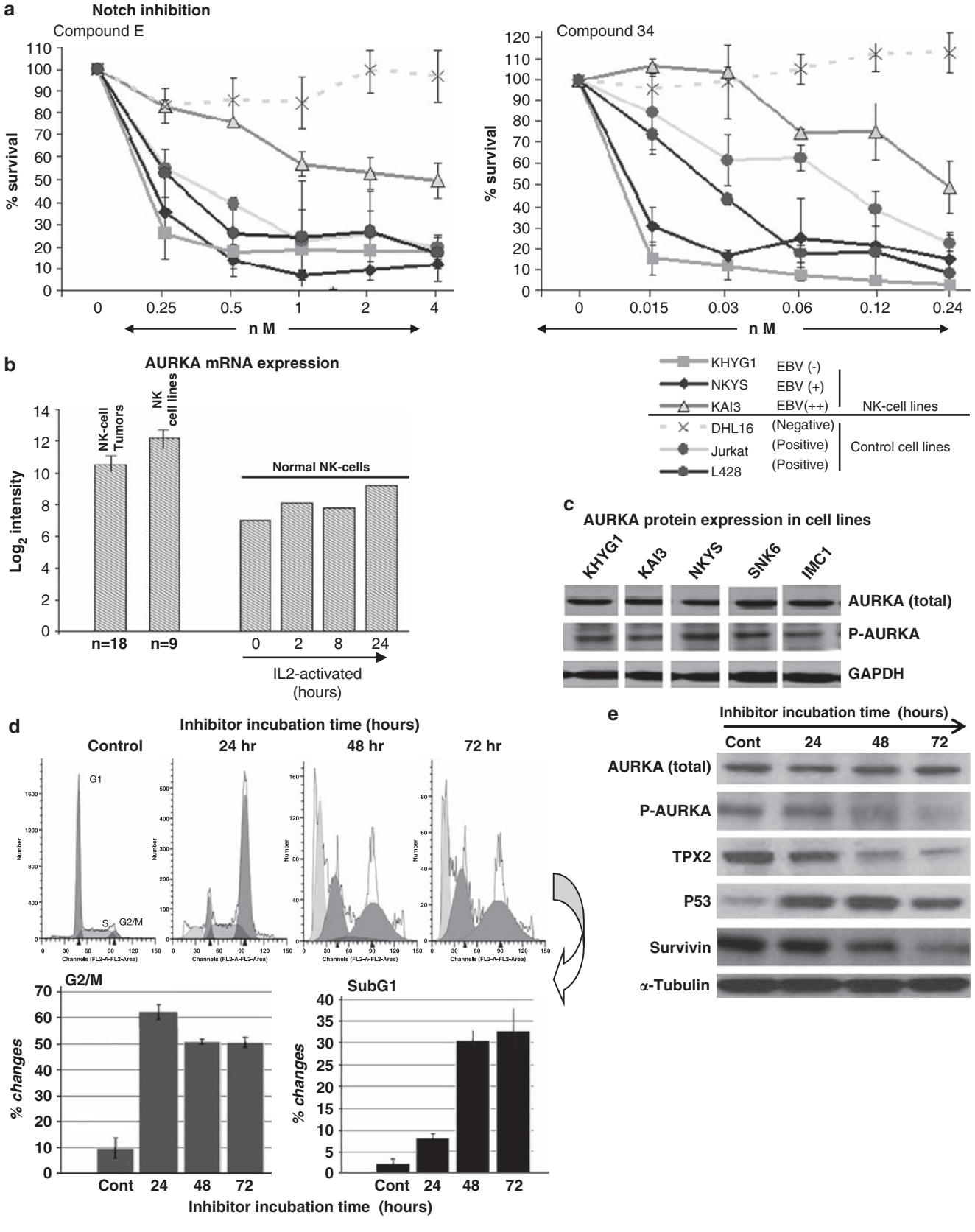
Pathways/gene signatures	*No. of genes*	P-*value*	*FDR*	*GSEA description*	*Reference/Broad institute' MSigDB*
Angiogenesis					
VEGF-regulated genes	94	0.002	0.135	Up-regulated genes after VEGF treatment	*Mol Hum Reprod* 2002; **8**: 855–863
Matrix metalloproteinase	30	0.004	0.115	Curated gene signature of MMPS	http://www.broad.mit.edu/gsea/msigdb
Hypoxia signaling mediated by HIF-1	94	0.022	0.115	Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1	*Blood* 2005; **15**: 659–669
Hypoxia signaling mediated by VHL-HIF	438	0.04	0.113	VHL-HIF-induced gene signature	*Mol Cancer Res* 2003; **1**: 453–462
Genotoxic stress					
Genotoxic stress by UV or γ-radiation	35	<0.0001	0.045	Curated gene signature of p53 during genomic stress	*Oncogene* 2005; **28**: 5026–5042
TP53-regulated genes	31	<0.001	0.155	Up-regulated by expression of p53 in p53-null, brca1-null MEFs	*Oncogene* 2003; **12**: 3749–3758
Genotoxin stress	34	0.008	0.182	Common genes regulated by (cisplatin, methyl methanesulfonate, mitomycin C, taxol, hydroxyurea and etoposide)	*Mutat Res* 2004; **18**: 5–27
P53-dependent p21 targets	49	0.036	0.115	Genes down-regulated by p21	*J Biol Chem* 2002; **7**: 36329–36337
Proliferation related					
Proliferation signature	203	0.006	0.113	Cell proliferation genes determined in zebra fish	*Proc Natl Acad Sci USA* 2005; **13**: 13194–13199
Myb-regulated genes	317	0.027	0.118	Positive and negative determinants of target gene specificity in myb transcription factors	*J Biol Chem* 2004; **9**: 29519–29527
Myc-up-regulated genes	53	<0.001	0.217	Genes up-regulated in hepatoma tissue of Myc transgenic mice	*Nat Genet* 2004; **36**: 1306–1311
Others					
NOTCH signaling	28	<0.001	0.067	Genes concomitantly modulated by activated Notch-1 in mouse and human primary keratinocytes	*Genes Dev* 2006; **15**: 1028–1042
WNT signaling	23	0.004	0.176	Genes up-regulated by Wnt-3A	*BMC Dev Biol* 2002; **2:** 8
TGF-β signaling	80	0.008	0.124	Up-regulated genes upon TGF-β treatment	*J Biol Chem* 2001; **18**

Abbreviations: FDR, False discovery rate; GSEA, Gene Set Enrichment Analysis; MMP, Matrix Metalloproteinase; TGF, Transforming growth factor; VEGF, Vascular endothelial growth factor.

Figure 4 (**a**) Treatment with Notch inhibitors (Compound-E and Compound-34) induced growth arrest in NK-cell lines and positive controls Jurkat and L428 (Hodgkin lymphoma cell line), but not in germinal center B-cell line (DHL16). (**b**) *AURKA* mRNA expression (*using probe sets 204092_S_at and 208079_S_at of HG U133 plus 2 Affymetrix array*) in NK-cell tumors and NK-cell lines in comparison with normal NK cells. (**c**) Protein expression of AURKA and p-AURKA in NK-cell lines. (**d**) Treatment of NK-cell lines with AURKA inhibitor (MK-8745) induced G2/M growth arrest and apoptosis subG1 arrest and representative NK-cell lines showing sensitivity to AURKA inhibition at 24 h after incubation. (**e**) AURKA inhibition led to p53 induction and TPX2 and survivin down-regulation by inhibition. *Antibody source*: AURKA (Sigma Aldrich, St Louis, MO, USA); phospho-AURKA & Survivin (Cell Signaling Technologies, Danvers, MA, USA); TPX2 & TP53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and α-tubulin (Cedarlane Laboratory, Burlington, NC, USA).

inhibitors induced significant concentration-dependent growth inhibition of the NK-cell lines, NKYS and KHYG1, whereas KAI3 was less sensitive (Figure 4a).

We observed high expression of *AURKA* mRNA in patient samples and cell lines. The expression of the protein was seen in all NK-lymphoma cell lines (Figure 4b and c). AURKA is located



at a frequently (>50%) amplified locus (20q13) in lymphomas derived from NKCL[31] and is involved in multiple pathways, promoting the proliferative function of MYC[32] and WNT signaling,[33] while inhibiting TP53.[34] Moreover, AURKA is up-regulated by hypoxia[35] that is frequently observed in ENKTL. Many of these signatures were significantly enriched in ENKTL, for example Myc-induced signature, and WNT signaling and genotoxic pathway (Table 3). The active phosphorylated form (p-AURKA) and its substrates (TPX2, TACC3 and EG5) could be shown. We, therefore, tested the NK-cell lines with a novel small-molecule AURKA inhibitor (MK-8745) and all cell lines showed a significant increase in apoptosis and cell cycle arrest (Figure 4d; Supplementary Figure 3a). This inhibition led to decrease in endogenous p-AURKA and its substrate TPX2, without affecting the level of total AURKA. The downstream target of AURKA, TP53, was significantly induced and there was a gradual decrease in survivin (target of TP53) level with time (Figure 4e).

Discussion

The clinicopathological features of ENKTL cases included in this study are consistent with previous studies.[7,36] The immunophenotype of tumor cells showed expression of NK-cell markers including CD56, cytoplasmic CD3ε, CT molecules (TIA1 and granzyme B) and presence of EBV in the majority of the cases, consistent with observations that NK-cell is indeed the cell of origin in the majority of ENKTL cases. In keeping with this observation, our molecular classifier included most of the up-regulated genes that coded for the distinctive phenotypic and functional characteristics associated with NK cells and showed high specificity in distinguishing NK-cell malignancies including ANKL from most PTCL cases. However, we also identified two cases of γδ T-cell lineage in ENKTL, which can be differentiated from their NK-cell counterpart by the high expression of CD3γ, CD3δ and TCRγ-associated transcripts. Despite the difference in cell lineage, these ENKTL cases share very similar morphological and clinical characteristics and as shown in this study, also share a very similar molecular profile. NK-cell neoplasms are characterized by the presence of EBV in tumor cells; however, tumor cases/cell lines without EBV have been reported.[25,37–40] In our series, EBV status has no impact on GEP either in tumor specimen or in cell lines.

Our molecular classifier not only identified γδ T-cell cases of ENKTL, but also a subset of γδ-PTCL (NHS) in other extranodal areas that showed a strikingly similar GEP to NK cells, NK-cell lines and to γδ T-cell lines derived from ENKTL.[18] The gene-set-enrichment-analysis findings of chromosome 6q gene enrichment in γδ-PTCL (NHS) compared with NKCL is likely related to the frequent deletion of 6q in the NK-cell counterpart.[31] This deletion is not observed in the γδ T-cell lines,[41] and may be similarly true for γδ-PTCLs (NHS) as well. Gene signatures associated with IL12 signaling, shown to be critical for γδ T-cell proliferation by resisting apoptosis,[42] were up-regulated in γδ-PTCL (NHS) compared with NKCL. The immunohistochemical staining and pathology review provided further evidence for γδ T-cell lineage in these cases. The γδ-PTCL (NHS) case presenting in the skin in our study resemble the primary cutaneous γδ T-cell lymphoma as defined in the current WHO.[3] It would be of interest to study more cases of primary cutaneous γδ T-cell lymphomas to determine whether they also share the GEP of the γδ-PTCL (NHS) cases we have defined in this study.

The term mucocutaneous or NHS γδ-PTCL mentioned in the WHO-EORTC classification scheme[12] would encompass the cases of γδ-PTCL described here, although the heterogeneity within this group of disorders has not been defined. Normal γδ T cells are enriched in mucosal surfaces[43] and exhibit many of the characteristics of the innate immune system similar to NK cells.[44] There are subsets of γδ T cells that differ in their ontogeny as reflected by their time of appearance in the thymus, TCR gene usage, TCR combinatorial complexity and their locations and functions in the body. There is also corresponding preferential γδ gene usage in γδ-PTCL at different sites such as Vδ1 in HSTCL[9,45], Vδ2 in primary cutaneous γδ-PTCL[46] and Vγ9Vδ2 in ENKTL and γδ-PTCL (NHS).[26,29] Consistent with these findings, the re-classified γδ-PTCLs in this study exhibited extranodal disease and expressed *Vγ9, Vδ2* mRNA at high levels. Different organ sites may be populated preferentially at different stages of development by γδ T-cell populations and there is likely to be further heterogeneity in the mucocutaneous γδ-PTCL. Therefore, a detailed study of a large series of γδ T-cell lymphomas from different sites is needed to decipher the molecular relationship among these γδ T-cell lymphomas.

The HSTCL appears to be distinct clinically, pathologically and by GEP from NKCL and γδ-PTCL of mucocutaneous sites presented in this study as also suggested by a previous observation.[47] Even after excluding the signatures contributed by the liver and spleen, or the differences in TCR usage, NKCL and γδ-PTCL (NHS) exhibit distinct GEP, especially the CT profile with HSTCL expressing *TIA-1*, but not *PRF, GZMA* and *GZMM*. These observations suggest diseases derived from γδ T cells in hepatosplenic and NHS sites are distinct.[16] It is possible that the γδ-PTCL (NHS) observed in this study are derived from a unique subset of γδ T cells with features very similar to NK cells compared with other γδ-PTCL. It is also probable that the clinicopathological and GEP characteristics of these γδ-PTCL are influenced by the microenvironment in which they are derived. These hypotheses need to be further tested by studying the TCR usage/sequence and the GEP of diverse subtypes of γδ-PTCL.

The NKCL shows a distinct GEP compared with CT(αβ)-PTCL defined previously.[17] The re-classified γδ-PTCL (NHS) and HSTCL are also distinct from CT(αβ)-PTCL. The latter entity exhibits high levels of *TCRα* and *β* transcripts, but not *TCRγ* and *δ* transcripts, and shows more functional characteristic of $CD8^+$ T cells. These cases tend to be more proliferative and show lower expression of *CD56, NCR1 (NKP46)* and *TCRδ* mRNA, but high expression of TCR signaling molecules. Clinically, all these CT subtypes of PTCL are highly aggressive and are associated with a short survival.

As patients with NK-cell malignancies have a poor outcome with current therapies,[4] novel approaches are needed to improve survival. Comparison of NKCL with PTCL-NOS yielded mostly pathways that are differentially expressed between NK and T cells and provided little insight into the biology of NKCL. Therefore, we compared NKCL with IL2-activated NK cells and NK-large-granular-lymphocytic proliferation cases. The enrichment of TGF*β* and other immunosuppressive pathways indicated an immunosuppressive microenvironment in NKCL that favors the survival of EBV infected/transformed NK cells. The significant enrichment of angiogenesis pathways in NKCL may be due the vascular destruction in these tumors resulting in hypoxia and activation of HIF1α. The genotoxic stress responsive gene signature indicated the presence of conditions that may activate TP53 function. *TP53* is negatively regulated by MDM2, an E3 ubiquitin ligase and positively by an ubiquitin-specific protease-7 (USP7).[48] EBV nuclear-antigen 1 can stably bind to USP7 and promote TP53 degradation by MDM2.[49] Thus, targeting MDM2 function may provide a therapeutic option in ENTKL.

We also observed activation of WNT and NOTCH-1 pathways that was also described by Huang *et al*[15] in a series of seven ENKTL cases. We tested two γ-secretase inhibitors in NK-cell lines, and observed growth and survival inhibition in the tested NK-cell lines validating the significance of the NOTCH pathway in neoplastic cells. We also tested an AURKA inhibitor as the phosphorylated AURKA (activated form) was present in all NK-cell lines and aside from its role in centrosome regulation and mitotic spindle formation,[50] AURKA can influence multiple signaling pathways that promote oncogenesis. Previous reports have shown that AURKA can phosphorylate TP53 at serine-215 resulting in its inactivation and degradation.[34] It also upregulates MYC and telomerase activity[32] and promotes WNT signaling.[33] Some of these signatures were significantly enriched in NKCL (Table 3). AURKA is also located at a frequently amplified locus (20q13) in NK-cell lymphoma.[31] The AURKA inhibitor MK-8745[51] induced cell cycle arrest and apoptosis in all NK-cell lines tested. There was a decrease in phosphorylated AURKA level with a concomitant decrease in its regulator TPX2.[52] AURKA inactivation was associated with increased TP53 level and decreased Survivin (TP53 target) level. Survivin is not only an anti-apoptotic factor, it is also essential for proper chromosome segregation and cytokinesis, thus augmenting AURKA.[53] These molecular events converge to induce cell cycle arrest and increased apoptosis. These findings suggest that MK-8745 could be a novel therapeutic agent for NK-cell-derived lymphomas.

In conclusion our molecular classifier identified lymphomas originating from NK cells as well as a subset of γδ T-cell with a very similar gene-expression profile to NK cells. These γδ PTCLs have distinct GEP compared with CT(αβ)-PTCLs and HSTCL and may be derived from an ontogenically and functionally distinct subset of γδ T cells. The Gene set enrichment analysis indicated an immunosuppressive microenvironment, genotoxic stress, angiogenesis and activation of NOTCH, and WNT signaling in ENKTL. The Notch inhibitors could inhibit growth of NK-cell lines, but the AURKA inhibitor was more effective probably because it affected multiple pathways. Pathway analysis and testing can rationally identify promising candidates for therapeutic trials in ENKTL.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)