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ORIGINAL RESEARCH

A Non-invasive Diagnosis of Histiocytic Necrotizing Lymphadenitis by Means of Gene Expression Profile Analysis of Peripheral Blood Mononuclear Cells

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Abstract Histiocytic necrotizing lymphadenitis (HNL), also called Kikuchi-Fujimoto disease, is a benign, self-limiting inflammatory disease with fever and painful cervical lymphadenopathy of unknown etiology. A lymph node biopsy is required for the definitive diagnosis because of no specific symptoms or laboratory findings for HNL. To establish the rapid non-invasive diagnostic method for this disease, we investigated genes specifically expressed in the patients by analyzing whole transcriptome using microarray analysis of peripheral blood mononuclear cells (PBMC). The top five up-regulated genes (*IFI44L*, *CXCL10*, *GBP1*, *EPSTI1* and *IFI27*) in HNL were interferon-induced genes (ISGs). The expression levels of the up-regulated genes by microarray were verified by quantitative PCR. High levels of serum CXCL10 concentration were confirmed at the symptomatic phase of HNL patients. The expression levels of these 5 genes positively correlated with each other (r^2=0.28–0.60). The genes were also highly expressed in HNL lymph nodes. The discriminant analysis using the expression levels of these five genes distinguished HNL with 84 % accuracy. The combination of up-regulated ISGs in HNL seemed to be a specific response induced by viral infections or autoantigens. An analysis of the gene expression profile of PBMC may provide a rapid non-invasive diagnosis of HNL.

Keywords Histiocytic necrotizing lymphadenitis · Kikuchi-Fujimoto disease · interferon-stimulated genes · gene expression · discriminate analysis

Abbreviations

ACTB	Beta actin
ADV	Adenovirus
AUC	Area under the curve
cDNA	Complementary DNA
C_T	Threshold cycle
CXCL10	Chemokine (C-X-C motif) ligand 10
EPSTI1	Epithelial stromal interaction 1 (breast)
FluA	Influenza type A virus
GBP1	Guanylate binding protein 1 interferon-inducible
HNL	Histiocytic necrotizing lymphadenitis
IFN	Interferon
IFI27	Interferon alpha-inducible protein 27
IFI44L	Interferon-induced protein 44-like
IL	Interleukin
IM	Infectious mononucleosis

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ISG	Interferon-stimulated gene
KD	Kawasaki disease
LNitis	Purulent lymphadenitis
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
SLE	Systemic lupus erythematosus
SoJIA	Systemic onset juvenile idiopathic arthritis

Introduction

Histiocytic necrotizing lymphadenitis (HNL), also called Kikuchi-Fujimoto disease, is a benign, self-limiting inflammatory disease with fever and tender cervical lymphadenopathy of unknown etiology [1, 2]. Severe HNL patients with hemophagocytic syndrome or prolonged fever need immunosuppressive therapy [3]. It is necessary to distinguish it from other febrile diseases with lymphadenopathy including leukemia, malignant lymphoma, infectious mononucleosis (IM), purulent/tuberculous lymphadenitis, Kawasaki disease (KD), systemic juvenile idiopathic arthritis (SoJIA), and systemic lupus erythematosus (SLE). Lymph node biopsies are required for the definitive diagnosis because of no specific symptoms or laboratory findings including imaging tests for HNL.

Histologic findings of involved lymph nodes include paracortical areas of coagulative necrosis with abundant karyorrhectic debris. Karyorrhectic foci consist of histiocytes, plasmacytoid dendritic cells, immunoblasts, and lymphocytes [1, 2]. Neutrophils and plasma cells are absent or scarce. Assessed by immunohistochemical analysis, histiocytes are positive for myeloperoxidase and CD68 antigen, and lymphocytes are predominantly $CD8^+$ cytotoxic T cells [1, 2, 4]. In the earlier stages, histiocytes and plasmacytoid dendritic cells (pDCs) are usually prominent in the lesions, suggesting that these cells have a close correlation with the pathogenesis of HNL [1, 2, 5]. By immunohistochemical analyses, it has been suggested that perforin and Fas pathways play important roles in the induction of apoptosis and necrotizing lesions [6, 7]. It was supported by the findings of microarray analysis which showed up-regulation of apoptosis- and cell cycle-associated genes in lymphnodes of HNL patients [8].

Some HNL cases were reported to occur in association with viral infections (e.g. Epstein-Barr virus, human herpes virus type 6, and human T-lymphotropic virus type 1) or autoimmune disease (SLE) [1, 9]. Increased serum concentrations of interferon (IFN)-γ, interleukin (IL)-6, and 2',5'-oligoadenylate synthetase as well as pyrexia and extranodal involvement of skin, bone marrow, and liver in the patients suggest the exaggerated systemic inflammatory response to viral pathogens or to autoantigens in the pathophysiology of HNL [10, 11].

To establish a rapid non-invasive diagnostic method for HNL, and to characterize the molecular pathophysiology of the disease, we investigated the gene expression profile of peripheral blood mononuclear cells (PBMC) by microarray analysis and performed discriminate analysis.

Materials and Methods

Patients

Twenty-four patients with HNL participated in this study. The clinical features of the patients are shown in Table I. The specimens were obtained from the patients before or without the treatment with steroids or immunosuppressive drugs etc. In addition, 93 disease controls and 34 healthy donors were included in this study (Table II). Cervical lymph node samples of patients with HNL (n=9) and reactive lymphadenopathy (n=4) were provided from Department of Pathology, Faculty of Medicine, Fukuoka University, Japan. For the analysis of serum concentrations of CXCL10, blood samples were obtained from 12 patients with HNL, 5 with KD, 4 with IM, and 5 with bacterial lymphadenitis. All patients were diagnosed by trained pediatricians and pathologists, according to the clinical manifestations, laboratory findings, and histological examinations. Informed consent was obtained from all participants for this study, according to the process approved by the Ethical Committee of Kyushu University, Fukuoka, Japan.

Total RNA Extraction and RNA Amplification

PBMC were separated from peripheral blood by density gradient centrifugation using Lymphocyte Separation Medium (MP Biomedicals LLC, CA, USA). Total RNA was extracted from PBMC or lymph nodes cells using an RNA extraction kit (Isogen) (Nippon Gene, Osaka, Japan), according to the manufacturer's instructions. An Amino Allyl MessageAmp aRNA Kit (Life Technologies, CA, USA) was used to amplify the total RNA.

Microarray Analysis

In order to characterize the gene expression profile of PBMC in HNL patients, microarray analysis of PBMC was performed using an AceGene Human Oligo Chip 30 K (Hitachi Solutions, Tokyo, Japan) that contains approximately 30,000 genes. The arrays were scanned by FLA-8000 (Fujifilm, Tokyo, Japan), and changed to the numerical values by ArrayVision Software (GE Healthcare, Buckinghamshire, UK). The numerical data were normalized using the LOWESS method, as described previously [12]. In the microarray analysis of PBMC, data from 2 patients with HNL, 5 with SoJIA, 3 with KD and a healthy donor were compared. Genes that were consistently up-regulated in PBMC of HNL patients, compared with patients with SoJIA or KD and a healthy donor, with more than

Table I Clinical manifestations and laboratory findings of HNL patients

Number of HNL patients	24
Gender male:female	11:13
Age at onset (years)	11.7 (3.5–14.3)*
Family history of HNL	0
Fever	24
Cervical lymphadenopathy	24
Biopsy of lymph node	8
Relapse of HNL	7
Association with autoimmune disease	1
WBC (/μL)	2840 (1340–6010)*
Platelet count (x10^9/L)	158 (86–308)*
LDH (IU/L)	625 (211–1179)*
CRP (mg/dL)	0.7 (0–3.7)*
Ferritin (ng/mL) (n=17)	262 (74–8123)
ESR (mm/1 h) (n=19)	31 (8–60)

*The variables expressed as median (range)

CRP C-reactive protein, *ESR* erythrocyte sedimentation rate, *LDH* lactate dehydrogenase, *WBC* white blood cell count

two-fold differences in the mean expression levels were selected. The data with low signal-to-noise ratios (S/N<2) were not used for further analysis. The data were analyzed using GeneSpring Software (Agilent Technologies, CA, USA).

Real-Time Quantitative PCR

First-Strand cDNA Synthesis Kit (GE Healthcare) with random hexamers was used to prepare the first-strand cDNA.

Table II The number of subjects in each group for discriminate analysis

Group	Number of subjects
1. Histiocytic necrotizing lymphadenitis (HNL)	24
2. Disease control (DC)	93
Kawasaki disease (KD)	11
Systemic onset juvenile idiopathic arthritis (SoJIA)	14
Systemic lupus erythematosus (SLE)	4
Measles	18
Varicella	5
Infectious mononucleosis (IM)	15
Adenovirus infection (ADV)	5
Influenza type A virus infection (FluA)	5
Purulent lymphadenitis (LNitis)	5
Sepsis	7
Lymphoid malignancy	4
3. Normal control (NC)	34

IFI44L, *CXCL10*, *GBP1*, *EPSTI1*, and *IFI27* mRNA expression levels were analyzed by TaqMan® gene expression assays Hs00199115_m1, Hs00171042_m1, Hs0026671_m1, Hs01566789_m1, and Hs00271467_m1, respectively (Life Technologies), and TaqMan Gene Expression Master Mix (Life Technologies). A TaqMan human *ACTB* (beta actin) endogenous control (Life Technologies) was used as an internal control. The mRNA expression levels of the interested and *ACTB* genes were quantified by a StepOnePlus™ Real Time PCR System and analyzed by using StepOne™ Software Version 2.1 (Life Technologies), as manufacturer's instruction. A comparative threshold cycle (C_T) method was used to determine the gene expression levels [13], by comparing the values of PBMC and lymph nodes from NHL patients with the median gene expression levels in 10 healthy donors and 4 patients with reactive lymphadenopathy, respectively. All experiments were carried out in triplicate.

Serum Concentration of CXCL10

Serum concentrations of CXCL10 (also known as IP-10) were measured by BD™ Human Chemokine Cytometric Bead Array Kit (BD Biosciences, NJ, USA) and an EPICS XL flow cytometer (Beckman Coulter, CA, USA) as manufacturers' instructions.

Statistical Analysis

Computation was carried out by using a statistical software; JMP version 8.0 (SAS Institute), according to JMP 8 Statistics and Graphics Guide, Second Edition. When we analyzed the data obtained by RT-PCR, logarithmically transformed values were used, because they showed log-normal distribution. Correlation coefficient was determined by Pearson's method. Statistical differences of the values of gene expression levels of PBMC and lymph nodes, and serum CXCL10 concentrations between HNL and controls were analyzed by Dunnett's test. Statistical difference of the serum CXCL10 concentrations between symptomatic and convalescent phases of HNL patients was analyzed by paired t-test. Canonical discrimination analysis was performed to distinguish HNL from disease controls and healthy donors by using the log-value of relative gene expression levels. The model was determined by stepwise procedure.

Results

Microarray Analysis

By microarray analysis of the mRNAs from PBMC in HNL patients, disease controls and healthy controls, one hundred and thirty seven up-regulated genes in HNL patients were

Table III Microarray analysis of peripheral blood mononuclear cells from patients with HNL, SoJIA, KD, and a healthy donor

The difference of mean gene expression levels between 2 HNL patients, 5 SoJIA patients, 3 KD patients and a healthy control donor in microarray analysis is given. One hundred and thirty-seven genes that showed more than two-fold expressional differences between HNL patients and SoJIA patients, KD patients as well as a healthy donor were selected, and the top 10 genes are listed

Gene name	Synonyms	Fold differences between a healthy donor		
		HNL	SoJIA	KD
Interferon-induced protein 44-like	*IFI44L*	12.99	3.32	0.39
Chemokine (C-X-C motif) ligand 10	*CXCL10*	12.04	2.21	0.56
Guanylate binding protein 1, interferon-inducible, 67 kDa	*GBP1*	8.30	2.51	1.12
Epithelial stromal interaction 1 (breast)	*EPSTI1*	7.10	3.01	1.05
Interferon, alpha-inducible protein 27	*IFI27*	6.90	2.14	1.05
Tumor necrosis factor (ligand) superfamily, member 10	*TNFSF10*	6.36	2.84	1.87
Immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	*IGJ*	6.26	2.08	2.29
Interferon-induced protein 44	*IFI44*	5.59	1.97	0.96
Interferon-induced protein with tetratricopeptide repeats 3	*IFIT3*	5.29	2.31	0.40
Proteasome (prosome, macropain) activator subunit 2 (PA28 beta)	*PSME2*	4.87	2.32	0.85

identified (data not shown), and the top 10 genes are shown in Table III. Nine of these genes, other than *IGJ*, were interferon-stimulated genes (ISGs) [14].

Quantitative RT–PCR Analysis

The 5 most up-regulated genes (*IFI44L*, *CXCL10*, *GBP1*, *EPSTI1*, and *IFI27*) in HNL patients were analyzed by RT-PCR to confirm the microarray data. As shown in Fig. 1, the expression levels of the 5 genes in HNL patients were significantly higher than those in normal controls. Gene expression profile of the relapsing patients was not significantly different from that of patients without relapse (data not shown). Interestingly, positive correlations were observed in the expression levels among the 5 genes (r^2=0.28–0.60 : Fig. 2), suggesting the up-regulation of these 5 genes by some common mechanism, possibly through the stimulation of type 1 IFNs. Although the expression levels of these genes in HNL patients were higher than those in normal controls, bacterial infections, and lymphoid malignancy, the expression levels of each gene were not specific for HNL: these were equivalent to or less than those of viral infections and SLE (Fig. 1).

Analysis with Lymph Node and Serum Samples of HNL Patients

We then investigated the expression levels of these ISGs in involved lymph nodes by quantitative PCR. The genes were expressed significantly higher in HNL than in reactive lymphadenopathy (Fig. 3). Next, we analyzed the serum concentration of CXCL10. Although serum CXCL10 concentrations of HNL patients were not significantly different from those of KD, IM, and purulent lymphadenitis patients (Fig. 4a), the CXCL10 levels at symptomatic phase diminished at convalescent phase in HNL patients (Fig. 4b).

Discriminate Analysis

For the purpose of classifying HNL based on the gene expression profile, we performed canonical discrimination analysis by using the log-value of relative gene expression levels of the 5 ISGs. The subjects were separated into 3 groups as shown in Table II for discriminate analysis. In the stepwise procedure, all 5 genes were selected as parameter. The canonical discriminate analysis was able to classify the subjects into 3 groups with 84.2 % accuracy (misclassified number: 24). The area under the curve (AUC) of HNL group was 0.975 (Fig. 5a and Table IV). The scoring coefficients in canonical plot were as follows:

$$\begin{aligned}\text{Canonical 1} &= -0.1947\text{IFI44L} - 0.2058\text{CXCL10} \\ &\quad + 0.4870\text{GBP1} - 0.1620\text{EPSTI1} + 0.2197\text{IFI27}, \\ \text{Canonical 2} &= 0.2485\text{IFI44L} + 0.2488\text{CXCL10} \\ &\quad - 0.2387\text{GBP1} - 0.2802\text{EPSTI1} + 0.1598\text{IFI27}\end{aligned}$$

In the 2 group model (HNL and disease control), top 3 genes (*IFI44L*, *CXCL10*, and *GBP1*) were selected as parameters and the statistical power was equal to 3 group model; the accuracy and AUC were 82.2 % (misclassified number: 21) and 0.942, respectively (Fig. 5b and Table IV). The scoring coefficients in canonical plot were as follows:

$$\begin{aligned}\text{Canonical 1} &= -0.2632\text{IFI44L} - 0.3061\text{CXCL10} \\ &\quad + 0.5101\text{GBP1}\end{aligned}$$

Discussion

In this study, we observed that ISGs (*IFI44L*, *CXCL10*, *GBP1*, *EPSTI1*, and *IFI27*) were up-regulated in PMBC as well as in involved lymph nodes of HNL patients (Table III, Fig. 1). The discriminate analysis showed that the expression levels of these genes were specific for HNL patients (Fig. 5).

The type I IFN response protects a host against the invasion of viral pathogens. The cellular factors mediating this defense are the products of the ISGs [14]. The involved lymph nodes of HNL are primarily composed of pDCs, histiocytes and T lymphocytes, and pDCs are known to be one of the major producers of type I IFNs [1, 2]. It is reported that CXCL10 and IL-18 were expressed in histiocytes, and CXCR3 and IFN γ were expressed in T lymphocytes by immunohistochemical staining, which suggested that the cytokine and chemokine pathways play important roles in the pathophysiology of HNL [15]. Elevated serum levels of CXCL10 (Figs. 1 and 4), as well as IFN-γ and IL-6 [11] suggest a systemic inflammation in HNL, which possibly contributed to the gene expression profile of PBMC. It is supposed that pDCs were the major producer of the type 1 IFNs which induced ISGs

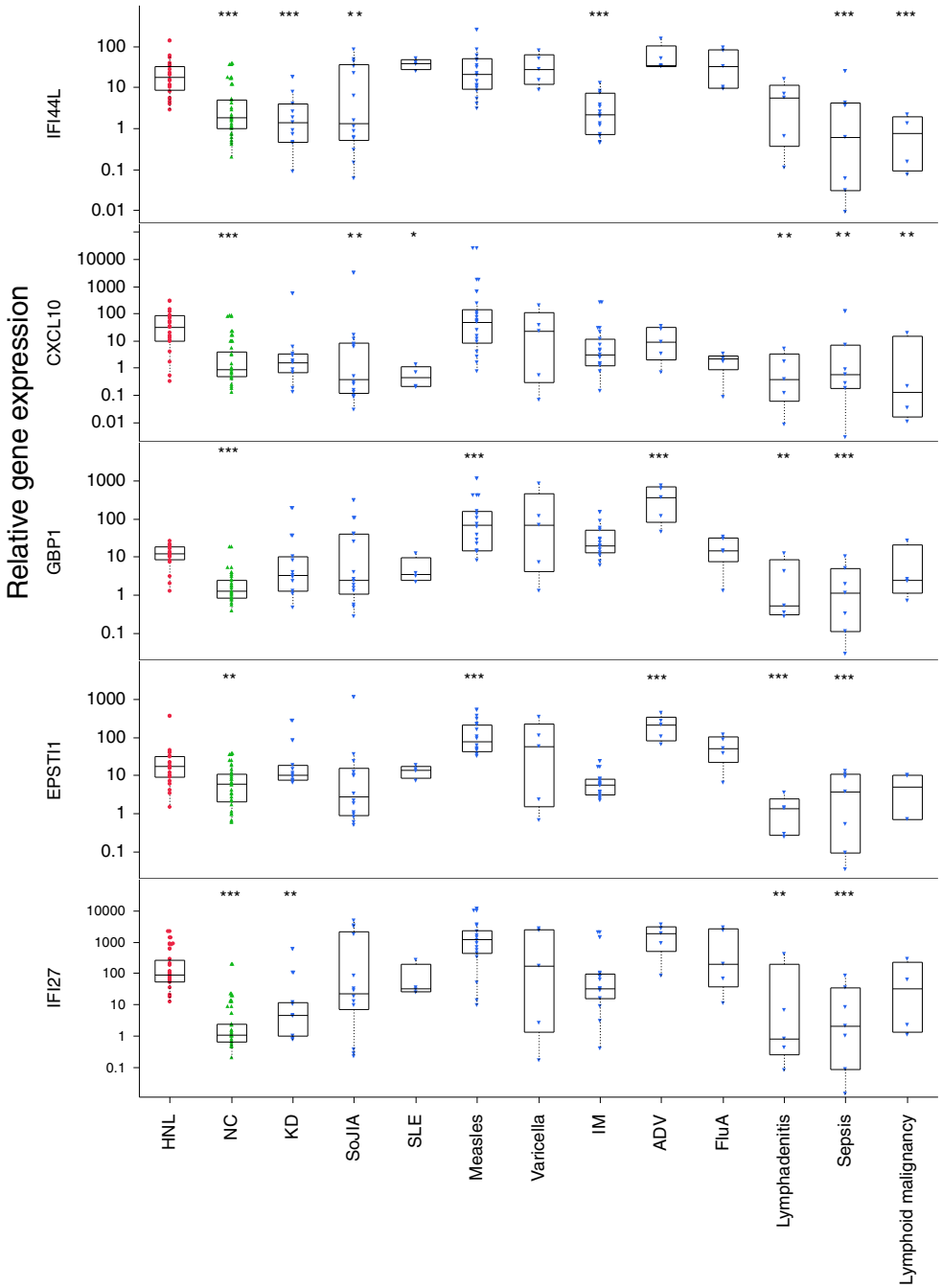

Fig. 1 Relative gene expression levels of mRNA from PBMC. The form of boxplot is as follows. The *bottom* and *top* of the box are the 25th percentile and the 75th percentile points, respectively. The *line* inside the box is the median. Each whisker extends up to 1.5 interquartile ranges from the end of the box. *$p<0.05$, **$p<0.01$, ***$p<0.001$

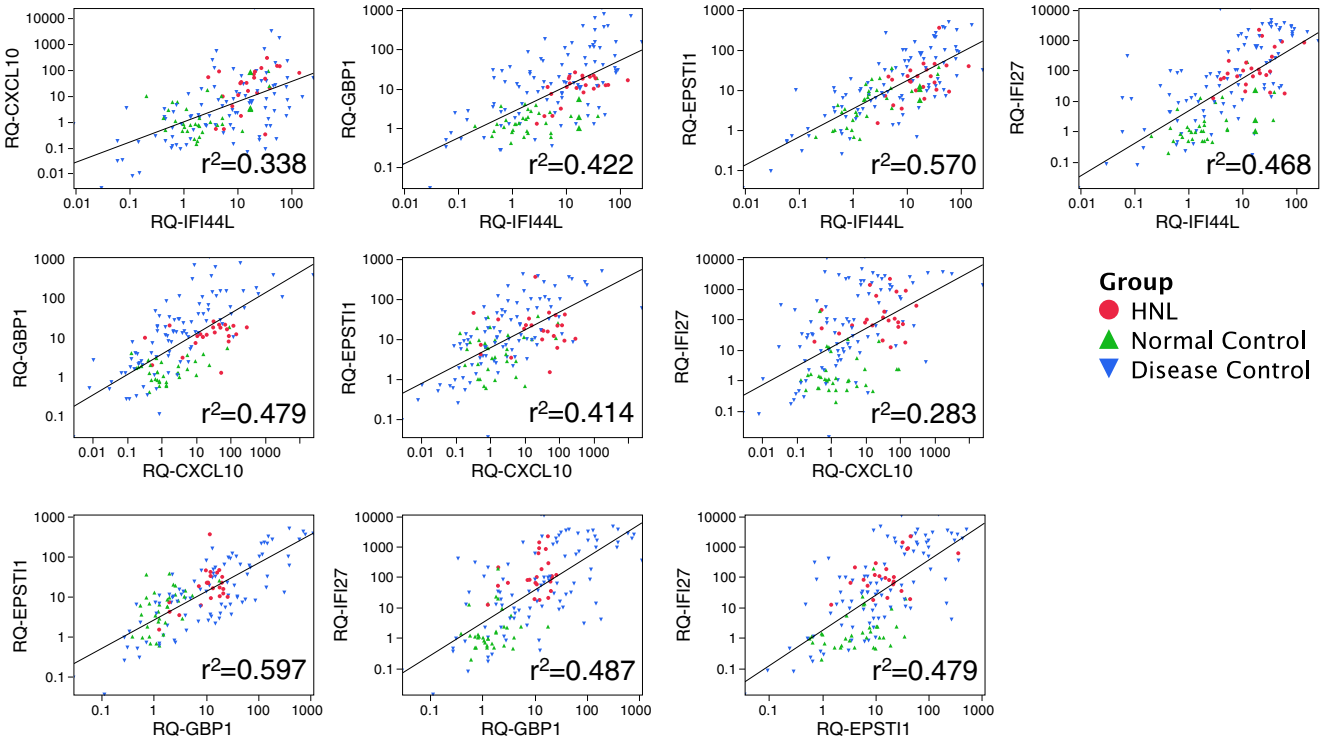

Fig. 2 Scatter plot of gene expression levels of 5 ISGs. The line is least-squares fit to data. *r2* correlation coefficient, *RQ* relative quantitation value

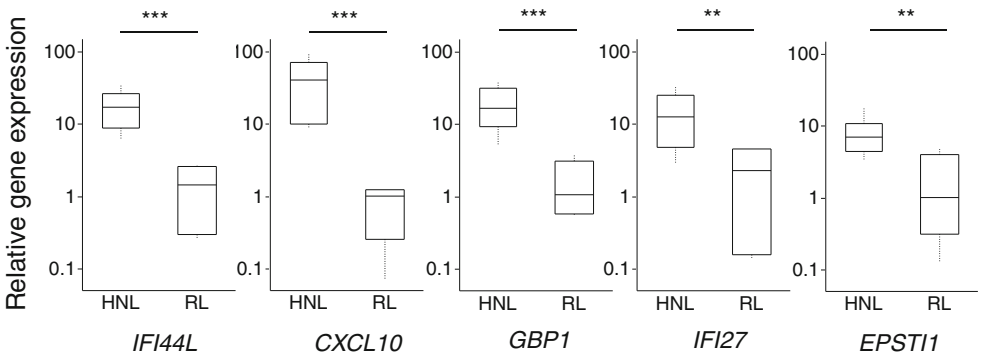

Fig. 3 Relative gene expression levels of mRNA from lymph nodes of patients with HNL and reactive lymphadenopathy. The form of box-plot is the same as Fig. 1. *RL* reactive lymphadenopathy **$p<0.01$, ***$p<0.001$

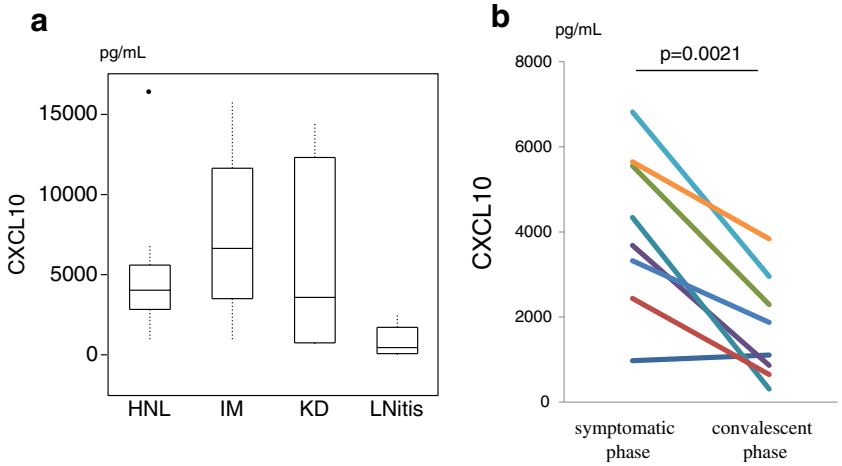

Fig. 4 Serum concentration of CXCL10 in HNL patients. **a** Serum concentration of CXCL10 at acute phase of HNL, IM, KD and LNitis. The form of box-plot is the same as Fig. 1. There was no significant difference between HNL ($n=12$) and disease control; IM ($n=4$), KD ($n=5$), and LNitis ($n=5$). **b** Diminishing serum CXCL10 concentration at convalescent phase of HNL ($n=8$)

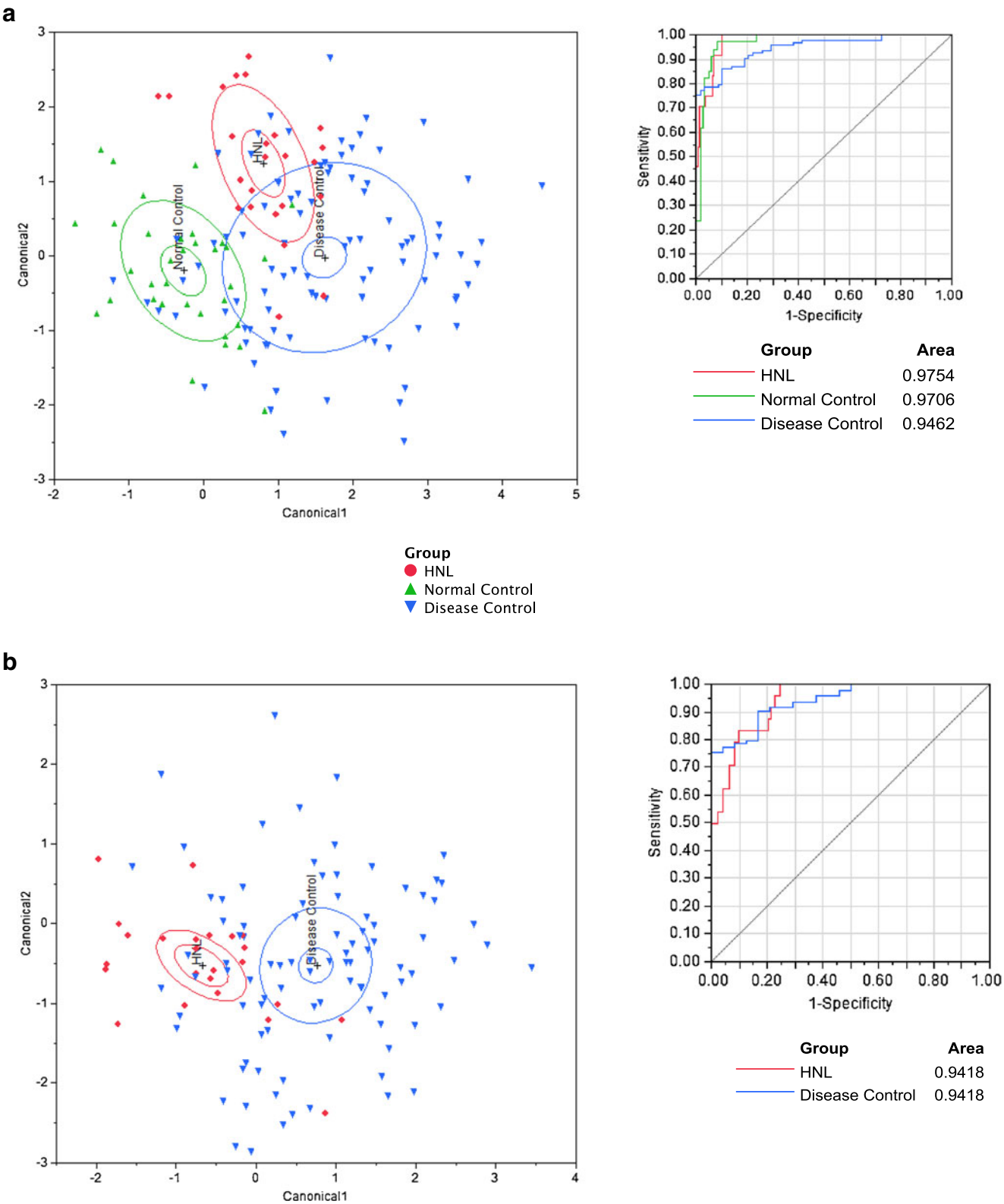

Fig. 5 Canonical plot and ROC curve in 3 groups; HNL (n=24), disease control (n=93), and normal control (n=34) (**a**), and in 2 groups; HNL and disease control (**b**). The inner and outer ellipses show 95 % and 50 % confidence regions, respectively

expression in this disease. On the other hand, these cytokines themselves were not highly up-regulated in PMMC of HNL patients in this study (data not shown). A paucity of pDCs in PBMC would account for it [16].

Table IV Numbers in discriminate analysis: actual rows by predicted columns

Three groups using 5 gene expression levels (Fig.5a)			
	HNL	Disease control	Normal control
HNL	22	2	0
Disease control	11	74	9
Normal control	1	1	32
Misclassified number: 24, accuracy: 84.2 %			
Two groups using 3 gene expression levels (Fig.5b)			
	HNL	Disease control	
HNL	20	4	
Disease control	17	77	
Misclassified number: 21, accuracy: 82.2 %			

Clinically, malignant lymphoma and leukemia are the most important disorders to be distinguished from HNL. A lymph node biopsy is often required for definitive diagnosis. CXCL10 expression may be enhanced in PBMC of the patients with these diseases because it was reported that CXCL10 expression was associated with the progression of leukemia and with the poor prognosis of lymphoma [17, 18]. In this study, we could distinguish HNL from these diseases by analyzing expression levels of other ISGs (Figs. 1 and 5). On the other hand, SLE presents the most challenging differential consideration, and sometimes its histologic presentation is almost identical to HNL [1, 2, 9]. Ishii et al. [16] reported that expression levels of *IFI27* and *EPSTI1* were increased in PBMC of SLE patients. GBP1 was expressed in lesional skin, and *IFI27*, *IFI44* and *IFI44L* were up-regulated in the synovium of patients with SLE [19, 20]. *IFI27* was also increased in PBMC of patients with Sjögren syndrome [21]. In our study, 4 ISGs, other than CXCL10, were up-regulated in SLE patients. Szturz et al. indicates that the pattern of serum cytokine levels in patients with HNL is similar to that of SLE patients [22]. These findings suggest that HNL and SLE are similar in the pathophysiology which includes immune responses mediated by type 1 IFNs.

Hundreds of ISGs were identified and different viruses are targeted by unique sets of ISGs. In addition, combined expression of pairs of ISGs showed additive antiviral effects [14]. We found that the expression levels of five ISGs (*IFI44L*, *CXCL10*, *GBP1*, *EPSTI1*, and *IFI27*) showed log-normal distribution and moderately positive correlation among them (r^2=0.28–0.60) (Figs. 1 and 2). These results suggest that the 5 genes are coordinately-induced in HNL. On the other hand, up-regulation of ISGs was similarly observed in patients with measles, varicella and other viral infections, and it was reported that GBP1, IFI27 and IFI44L could suppress hepatitis C virus replication [14, 23]. These findings indicate that HNL might be related with certain viral infections.

There are two kinds of ISGs, broad-acting effectors like interferon regulatory factor 1 (IRF1), retinoic acid-inducible gene-I (RIG-I), and melanoma differentiation-associated protein 5 (MDA5), and specific effectors which include IFI44L. It is known that unique sets of ISGs are important for specific antiviral effects [14]. The combination of up-regulated ISGs in HNL seemed to be a specific response induced by viral infections or autoantigens, which would be helpful for a non-invasive diagnosis for HNL.

There is a limitation for the availability of this model in daily routine for the diagnosis of HNL. Although it can not give the direct definitive diagnosis of HNL itself, the evaluation of the ISGs mRNA expression levels of peripheral blood seems to be helpful. Further research with more patients would be necessary for the early, non-invasive, and definitive diagnosis for HNL.

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