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Comprehensive gene expression profiles of NK cell neoplasms identify vorinostat as an effective drug candidate

Kennosuke Karube [a], Shinobu Tsuzuki [a], Noriaki Yoshida [a,b], Kotaro Arita [a], Harumi Kato [a], Miyuki Katayama [a], Young-Hyeh Ko [c], Koichi Ohshima [d], Shigeo Nakamura [e], Tomohiro Kinoshita [f], Masao Seto [a,b,*]

[a] Division of Molecular Medicine, Aichi Cancer Center Research Institute, Nagoya, Japan
[b] Department of Cancer Genetics, Nagoya University Graduate School of Medicine, Nagoya, Japan
[c] Department of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Republic of Korea
[d] Department of Pathology, School of Medicine, Kurume University, Kurume, Japan
[e] Department of Pathology and Clinical Laboratories, Nagoya University Hospital, Nagoya, Japan
[f] Department of Hematology and Cell Therapy, Aichi Cancer, Nagoya, Aichi, Japan

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ABSTRACT

NK cell neoplasms are lymphoid malignancies with an aggressive clinical course. In the present study, we analyzed gene expression profiling of NK cell neoplasms and attempted to identify important molecular pathways and new effective drugs. Pathway analysis of gene expression profiles suggested the important roles of the JAK-STAT pathway, NF-κB pathway or Wnt pathways in NK cell neoplasms. Notably, western blot analysis revealed that STAT3 was expressed and phosphorylated at a higher level in NK cell lines than in normal NK cells or other cell lines. These findings indicate the occurrence of JAK-STAT activation in NK cell neoplasms. Connectivity Map (CMAP) analysis of gene expression profiles identified candidate drugs against NK cell neoplasms. Among the drugs suggested by CMAP analysis, we focused on puromycin, phenoxybenzamine, LY294002, wortmannin, vorinostat and trichostatin A because they exhibited high enrichment scores. We added these drugs to NK cell lines and other cell lines. Among the drugs, vorinostat suppressed NK cell line proliferation at a significantly lower concentration compared to other cell lines. Suppression of the JAK-STAT pathway appeared to contribute to this effect. Vorinostat may be a good candidate for use in the therapy against NK cell neoplasms.

1. Introduction

NK cell neoplasms are lymphoid malignancies with an aggressive clinical course. NK cell neoplasms are more prevalent in Asians and native Americans than in people from western countries [1]. The WHO classification defines NK cell neoplasms as two major disease entities, aggressive NK cell leukemia (ANKL) and extranodal NK/T cell lymphoma of a nasal type (ENKTL) [1,2]. Neoplastic cells of both diseases express the NK cell markers CD16 and CD56, cytotoxic molecules such as granzyme B and T cell-restricted intracellular antigen (TIA1), and the EB virus-associated molecules EBV-LMP1 and EBER [1,2].

Chemotherapy and radiation therapy are two major therapies against NK cell neoplasms. Tumor cells of NK cell neoplasms generally express P-glycoprotein, which is a product of the MDR1 gene and is related to multi-drug resistance [3,4]. Therefore, combinations of some anti-tumor drugs are needed for therapy against NK cell neoplasms. Prognosis of patients with NK cell neoplasms is improved by a combination of radiation therapy and chemotherapy for patients at the early stage [5]. However, prognosis of patients at the advanced stage is still poor [6,7]. Current chemotherapies against NK cell neoplasms utilize several existing drugs [8]. Although various new anti-tumor drugs have been developed recently, few reports describe the effects of these new drugs on NK cell neoplasms [9–12].

Recent analyses involving gene expression profiling, or array CGH are gradually clarifying the molecular pathogenesis of NK cell neoplasms [10,13,14]. Ng et al. [15] indicated that some microRNAs such as miR-101, miR-26a and miR26b function as tumor suppressors. Koo et al. [16] demonstrated that the JAK3 mutation (A572V or A573V) was found in about 35% of ENKTL cases and that this mutation induced constitutive activation of JAK3. However, the association between molecular pathogenesis and therapeutic

* Corresponding author at: Division of Molecular Medicine, Aichi Cancer Center Research Institute, Chikusa-ku, Kanokoden 1-1, Nagoya 464-8681, Japan. Tel.: +81 52 762 6111; fax: +81 52 764 2982.
E-mail address: mseto@aichi-cc.jp (M. Seto).

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methods has not been adequately discussed until now. In the present study, we analyzed gene expression profiling of NK cell neoplasms and attempted to detect important molecular pathways associated with these phenomena. Connectivity Map (also known as CMAP) is a collection of genome-wide transcriptional expression data from cultured human cells treated with bioactive small molecules [17]. We searched for effective drugs against NK cell neoplasms by analyzing the gene expression profile of NK cell neoplasms using CMAP, and identified vorinostat as a candidate drug against NK cell neoplasms. *In vitro* analyses also demonstrated the inhibitory effects of vorinostat on NK cell lines.

2. Methods

2.1. Samples and cell lines

RNA samples from 11 clinical cases, seven cell lines, and normal NK cells were obtained by a Trizol RNA extraction solution (Life Technologies Japan, Tokyo, Japan). These samples were the same as those previously reported [13]. The samples were taken from patients at Samsung Medical Center, Aichi Cancer Center, Nagoya University Hospital, and collaborating institutions. This study was performed at Aichi Cancer Center with the approval of the Institutional Review Board. The diagnoses were based on the WHO classification, 4th edition. Seven cell lines of NK cell neoplasms (NK-YS [18], SNK6 [19], NK92 [20], NKL [21], HANK-1 [22], KHYG-1 [23] and SNK10 [24]) were obtained from each institute where they were established. In addition to the NK cell lines, we also analyzed some other common cell lines: T cell lines (Jurkat, Hut78, MOLT4 and SU9T01 [25,26]), B cell lines (Jeko1, Raji, SUDHL6 and Reh), a Hodgkin cell line (HDLM2), a myeloid cell line (K562), and epithelial cell lines (293T, Hela, MKN45 and MCF7). HDLM2 was purchased from DSMZ (Braunschweig, Germany). SU9T01 was kindly provided by Dr. Arima from Kagoshima University (Kagoshima, Japan) [25,26]. MKN45 was purchased from the JCRB Cell Bank (Osaka, Japan). HANK1 and SNK10 were maintained in Iscove modified Dulbecco medium (IMDM) with 10% human serum, penicillin (50 U/mL), streptomycin (50 μg/mL), and interleukin-2 (50 U/mL) at 37 °C in 5% carbon dioxide, while the other NK cell lines were maintained in IMDM with 10% fetal calf serum, 100 U/ml of interleukin-2 and antibiotics. All other common cell lines, except for MKN45, were maintained in IMDM with 10% fetal calf serum and antibiotics. MKN45 was maintained in RPMI medium with 10% fetal calf serum and antibiotics.

Normal NK cells from three healthy volunteers were isolated and analyzed as control samples. Details of the method employed for the isolation are described in a previous report [13].

2.2. Gene expression profiling

The Low RNA Input Linear Amplification and Gene Expression Hybridization kits (Agilent Technologies) were used for RNA labeling and amplification. The Whole Human Genome 4X44k Oligomicroarray Kit which contains 43,377 60-mer probes and Gene Expression Hybridization kit (Agilent Technologies) were used for the hybridization of labeled RNA. The experimental protocol employed paralleled the manufacturer's protocol (www.agilent.com). Statistical analysis of gene expression profiles including Gene set enrichment analysis (GSEA) [27] and use of the Web-based gene set analysis toolkit (Webgestalt) (http://bioinfo.vanderbilt.edu/webgestalt/) were performed using normalized data. A detailed description of normalization and these analyses has been reported [13]. CMAP analysis (Broad Institute) [17] was performed with the latest dataset version (Build 02), which contains 6100 expression profiles representing 1309 compounds (http://www.broad.mit.edu/cmap/). The up-regulated 716 probes (486 genes) and down-regulated 637 probes (511 genes) in neoplastic samples compared with normal NK cells were used for the comparison of neoplastic samples and normal NK cells. These genes showed the largest fold changes between neoplastic samples and normal NK cells, exhibiting a z-score of more than one [13]. Vorinostat was added to NKL and NKYS, representative NK cell lines. We also analyzed the change of gene expression profiling by vorinostat. At first, down- or up-regulated genes with a z-score of more than 0.5 following vorinostat treatment were selected in NKL and NKYS, respectively. Commonly down- or up-regulated genes were then selected. 292 genes and 175 genes were commonly down-regulated or up-regulated by vorinostat, respectively. These gene lists were used for each statistical analysis.

A heatmap of genes associated with the JAK-STAT pathway was built. Webgestalt analysis identified the following JAK-STAT related gene sets with significance ($P < 0.05$): JAK-STAT signaling pathway ($P = 0.012$), hsa_TTCYNRGAA_V$STAT5B_01 ($P = 0.003$), hsa_V$STAT5B_01 ($P = 0.014$), hsa_V$STAT_01 ($P = 0.015$), hsa_V$STAT5A_01 ($P = 0.03$), hsa_V$STAT4_01 ($P = 0.03$), hsa_V$STAT_Q6 ($P = 0.03$), hsa_V$STAT6_02 ($P = 0.03$), and hsa_V$STAT1_03 ($P = 0.03$). 16 genes were included in these gene sets. The value of a gene with multiple probes was calculated as an average score of the probes. Cluster v3.0 (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster) and Java Treeview (http://sourceforge.net/projects/jtreeview) were used to make the heatmap [28]. Gene normalization was applied in this analysis.

2.3. Cell proliferation analysis and reagents

Cells were seeded in triplicate in 96-well plates (5000 per well), incubated for 24 h, and then treated for 72 h with a six-point dilution series of compounds dissolved in DMSO (Wako, Osaka, Japan). Cell proliferation was measured using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions, except for Reh and Jurkat, in which the assay methods did not work. Cell numbers for Reh and Jurkat were determined using the trypan blue exclusion assay. Percent growth was determined relative to untreated controls. After 24 h, DMSO, puromycin (Sigma, St. Louis, MO), phenoxybenzamine (Alexis Biochemicals, San Diego, CA), LY294002 (Cell Signaling Technology; Danvers, MA), wortmannin (Sigma), trichostatin A (Wako), and vorinostat (Toronto Research Chemicals, North York, Canada) were added to achieve the indicated drug concentration, and the cells were incubated for an additional 72 h. A colorimetric assay was done after addition of 10 μL of Cell Counting Kit-8 to each well, and the plates were incubated at 37 °C for 1 h. The absorbance was read at 450 nm using a multiplate reader. Percent growth was determined relative to untreated controls. The Student's *t*-test was used to determine significant differences.

Concentration ranges were selected in accordance with those shown in the literature for puromycin (0–3 μmol/L [29]), Ly294002 (0–60 μmol/L [30]), wortmannin (0–60 μmol/L [31]), trichostatin A (0–0.6 μmol/L [32]), and vorinostat (0–3 μmol/L [33]). We used a concentration range of 0–60 μmol/L for phenoxybenzamine, as this compound had not yet been used for the treatment of cancer cells. The concentration of trichostatin A was increased for K562 (0–20 μM) because K562 was more refractory to trichostatin A compared to other cell lines.

2.4. Western blot analysis

Western blot analysis was performed essentially as previously described [34]. The transferred polyvinylidene fluoride membranes were incubated overnight with anti-actin antibody (AC-40; Sigma–Aldrich), anti-STAT3 antibody (Cell Signaling Technology), and anti-phospho-STAT3 Tyr 705 (pSTAT3) antibody (Cell Signaling Technology) in blocking buffer at 4 °C. These were then washed extensively in PBS containing 0.05% Tween 20 T-PBS and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Amersham, Arlington Heights, IL) diluted at 1:1000 in blocking buffer without 0.05% NaN_3. Antibody binding was visualized using an enhanced chemiluminescence detection kit (Amersham). The intensity of each band in the agarose gel image was calculated using Image J software (http://rsbweb.nih.gov/ij/).

2.5. Apoptosis and cell cycle assay

Apoptotic cell death was determined using the Annexin V APC Apoptosis Detection Kit (eBioscience) according to the manufacturer's instructions. Briefly, cells were washed in serum(-) medium and stained for 15 min at room temperature. For the cell cycle assay, propidium iodide (PI) staining was performed according to the method described in a previous report with some modifications [35]. Briefly, 5×10^5 cells were incubated in 1 ml PBS containing 0.1% Triton X-100 (Sigma–Aldrich), 0.5% RNase A (Qiagen) and 20 mg/ml PI (Wako, Osaka, Japan). Analyses were performed using a FACSCalibur (BD Biosciences) instrument and FlowJo software (Tommy Digital Biology, Tokyo, Japan).

2.6. Mutation analysis

Coding regions including A572 and A573 of JAK3 were amplified from genomic DNA by PCR. PCR primers were synthesized based on previous reports [16]. All seven cell lines used in this study were analyzed. Direct sequencing of PCR products was performed through capillary electrophoresis using the ABI3100 sequencer (Applied Biosystems).

2.7. Semi-quantitative RT-PCR

Semi-quantitative determination of ASPM and ID3 gene expression was assessed and compared with the β-actin gene as an internal control following the method previously described [13]. The forward and reverse primers used for RT-PCR of ASPM and ID3 were as follows: ASPM; forward (5′-ATCTCAAACGCCATCAGG-3′) and reverse (5′-CATTTTACGTTGCTTCCATTT-3′), ID3; forward (5′-AAAGGAGCTTTTGCCACTGA-3′) and reverse (5′-TCTCCAGGAAGGGATTTGGT-3′). The PCR conditions comprised 95 °C for 3 min and then 25 cycles (β-actin) or 35 cycles (ID3) at 95 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min. Touchdown PCR was adopted for ASPM. Annealing conditions for the touchdown protocol involved a reduction of the initial annealing temperature of 63 °C by 1 °C after every two cycles to reach 58 °C for the final 25 cycles.