Gene expression analysis of hypersensitivity to mosquito bite, chronic active EBV infection and NK/T-lymphoma/leukemia

Kana Washio[1,2], Takashi Oka[2], Lamia Abd Al Kader[2,6], Michiko Muraoka[1], Akira Shimada[1], Megumi Oda[1], Hiaki Sato[2], Katsuyoshi Takata[2], Yoshitoyo Kagami[3], Norio Shimizu[4], Seiichi Kato[5], Hiroshi Kimura[7], Kazunori Nishizaki[8], Tadashi Yoshino[2], Hirokazu Tsukahara[1]

[1]Department of Pediatrics, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; [2]Department of Pathology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; [3]Division of Molecular Medicine, Aichi Cancer Center Research Institute, Nagoya, Japan; [4]Department of Virology, Division of Virology & Immunology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan; [5]Department of Pathology and Laboratory Medicine, Nagoya University Hospital, Nagoya, Japan; [6]Department of Pathology, Faculty of Medicine, Mansoura University, Egypt; [7]Department of Virology, Nagoya University Graduate School of Medicine, Nagoya, Japan, [8]Department of Otorhinolaryngology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Corresponding author: Takashi Oka, PhD., DMSc.

Address: Department of Pathology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 2-5-1 Shikata-chou, Kita-ku, Okayama 700-8558, Japan

E-mail:oka@md.okayama-u.ac.jp, Phone:(+81) 86-235-7150, Fax:(+81) 86-235-7156

Running title: Gene expression profile of NK-cell disorders

Total word count: 3,492, Abstract word count: 148, Tables:2, Figures: 4, Supplementary Figures:4, Supplementary Tables: 9, Number of reference: 44

Keywords: CAEBV, HMB, NK-LPD, NK-lymphoma/leukemia, microarray

ABSTRACT

The human herpes virus, Epstein-Barr virus (EBV), is a known oncogenic virus and plays important roles in life-threatening T/NK-cell lymphoproliferative disorders (T/NK-cell LPD) such as hypersensitivity to mosquito bite (HMB), chronic active EBV infection (CAEBV), and NK/T-cell lymphoma/leukemia. During the clinical courses of HMB and CAEBV, patients frequently develop malignant lymphomas and the diseases passively progress sequentially. In the present study, gene expression of $CD16^{(-)}CD56^{(+)}$-, $EBV^{(+)}$HMB, CAEBV, NK-lymphoma, and NK-leukemia cell lines, which were established from patients, was analyzed using oligonucleotide microarrays and compared to that of $CD56^{bright}CD16^{dim/-}$NK cells from healthy donors. Principal components analysis showed that CAEBV and NK-lymphoma cells were relatively closely located, indicating that they had similar expression profiles. Unsupervised hierarchal clustering analyses of microarray data and gene ontology analysis revealed specific gene clusters and identified several candidate genes responsible for disease that can be used to discriminate each category of NK-LPD and NK-cell lymphoma/leukemia.

INTRODUCTION

The human herpes virus, Epstein-Barr virus (EBV), is a known oncogenic virus. EBV plays important roles in the oncogenesis of some tumors such as Burkitt's lymphoma, nasopharyngeal carcinoma, and gastric cancer. Since the late 1970s, EBV has been thought to be associated with life-threatening T/natural killer-cell lymphoproliferative disorders (T/NK-cell LPD) such as hypersensitivity to mosquito bite (HMB), hydroa vacciniforme, chronic active EBV infection (CAEBV), and NK/T-cell lymphoma/leukemia [1-8].

T/NK-cell LPD are often complicated with hemophagocytic syndrome and become fatal. Additionally, patients frequently develop malignant lymphomas during the clinical courses of HMB and CAEBV. Indeed, there have been some reports of fatal cases in which CAEBV developed from HMB and progressed to nasal NK/T-cell lymphoma, suggesting that T/NK-cell LPD are sequential diseases [9-13]. Difficulties associated with the study of EBV-associated T/NK-cell LPD include:

1) Unknown pathogenesis; studies have shown that, in these diseases, EBV somehow infects T or NK cells, but the mechanism(s) of infection, and how EBV affects these cells as an oncogenic virus, remain unclear [1, 2, 3].

2) Poor prognosis; severe HMB and CAEBV are treated with immunosuppressant and anti-cancer drugs, but the efficacy of these drugs is very limited, and hematopoietic stem cell transplantation (HSCT) is the only curative therapy. The five-year overall survival of NK/T-cell lymphoma/leukemia patients ranges from 8 to 55% [11, 13, 14]. As such, the prognosis of EBV-associated T/NK-cell LPD is very poor.

3) Differences in disease prevalence between areas, and rarity of the disease; EBV-associated T/NK-cell LPD are very rare, especially in Europe and North America, and they are usually observed in East Asian countries, including

Japan. The areas and patient numbers for these diseases are limited, which might explain why there are so few investigations of T/NK-cell LPD.

Human NK cells are divided into different subpopulations based on relative expression of the surface markers CD16 and CD56 [2, 6, 15]. The two major subsets are $CD56^{bright}CD16^{dim/-}$ and $CD56^{dim}CD16^{+}$. $CD56^{bright}CD16^{dim/-}$NK cells comprise the majority of the NK cell population in secondary lymphoid tissues such as the lymph nodes, tonsils and spleen. Although they produce many cytokines, they have poor cytotoxic ability compared to $CD56^{dim}CD16^{+}$NK cells, which comprise the majority of the NK cell population in peripheral blood [2, 6, 16, 17].

In the present study, we focused on EBV-associated NK-LPD using the surface phenotype $CD56^{bright}CD16^{dim/-}$ of EBV-associated T/NK-cell LPD cell lines. Some of the EBV-negative T/NK-cell LPD cell lines had a $CD56^{dim}CD16^{+}$ surface phenotype. We compared the gene expression of the EBV-associated $CD56^{bright}CD16^{dim/-}$T/NK-cell LPD cell lines that we analyzed using oligonucleotide microarrays with that of control $CD56^{bright}CD16^{dim/-}$NK cells, which were freshly collected from the tonsils of healthy donors, and of peripheral blood mononuclear cells (PBMCs) from healthy volunteers. Several comprehensive expression analyses of CAEBV have been reported, including an EBV DNA microarray and an oligonucleotide DNA microarray of nasal NK/T lymphoma and CAEBV [18-21]. However, to the best of our knowledge, the present study is the first to use a microarray to investigate expression profiles of EBV-associated $CD56^{bright}CD16^{dim/-}$T/NK-cell LPD, including the sequential diseases of HMB, CAEBV, NK-lymphoma, and NK-leukemia.

We isolated several candidate genes responsible for disease in each step of the sequential disease transformation in the present gene expression profile analyses.

MATERIALS AND METHODS

Patient Samples

Paraffin blocks of lymphoma samples from the archives of the Department of Pathology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, the Department of Pathology and Laboratory Medicine, Nagoya University Hospital, and related hospitals were used. Reactive lymphoid hyperplasia (RLH) and tonsils were used as normal controls. Informed consent was obtained from all patients for the analysis of their tissue samples. This study was approved by the Institutional Review Board of the Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University and related hospitals, in accordance with the Declaration of Helsinki.

Cell Culture

Five EBV-positive NK tumor cell lines were used (Table S1). KAI3, SNK10 and NK-YS were established from the peripheral blood of a patient with HMB [22], with CAEBV [23] and with nasal NK/T lymphoma in a leukemic state [24], respectively. SNK6 and HANK1 were derived from the primary lesion of a nasal NK/T lymphoma [23, 25]. KAI3 was maintained in AIM-V (GIBCO, Grand Island, NY) supplemented with 10% bovine serum (Sankou Junyaku, Chiba, Japan) and 50 U/ml interleukin (IL)-2 (Strathman Biotech GMBH, Hannover, Germany). SNK10 and SNK6 were maintained in Gm^{+}RPMI (GIBCO) supplemented with 10% human serum and 700 U/ml IL-2. HANK1 and NK-YS were maintained in IMDM (GIBCO) supplemented with 10% bovine serum and 100 U/ml IL-2.

PBMCs were obtained from healthy donors using Ficoll-Paque Plus density gradient centrifugation. $CD16^{(-)}$NK cells were isolated from the tonsils of healthy donors using the MACS $CD56^{(+)}CD16^{(-)}$NK Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

Microarray Analysis

Total RNA was isolated from cells using the RNeasy Mini or Micro Kit (Qiagen, Düsseldorf, Germany), followed by DNase treatment to remove contaminating genomic DNA. RNA quality was verified using agarose gel electrophoresis. The ratios of the optical densities measured at 260 nm and 280 nm (OD260/OD280) were between 1.8 and 2.1.

Synthesis of cDNA, cRNA labeling, and hybridization, were performed using the GeneChip One-cycle Target Labeling Kit and Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA).

The Affymetrix Microarray Suite 5.0 Statistical Algorithm was used for calculation of the signal intensity and the detection calls for each gene. Probes associated with low expression, and probes classified as having absent detection calls in all samples, were excluded. Probe datasets were normalized through the following steps using the Subio Platform software (Subio, Nagoya, Japan): 1) shifting of low signals of <1.0 to 1.0; 2) transforming into log2; 3) applying global normalization to the 75th percentile; and then 4) calculating ratio against the mean of PBMC1 and PBMC2. Principal Component Analysis (PCA) and Hierarchal Clustering Analyses (HCA) were performed using the differentially expressed probe sets between the six groups identified by one-way analysis of variance (ANOVA; $p < 0.05$) as previously described [26]. Differentially expressed genes were subjected to gene ontology (GO) analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID). Pathway-specific analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway database.

Immunohistochemical Staining

Paraffin blocks were retrieved and new CAEBV and NK lymphoma sections were used to ensure staining quality. Mouse monoclonal antibodies against CD56, CD3 (NCL-CD56-1B6 and NCL-L-CD3-565, respectively; Novocastra, Newcastle upon

Tyne, UK) and CD16 (No.7530, Becton Dickinson, San Jose, CA, USA); rabbit monoclonal anti-survivin antibody (#2808, Cell Signaling Technology, Beverly, MA, USA); and rabbit polyclonal anti-SHP2 (GTX101062, GeneTex, San Antonio, TX, USA) and anti-SHP1 (sc287, Santa Cruz Biotechnology, Santa Cruz, MA, USA) antibodies were used. Staining was performed with a Leica BOND-MAX™ autostainer (Leica Biosystems, Melbourne, VIC, Australia). Both staining intensity and the percentage of positive tumor cells were evaluated in ten high-power fields by three investigators.

Fluorescent immunohistochemical staining

The procedure was as described previously [27]. Rabbit monoclonal anti-Fas (ab133619, Abcam, MA, USA), mouse monoclonal anti-CD56, anti-mouse IgG Alexa Flour 555 and anti-rabbit IgG Alexa Flour 488 (Invitrogen, Oregon, USA) antibodies were used for this analysis.

Western blotting analysis

Western blot analyses of the cell lines were performed as described previously [28]. Antibodies used for these analyses were: anti-SHP1, anti-SHP2 and anti-survivin antibodies as described for immunohistochemical staining, and rabbit monoclonal anti-Cyclin D1, anti-Cyclin A2 (#2978 and #4656 respectively, Cell Signaling, Beverly, MA, USA), anti-Cyclin E2 (ab40890, Abcam) and anti-Fas (ab133619, Abcam) antibody; mouse anti-beta-actin (A5441, Sigma Aldrich, St. Louis, USA) antibody; and HRP-labeled anti-rabbit or anti-mouse (NA9340 and NA9310, respectively, GE Healthcare, Little Chalfont, Buckinghamshire, UK) antibodies.

Flow cytometric (FCM) analysis of the cell cycle

Cells were stained with propidium iodide (PI), and the cell cycle was analyzed using the FACS Calibur (BD BioScience, CA, USA) as described previously [29].

RESULTS

Principal Component Analysis and Unsupervised Hierarchal Clustering Analyses.

PCA of gene expression indicated some distance between three groups of cells; 1) normal PBMCs, 2) NK-type cells including normal $CD16^{(-)}$NK cells, HMB, CAEBV and NK-lymphoma, and 3) NK-leukemia, according to the first two principal components (Figure 1A). PCA analysis of the NK-type cells showed that normal $CD16^{(-)}$NK cells are distantly related to NK-LPD cells. NK-LPD cells are subdivided into three categories: 1) HMB, 2) CAEBV and NK-lymphoma, and 3) NK-leukemia. The first two principal components explained 44.4% of the variance. CAEBV and NK-lymphoma cells were relatively closely located on the scatterplot, indicating that they had similar expression profiles (Figure 1B).

HCA identified seven gene clusters according to characteristic gene expression patterns: Cluster A-1 showed downregulation and Cluster A-2 showed upregulation in HMB, CAEBV, NK-lymphoma and NK-leukemia versus normal NK cells; Cluster B-1 showed downregulation and Cluster B-2 showed upregulation in CAEBV, NK-lymphoma and NK-leukemia versus normal NK cells and HMB; Cluster C showed downregulation in NK-lymphoma and NK-leukemia versus normal NK cells, HMB and CAEBV. Cluster D-1 showed downregulation and Cluster D-2 showed upregulation in NK leukemia versus normal NK cells, HMB, CAEBV and NK-lymphoma; Clusters B, C and D showed differential expression profiles among each node of HMB, CAEBV, NK-lymphoma and NK-leukemia that discriminated each disease (Figure 1C, Table S2, S3, S4, S5, S6, S7, S8).

Gene Ontology (GO) Analyses of Differentially Expressed Genes.

GO analysis indicated significantly enriched GO categories in biological processes for each differentially expressed cluster; the top seven enriched GO term categories for each cluster are summarized in Table I.

Genes in Cluster A-1 shared common GO terms related to the “Src-homology-3

domain", "Immunoglobulin subtype", "Cytoskeleton", "Neutrophin signaling pathway", "Circadian rhythm" and "Regulation of transcription". Representative genes included *GPR82, MYO18B, CYP1B1, S100A8, HBA1 &2, NRGN, NR4A2, PTPN6, HBB, CD6, LYZ, PPBP, RNF130, KLF4, RASA3* and *LY9*.

Cluster A-2 was enriched for GO term categories such as "Membrane-encoded lumen", "DNA replication and DNA metabolic process", "RNA binding and RNA processing" and "Cell cycle and cell division", and included *RFC2, PTP4A1, JAK3, STAT3, CISH(CIS), DHFR, FOXM1, CDC45L, CXCL10, FDXR, PTPN11, BIRC5, DHRS2, GEMIN4* and *GINS3*. Cluster B-1 was enriched for GO term categories such as "Lymphocyte mediated immunity", "Membrane", "Disulfide bond", "Phosphorus metabolic process" and "Regulation of apoptosis", and included *PRKCD, B4GALT6, LGR4, MXRA7, MYOF, PTPRN2, SEPT9, AKT3, SHC4, CRTAM, CADM1* and *OSMR*. "Regulation of apoptosis" included *CADM1, CRTAM* and *PROK2*. Genes in Cluster B-2 shared common GO terms related to the "Endoplasmic reticulum", "Intracellular signaling cascade", "Membrane fraction", "Phosphate metabolic process" and "Ion transport" and included *TNFRSF10A, CCDC125, NHEDC2, CCND2, FADS2, PDGFRA, RCAN2, FADS1, HGF, UTP18* and *ATL3*.

Cluster C was enriched for GO term categories such as "Regulation of transcription", "C2 membrane targeting protein", "Oxidation reduction" and "Regulation of Wnt-receptor signaling pathway", and included *TC2N, SNX20, ZNF569, DDX3Y, PALLD, SOX4, TJP2, DHRS3, PHACTR2, CRY1, SEMA4A, LEF1, GPD2, SYTL1* and *PTPLAD2*. "Oxidation reduction" included *RFESD, ALKBH3, DHRS3, GPD2, IDG2* and *NPHP3*. "Regulation of Wnt receptor signaling pathway" included *SOX4, ADCY9, CTNNBIP1, KLRB1, LEF1, IPAR6* and *USP9Y*.

Cluster D-1 was enriched for GO term categories such as "MHC-II protein complex or Cell adhesion molecules (CAMs)", "Transmembrane", "Negative regulation of

biosynthetic process", "Transcription repressor activity" and "Negative regulation of RNA metabolic process", and included *CCDC141, SLC35E4, TMEM189, HLA-DPB1, PEPD, GNAQ, SLC16A3, BIN1, NINJ1, MYO6, BCL6, ATXN1, CRIP1, HLADRB4, TP63, ICOS, HLADRA, RHOB* and *FUNDC1*. Some genes in Cluster D-1 were categorized in "MHC-II complex or Cell adhesion molecules (CAMs)", including *CD2, ICOS, ITGB7, HLA-DMA, HLA-DOA, HLA-DPA1, HLA-DQA1* and *HLA-DRA*.

Cluster D-2 shared common GO terms related to "Proto-oncogene", "Pathway of cancer", "Embryonic morphogenesis", "Positive regulation of growth" and "Adhesion junction", and included *ATAD3A, NOX5, SNTG2, LOC643201, TJP1, SSX1, SSX2, SSX3, SPANXB1, MECOM, SPANXA2* and *NEFH*. "Pathway in cancer" included *ITGA6, SPHK1, SFN, TJP1, FGF9* and *TMP3*. "Proto-oncogene or Regulation of transcription" included *CSF1R, MECOM, SSX1, SSX2, SSX2B, SSX5 TIMP3* and *ZNF320*.

Pathway Analysis

The clusters extracted from HCA were applied to pathway analysis. Some pathways in which several genes showed altered expression patterns were extracted.

Cell cycle pathway

Many genes belonging to Cluster A-2 appear in the cell cycle pathway (Figure 2A,B). The expression levels of genes that promote cell cycle progression such as CDKs (*CDK8, CDK2, BCCIP,* and *CDKN2AIP*), cyclins (*CCNA2* (CycA), *CCNE2* (CycE), *CCNB1* (CycB), and *CCND2* (CycD)), a CDK substrate (*NUCKS1*), and *SKP2, MCM3/4/5, MYC, MAX, MINA, CDC42, CDC42SE1, CDC23, CDC6* and *CDC45L* increased as disease progressed. Genes involved in DNA mismatch repair (*MLH1, MSH6, EXO1, RPA3,* and *LIG1*) and cell cycle arrest, such as genes in the P53 signaling pathway (*TP53, TP53BP2, TRIAP1, MDM2, CDKN1A* (p21/Cip1), *CASP3, FAS, BAX*, and *CHK1/2*), showed the same expression pattern as the cell cycle progression genes.

Apoptosis

Both apoptosis-inducing and -evading gene transcription was increased in EBV-positive NK-LPD (Figure S1). The transcription of pro-apoptotic genes in the death-signaling pathway (*TNFSF10* (TRAIL), *TNFRSF10B* (TRAILR), *TNFRSF8*, *IRAK1*, *IKIP*, *BAX*, *CASP3* and *FAS)* was increased; these genes were categorized in Cluster A-2. *TNFSF10* (TRAIL), *TNFRSF10B* (TRAILR), *IRAK1*, *IKIP*, and *BAX* transcription was highest in HMB and/or CAEBV, and was decreased in EBV-positive NK-leukemia. Some apoptosis-evading genes, such as *BCL2L1* (BCLXL), *BCL2L12*, *XIAP* and *BIRC5* (survivin), were strongly expressed in EBV-positive NK-LPD versus normal NK cells. The expression levels of *TNFRSF8* and *BIRC5* increased as disease progressed.

JAK/STAT pathway

In the *JAK/STAT* pathway, the transcription levels of *JAK3* and *STAT3* were increased in EBV-positive NK-LPD versus normal NK cells (Figure 2C,D). *CSF2RB* and *IL-21R*, genes that encode cytokine receptors upstream of the *JAK/STAT* pathway, and *CIS*, *NRAS*, *SOCS3* and *Pim-1*, genes that encode downstream molecules of this pathway, showed the same expression pattern as that of *JAK3* and *STAT3*, suggesting that enhancement of this signaling pathway may induce cell proliferation. Many related genes were categorized in Cluster A-2. In EBV-positive NK-LPD, expression of the gene *PTPN6* that encodes SHP1, a negative regulator of the *JAK/STAT* pathway, was decreased, whereas expression of the gene *PTPN11* that encodes SHP2, a positive regulator of this pathway, was strongly increased.

Western blotting analysis

Western blotting analysis of the five cell lines indicated that Cyclin-D1, -E2, -A2 and SHP2 protein expression gradually increased, whereas SHP1 protein expression gradually decreased, with disease progression. Fas protein expression was strongest in CAEBV (SNK10) cells. Survivin protein expression was highest in NK-lymphoma

(HANK1, SNK6) cells (Figure 3A).

Effects of culture medium components on protein expression and the cell cycle

The effects of the different culture media used for culture of the five cell lines on the cell cycle and related gene expression were investigated. Western blotting and cell cycle analyses showed that differences between the effects of the different culture media on cell cycle and Cyclin-D1 expression were much smaller than the changes in these parameters among the cell lines (Figure S2 & Table S9).

Immunohistochemical analysis

Patient specimens of NK/T-lymphoma, CAEBV and RLH were immunohistochemically stained for protein expression of SHP-1, SHP-2, and survivin, which are encoded by *PTPN6*, *PTPN11* and *BIRC5*, respectively (Figure 3B, Table II). SHP-1 staining was positive in both normal NK (72%) and T (98%) cells in RLH, and the strength and percentage of SHP-1 staining was lower in NK/T-lymphoma (20.0% SHP-1 positive), and in NK- or T-cell-type CAEBV (0% and 40.0% SHP-1 positive, respectively). On the other hand, SHP-2 staining was negative in both normal NK (3.5%) and T (2.3%) cells in RLH, whereas the strength and percentage of SHP-2 staining gradually increased from NK- to T-cell-type CAEBV, to NK/T-lymphoma (28.6%, 40.0% and 60.0% SHP-2 positive, respectively). Survivin staining was faintly positive in both normal NK (6%) and T (1.3%) cells in RLH, and the strength and percentage of survivin staining was much higher in NK- or T-cell-type CAEBV (85.7% and 100% survivin positive respectively) and in NK/T-lymphoma (80.0% survivin positive). Fas staining was positive in NK-type CAEBV (71.4%) and that was lower in NK/T-lymphoma (20.0%) and normal NK cells in RLH (5.0%).

DISCUSSION

PCA showed that the distance between $CD56^{(+)}$CAEBV and NK-lymphoma cells in the first two principal components was very small, indicating that $CD56^{(+)}$CAEBV and

NK-lymphoma have a closely related expression profile (Figure 1). However, unsupervised HCA and GO analysis indicated differential expression patterns of some of the specific genes in Clusters B-1 and C between these cells. Thus, several candidate genes responsible for disease were identified (Table I, S4 and S6) that may discriminate two categories of disease as well as predict clinical outcome.

NK-lymphoma/leukemia showed increased expression of genes involved in cell cycle progression, such as CDKs, cyclins, and a CDK substrate, with disease progression (Figure 2A,B). The anti-apoptotic genes *BIRC5* (survivin) and *BCL-XL,* were strongly expressed, especially in NK-lymphoma/leukemia, indicating that NK-lymphoma/leukemia have more severe malignant characteristics than the other cell lines. In contrast, EBV-positive HMB and/or CAEBV showed gene expression patterns characteristic of both infection, e.g., expression of genes involved in cell cycle arrest to allow virus replication, and also of malignancy e.g., expression of genes involved in enhancement of cell cycle progression and in anti-apoptosis leading to uncontrolled cell proliferation. Transcription of pro-apoptotic genes in the death-signaling pathway (*TNFSF10* (TRAIL), *TNFRSF10B* (TRAILR), *BAX* and *FAS*) were highest in HMB and/or CAEBV, indicating that HMB and/or CAEBV are competent to induce apoptosis and that they are intermediate diseases between premalignant and malignant (Figure S1,3A). This conclusion is consistent with the proposal of Oshima et al. [1] that CAEBV is pathologically categorized as a continuous spectrum ranging from a smoldering phase to overt leukemia/lymphoma. These gene expression patterns are consistent with the clinical features of EBV-positive NK-LPD, such as viral genome increases in peripheral blood or the sequential progression of disease [9-13].

The expression patterns of some genes involved in the JAK/STAT pathway were altered in EBV-positive NK-LPD, especially of *PTPN6* (SHP1) and *PTPN11* (SHP2), which encode non-receptor type protein tyrosine phosphatases that are reported to be

involved in some malignant diseases [28,30-36]. SHP1 is a negative-regulator of JAK/STAT signaling [28,30,31]. Decreased expression of SHP1 in HMB (KAI3) cells may be responsible for the hypersensitivity to mosquito bite and prolonged inflammation. Downregulation of *PTPN6* gene expression by CpG-island DNA hypermethylation and the loss of heterozygosity are frequently observed in many lymphomas/leukemias [28,30-35]. *PTPN11* was isolated as the gene whose germline mutation is responsible for Noonan syndrome [37]. Somatic mutations in *PTPN11* upregulate SHP2 expression and result in juvenile myelomonocytic leukemia, myelodysplastic syndrome or acute myeloid leukemia in pediatric patients and in gastric cancer with CagA-positive *Helicobacter pylori* infection [38-40]. SHP2 is implicated in multiple cell signaling pathways such as the Ras/MAPK, JAK/STAT, PI3K-AKT, NFkB, and NAFT pathways [39,41]. In the present study, the gene expression profiles of EBV-positive NK-LPD cell lines revealed suppression of *PTPN6* but significantly increased *PTPN11* expression even in HMB early stage cells versus normal NK cells, which were confirmed by Western blotting analysis. These evidences suggest that SHP1 suppression and SHP2 overexpression are a key event in the tumorigenesis of EBV-positive NK-LPD, similar to the mechanism of H pylori(+), EBV(+) gastric cancer [42].

Finally, we propose a hypothetical scheme for the mechanism of EBV-positive NK-LPD onset as follows (Figure 4). After EBV infection, SHP1 and SHP2 expression dramatically changes, which might affect the JAK/STAT and RAS/MAPK pathways, leading to abnormal proliferation. The increased expression of cyclins and CDKs may enhance these events. Viral genes, such as *LMP1* and *ZEBRA*, also affect the cell cycle and increase the expression of DNA checkpoint genes, such as *p21*, *p53* and *chk1/2*, for virus replication [17,43,44]. However, HMB and CAEBV still display some characteristics of normal cells and the expression of pro-apoptotic genes is increased in

these diseases versus normal NK cells. As progression to NK-lymphoma/leukemia, pro-apoptotic gene expression is reduced and the expression of anti-apoptotic genes increase. These characteristic expression pattern changes correspond to the typical clinical course of EBV-positive NK-LPD; NK-LPD sometimes onsets as HMB and progresses to CAEBV, then to NK-lymphoma, and ultimately to NK-leukemia.

EBV-positive NK-LPDs diseases are only observed in East Asian countries and the mechanisms underlying disease onset and progression remain to be elucidated. The prognosis of these diseases is not good. Over the long clinical course of CAEBV, almost all patients encounter severe complications such as hemophagocytic syndrome, myocarditis, NK-lymphoma or NK-leukemia, which worsen if not given HSCT with high-dose chemotherapy. In NK-lymphoma, the novel and most promising chemotherapy SMLE can achieve complete remission in only 50% of patients [44]. The present study revealed several gene clusters, key molecules and relevant pathways that are related to the tumorigenesis or progression of EBV-positive NK-LPD. These findings provide important information for understanding the molecular mechanisms underlying the sequential transformation caused by EBV infection in EBV-associated NK-LPD, and may also contribute to the development of new therapeutic strategies for these fatal diseases.

Acknowledgements

The authors gratefully acknowledge Dr. K. Iwatsuki (Okayama University, Japan) for kindly providing the KAI3 cell line. We also thank Ms. H. Nakamura and Ms. M. Shiotani for their technical support.

Disclosure/Conflict of Interest

This work was supported by a grant from The Ministry of Education, Culture, Sports, Science and Technology of Japan to T.O. (#22590312). A patent entitled “Measurement method to assist Laboratory testing for progression from EBV(+)

T/NK-cell lymphoproliferative disorders to NK/T-cell lymphoma/leukemia", is pending (The original patent is in Japanese) (TO, KW, TY).

References

1. Oshima K, Kimura H, Yoshino T, Kim CW, Ko YH *et al*. Review article Proposed categorization of pathological states of EBV-associated T/natural killer-cell lymphoproliferative disorder (LPD) in children and young adults: Overlap with chronic active EBV infection and infantile fulminant EBV-LPD. *Pathol Int* 2008; **58**: 209-217

2. Park S, Ko YH. Epstein-Barr virus-associated T/natural killer-cell lymphoproliferative disorders. J Dermatol. 2014 Jan; 41(1):29-39.

3. Ohga S, Ishimura M, Yoshimoto G, Miyamoto T, Takada H, Tanaka T, Ohshima K, Ogawa Y, Imadome K, Abe Y, Akashi K, Hara T. Clonal origin of Epstein-Barr virus (EBV)-infected T/NK-cell subpopulations in EBV-positive T/NK-cell lymphoproliferative disorders of childhood. *J Clin Virol*. 2011 May; 51(1): 31-7. doi: 10.1016/j.jcv.2011.01.014.

4. Lee WI, Lin JJ, Hsieh MY, Lin SJ, Jaing TH, Chen SH, Hung IJ, Yang CP, Chen CJ, Huang YC, Li SP, Huang JL. Immunologic difference between hypersensitivity to mosquito bite and hemophagocytic lymphohistiocytosis associated with Epstein-Barr virus infection. *PLoS One*. 2013 Oct 18; 8(10): e76711. doi: 10.1371/journal.pone.0076711.

5. Asada H[1]. Hypersensitivity to mosquito bites: a unique pathogenic mechanism linking Epstein-Barr virus infection, allergy and oncogenesis. *J Dermatol Sci*. 2007 Mar; 45(3): 153-60. Epub 2006 Dec 13.

6. Hiroshi Kimura. Pathogenesis of chronic active Epstein-Barr virus infection: Is this an infectious disease, lymphoproliferative disorder, or immunodeficiency? *Rev. Med. Virol*. 2006; 16: 251–261.

7. Lee WI, Lin JJ, Hsieh MY, Lin SJ, Jaing TH, Chen SH, Hung IJ, Yang CP, Chen CJ, Huang YC, Li SP, Huang JL. Immunologic difference between hypersensitivity to mosquito bite and hemophagocytic lymphohistiocytosis associated with Epstein-Barr

virus infection. *PLoS One*. 2013 Oct 18; 8(10): e76711. doi:10.1371/journal.pone.0076711. eCollection 2013.

8. Takahashi E, Ohshima K, Kimura H, Hara K, Suzuki R, Kawa K, Eimoto T, Nakamura S; NK-cell Tumor Study Group. Clinicopathological analysis of the age-related differences in patients with Epstein-Barr virus (EBV)-associated extranasal natural killer (NK)/T-cell lymphoma with reference to the relationship with aggressive NK cell leukaemia and chronic active EBV infection-associated lymphoproliferative disorders. *Histopathology*. 2011 Oct; 59(4): 660-71. doi: 10.1111/j.

9. Nitta Y, Iwatsuki K, Kimura H, Kojima S, Morisima T *et al*. Fatal natural killer cell lymphoma arising in a patient with a corp of Epstein-Barr virus-associated disorders. *Eur J Dermatol* 2005; **153**: 981-986

10. Picard C, Gouarin S, Comoz F, Barreau M, Verneuil L, Troussard X, Dompmartin A. Chronic active Epstein-Barr virus infection with cutaneous and sinus lymphoproliferation in a white female patient with 25 years' follow-up: an original case report. *Br J Dermatol*. 2015 Jul 4. doi: 10.1111/bjd.14014.

11. Fujiwara S, Kimura H, Imadome K, Arai A, Kodama E, Morio T, Shimizu N, Wakiguchi H. Current research on chronic active Epstein-Barr virus infection in Japan. *Pediatr Int*. 2014 Apr; 56(2):159-66.

12. Ishihara S, Okada S, Wakiguchi H, Kurashige T, Hirai K, Kawa-Ha K. Clonal lymphoproliferation following chronic active Epstein-Barr virus infection and hypersensitivity to mosquito bites. *Am J Hematol*. 1997 Apr; 54(4): 276-81.

13. Hiroshi Kimura, Yoshinori Ito, Shinji Kawabe, Kensei Gotoh, Yoshiyuki Takahashi, Seiji Kojima, Tomoki Naoe, Shinichi Esaki, Atsushi Kikuta, Akihisa Sawada, Keisei Kawa, Koichi Ohshima, and Shigeo Nakamura. EBV-associated T/NK–cell lymphoproliferative diseases in nonimmunocompromised hosts: prospective analysis of 108 cases. *Blood* 2012; 119(3): 673-686

14. Suzuki R. Treatment of advanced extranodal NK/T cell lymphoma, nasal type and aggressive NK-cell leukemia. *Int H Hematol* 2010; 92: 697-701

15. Caligiuri MA. Human natural killer cells. *Blood* 2008; **112**: 461-469

16. Poli A, Michel T, Thérésine M, Andrès E, Hentges F *et al*. CD56^{bright} natural killer (NK) cells: an important NK subset. *Immunology* 2009; **126**: 458-465

17. Yoshihito Kasahara, Akihiro Yachie. Cell type specific infection of Epstein/Barr virus (EBV) in EBVassociated hemophagocytic lymphohistiocytosis and chronic active EBV infection. *Critical Reviews in Oncology/Hematology* 44 (2002) 283-294

18. Zhang Y, Ohyashiki JH, Takaku T, Shimizu N, Ohyashiki K. Transcriptional profiling of Epstein-Barr virus (EBV) genes and host cellular genes in nasal NK/T-cell lymphoma and chronic active EBV infection. *Br J Cancer*. 2006; 94(4): 599-608.

19. Ito Y, Shibata-Watanabe Y, Ushijima Y, Kawada J, Nishiyama Y, Kojima S, Kimura H. Oligonucleotide microarray analysis of gene expression profiles followed by real-time reverse-transcriptase polymerase chain reaction assay in chronic active Epstein-Barr virus infection. *J Infect Dis*. 2008 Mar 1; 197(5): 663-6.

20. Murakami M, Hashida Y, Imajoh M, Maeda A, Kamioka M, Senda Y, Sato T, Fujieda M, Wakiguchi H, Daibata M. PCR array analysis of gene expression profiles in chronic active Epstein-Barr virus infection. Microbes *Infect*. 2014 Jul; 16(7): 581-6.

21. Kawano Y, Iwata S, Kawada J, Gotoh K, Suzuki M, Torii Y, Kojima S, Kimura H, Ito Y. Plasma viral microRNA profiles reveal potential biomarkers for chronic active Epstein-Barr virus infection.*J Infect Dis*. 2013 Sep 1; 208(5): 771-9. doi: 10.1093/infdis/jit222.

22. Tsuge I, Morisima T, Morita M, Kimura H, Kuzushima K *et al*. Characterization of Epstein-Barr virus (EBV)-infected natural killer (NK) cell proliferation in patients with severe mosquito allergy; establishment of an IL-2-dependennt NK-like cell line. *Clin Exp Immunol* 1999; **115**: 385-392

23. Zhang Y, Nagata H, Ikeuchi T, Mukai H, Oyoshi MK *et al*. Common cytological and cytogenetic features of Epstein-Barr virus (EBV)-positive natural killer (NK) cells and cell lines derived from patients with nasal T/NK-cell lymphomas, chronic active EBV infection and hydroa vacciniform-like eruptions. *British Journal of Haematology* 2003; **121**: 805-814

24. Tsuchiyama J, Yoshino T, Mori M, Kondoh E, Oka T *et al*. Characterization of a Novel Human Natural Killer-Cell Line (NK-YS) Established From Natural Killer Cell Lymphoma/Leukemia Associated with Epstein-Barr Virus Infection. *Blood* 1998; **92**: 1374-1383

25. Kagami Y, Nakamura S, Suzuki R, Iida S, Yatabe Y *et al*. Establishment of an IL-2-dependent cell line derived from ‘nasal-type’ NK/T-cell lymphoma of CD2+, sCD3-, CD3 ε +, CD56+ phenotype and associated with the Epstein-Barr virus. *Journal of haematology* 1998; **103**: 669-677

26. Tsuge M, Oka T, Yamashita N, Saito Y, Fujii Y, Nagaoka Y, Yashiro M, Tsukahara H and Morishima T, Gene expression analysis in children with complex seizures due to influenza A(H1N1)pdm09 or rotavirus gastroenteritis. J Neurovirol. 2014:73-84. doi: 10.1007/s13365-013-0231-5.

27. Abd Al Kader L, Oka T, Takata K, et al. In aggressive variants of non-Hodgkin lymphomas, Ezh2 is strongly expressed and polycomb repressive complex PRC1.4 dominates over PRC1.2, Virchows Arch. 2013; 463(5): 697-711.

28. Oka T, Ouchida M, Koyama M *et al*. Gene silencing of the tyrosine phosphatase SHP1 gene by aberrant methylation in leukemias /lymphomas. *Cancer Res*. 2002; **62**: 6390-6394

29. Deitch AD, Law H, deVere White RA stable propidium iodide staining procedure for flow cytometry. J Histochem Cytochem. 1982 Sep;30(9):967-72.

30. Xu D, Qu CK, Protein tyrosine phosphatase in the JAK/STAT pathway. *NIH Public*

Access 2008; **13**: 4925-4932

31. Han Y, Amin HM, Franko B, Franz C, Shi X *et al*. Loss of enhances JAK3/STAT3 signaling and decreases proteasome degradation of JAK3 and NPM-ALK in ALK+ anaplastic large-cell lymphoma. *Blood* 2006; **108**: 2796-2803

32. Oka, T., Yoshino T., Hayashi K., Ohara N., Nakanishi T., Yamaai Y., Hiraki A., Aoki-Sogawa C., Kondo E., Teramoto N., Takahashi K., Tsuchiyama J., and Akagi T. Reduction of hematopoietic cell-specific tyroshine phosphatase SHP-1 gene expression in natural killer cell lymphoma and various types of lymphomas/leukemias: Combination analysis with cDNA expression array and tissue microarray *American J Pathol* 159: 1495-1505, (2001)

33. Oka T., Yoshino T., Hayashi K. and Akagi T. Abnormal expression of protein-tyrosine phosphatase SHP1 gene in malignant lymphomas and leukemias. *Jpn. J. Cancer Clin* 2002; 48(10): 561-568

34. Koyama M, Oka T, Ouchida M, Nakatani Y, Nishiuchi R, Yoshino T, Hayashi K, Akagi T and Seino Y. Activated proliferation of B-cell lymphomas/leukemias with the *SHP1* gene silencing by aberrant CpG methylation. *Lab Invest*. 2003, 83(12), 1849-1858

35. T. Oka., M. Ouchida., M. Tanimoto., K. Shimizu and T. Yoshino., High frequent gene silencing of hematopoietic cell specific protein tyrosine phosphatase SHP1 in hematopoietic cell malignancies. In “Gene silencing: New research”., edited by G.W. Redberry, pp1-34, 2006, Nova Science Publishers, Inc.(New York)

36. Kang X, Kim J, Deng M, John S, Chen H, Wu G, Phan H, Zhang CC. Inhibitory leukocyte immunoglobulin-like receptors: immune checkpoint proteins and tumor sustaining factors. *Cell Cycle.* 2015 Dec 4:0. [Epub ahead of print]

37. Tartaglia M, Mehler EL, Goldberg R, Zampino G, Brunner HG *et al*. Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome.

Nat Genet 2001; **29**: 465-8

38. Tartaglia M, Niemeyer CM, Fragale A, Song X, Buechner J *et al*. Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. *Nat Genet* 2003; **34**: 148-50

39. Xu D, Qu CK, Protein tyrosine phosphatase in the JAK/STAT pathway. *NIH Public Access* 2008; **13**: 4925-4932) (Tonks, N. K. Protein tyrosine phosphatases: from genes, to function, to disease. *Nat. Rev. Mol. Cell. Biol.* 2006; **7**: 833-846

40. Hatakeyama M, Higashi H. Helicobacter pylori CagA: a new paradigm for bacterial carcinogensis. *Cancer Sci* 2005; **96**: 835-43

41. Neel BG, Gu H, Pao L. The 'Shp' ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. *Trends Biochem Sci* 2003; **28**: 284-93

42. Saju P, Murata-Kamiya N, Hayashi T, Senda Y, Nagase L, Noda S, Matsusaka K, Funata S, Kunita A, Urabe M, Seto Y, Fukayama M, Kaneda A, Hatakeyama M. Host SHP1 phosphatase antagonizes Helicobacter pylori CagA and can be downregulated by Epstein-Barr virus. Nat Microbiol. 2016 Mar 14;1:16026. doi: 10.1038/nmicrobiol.2016.2

43. Ito T, Kawazu H, Murata T, Iwata S, Arakawa S, Sato Y, Kuzushima K, Goshima F, Kimura H. Role of latent membrane protein 1 in chronic active Epstein-Barr virus infection-derived T/NK-cell proliferation. *Cancer Med*. 2014 Aug; 3(4):787-95.

44. Yamauchi Y, Tachiband Y, Maeda A, Wakiguchi H, Usui M, Kurata T, Sairenji T. Evaluation of antibodies to the Epstein-Barr virus immediate early gene product ZEBRA by a new enzyme-linked immunosorbent assay. *Intervirology*. 1998;41(6):278-84.

Figure legends

Figure 1. ***Principal component analysis (PCA) of the gene expression in HMB,***

CAEBV, NK/T-lymphoma and NK/T-leukemia cell lines, CD16$^{(-)}$normal NK cells and healthy PBMCs. **A)** There is some distance between three groups, 1) healthy PBMCs, 2) NK-type cells, including normal CD16$^{(-)}$NK cells, HMB, CAEBV and NK-lymphoma, and 3) NK-leukemia, according to the first two principal components, indicating that HMB, CAEBV and NK-lymphoma cell lines retain normal CD16$^{(-)}$NK cell characters. **B)** PCA analysis of NK-type cells showing that normal CD16$^{(-)}$NK cells are distantly related to NK-LPD cells. NK-LPD cells are subdivided into three categories: 1) HMB, 2) CAEBV and NK-lymphoma, and 3) NK-leukemia. CAEBV cells show a close relation to NK-lymphoma cells. ***Hierarchical clustering analysis (HCA) of the gene expression in HMB, CAEBV, NK/T-lymphoma and NK/T-leukemia cell lines, CD16$^{(-)}$normal NK cells and healthy PBMCs.*** **C-I)** ***Clusters were classified into seven categories according to characteristic expression patterns.*** **C-II)** Cluster A-1 shows downregulation and A-2 shows upregulation in HMB, CAEBV, NK-lymphoma and NK-leukemia when compared to normal NK cells. Cluster B-1 shows downregulation and B-2 shows upregulation in CAEBV, NK-lymphoma and NK-leukemia when compared to normal NK cells and HMB. Cluster C shows downregulation in NK-lymphoma and NK-leukemia when compared to normal NK cells, HMB and CAEBV. Cluster D-1 shows downregulation and D-2 shows upregulation in NK-leukemia when compared to normal NK cells, HMB, CAEBV and NK-lymphoma.

Figure 2. ***KEGG pathway analysis of the cell cycle.*** **A)** Gene expression changes among cell lines and normal NK cells were mapped on to the cell cycle pathway. The colored boxes indicate significant upregulation (red) or downregulation (blue) of transcripts in normal PBMCs, normal NK cells, HMB, CAEBV, NK-lymphoma and NK-leukemia. **B)** The graphs show the mRNA expression levels of representative genes. Many genes belonging to Cluster A in Figure **1 C-II** appear in the cell cycle pathway.

The expression levels of cell cycle progression genes, such as for CDKs and cyclins, increase with disease progression. Genes involved in DNA damage checkpoints and cell cycle arrest, such as *p53*, *p21* and *Chk1/2*, show the same expression pattern as cell cycle progression genes. ***KEGG pathway analysis of JAK/STAT signaling*. C)** Gene expression changes among cell lines and normal NK cells were mapped on the JAK/STAT signaling pathway. The colored boxes indicate significant upregulation (red) or downregulation (blue) of transcripts in normal PBMCs, normal NK cells, HMB, CAEBV, NK-lymphoma and NK-leukemia. **D)** The graphs show the mRNA expression levels of representative genes. The expression levels of JAK3 and STAT3 are increased in EBV-positive NK-LPD, and the cytokine receptors upstream of JAK/STAT show the same expression pattern as JAK3/STAT3. SHP-1 expression is decreased and SHP-2 expression is remarkably increased in EBV-positive NK-LPD.

Figure 3. ***Western blot analyses of Cyclin-D1, -E2, -A2, Fas, Survivin, SHP1 and SHP2 in EBV(+)LPD, EBV(+)NK lymphoma/leukemia cell line and control cells.***

A) Expression of Cyclin-D1, -E2, -A2 and SHP2 was gradually increased according to the progression of diseases in contrast to almost no expression in normal control of healthy PBMCs and healthy CD16(-)NK cells. Positive control of HeLa cells, a cervical cancer cell line, showed positive for these proteins. Fas showed high expression in CAEBV (SNK10) cells and gradually decreased in NK lymphoma/leukemia cells (HANK1, SNK6 & NK-YS) and showed no expression in normal PBMCs and CD16(-)NK cells. Survivin showed highest expression in NK lymphoma cells (HANK1 & SNK6). In contrast, SHP1 showed high expression in normal PBMCs and CD16(-)NK cells, gradually decreased from CAEBV, NK lymphoma to NK leukemia cells. HMB cell line (KAI3) showed no expression of SHP1. ***Immunohistochemical staining of SHP-2 and Survivin and immunofluorescent double staining of Fas in RLH, CAEBV and NK/T lymphoma.*** **B)** Representative data of Immunohistochemical

staining of SHP-2 and Survivin were shown. The staining of SHP-2 was negative in ***a)*** $CD56^{(+)}$normal NK cells, but positive in both ***b)*** $CD56^{(+)}$CAEBV and ***c)*** CD56(+)NK/T-lymphoma. The staining of survivin is very faint in ***d)*** $CD56^{(+)}$normal NK cells, and positive in ***e)*** $CD56^{(+)}$CAEBV and ***f)*** $CD56^{(+)}$NK/T-lymphoma. (Magnification ×400; Red color: CD56; Brown color: SHP2 and survivin).

Figure 4. ***Hypothetical mechanism underlying the onset of EBV-positive NK-LPD.*** After EBV infection, LMP1 or ZEBRA is expressed in the infected cells, resulting in an increase in ZEBRA-dependent DNA checkpoint genes, such as *p21* and *p53,* and cell cycle arrest to allow virus replication. LMP1 activates the MAPK and/or NFkB pathway and induces c-myc expression, eventually promoting cell cycle progression and suppressing apoptosis. Strong repression of SHP1 expression induces the continuous activation of the JAK/STAT and RAS/MAPK pathways, which leads to aberrant cell proliferation. The increased expression of SHP2 in NK-LPD may also activate the RAS/MAPK pathway. Increased survivin expression in NK leukemia may suppress CASPA9, leading to resistance against apoptosis.

A List of Supporting Information

Table S1. Origin and characters of cell lines and normal CD16(-) NK cells.

Table S2. Cluster A-1, which shows down regulation in HMB, CAEBV, NK lymphoma and NK leukemia compared to Norm NK.

Table S3. Cluster A-2, which shows up regulation in HMB, CAEBV, NK lymphoma and NK leukemia compared to Norm NK.

Table S4. Cluster B-1, which shows down regulation in CAEBV, NK lymphoma and NK leukemia compared to Norm NK/HMB.

Table S5. Cluster B-2, which shows up regulation in CAEBV, NK lymphoma and NK leukemia compared to Norm NK and HMB.

Table S6. Cluster C, which shows down regulation in NK lymphoma and NK leukemia compared to Norm NK, HMB and CAEBV.

Table S7. Cluster D-1, which shows down regulation in NK leukemia compared to Norm NK. HMB, CAEBV and NK lymphoma.

Table S8. Cluster D-2, which shows up regulation in NK leukemia compared to Norm NK, HMB, CAEBV and NK lymphoma.

Table S9, Cell cycle analysis of NK cell lines in the different medium condition.

Figure S1. ***KEGG pathway analysis of apoptosis.*** **A)** Gene expression changes among cell lines and normal NK cells were mapped on the apoptotic pathway. The colored boxes indicate significant upregulation (red) or downregulation (blue) of transcripts in normal PBMCs, normal NK cells, HMB, CAEBV, NK-lymphoma and NK-leukemia. **B)** The graphs show the mRNA expression levels of representative genes. The expression levels of both apoptosis-inducing and -evading genes are increased in EBV-positive NK-LPD.

Figure S2. ***Effects of culture medium condition on cyclin-D1 expression.*** **A)** In order to investigate the effects of culture medium condition on the expression of cell cycle related genes, Western blot analyses of Cyclin-D1 was performed. Cyclin–D1 expression showed gradually increased from KAI3, SNK10, HANK1, SNK6 and NKYS as disease progression in contrast to no expression in normal controls. **B)** On the other hand, Cyclin-D1 expression showed almost no difference in the different culture condition of each cell line, indicating that different medium condition induced almost no effects on Cyclin-D1 expression. Medium1: Gm+RPMI supplemented with 10% human serum and 700 U/ml IL-2, Medium2: AIM-V supplemented with 10% bovine serum and 50 U/ml IL-2, Medium3: IMDM supplemented with 10% bovine serum and 100 U/ml IL-2.

Figure S3. ***Effects of culture medium condition on cell cycle.*** In order to investigate the effects of culture medium condition on cell cycles of each cell line, Flow cytometry (FCM) analyses were performed, showing that the difference of medium condition does not affect so much on cell cycle in each cell line in contrast to the large difference among cell lines. Detailed data of cell cycle states were summarized in Table S9.

Figure S4 ***Immunofluorescent double staining of Fas and CD56 in RLH, CAEBV and NK/T lymphoma.*** **a)** RLH patient specimen was treated with rabbit anti-Fas and**, b)** mouse anti-CD56 antibodies followed by anti-rabbit IgG Alexa488 (green) and anti-mouse IgG Alexa555 (red) and **c)** stained with DAPI (blue). **d)** Images of a), b), and c) were merged. No CD56(+)NK cells showed Fas-positive in RLH. **e)** CAEBV patient specimen was treated with rabbit anti-Fas antibody**, f)** mouse anti-CD56 antibody followed by second antibodies staining as in RLH and **g)** stained with DAPI. **h)** Images of e), f), and g) were merged. CD56(+)NK cells in CAEBV showed Fas-positive (arrows). **i)** NK/T lymphoma patient specimen was treated with rabbit anti-Fas antibody**, j)** mouse anti-CD56 antibody followed by second antibodies staining and **k)** stained with DAPI. **l)** Images of i), j), and k) were merged. No CD56(+)NK cells showed Fas-positive in NK/T lymphoma (bar indicates 20 micron)

<u>Supplemental information</u>

Supplemental explanation about microarray analysis methods specifically about 1) why were low signals transformed to 1.0 instead of eliminated? 2) Why was global normalization chosen to the 75th percentile rather than to housekeeping genes, which would better account for both cell sample and cDNA quality?

1) This analysis method has been used in many articles in the field of microarray. The reason why low signals transformed to 1.0 instead of eliminated, is data was normalized with 75th percentile later and this transformation will affect to these

process. The 75th percentile is reasonable to be 75th percentile of all measured values. If low signals are eliminated, data will shift from 75th percentile. The total number of probe sets, showing less than 1.0, is different among each array. The 75th percentile becomes different among arrays if low signals are eliminated. Additionally, low signals are eliminated in the filtering process in later, so we do not need to eliminate low signals in this step. For example, the expression pattern such as 0.1, 100, 20 is better to transform to 1, 100, 20, because in the case of NA (eliminated value is transformed to Not Applicable), 100, 20 become impossible to compare the values between 1st and 2nd values, or 1st and 3rd values.

2) The global normalization has been accepted to be better than the normalization with housekeeping genes in the case of genome wide analyses.

Why was one-way ANOVA used to generate the list from the probe-set instead of two-way?

The reason why we chose one-way ANOVA, is that the parameter is only one. One-way ANOVA is appropriate in the case of one-dimensional analysis.