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IV. 研究成果の刊行物・別刷

Original Article

Activated Janus Kinase 3 expression not by activating mutations identified in Natural Killer/T-cell lymphoma

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Janus Kinase 3 (JAK3) is a non-receptor tyrosine kinase, predominantly expressed in hematopoietic cells, that plays an essential role in hematopoiesis during T cell development. JAK3 somatic-activating mutations were identified in extranodal natural killer/T cell lymphomas (ENKTL) in recent cases in Singapore. We hypothesized these mutations might play an important role in the pathogenesis of T and NK cell neoplasms in other areas of the world. We performed JAK3 exon13 sequencing for different types of T and NK cell neoplasms including ENKTL (59 cases total). We identified four mutations in three (5.0%) cases. All of the mutations were from ENKTL cases (15.8%). Among the four newly found mutations, three are silent mutations and one introduces a stop codon, which was not an activating mutation as in the cases in Singapore. We detected four (30.8%) cases positive for phosphorylated JAK3 expression among 13 NKTCL cases when we performed JAK3 (phospho Y785) immunostaining on sections of ENKTL samples. It seems that phosphorylated JAK3 expression does not necessarily harbor exon 13 mutations. The mechanism responsible for activating expression of the gene will be a topic for further research.

Key words: JAK 3, mutation, natural killer/T cell lymphoma, T and NK cell lymphomas

JAK3 is a non-receptor tyrosine kinase, predominantly expressed in hematopoietic cells, that plays an essential role in hematopoiesis during T cell development.[1] The JAK proteins are composed of FERM, SH2, pseudokinase and kinase domains.[1] The pseudokinase domain structurally resembles a kinase domain, although it does not function as one, and is thought to have regulatory function.[2] This domain is a hotspot for most of the mutations described in JAK1-3 in myeloproliferative disorders (MPDs) and leukemias.[2] Recently, JAK3 activating mutations in this domain were found in 35.4% of ENKTL cases in Singapore.[3] We hypothesized the mutation might also play an important role in the pathogenesis of T and NK cell neoplasms in other geographic areas. So we check the new found JAK3 mutation in ATLL, AITL, PTCL NOS, CAEBV and ENKTL samples. We performed immunostaining for phosphorylated JAK3 in ENKTL in order to examine if the gene mutation impacted protein expression.

MATERIALS AND METHODS

Tissue samples

The cases are composed of 40 T cell lymphoma DNA samples (ATLL: 14 cases, AITL: 11 cases, PTL: 11 cases, CAEBV: 4 cases) from Kurume University, and 19 formalin-fixed paraffin-embedded (FFPE) ENKTL samples from Siriraj Hospital, Mahidol University, Thailand. All patients provided informed consent in accordance with the Declaration of Helsinki, and use of patient materials and information was approved by the institutional review boards of Kurume University and Mahidol University.

Mutation validation by Sanger sequencing

The DNA samples from Kurume university, Japan were isolated from fresh or frozen tissue according to standard procedures and stored prior to this study. DNA of FFPE samples from Mahidol University, Thailand, was extracted using a commercial kit (Blood and Genomic Extraction Miniprep System, Viogene, Sunnyvale, CA, USA). Sanger sequencing was used to confirm the JAK3 mutations and validate their prevalence in T and NK cell neoplasms. Semi-nested PCR

was used to amplify JAK exon 13 from all samples. The amplification primers sequences used for validation are as follows:

JAK3-EX13-FW(10737–10760)
5′-AAGGC′AGGTC′TGTGA′GCACA′AAA-3′
(1st) JAK3-EX13-RV-S1(10935–10960)
5′-AGAGGTGGGAAGAACAGCCTAGACTT-3′
(2nd)JAK3-EX13-RV1(10942–10964)
5′-GGAAGAACAGCCTAGACTTGGGT-3′

For Sanger sequencing, PCR was carried out with AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) and cycled at 95°C for 10 min; 40 cycles of 95°C for 30 s; 65°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 10 min. Sequencing PCR was carried out with an ABI BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) and cycled at 96°C for 1 min; 25 cycles of 96°C for 10 s; 50°C for 5 s, and 60°C for 4 min. The resulting products were run on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Immunohistochemistry for JAK3

All ENKTL cases for which unstained slides were available were examined with rabbit polyclonal antibody for JAK3 (phospho Y785) (Abcam Ltd, Cambridge, UK) following a previously described method.[4]

RESULTS

Validation of JAK3 mutations

In total we found JAK3 mutations in three of 59 (5.0%) T and NK cell neoplasms. No mutations were observed in subtypes of T and NK cell neoplasms, including 14 ATLLs, 11 AITLs, 11 PTCLs and 4 CAEBVs except for ENKTL, where three of 19 (15.8%) samples carried four JAK3 mutations. These mutations were found to be new mutations and are not SNPs, when compared with a SNP bank. These mutations are in the JH2 pseudo-kinase domain including T-10: 579V(GTG)– 579V(GTA), T-11:578Q(CAA)–stop(TAA), T-11:581Y(TAC)– 581Y(TAT); T-24: D(GAC)–D(GAT). Three of them are silent mutations and one ends transcription by inserting a premature stop codon. All of these mutations were detected in cases from Thailand (Table 1, Fig. 1).

We conducted Epstein–Barr virus-encoded RNA (EBER) testing prior to this study on part 48 of these cases by *in situ* hybridization. The positivity was as follows: ATLL: (1/8, 12.5%), AITL: (2/7, 28.6%), PTL: (0/10, 0%), CAEBV: (4/4, 100%), ENKTL:(19/19) 100%.

Table 1 (a) JAK3 mutations in Kurume University cases and (b) JAK3 mutation detected in Aichi Cancer Center samples

Diseases	JAK3 mutation
(a)	
Kurume university cases (Japan)	
Adult T-cell leukemia/lymphoma (ATLL)	0/15
Angioimmunoblast T cell lymphoma (AITL)	0/11
Peripheral T cell lymphoma, not otherwise specified (PTCL-NOS)	0/10
Chronic active EB virus infection of T cell or NK cell type (CAEBV)	0/4
Mahidol university cases (Thailand)	
Natural killer/T-cell lymphoma (NKTCL)	3/19
Total	3/59
(b)	
ENKTL	0/17
ANKL	0/10
EBV+T/NK LPD	0/15
Total	0/42
Cell lines	
NK-YS SNK6 NK92 NKL HANK-1 KHYG-1 SNK10	0/7

JAK3 immunohistochemistry in ENKTL samples

There were four (30.8%) cases positive for phosphorylated JAK3 among 13 ENKTL cases. The positive cases show immunostaining of the tumor cell cytoplasm. This provides evidence that JAK3 is constitutively phosphorylated. The percentage of positive cells was usually high (more than 60%), with a strong cytoplasmic staining. (Fig. 2). These four cases do not carry JAK3 exon 13 mutations. The cases with JAK3 mutations have no expression of phosphorylated JAK3.

DISCUSSION

JAK3 belongs to a subfamily of non-receptor tyrosine kinases, along with JAK1, JAK2, and Tyk2. Although JAK1, JAK2, and Tyk2 are expressed ubiquitously, JAK3 expression is restricted to hematopoietic lineage cells.[5,6] It plays an important role in several signaling pathways involved in haematopoietic cell proliferation and survival. JAK3 activating mutations have been found in acute megakaryoblastic leukemia, lymphoproliferative diseases and adult T cell leukemia.[1,7,8]

Recently a group in Singapore identified JAK3 activating mutations in ENKTL patients. They found JAK3 somatic activating mutations (A572 and A573V) in 35.4% of NKTCL cases; this was the first report describing JAK3 mutations in NKTCL. In order to confirm the new-found mutations in T/NK cell lymphomas, we studied 59 cases of T/NK cell lymphomas (ATLL, AITL, PTCL-NOS, CAEBV) and ENKTL cases. Unfortunately, we found no cases that had activating JAK3 mutations. A study from the Aichi Cancer Center[9] (from patients
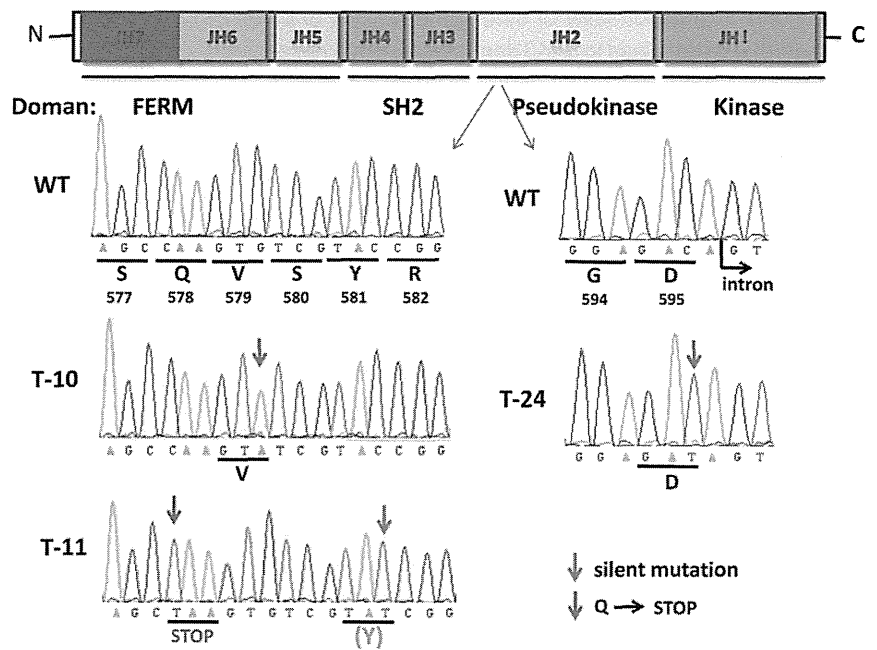
Figure 1 JAK3 (Exon 13) mutation sites detected by Sanger sequencing. Four mutations in the JH2 pseudo-kinase domain are found from three cases of ENKTL from Thailand. The mutations included T-10: 579V(GTG)–579V(GTA), T-11:578Q(CAA)–stop(TAA), T-11:581Y (TAC)–581Y(TAT); T-24: D(GAC)–D(GAT). Three of them are silent mutations and one introduces a premature stop codon. T10, T11, T24 are serial numbers of the samples.
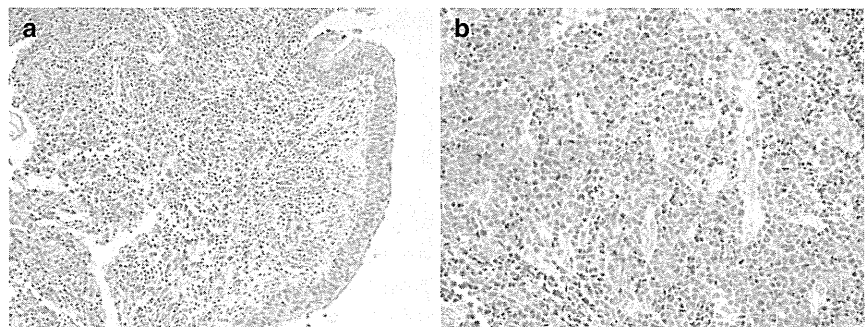

Figure 2 Immunostaining of JAK3 (phospho Y785) against NKTCL. Immunohistochemistry analysis of tumor sections in two representative patients show constitutive phosphorylation of JAK3 at tyrosine 785 in neoplastic cells from 4 of the 13 extranodal, natural killer/T-cell lymphoma samples. Panel (a) shows magnification of 100X and (b) shows magnification of 200X.

who gave consent to share their private data) included 42 cases of NK/T cell neoplasms and seven NK/T cell lines and detected no mutations by sequencing of exon 13 of the JAK3 gene (those cases are all EBER positive). In all, it seems that the activating JAK3 exon 13 mutation in the JH2 domain is not a frequent mutation event in T/NK cell lymphomas. However, we did detect four mutations (including three silent mutations and one stop codon introduction) in three NKTCLs. Notably, these mutations were not found in ATLL, AITL, PTCL-NOS, or CAEBV. These results suggest that the JAK3 mutations occur exclusively in ENKTL, although activating mutations are rare. In this study, we detected the activated expression of JAK3 by immunohistochemistry against ENKTL samples. We found four cases positive for JAK3 from 13 ENKTL patients, but none of the positive cases carried any JAK3 mutations. The cases with JAK3 mutations did not show aberrant JAK3 expression. Thus, the activated expression of JAK3 has no relation with the JAK3 JH2 mutations found in our study. We found another new report from France about JAK3 activated expression and

activating mutations in ENKTL after we finished our study.[10] In this study they found 87% of the cases were positive for activated JAK3 but only 21% of the patients harbored acquired mutations in the JAK3 gene. The discrepancy of the positivity of JAK3 activated expression may due to different phosphorylated JAK 3 antibodies used in the two studies or because of different populations. There are two currently available commercial antibdies to detect phosphorylated JAK3: Anti-JAK3 (phospho Y785) antibody, clone ab61102 (Abcam Ltd) and Anti-JAK3 (phospho Y980), clone sc-16567 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). These two antibodies detect correspondingly phosphorylated JAK3. Our results of immunostaining were similar to the above French study in that the cytoplasm of malignant cells was stained by an aiti-phospho-JAK3 antibody, providing evidence that JAK3 is constitutively phosphorylated. But we don't know the relationship of the positivity of these two antibodies. In any case, the two studies found a similar phenomenon: cases with activated expression of JAK3 do not necessarily harbor mutations in the

JAK3 gene. The abnormality of other gene regions or other mechanisms may be lead to JAK3 deregulation in the absence of mutations. The downstream effects of activated JAK3 and their potential role in the pathogenesis of ENKTL is a topic for future studies.

Extranodal Natural killer/T cell lymphoma is particularly prevalent in Asian countries and some parts of Latin America. EBV is believed to play an important role in lymphomagenesis.[11] On the other hand, no recurrent chromosomal or genetic abnormalities have been identified for ENKTL or ANKL, except for the new found JAK3 mutations.[3,10] Chromosome 6q loss frequently occurs in ENKL,[12–14] but is rather rare in ANKL.[14,15] The PRDM1 gene, also known as BLIMP1, was reported to be deleted or mutated in ENKTL lymphoma cells.[16] However, this gene is not lineage-specific to NK/T-cell lymphomas. From our study and the Aichi Cancer Center's study, we did not find the same JAK3 JH2 domain mutations as in the former study.[3] This means this new-found mutation is not a frequent mutation event among ENKTL patients from Thailand and Japan. However, we cannot deny that the activating mutation may occur in other regions of JAK3 or different activating mutations may occur in other geographical regions. Further investigations are required to identify driver genetic abnormalities for NK/T cell neoplasms.

ACKNOWLEDGMENTS

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Microarray analysis of gene expression by microdissected epidermis and dermis in mycosis fungoides and adult T-cell leukemia/lymphoma

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Abstract. The characteristic histopathological feature of mycosis fungoides (MF) and adult T-cell leukemia/lymphoma (ATLL) is epidermotropism. To identify the mechanism for epidermotropism of lymphoma cells, total RNAs were obtained from skin biopsies of epidermis and dermis of MF and ATLL patients by means of laser capture microdissection, and used for subsequent complementary DNA (cDNA) microarray experiments. This procedure has made it possible for us to observe and evaluate the regional environment of MF and ATLL. Hierarchical cluster analysis revealed that the cDNAs could be clearly differentiated into MF and ATLL. CCL27 was expressed in the dermis generated from keratinocytes, CCR4/CCR6/CCR7/CCR10/cutaneous lymphocyte-associated antigen (CLA) lymphoma cells in the dermis, and CCL21 in the extracellular matrix (stroma). Lymphotoxin (LT) β and CCL21 expression was significantly higher and that of CCR10 relatively for MF, while CCR4 and CLA expression was relatively higher for ATLL. In the epithelium, keratinocytes expressed CCL20/CCL27, and lymphoma cells CCR4/CCR6/CCR10, while CCR4, CCR6, CCL20 and CCL27 expression was relatively higher for ATLL than MF. The dermis of MF, but not that of ATLL, showed correlation between CCR7 and CCL21. These findings support the suggestion that chemokines and chemokine receptors are involved in the pathogenesis of

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MF and ATLL, indicate that cutaneous homing seems to be different for MF and ATLL, and point to the possibility that cutaneous T-cell lymphomas originate in regulatory T cells, especially in the case of ATLL.

Introduction

Mycosis fungoides (MF) is the most common form of cutaneous T-cell lymphomas (CTCL), which is a heterogeneous group of non-Hodgkin's lymphomas thought to result from malignancies of skin homing T cells (1) and presenting with patches, plaques and tumors.

While the early stage shows a wide clinical spectrum that overlaps with several inflammatory dermatoses, clinical as well as pathological findings indicate various overlaps between MF and inflammatory diseases, with epidermotropism disproportionate to the degree of spongiosis being one of the most useful pathological distinguishing features.

Adult T-cell leukemia/lymphoma (ATLL) is a human malignancy associated with human T-cell lymphotropic virus-type I (HTLV-1). Cutaneous lesions of ATLL consist of papules and nodules/tumors. While survival of ATLL cases with skin manifestations is reportedly significantly shorter than that of MF cases (2), histological findings for the two diseases are similar.

The mechanism of epidermotrophism seen in MF and ATLL has not yet been clearly identified. What is known is that chemokines regulate multiple cell functions, including cell chemotaxis, proliferation and apoptosis, and are involved in leukocyte transendothelial migration and homing to tissues. Previous studies have reported that positivity for CCR4 is significantly associated with skin involvement in MF and ATLL, and CCR10 is expressed by malignant cells in MF and Sézary syndrome (SS) (3). Malignant MF and SS cells also show high expression of CCR7. The cutaneous lymphocyte-associated antigen (CLA) recognized by the HECA-452

antibody is an adhesion molecule selectively expressed by a subset of circulating memory T-cells, normal T cells in inflamed skin and by the vast majority of CTCL (4-6). A previous study found that CLA was expressed on epidermotrophic lymphoma cells in all early stages MF. As for ATLL, CLA$^+$ cases showed a significant preference for skin involvement when compared with the CLA$^-$ cases as also previously reported by other investigators (7).

Complementary DNA (cDNA) microarray technology is a powerful tool for gaining insight into the molecular complexity and pathogenesis of various diseases and makes it possible to identify differences in numerous gene expressions.

Since the skin consists of epidermis [including keratinocytes and dendritic cells (DCs)], dermis (stroma and vessels) and subcutaneous tissue with or without inflamed and/or inflammatory cells, gene expression of epidermis and dermis should be analyzed separately to gain a better understanding of the contribution made by each component of a tissue to the pathogenesis of epidermotrophism. To this end, laser capture microdissection (LMD) was used in our study to allow us to focus on the differences in gene expression levels between epidermis and dermis in MF and ATLL and to subsequently determine these expressions immunohistochemically in patients.

It is believed that this technique, which combines LMD and microarray technology for the analysis of CTCL gene expression in epidermis and dermis, can improve our understanding of epidermotrophism at the molecular level.

Materials and methods

Eighteen skin samples obtained from patients treated at the Department of Dermatology, Kurume University School of Medicine consisted of five epidermal and three dermal samples each from MF and ATLL as well as two epidermal samples from dermatitis patients used as controls. Skin biopsies were performed after informed consent had been obtained from all patients or their guardians in accordance with the Declaration of Helsinki. Both paraffin-embedded and frozen tissues were used. Histopathological diagnoses were based on the World Health Organization classification (WHO) and performed by five pathologists (K.H., J.K., Y.K., H.S. and K.O.). The clinical data for all patients obtained from the medical records are summarized in Table I, which shows that both epidermal and dermal samples were harvested from two MF (E-M3/D-M1 and E-M5/D-M2) and two ATLL (E-A2/D-A1 and E-A3/D-A2) patients. The other samples were either epidermal or dermal. This study was approved by the Kurume University Institutional Review Board.

Laser microdissection. The samples were embedded in an optical cutting temperature compound (Sakura Finetechnical, Tokyo, Japan), immediately frozen in liquid nitrogen, and stored at -80°C for microdissection. A series of 10-μm thick sections were cut from frozen tissue specimens at -20°C, and mounted on 2.0-μm-thick PEN-Membrane slides (MicroDissect GmbH, Herborn, Germany). The epidermal and/or dermal regions were microdissected from the about 70 cryosections by means of LMD. After fixation in 100% ethanol, the slides were stained in turn with Mayer hematoxylin and eosin, washed

in diethylpyrocarbonate-treated water at each phase of the process and then air-dried with a fan. The frozen sections were microdissected with a Leica LMD6000 laser microdissection system (Leica, Wetzlar, Germany) in accordance with the company's protocol. Finally, the microdissected fragments were dropped into 0.5 ml tube caps containing 50 μl lysis buffer for RNA extraction (8).

RNA extraction and biotinylated cRNA amplification. Total RNA was extracted from the samples collected by means of LMD and with an RNAqueous-Micro kit (AM1931; Ambion, Austin, TX) according to the manufacturer's instructions. Complementary RNA (cRNA) amplification and labeling with biotin were used for gene expression profiling by microarray analysis. Briefly, 500 ng total RNA was amplified overnight (14 h) with the Illumina Total Prep RNA Amplification kit (AMIL1791; Ambion) according to the manufacturer's protocol. Reaction cRNA was biotinylated during *in vitro* transcription.

Illumina BeadChips microarray. Sentrix Human WG-6 v3.0 Expression BeadChips were purchased from Illumina, Inc. (San Diego, CA). More than 48,000 different bead types, each with a 50-base gene-specific probe, are represented on a single Beadchip. For each probe represented on the array, beads are assembled with an average 30-fold redundancy. A hybridization mixture containing 1.5 μg biotinylated cRNA was hybridized to the beadchips at 58°C overnight (18 h) before being washed and stained with streptavidin-Cyanine-3 (Cy3) (PA23031; GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol. Beadchips were scanned on Illumina BeadStation 500 and fluorescent hybridization signals were assessed with Illumina Beadstudio software.

The data discussed in this publication have been deposited in National Center for Biotechnology Information (NCBIs) Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih. gov/geo/) and are accessible through GEO Series accession number GSE40639.

Statistical analysis. For the pre-processing step, variance in the data was first stabilized with the variance stabilizing transform method (9) and then normalized with a robust spline normalization method, both of which are used with the Lumi BioConductor package (Illumina) (10). Effectively absent transcripts were filtered out to reduce false positives. Detection of transcripts was considered to be achieved if the detection p-value calculated from the background with the Illumina BeadStudio was <0.05 for all hybridizations. The Significance Analysis of the Microarrays statistical test (11), which is used as part of the bioconductor 'samr' package and takes multiple testing into account by estimating the false discovery rate, was used to identify differentially expressed transcripts for a comparison of MF and ATLL patients. If a transcript was up- or downregulated by a factor of ≥2 and had a Significance Analysis of Microarrays q-value (false discovery rate) of <0.001, we regarded the expression of this transcript in MF patients as different from that in ATLL patients. To obtain reproducible clusters for classifying the 16 samples (all samples detailed above except for the two control samples), expression data were analyzed with

Table I. Summary of patients enrolled in this study: number, age, gender and stage.

Sample no.	Age (years)	Gender	TNM	Stage
MF				
E-M1	78	Male	T2aN0M0	IB
E-M2	62	Female	T2bN0M0	IB
E-M3,D-M1	57	Male	T1bN0M0	IA
E-M4	22	Male	T1bN0M0	IA
E-M5, D-M2	64	Male	T2bN0M0	IB
D-M3	61	Male	T2bN0M0	IB
ATLL				
E-A1	56	Male	T2aN2M0	IIA
E-A2, D-A1	70	Male	T2cN0M0	IB
E-A3, D-A2	32	Female	T2cN0M0	IB
E-A4	63	Male	T3aNxM0	IIB
E-A5	79	Male	T3bN0M0	IIB
D-A3	54	Male	T3aN1M0	IIB
Control				
N1	66	Male		
N2	71	Female		

The samples were obtained from six MF and ATLL patients each, and from two dermatitis patients as controls. The five epidermal (E) samples of MF (-M) patients were numbered E-M1 through E-M5, and the three dermal (D) samples of MF (-M) patients were numbered D-M1 through D-M3. Similarly, the ATLL (-A) samples were numbered E-A1 through E-A5 and D-A1 through D-A3. The two epidermal samples from dermatitis patients used as controls were numbered N1 and N2.

GeneSpring 7.2 software (Silicon Genetics, Redwood City, CA), which was also used to generate heatmaps of certain genes of interest.

Immunohistochemistry. The paraffin-embedded specimens were used for manual immunohistochemical analysis of CCR4 (Pharmingen, San Diego, CA), CCR7, CCR6 and CCR10 (all from Medical and Biological Laboratories Co., Ltd., Nagoya, Japan), CCL20, CCL27, lymphotoxin (LT) β and TNF receptor (TNFR) 2 (all from R&D Systems, Minneapolis, MN), CCL21 (Santa Cruz Biotechnology, Santa Cruz, CA), β-defensin (BDF) 1 (Phoenix Pharmaceuticals, Inc., Burlingame, CA) as previously described (12). Appropriate positive and negative control experiments were run simultaneously. Heat-mediated antigen retrieval was used for all analyses except those of CCR6 and BDF1. The staining results were evaluated semiquantitatively by three independent observers, and scored comprehensively in view of intensity, expression pattern and number of positive cells, and so on. Images were visualized with an Olympus AX80 microscope (Olympus, Tokyo, Japan), equipped with an Olympus Planapo 40x/0.95 numerical aperture objective. Images were captured with an Olympus DP70 camera and Olympus DP controller software, and were processed with Olympus DP manager software.

Results

Following appropriate normalization and standardization procedures, the data on each chip were compared with each other by using a hierarchical clustering method (Fig. 1). Genes differentially expressed in MF and ATLL were organized by means of Ingenuity Pathway Analysis into an interactome network, which was then used for an comparison of the gene expression (transcriptional profile) of epidermal or dermal MF with that of ATLL. In the epidermis, 580 probe sets were identified as upregulated or downregulated on a heat map (Fig. 1A), while in the dermis, 234 probe sets were found to be up- or downregulated (Fig. 1B). The first 10 genes showing the highest upregulation are listed in Table II. In the dermis, LTβ and CCL21 in particular showed different expressions in MF and ATLL.

Next, we focused on the array data that could identify the possible involvement of specific chemokine receptors and ligands, including CCR4, CCR6, CCR7 and CCR10 and others, in the pathophysiology of CTCL, MF and ATLL. CCR4 expression was especially high in ATLL, while CCL27 expression in the epidermis was high in both MF and ATLL, and CCR10, a receptor of CCL27 detected in the dermis, was more highly expressed in MF than in ATLL. Also in the dermis, expression of the LN homing molecule CCR7 was high in both MF and ATLL, while a close correlation between CCR7 and CCL21, a chemokine receptor and its ligand, was observed in MF. CCR6 expression was elevated in the epidermis and dermis of both MF and ATLL, while expression of CCL20, a CCR6 ligand, showed no correlation with CCR6. BDFs were identified as not only antibacterial peptides but also as CCR6 ligands. BDF 1 expression was reduced in the epidermis of MF and ATLL, while BDF 3 expression in the epidermis of MF was higher than in controls, which was similar to the finding of a previous study (13).

We also focused on CLA, which is recognized as an adhesion molecule selectivity expressed by a subset of circulating memory T-cells, normal T cells in inflamed skin and by the vast majority of CTCL (4-6). CLA was expressed in the dermis of both MF and ATLL, with a particularly high expression in ATLL.

In order to confirm the expression levels of the proteins, CCR6, CCR7, CCR10, CCL20, CCL21, CCL27, BDF1, LTβ and TNFR2 on MF and ATLL tissue sections were subjected to immunohistochemistry. The findings of the immunohistochemical analysis were basically consistent with the results of the microarray analysis. A representative stain is shown in Fig. 2 and the immunohistochemical scores are presented graphically in Fig. 3.

In principle, CCR4, CCR6, CCR7, CCR10 and/or CLA were all expressed in the lymphoma cells. CCR4, CCR6 and CCR10 were identified in both dermis and epidermis, while CCL27 and CCL20 were expressed in keratinocytes and CCL21 was expressed in the extracellular matrix (stroma). In addition, CCL27 was also stained in dermis released from keratinocytes.

In controls, epidermal keratinocytes weakly expressed CCL27 within their cytoplasm, and mainly in the spinous

Heat maps

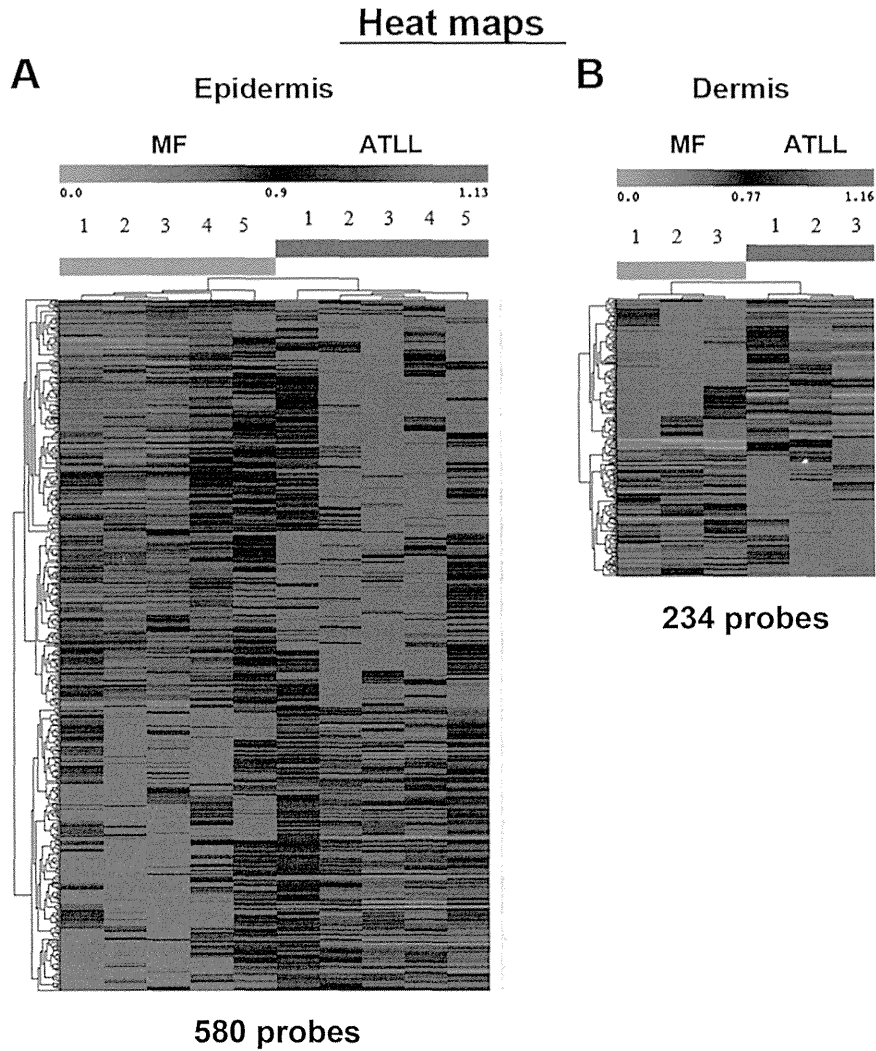**A** Epidermis

B Dermis

580 probes

234 probes

Figure 1. Gene expression profiles and clustering. The gene expression of MF was compared with that of ATLL by using a hierarchical clustering method. (A) In the epidermis, 580 probe sets were identified as up- or downregulated. (B) In the dermis, 234 probe sets were identified.

layers of the epidermis. However, positive lesions of epidermis keratinocytes were stronger in thicker layers in most MF and ATLL patients than in controls. Moreover, CCL27 was expressed not only in cytoplasm but also occasionally in membrane of MF and ATLL, so that there was in fact not much difference between these two types of CTCL. Faint CCL27 expression in dermis was detected in MF, ATLL and in one of the controls. The differences in CCL27 expression levels in dermis were immunohistochemically imperceptible. CCR10 was stained weakly in both epidermis and dermis, but in the dermis usually more strongly in MF than in ATLL cells, which correlated moderately with the overall gene expression profile. CCR6 expression was more elevated in the epidermis and dermis of both MF and ATLL than in controls, and the immunohistochemical score correlated with the result of the microarray analysis. CCL20 was expressed very weakly in the cytoplasm of keratinocytes, stained in the granular layer of all samples and in all layers of the epidermis of some samples.

Although each one of the controls and MF samples showed high levels of CCL20, all ATLL samples displayed high expression in the epidermis. Expression of BDF1 in nucleus

and/or nuclear membrane was diminished in the epidermis of MF and ATLL. Because the stain was quite faint, it was difficult to determine even whether CCL20 and BDF1 in the dermis were positive or negative. The cell membrane of infiltrating lymphoma cells stained for CCR7, while staining for CCL21 was observed within the cytoplasm of epidermal keratinocytes and was diffusely distributed on the dermal extracellular matrix. The intensity and density of CCR7$^+$ infiltrating lymphoma cells correlated with the intensity of CCL21 in stroma in the dermis of MF cases. LTβ positive cells were scattered throughout the dermis and their expression was homogeneous in the nucleus or the surface of the nucleus of lymphoma cells, while LTβ expression levels in the dermis were higher for MF than for ATLL. No correlation with LTβ was observed for TNFR2, which is one of the soluble secreted LT homotrimers (LTα3) that trigger TNFRs.

Discussion

The microarray and immunohistochemistry findings of our study showed high expression of LTβ and CCL21, and correl-

Table II. Genes differentially regulated in MF and ATLL.

A, Upregulated genes in MF compared with genes in ATLL

Epidermis	Gene symbol	Gene name	Exp. value
1	ZSCAN18	Zinc finger and SCAN domain containing 18	1.392
2	COL16A1	Collagen	1.217
3	EID2B	EP300 interacting inhibitor of differentiation 2B	1.167
4	HSPA1A	Heat shock 70 kDa protein 1A	1.046
5	GGA1	Golgi associated	1.038
6	LRIG1	Leucine-rich repeats and immunoglobulin-like domains 1	1.010
7	XPNPEP3	X-prolyl aminopeptidase (aminopeptidase P) 3	1.003
8	SMARCA2	SWI/SNF related	0.979
9	ID2	Inhibitor of DNA binding 2	0.968
10	ARL6IP5	ADP-ribosylation-like factor 6 interacting protein 5	0.950

Dermis	Gene symbol	Gene name	Exp. value
1	LTB	Lymphotoxin β	2.946
2	CCL21	Chemokine (C-C motif) ligand 21	2.189
3	IFITM3	Interferon induced transmembrane protein 3	1.666
4	THY1	Thy-1 cell surface antigen	1.594
5	ACTN1	Actinin	1.585
6	CHST15	Carbohydrate	1.449
7	PON2	Paraoxonase 2	1.447
8	CYR61	Cysteine-rich	1.436
9	TAGLN	Transgelin	1.436
10	PRRX1	Paired related homeobox 1	1.412

B, Downregulated genes in MF compared with genes in ATLL

Epidermis	Gene symbol	Gene name	Exp. value
1	GPX3	Glutathione peroxidase 3	-2.097
2	PSMC1	Proteasome	-1.668
3	CALML3	Calmodulin-like 3	-1.587
4	NDRG4	NDRG family member 4	-1.169
5	PRSS3	Protease	-1.113
6	ADM	Adrenomedullin	-1.063
7	SMOX	Spermine oxidase	-1.056
8	CBX3	Chromobox homolog 3	-1.044
9	WBP5	WW domain binding protein 5	-0.970
10	LRRC20	Leucine rich repeat containing 20	-0.967

Dermis	Gene symbol	Gene name	Exp. value
1	TOX2	TOX high mobility group box family member 2	-2.925
2	ADAP1	ArfGAP with dual PH domains 1	-2.848
3	AKAP7	A kinase anchor protein 7	-2.449
4	CADM1	Cell adhesion molecule 1	-2.293
5	ZNF365	Zinc finger protein 365	-2.157
6	RGS2	Regulator of G-protein signaling 2	-2.129
7	MTX3	Metaxin 3	-2.080
8	SH3KBP1	SH3-domain kinase binding protein 1	-1.871
9	CHST7	Carbohydrate sulfotransferase 7	-1.792
10	CST7	Cystatin F (leukocystatin)	-1.739

The 10 most significant signatures of gene expression are shown. LTβ and CCL21 in the dermis showed higher expressions in MF than in ATLL.
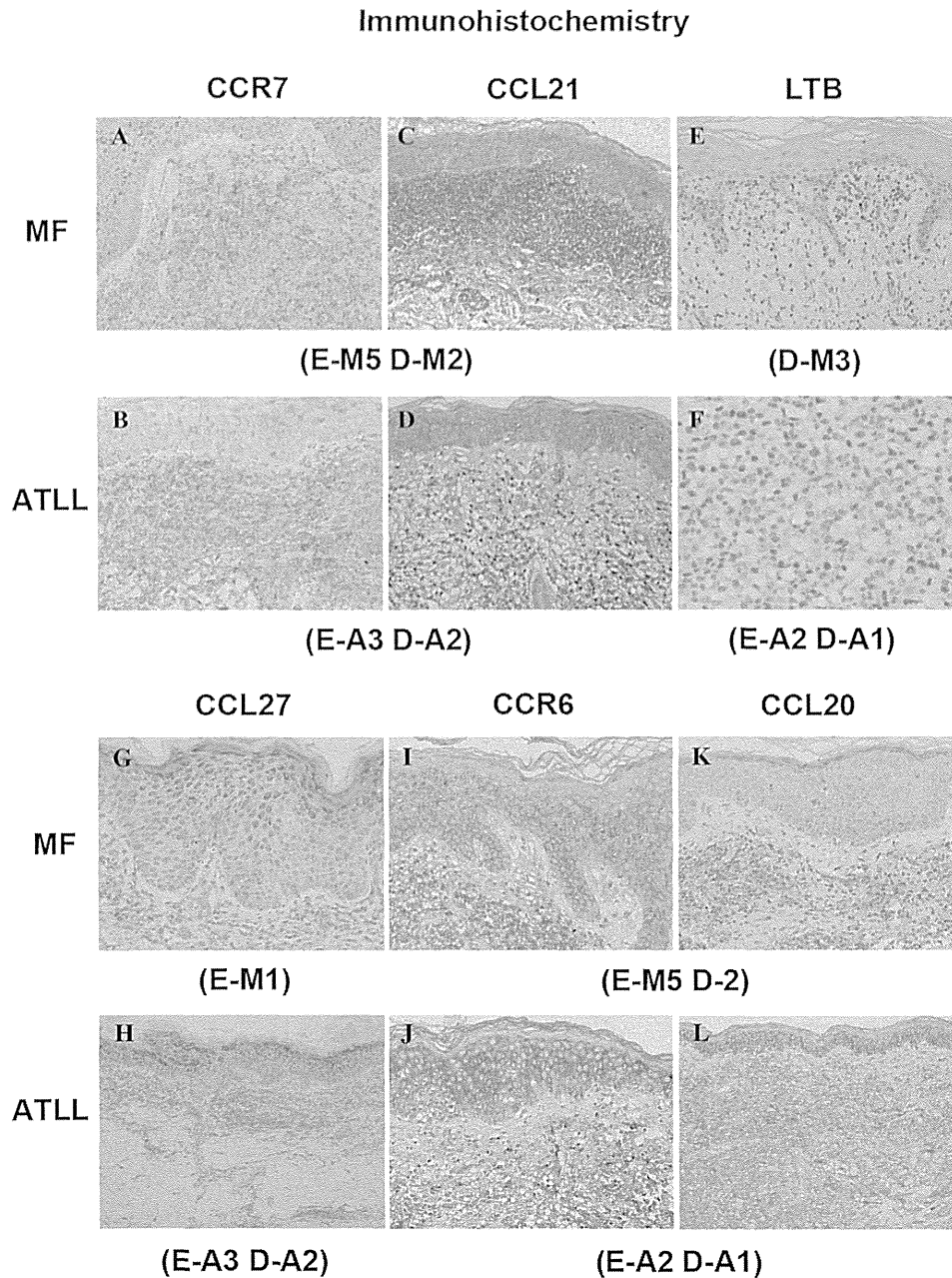
Immunohistochemistry

Figure 2. Representative immunohistochemical stain. CCR7 was expressed in the lymphoma cells (A, B) and CCL21 in the extracellular matrix (stroma) (C, D). CCL21 and LTβ were higher in the dermis in MF (C, E) than in ATLL (D, F). CCL27 (G-H) and CCL20 (K-L) were expressed in keratinocytes. CCR6 expression in the epidermis and dermis was elevated in both MF and ATLL (I-J). CCL27, CCR6 and CCL20 in the epidermis were relatively higher in ATLL than in MF. (Original magnification x100). (A, C, I, K) were from sample no. E-M5 D-M2 and (B, D, H) from E-A3 D-A2, (E) was from D-M3, (F, J, L) were from E-A2 D-A1 and (G) was from E-M1.

tion between CCL21 and CCR7, a receptor of CCL21 which is the LN homing molecule, was observed in the dermis of MF. CCR7 expression was also high in the dermis of ATLL.

LTβ, which was indentified in the dermis of MF samples in our study, is a member of the tumor necrosis factor superfamily. LTβ in human cells contains a transmembrane domain, and the surface LT form is most likely a trimer with an LTα1β2 stoichiometry, while the LTα1β2 heterotrimer binds the LTβ receptor (LTβR) (14).

LTs are expressed by activated T-, B-, NK- and lymphoid tissue inducer cells (15-17) and are crucial for organogenesis and maintenance of lymphoid tissues (18,19). LTβR, on the other hand, is expressed on many different cell types including cells of epithelial lineages, while ligation of LTβR results in NF-κB activation (20-22) and leads to secondary lymphoid organogenesis and homeostasis. Signaling via LTβR is involved not only in host defense and autoimmune diseases, but also in tumor cell proliferation (23). Furthermore, NF-κB stimulates proliferation and blocks programmed cell death (apoptosis) in various cell types (24,25).

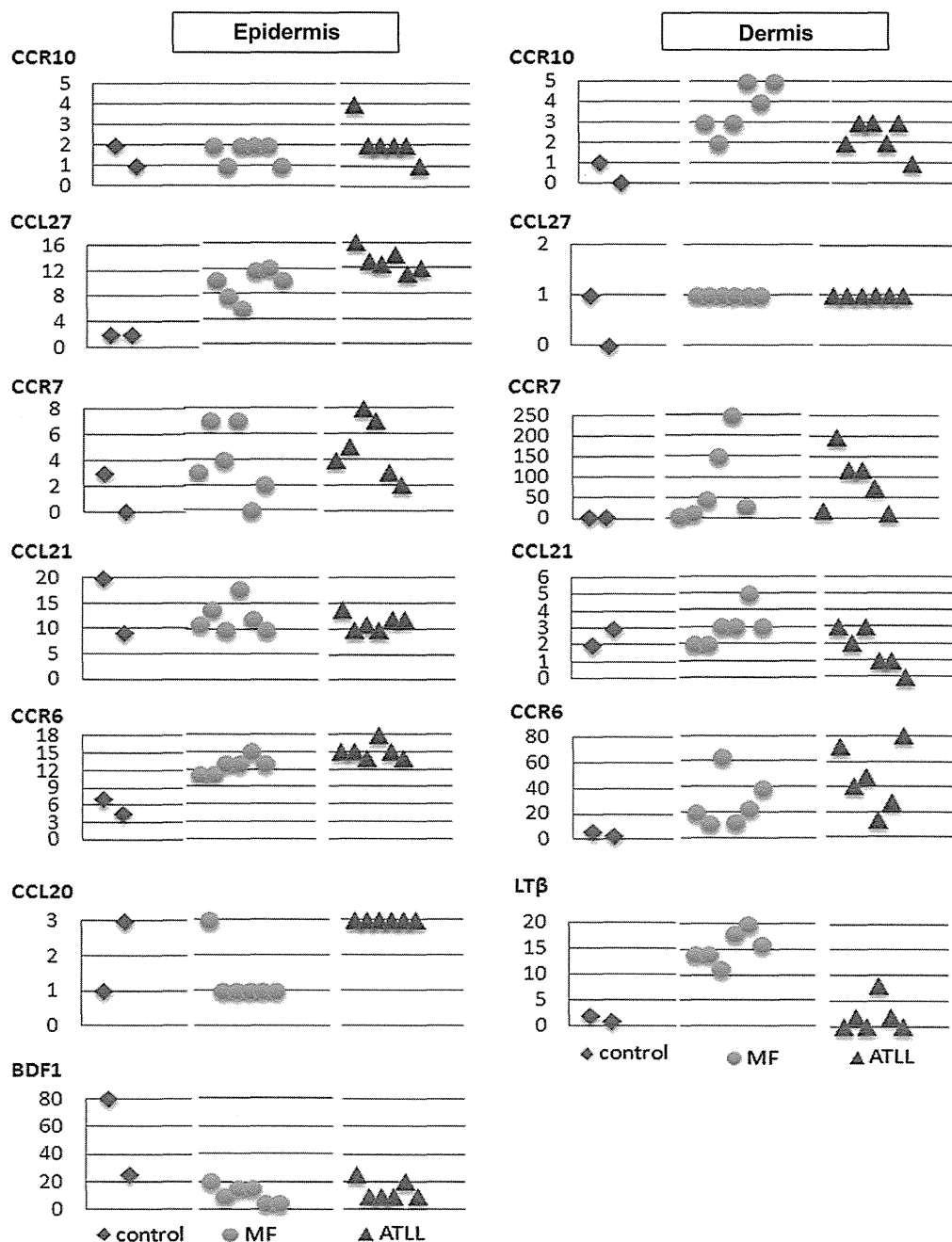Previous reports have suggested that the activation of the NF-κB signaling pathway via LTβR results in the prolifer-

Figure 3. Immunohistochemical scores. Scores for LTβ and CCR10 in the dermis were usually higher for MF than ATLL. CCR6 was more elevated in the epidermis as well as dermis of both MF and ATLL than of controls. CCL20 showed a high score in the epidermis of all ATLL samples. Controls are shown as rhomboids, MF cases as dots and ATLLs as triangles.

ation of melanoma (26) and hepatocellular carcinoma (27). NF-κB activation was also detected in lymphoid malignancies (28,29). In this connection, it was found that peripheral T cell lymphomas/not otherwise specified (PTCLs/NOS) with NF-κB inactivity showed better survival (30-32).

Another study reported that LTβR organizes the recruitment and survival of CD4 T cells through the induction of CCL21 expression which is produced exclusively by stroma (33). In our study we found that expression levels of both the LTβ and CCL21 gene are high in the dermis of MF. These findings seem to suggest that LTβR, which can be expressed

in epidermis, organizes the recruitment and survival of CD4-positive MF cells through the induction of CCL21.

CCR7 (the receptor of CCL21) physiologically regulates the lymphoid homing of T-cells and probably plays an important role in the tropism of CTCL cells to peripheral LNs, which constitutively synthesize CCR7 ligands, CCL19 and CCL21 (34). It has been further hypothesized that CCR7, which is expressed at fairly high levels in SS cells (35), may function as a mediator of LN infiltration of CTCL. In our study, CCR7 expression was detected in the dermis of MF and ATLL. Interaction between CCR7 and CCL21 in the dermis

of MF probably functions to maintain localization to the skin for a long time. On the other hand, T cells infected by HTLV-1 circulate in the bloodstream from the outset, while ATLL cells, expressing the skin-homing properties of CCR4, CLA, CCR6 and CCR10, may affect cutaneous involvement in smorderling or cutaneous type of ATLL patients.

While T cells infected by HTLV-1 are circulating in the peripheral blood, they can spontaneously encounter CCL21 produced by LNs, and CCR7+ ATLL cells can be trapped in the LNs. Migration to the LNs can thus occur at an earlier stage in ATLL than in MF.

CTCL is characterized by clonal expansion of a mature CD4-positive clone of the Th phenotype, putatively from a skin homing subset of memory T cells (36) whose migration to respective tissues is tightly regulated by adhesion molecules and chemokine receptors. For example, memory T cells that infiltrate the skin express a unique adhesion molecule known as CLA. Chemokines and their receptors have also been associated with tumor metastasis (37-39) and possibly trafficking of lymphoma cells (40). In addition, chemokines are produced by not only malignant T cells but also epidermal keratinocytes, DCs and dermal vessels. We therefore did not use fractionated malignant T cells, but the microdissected epidermal and dermal affected regions in MF and ATLL to investigate the expression profiles of CCR4, CCR6, CCR7, CCR10, CCL20, CCL21, CCL27, BDFs and CLA by means of microarray analysis and/or immunohistochemistry. We found that CCL27 expression in the epidermis was high in both MF and ATLL. CCR10 expression observed in the dermis was higher in MF than ATLL, and the microarray analysis showed that CCR4 expression was especially high in ATLL. Previous studies have reported that CCR4 expression was detected much more frequently in both MF and ATLL than in healthy controls (41,42).

In addition, findings of a previous study indicated that DCs synthesize CCR4 ligands, which rapidly stimulate chemotaxis of, or conjugate formation with, normal T cells (43,44). A characteristic histopathological marker, Pautrier's microabscesses of CTCL, which are known to be DC-malignant T cell conjugates (45), may be initiated by DC-derived chemokines. Other data indicated that preferential expression of CCR4 constitutes a sign of worse prognosis, and CCR4 expression was especially high in ATLL in our study. This finding corresponds to the pathological feature of more visible and larger Pautrier's microabscesses as well as the clinical feature of worse prognosis for ATLL than for MF.

CCR10 is enriched in CLA+ skin-homing T cells of psoriasis, atopic dermatitis and CTCL patients, while it is only rarely expressed on peripheral blood T cells and skin samples from healthy persons (3,46). Moreover, CCL27 (CCR10 ligand) is constitutively present in epidermal keratinocytes (basal layer) under basal, non-inflammatory conditions and can be rapidly released from activated keratinocytes (46). CCL27-CCR10 interactions thus play an early role in the pathophysiology of MF from the patch stage (47), and an increase in CCL27 in the serum of MF patients was observed in previous studies (48). Our data for CCL27-CCR10 interactions agreed with the pathophysiology outlined above.

Our findings also indicate that CCR4 in ATLL as well as CCR10 in MF may play a much more prominent role in

epidermotrophism. However, the origin of malignant T cells in MF and ATLL remains to be fully elucidated. High-level expressions of CCR4, CLA and CCR6 indicate the possibility of not only Th but also Treg origin especially in ATLL. Treg action suppresses activation of the immune system and tolerance for self-antigens. The phenotype of CD4+CD25+ and the expression of Foxp3, a master transcriptional regulator of Treg development and function, suggest that human skin-resident T cells appear to represent Tregs. Tregs also express functional skin-homing receptors. Although CCR6 does not seem to be a functional marker for Treg, it may facilitate CLA+ Treg migration to skin. As for Th, there are conflicting reports about Th1 and Th2 profiles of CCR4 in MF. Berger *et al* demonstrated that malignant CD4+ T cells can be induced to express a CD25+ Treg phenotype (49). Our analyses, however, disclosed that CCR4 and CCR6 in both epidermis and dermis and CLA in dermis were expressed in MF and ATLL, with the expression being especially high in ATLL. Our data therefore support the possibility that the origin of CTCL is Treg (particularly in ATLL) as also previously reported (50). Further investigation will be required to determine whether CTCL shows Th1/Th2/Treg-type polarization in lesional skin.

While the mechanisms involved in tumor homing have not yet been fully identified, it has been suggested that chemokines and chemokine receptors are involved in the pathogenesis of tumor homing. There are no previous reports on the use of the microdissection method for a comparison of epidermal and dermal gene expression levels in MF and ATLL by means of microarray analysis, a procedure which has made it possible to observe and evaluate the regional environment of MF and ATLL, including malignant and inflamed T-cells, DCs, keratinocytes and dermal vessels. DNA microarray analysis enabled us to comprehensively identify differences and patterns in gene expression in MF and ATLL, while our findings support the notion that CCR10 and its ligand CCL27 may contribute to the skin infiltration of malignant T-cells in MF and ATLL. However, further studies are needed to clarify the mechanism for epidermotrophism because of the complexity of its interactions.

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Japan Clinical Oncology Group (JCOG) prognostic index and characterization of long-term survivors of aggressive adult T-cell leukaemia-lymphoma (JCOG0902A)

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Summary

This study evaluated the clinical features of 276 patients with aggressive adult T-cell leukaemia-lymphoma (ATL) in 3 Japan Clinical Oncology Group (JCOG) trials. We assessed the long-term survivors who survived >5 years and constructed a prognostic index (PI), named the JCOG-PI, based on covariates obtained by Cox regression analysis. The median survival time (MST) of the entire cohort was 11 months. In 37 patients who survived >5 years, no disease-related deaths in 10 patients with lymphoma-type were observed in contrast to the 10 ATL-related deaths in other types. In multivariate analysis of 193 patients, the JCOG-PI based on corrected calcium levels and performance status identified moderate and high risk groups with an MST of 14 and 8 months respectively (hazard ratio, 1·926). The JCOG-PI was reproducible in an external validation. Patients with lymphoma-type who survived >5 years might have been cured. The JCOG-PI is valuable for identifying patients with extremely poor prognosis and will be useful for the design of future trials combining new drugs or investigational treatment strategies.

Keywords: adult T-cell leukaemia-lymphoma, Japan Clinical Oncology Group trials, long-term survivors, prognostic index.

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Adult T-cell leukaemia-lymphoma (ATL) is a distinct peripheral T-lymphocytic malignancy associated with human T-cell lymphotropic virus type I (HTLV-1) (Uchiyama *et al*, 1977; Poiesz *et al*, 1980; Hinuma *et al*, 1981; Miyoshi *et al*, 1981; Yoshida *et al*, 1982). Classification of clinical subtypes into acute, lymphoma, chronic and smouldering was proposed based on prognostic factors, clinical features and the natural history of the disease (Shimoyama, 1991). Patients with aggressive ATL (i.e., acute, lymphoma and unfavourable chronic types) have frequently been treated as a subtype of aggressive non-Hodgkin lymphoma (NHL), whereas those with indolent ATL (i.e., favourable chronic and smouldering types) have been managed as a subtype of chronic lymphoid leukaemia (Shimoyama, 1994; Tobinai & Watanabe, 2004). Aggressive ATL typically has a very poor prognosis compared with aggressive B-cell lymphomas, such as diffuse large B-cell lymphoma and peripheral T-cell lymphoma excluding ATL (The International Non-Hodgkin's Lymphoma Prognostic Factor Project's, 1993; Shimoyama, 1994; Gallamini *et al*, 2004; Watanabe *et al*, 2010). In the 1980's, patients with aggressive ATL were reported to have a median survival time (MST) of approximately 8 months, with a 2-year survival rate of <5% because of the multidrug-resistant phenotype of their malignant tumour cells, rapid proliferation of the tumour cells, a large tumour burden with multi-organ failure, hypercalcaemia, and/or frequent opportunistic infections (Lymphoma Study Group, 1991; Shimoyama, 1991, 1994; Tobinai & Watanabe, 2004).

The Japan Clinical Oncology Group (JCOG)-Lymphoma Study Group (LSG) has conducted consecutive clinical trials to improve the survival of patients with ATL. Earlier trials

(JCOG7801, JCOG8101, and JCOG8701) revealed poor prognosis of ATL compared with other aggressive NHLs (Shimoyama *et al*, 1988; Tobinai *et al*, 1994). Furthermore, the disappointing results with conventional chemotherapies in the 1980s and the proposal for a subtype classification of ATL led us to conduct clinical trials with new agents that exclusively targeted aggressive ATL. The first phase II trial, JCOG9109 (1991–1993), evaluated combination chemotherapy with deoxycoformycin, an inhibitor of adenosine deaminase, which had been effective as a single agent against relapsed or refractory ATL (Tobinai *et al*, 1992). However, the results were disappointing with an MST of 7 months, similar to the findings of previous JCOG-LSG trials (Tsukasaki *et al*, 2003). The next phase II trial, JCOG9303 (1994–1996), evaluated the chemotherapy regimen VCAP-AMP-VECP (LSG15) against aggressive ATL. This dose-intensified multi-agent chemotherapy consisted of vincristine, cyclophosphamide, doxorubicin (DXR) and prednisone (PSL) for VCAP, DXR, ranimustine and PSL for AMP, and vindesine, etopside, carboplatin and PSL for VECP, supported by granulocyte colony-stimulating factor and intrathecal (IT) prophylaxis with methotrexate (MTX) and PSL. This phase II trial showed promising results, with complete remission (CR) and overall response rates of 36% and 81%, respectively, and an MST of 13 months at the expense of haematological and other toxicities (Yamada *et al*, 2001). Based on these results, we proceeded to the phase III trial JCOG9801 (1998–2003), which compared a modified VCAP-AMP-VECP regimen (shortened from 7 to 6 courses), to which cytarabine was added to the IT prophylaxis, *versus* CHOP (cyclophosphamide, DXR, vincristine and PSL)-14 supported by granulocyte

colony-stimulating factor and IT prophylaxis identical to the former regimen. The CR and 3-year overall survival (OS) were higher in the modified VCAP-AMP-VECP arm than in the CHOP-14 arm (40% vs. 25% and 24% vs. 13% respectively), suggesting that the former is a more effective regimen at the expense of greater toxicity for patients with newly diagnosed aggressive ATL (Tsukasaki et al, 2007).

Through these 3 JCOG trials for patients with aggressive ATL, the 5-year OS was improved, from 5% in the 1980's to 15% in the 1990s. To characterize the long-term survivors of aggressive ATL and to develop a new prognostic index (PI) for the disease, we performed a combined analysis (JCOG0902A) of all the patients enrolled in the 3 JCOG trials.

Methods

Study population

A total of 276 patients who were registered in the 3 JCOG trials described above were enrolled in this study (Yamada et al, 2001; Tsukasaki et al, 2003, 2007). Some patients did not receive anti-viral therapy using interferon-alpha and zidovudine because these drugs for ATL was not covered by the National Health Insurance in Japan. The eligibility criteria for the 3 JCOG trials were detailed in previous reports (Yamada et al, 2001; Tsukasaki et al, 2003, 2007). Briefly, patients were eligible to participate if they had aggressive ATL (i.e., acute, lymphoma, or unfavourable chronic type) with no prior chemotherapy, were aged 15–69 years and had preserved organ functions, no proven central nervous system (CNS) involvement and a performance status (PS) of 0–3 or 4 due to hypercalcaemia caused by ATL. The diagnosis of ATL was made based on seropositivity for HTLV-1 antibody and histologically and/or cytologically proven peripheral T-cell malignancy. Monoclonal integration of HTLV-1 provirus was analysed in 104 of 276 patients studied. Among these 104 patients, integration was detected in 100 patients and not detected in four patients.

The PI for the JCOG trials, which we refer to as the JCOG-PI, was constructed from the data of patients who participated in these trials (training set) and was then applied to an external validation set. The external validation set consisted of 136 patients who had not participated in prior JCOG studies but had received anthracycline-containing regimens as initial chemotherapy at three sites (Nagasaki University Hospital, Nagasaki Medical Centre, and Sasebo City General Hospital) under the remit of the JCOG-LSG. These patients were a subset of those from a previous retrospective study (Katsuya et al, 2012) and their OS and corrected calcium levels were reviewed.

Data and analysis sets

The endpoint of this study was OS, defined as the duration between registration to each JCOG trial and death from any

cause or censored at the last follow up in living patients. For the validation data set, we substituted the date of treatment initiation for the date of registration.

Candidate covariates were sex, age, Eastern Cooperative Oncology Group (ECOG) PS, B symptoms, clinical stage, liver involvement, lactate dehydrogenase, blood urea nitrogen (BUN), corrected calcium levels, serum total protein, serum albumin, white blood cell count, total (normal and abnormal) lymphocyte count, neutrophil count and platelet count. We excluded the treatment regimen from the covariates because our aim was to create an index that could stratify the patients' prognosis and be applicable to future clinical trials evaluating various promising regimens. Cut-off values were determined clinically by dividing the continuous biological and laboratory test variables into no more than three categories. The data of 193 patients with a complete set of candidate covariates were used for the training set (Fig 1).

The protocol of this study was reviewed and approved by the JCOG Protocol Review Committee.

Statistical analysis

Patients who survived >5 years were categorized according to ATL subtype (acute, lymphoma or unfavourable chronic types). In addition, to evaluate the ATL-related death events for each subtype, a disease-specific mortality curve was estimated, for only those patients who survived >2 years, by means of a competing risks framework (Kalbfleisch & Prentice, 2002). The proportion of patients who survived >5 and >10 years was calculated to evaluate the association between long-term survival and CR (including CR unconfirmed) for initial treatment. The proportion of cases with
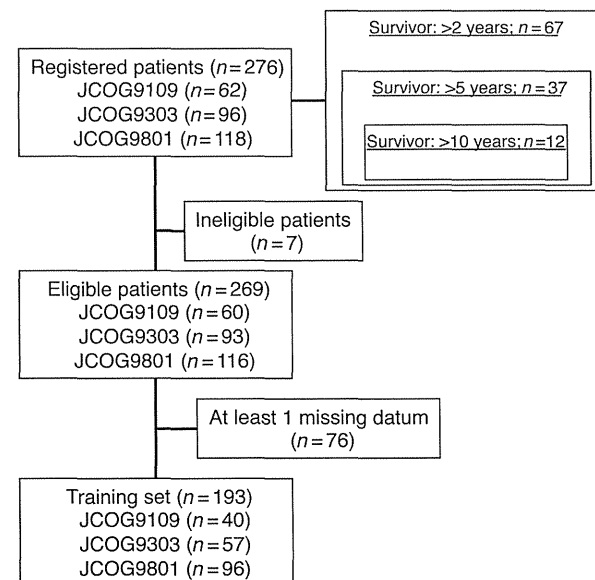

Fig 1. Patient disposition of the training set.

CNS involvement was compared among the JCOG trial regimens in an exploratory evaluation of the efficacy of prophylactic IT treatment. The prophylactic IT treatments against CNS involvement were: none in JCOG9109, MTX and PSL in JCOG9303, and MTX, cytarabine and PSL in both regimens in JCOG9801. Confidence intervals (CIs) for all the above proportions were computed using the Clopper–Pearson method (Clopper & Pearson, 1934).

Analyses for the development and validation of the JCOG-PI were performed according to a pre-specified analysis plan. The JCOG-PI consisted of risk groups that were developed using Cox's proportional hazards model. Before constructing the JCOG-PI, covariates with several definitions were selected for those with the smallest Akaike's Information Criteria (Akaike, 1973) on univariate analysis. Next, we verified the correlations between covariates to avoid multi-colinearity. Stepwise Cox regression analysis was then performed to identify unfavourable prognostic factors for constructing the JCOG-PI. The entry criterion was $P < 0.20$ and the removal criterion was $P > 0.15$.

The maximum number of risk group strata was set at three, based on the opinions of JCOG-LSG members who commented that too many strata were impractical for evaluating risk. The risk group was divided with patients equally distributed. The log-rank test was used to assess the discrepancy between the risk groups and the Kaplan–Meier method was applied to estimate OS.

All statistical analysis was performed using SAS Release 9·1 (SAS Institute, Inc, Cary, NC, USA). All reported P values are two-sided and $P < 0.05$ was considered statistically significant.

Results

Patient characteristics

A total of 276 patients were registered in the 3 trials (JCOG9109, $n = 62$; JCOG9303, $n = 96$; and JCOG9801, $n = 118$) from 58 institutions in Japan. The MST and the 5-year OS of all patients were 11 months and 14% respectively (Fig 2A). The OS of each treatment regimen during the long follow up reconfirmed the findings of each original report (Fig 2B) (Yamada *et al*, 2001; Tsukasaki *et al*, 2003, 2007). Clinical characteristics are shown in Table I.

Long-term survivors according to subtype and initial response

The disease-specific mortality curve of patients who survived >2 years according to subtype is presented in Fig 3. Among the 37 patients (acute, $n = 22$; lymphoma, $n = 8$; unfavourable chronic, $n = 7$) who survived >5 years, there were no ATL-related deaths in lymphoma type, which was in contrast to the 10 ATL-related deaths in the acute and unfavourable chronic types after 5 years.
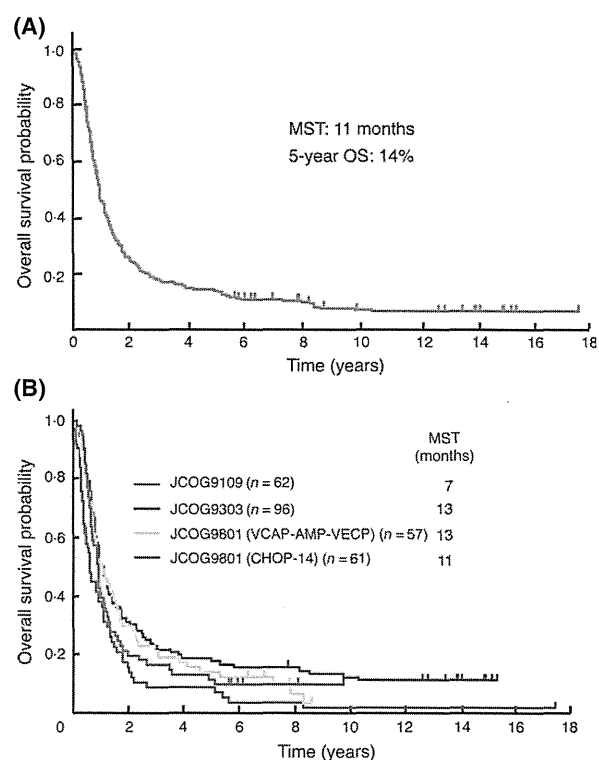

Fig 2. Overall survival (OS) of all registered patients in 3 Japan Clinical Oncology Group (JCOG) trials and according to treatment regimens. (A) OS of all 276 registered patients. Median survival time (MST) and the 5-year OS were 11 months and 14%, respectively.(B) OS according to different treatment regimens. MST was 7 months in JCOG9109, 13 months in JCOG9303, 13 months in VCAP-AMP-VECP of JCOG9801 and 11 months in CHOP-14 of JCOG9801.

Of the 276 patients, 88 (32%) achieved CR with initial treatment. Of these 88 patients, 24 (27%) patients had survived >5 years and 11 (13%) patients had survived >10 years. Of the remaining 188 patients who did not achieve CR, 13 (17%) patients who survived >5 years and only 1 (0·5%) patient survived >10 years.

CNS involvement by treatment regimen

CNS involvement was 1·6% (95% CI, 0·04–8·7) in JCOG9109, 6·3% (95% CI, 2·3–13·1) in JCOG9303, and 3·5% (95% CI, 0·4–12·1) in the VCAP-AMP-VECP arm and 8·2% (95% CI, 2·7–18·1) in the CHOP-14 arm of JCOG9801. No significant differences in the proportion of CNS involvement were observed among the regimens.

Development of the PI

In univariate analyses, three covariates showed significant associations with OS, namely PS, corrected calcium level and serum total protein (all $P < 0.05$; Table II). Stepwise Cox regression analysis returned three unfavourable prognostic

Table I. Clinical characteristics of 15 covariates in all 276 registered patients.

		JCOG9109 (n = 62)	JCOG9303 (n = 96)	JCOG9801 (n = 118)	Total (n = 276)
Initial date of registration		November 1991	January 1994	July 1998	
Final date of registration		July 1993	December 1996	October 2003	
Number of sites		30	20	27	49
Sex	Male/female	38/24	54/42	61/57	153/123
Age, years	≥20, <30	0	1	0	1
	≥30, <40	2	7	6	15
	≥40, <50	14	29	20	63
	≥50,<60	27	24	44	95
	≥60, <70	19	35	48	102
PS	0/1	23/22	19/25	49/46	91/93
	2/3/4/NE	7/9/1/0	17/9/8/18	18/4/1/0	42/22/10/18
B symptoms	+/−/NE	22/36/4	39/57/0	45/73/0	106/166/4
Stage	I/II/III/IV	1/4/8/49	2/6/14/74	0/4/8/106	3/14/30/229
Liver invasion	+/−	10/52	20/76	25/93	55/221
LDH, iu/l	<−1 × ULN/>	9/53	10/86	20/98	39/237
BUN, mmol/l	<−1 × ULN/>/NE	47/14/1	80/15/1	107/11/0	234/40/2
Corrected Ca, mmol/l	<2·75/≥/NE	49/9/4	75/16/5	93/25/0	217/50/9
Serum protein, g/l	<60/≥/NE	18/44/0	27/69/0	30/87/1	75/200/1
Albumin g/l	<35/35–40/≥40/NE	18/26/15/3	35/39/18/4	28/64/26/0	81/129/59/1
WBC ($\times 10^9$/l)	<3/≥	48/14	77/19	104/14	229/47
Lymphocytes ($\times 10^9$/l)*	<4/4–15/≥15/NE	28/16/14/4	54/19/23/0	64/33/20/1	146/68/57/5
Neutrophils ($\times 10^9$/l)	<8/≥/NE	49/12/1	75/21/0	94/24/0	218/57/1
Platelets ($\times 10^9$/l)	<150/≥	16/46	19/77	19/99	54/222

B symptoms: fever, night sweats, and weight loss.
JCOG, Japan Clinical Oncology Group; ECOG PS, Eastern Cooperative Oncology Group performance status; Ca, calcium level; WBC, white blood cell count; ULN, upper limit of normal; NE, not evaluated.
*total (normal + abnormal) lymphocyte count.

factors associated with OS, namely a high, corrected calcium level, high PS (2–4), and the existence of B symptoms, although the third factor was not statistically significant (Table II). Table II also presents the results of the model when the two significant factors of corrected calcium and ECOG PS were included. The hazard ratios (HRs) estimated by this model were 1·574 (95% CI, 1·088–2·277; $P = 0·016$) for corrected calcium and 1·554 (95% CI, 1·120–2·157; $P = 0·008$) for ECOG PS.

The four categories consisting of the two prognostic factors (corrected calcium level and PS) were combined into a dichotomous PI, named the JCOG-PI, by considering its potential for clinical use. Similarly, we constructed a dichotomous PI including B symptoms with two prognostic factors. We excluded B symptoms from further assessment because the Akaike Information Criteria of JCOG-PI (1537·8) was smaller than that of PI (1545·6).

According to the JCOG-PI, the MST and 5-year OS were 14 months and 18% in patients with both corrected calcium <2·75 mmol/l and a PS of 0 or 1 (moderate-risk group) and were 8 months and 4% in patients with corrected calcium ≥2·75 mmol/l and/or a PS of 2–4 (high-risk group) respectively (Fig 4A). The HR and 95% CI were 1·926 and 1·423–2·606 respectively ($P < 0·0001$).

External validation

Nine patients in the validation set of 136 patients had missing corrected calcium or PS data, resulting in 127 evaluable patients (Fig 5). The median and longest follow-up periods were 9 months and 97 months, respectively. The HR was 2·138 (95% CI, 1·414–3·233, $P = 0·0003$) with an MST of 18 months and 6 months in the moderate- and high-risk groups respectively and JCOG-PI showed good reproducibility (Fig 4B).

Discussion

In this first prospective analysis of a large cohort of aggressive ATL patients from prospective clinical trials conducted after the clinical subtype classification of ATL was introduced, we constructed the JCOG-PI based on corrected calcium level and PS and validated it with external data. The ascertained discrepancy was stronger among the external validation set. In addition, OS of high-risk patients was worse in the external validation set than in the training set, probably reflecting poor organ functions and other unfavourable prognostic factors in patients not participating in clinical trials. The OS of the moderate-risk patients was better in the