

Original Articles

Gene Expression in Adult T Cell Leukemia/Lymphoma : Up-Regulation of Matrix Metalloproteinase 2 in Skin Lesions

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The neoplastic cells of adult T-cell leukemia/lymphoma (ATLL) invade various body organs, typically the lymph nodes (LN) and skin. The present study was designed to clarify the mechanism of invasion, by using a DNA array that allowed comparison of gene expression among ATLL cells in different sites, namely peripheral blood (PB), LN, and skin. The expression of matrix metalloproteinase 2 (MMP-2) mRNA was significantly up-regulated in skin relative to PB. Immunohistochemistry showed higher MMP-2 expression in ATLL cells found in the skin than in LN. Expression of membrane-type1-MMP (MT1-MMP), an activator of pro-MMP-2, was detected in fibroblasts present in the skin stroma. The proportion of MMP-2-positive cells in PB was not different between patients with or without skin invasion. Our results indicate that MMP-2 does not account for migration of ATLL cells to the skin, but once these cells reach the skin, this molecule seems to play an important role in proliferation of ATLL cells and/or their invasion of skin tissue.

Key words Adult T cell leukemia/lymphoma, matrix metalloproteinase 2, skin, invasion

INTRODUCTION

Adult T cell leukemia/lymphoma (ATLL) is a lymphoproliferative disorder of helper/inducer T cell origin, caused by human T lymphotropic virus type 1 infection (HTLV-1)¹⁻³. ATLL is prevalent in areas endemic for HTLV-1, including south-western Japan and the Caribbean basin⁴. HTLV-1 infection mainly occurs early in life via the breast milk of carrier mothers⁵, and approximately 4-5% of HTLV-1 carriers are thought to develop ATLL after long latency

periods, usually 40 to 50 years⁶.

In ATLL, leukaemic cells frequently infiltrate organs such as lymph nodes, spleen, liver, and skin⁷. In particular, skin invasion represents a significant difference from other types of lymphomas or leukemias⁸. However, little is known about the underlying molecular mechanisms of this phenomenon, although there have been many reports on molecules associated with ATLL carcinogenesis such as matrix metalloproteinase-9 (MMP-9)⁹, a T-cell differentiation antigen, MAL¹⁰, p21/waf1¹¹, and chemokine I-309¹². In most of the above reports, a cell line or peripheral blood samples were analyzed. There is therefore a need for studying tissue samples to identify molecules that could account for ATLL cell invasion into tissue. We used gene expression profiling as an initial step in an attempt to identify molecules differentially expressed in lesions in skin, lymph nodes (LN), and peripheral blood (PB). Our results showed that matrix-metalloproteinase-2 (MMP-2) was up-regulated

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in ATLL cells in LN and skin compared with PB. Based on these findings, we also used immunohistochemistry and flow cytometry to characterize the expression of this protein in ATLL skin lesions.

MATERIALS AND METHODS

Patient samples

Studies were conducted in 29 ATLL patients, including 9 skin sections, 5 LN sections, and 15 PB samples. ATLL was diagnosed according to the following criteria: positivity of serum antibodies against HTLV-1; morphologic characteristics showing highly convoluted nuclei; CD3+ and CD4+ phenotypes; and monoclonal integration of the HTLV-1 proviral genome¹³.

Tissue processing and cell samples from peripheral blood

Diagnostic tissue samples from skin and LN were fixed in buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin for routine histologic examination (Fig. 1(A), (B), (C), (D)). Five skin sections and four LN sections were used for gene expression profiling analysis. These portions of the specimens were precooled in isopentane and frozen in liquid nitrogen. Peripheral blood mononuclear cells (PBMC) were obtained from 15 patients with ATLL. In PB, over 90% of the PBMC were atypical ATLL cells. Five samples were used for gene expression analysis, and the others were analyzed with a monoclonal antibody (mAb) for MMP-2 (Daiichi Fine Chemical Co., Takaoka JAPAN), using a FACScan analyzer (Becton-Dickinson, Franklin Lakes, NJ).

Immunostaining

In these studies, mAbs for MMP-2 and MT-1 MMP (Daiichi Fine Chemical Co.) were used for paraffin-embedded samples.

Microarray procedures

Total RNA was prepared from frozen biological samples by the guanidinium thiocyanate-phenol-chloroform method using the Total RNA Separator kit (Clontech, Paolo Alto, CA). All

cDNA microarray analyses were performed using total RNA (3DNA™ Submicro Expression Array Detection Kit; Genisphere Inc. Montvale, NJ; <http://www.arrayit.com/DNA-Microarray-Protocols/>). Briefly, total RNA was reverse transcribed using the included deoxynucleotide triphosphate mix and reverse transcriptase (RT) primer oligo. The cDNA was mixed with the fluorescent 3DNA™ reagent, which included a capture sequence for the 5' end of the RT primer, and then hybridized to DNA chips. In each experiment, fluorescent cDNA probes were prepared from an experimental total RNA sample (Cy5-labelled) and a control total RNA sample (Cy3-labelled) mixed from a pool of total RNA from PBMC from 20 healthy volunteers. The use of a common control cDNA probe allowed the relative expression of each gene to be compared across all samples.

The cDNA clones were from Cancer Chip ver. 2.1 (Takara Bio, Otsu, JAPAN) (557 genes). Fluorescent images of hybridized DNA chips were obtained using a Scan Array 4000 scanner (<http://www.gsilumonics.com/>, GSI Lumonics, Boston, MA). Images were analyzed with QuantArray (GSI Lumonics), and fluorescence ratios (along with numerous quality control parameters) were stored in a custom database. Single spots or areas of the array with obvious blemishes were flagged and excluded from subsequent analysis. A set of clones that consistently behaved poorly across arrays was identified and excluded from all analyses. Fluorescence ratios were calibrated independently for each array by applying a single scaling factor to all fluorescent ratios from each array. This scaling factor was computed so that the median fluorescence ratio of well-measured spots on each array was 1.0.

Data analysis

All non-flagged array elements for which the fluorescence intensity in each channel was greater than 1.4 times the local background were considered well measured. The ratio values were stored in a table (rows, individual cDNA clones; columns, single total RNA samples). We calculated the mean value of each cDNA probe in skin lesions, LN lesions, and PB. Hierarchical clustering was applied to both axes using the weighted pair-group method with centroid average as implemented in the J-Express ([J. Clin. Exp. Hematopathol
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Matrix metalloproteinase 2 expression in ATL skin lesions

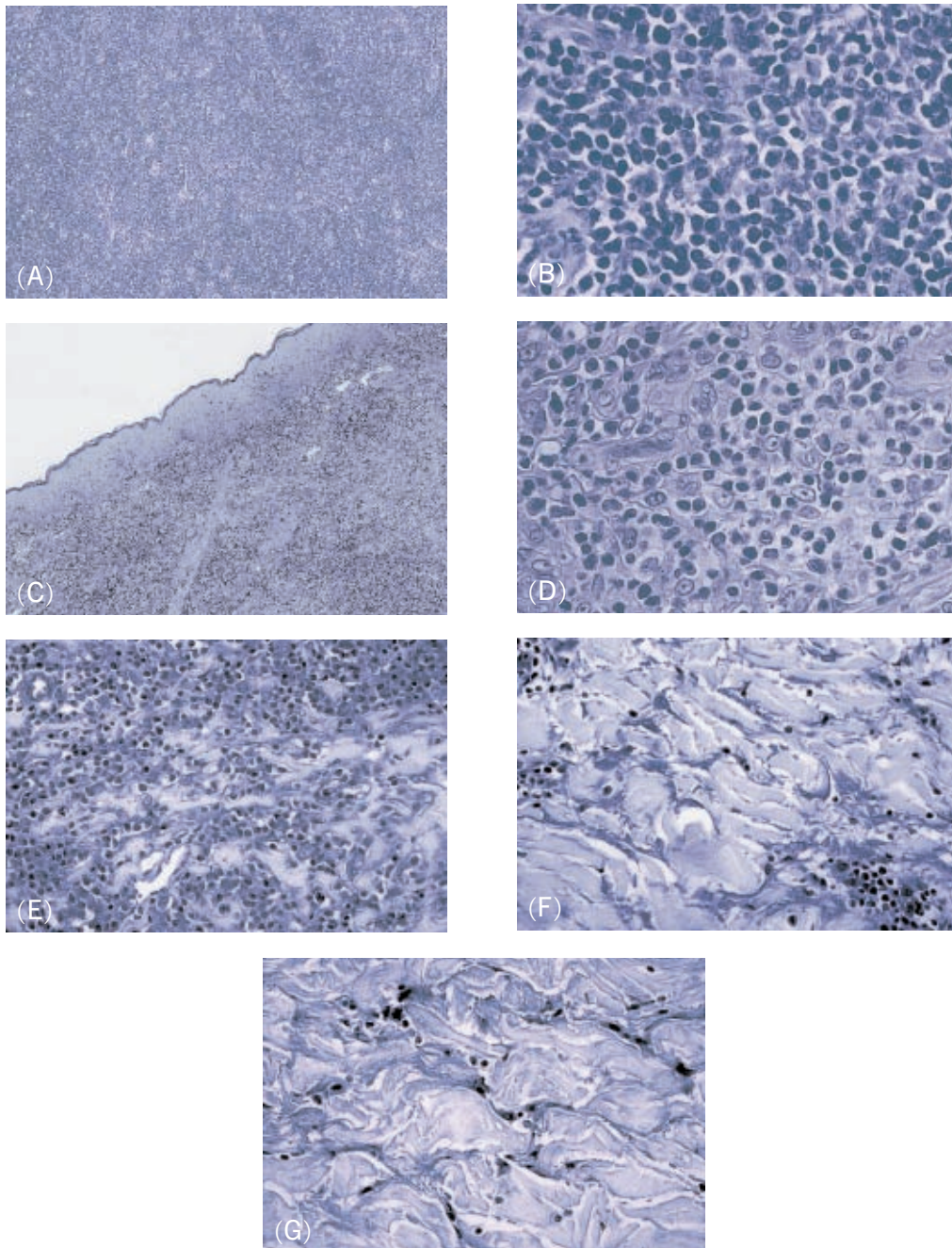


Fig. 1. ATLL morphology and phenotype in each tissue type. (A and B): Lymph node (hematoxylin and eosin), (C and D): Skin, (hematoxylin and eosin), (E and F): Immunostaining with antibodies against MMP-2 in skin lesions. Note the presence of MMP-2-positive lymphoma cells (E) and fibroblasts in the stroma (F). (G): Immunostaining with antibodies against MT1-MMP in skin lesions. Note the positively-stained fibroblasts in the stroma. All photographs were prepared from paraffin sections. Original magnification: A and C, $\times 20$; B and D, $\times 200$; E, F, and G, $\times 100$.

mine.com/index.j.html, <http://www.ii.uib.no/bjarted/iexpress/>, Dr. MolMine; contact@molmine.com). The distance matrices used were Pearson correlation for clustering the arrays and the inner product of vectors normalized to magnitude 1 for the genes. Results were analyzed with tree view (Dr. MolMine; <<http://www.nature.com/cgi-taf/DynaPage.taf?file=/nature/journal/v403/n6769/full/>>).

RESULTS

Gene profiling of ATLL cells from lymph nodes, skin, and peripheral blood

LN samples showed diffusely up-regulated gene expression, whereas skin lesions showed a mixed pattern of up- and down-regulated genes. We then compared the profiles of 10 up-regulated and 10 down-regulated genes in skin relative to peripheral blood and LN (Table 1). The expression of MMP-2 was significantly higher in skin than in PB (about 1.8 fold) (p-value=0.044, by

Student's t test).

Clustering and classification analysis

In the tree view of DNA chips from all cases, each site showed a near-perfect segregation. The LN samples exhibited a diffusely up-regulated pattern of gene expression (Fig. 2). Skin samples also showed gene clustering. However, two cases showed diffusely up-regulated gene expression, whereas the other three cases showed a mixed pattern of up- and down-regulated genes. There was no significant difference in the proportion of lymphoma cells between the two groups of skin lesions. The PB samples showed close clustering and a mixed pattern of up- and down-regulated genes (data not shown).

Next, we selected 20 genes that were most different between (1) skin and PB and (2) LN and PB, and performed clustering analysis. In the tree view comparing skin and PB, skin samples exhibited clearly different cluster patterns from

Table 1 Comparison of upregulated and downregulated genes in lymph nodes (LN), skin lesions and peripheral blood (PB).

LN vs PB		Skin vs PB	
Up-regulated			
Name	LN/PB	Name	Skin/PB
EphB2	70.13435	keratin 1	10.8898
monokine induced by gamma interferon	13.23423	keratin 5	5.31798
topoisomerase (DNA) II alpha (170kD)	7.083324	keratin 14	4.51169
cyclin E1	6.976491	keratin 10	4.090596
collagen, type I, alpha 2	6.353322	collagen, type III, alpha 1	3.926118
catenin (cadherin-associated protein), alpha 2	6.349954	collagen, type I, alpha 2	2.280633
formyl peptide receptor 1	6.242536	small inducible cytokine subfamily B (Cys-X-Cys), member 10	1.932534
keratin 14	6.203431	monokine induced by gamma interferon	1.848092
lumican	6.056085	matrix metalloproteinase2	1.795205
thrombopoietin	6.0262	collagen, type VI, alpha 3	1.729905
Down-regulated			
interleukin 8	0.286374	jun B proto-oncogene	0.211428
general transcription factor IIIH, polypeptide 2	0.405093	interleukin 1, beta	0.251978
BCL2-related protein A1	0.417415	FBJ murine osteosarcoma viral oncogene homolog B	0.352478
jun B protooncogene	0.446486	pro-platelet basic protein	0.366725
interleukin 1, alpha	0.483079	Rho guanine nucleotide exchange factor (GEF) 1	0.376712
fragile histidine triad gene	0.529086	plasminogen activator, urokinase receptor	0.401713
aldolase A, fructose-bisphosphate	0.553062	selectin L (lymphocyte adhesion molecule 1)	0.426048
paxillin	0.558565	ras homolog gene family, member G (rho G)	0.441275
ankyrin 1, erythrocytic	0.58108	interleukin 8	0.452102
pro-platelet basic protein	0.585744	BCL2-related protein A1	0.455703

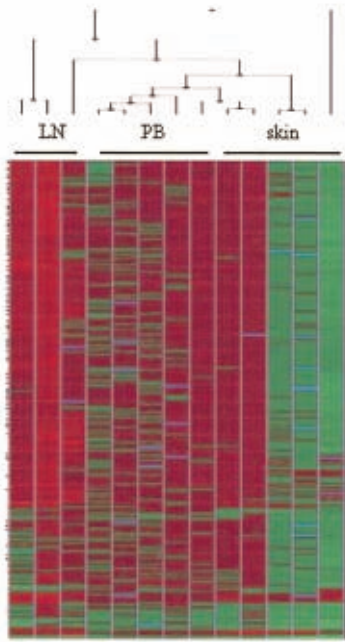


Fig. 2. Gene expression profiles of the three ATLL lesions using 557 genes (Full genes on the Cancer chip 2.1). Dendrogram showing hierarchical clustering of gene expression data (see Methods for algorithm) from three lymph nodes (LN), five skin lesions, and five peripheral blood (PB) samples obtained from patients with ATLL.

PB cases (Fig. 3a). In the tree view comparing LN and PB, LN cases showed close clustering and differed from PB samples (Fig. 3b). Based on the results of gene profiles, we then focused on the difference between the skin and PB. For the 10 up-regulated genes in the skin, almost all increased molecules were associated with basic skin structure, such as keratin and collagen. However, we also detected MMP-2, an important molecule associated with malignant tumour progression and metastasis^{14,15}. Accordingly, we analyzed this molecule in more detail.

Immunohistochemistry

Immunohistochemistry revealed strong staining for MMP-2 in the skin (Fig. 1E and F) compared with LN (Table 2). MT1-MMP was expressed in fibroblasts but not in lymphoma cells in the skin (Fig. 1G, Table 2). No MT1-MMP expression was noted in LN. Both

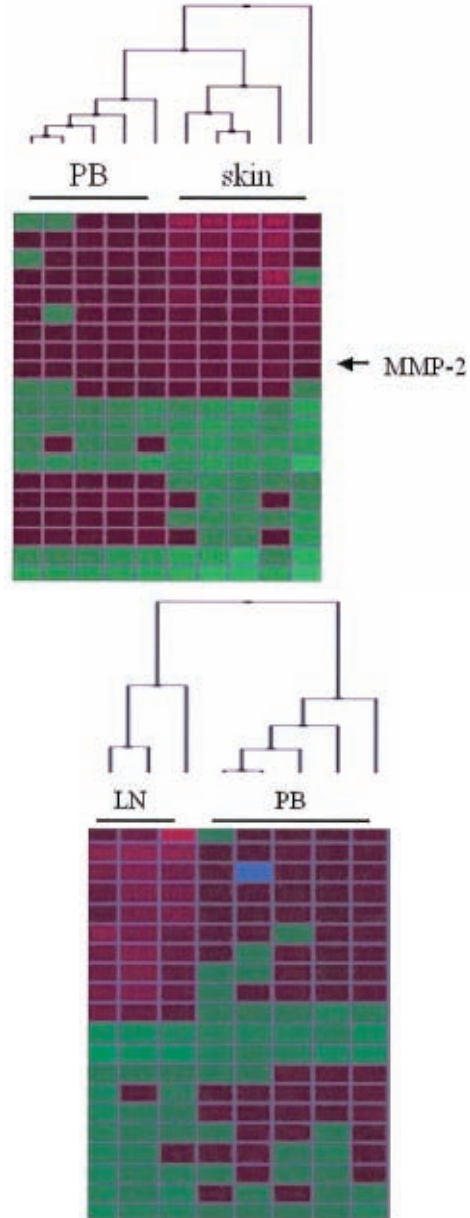


Fig. 3. Comparisons of gene expression profiles from the skin and peripheral blood (PB) (a) and lymph node (LN) and PB (b) of ATLL using the most up- and down-regulated 10 genes. Note that the clarity of each cluster is better than in Fig. 1.

lymphoma cells and stroma cells were negative for MT1-MMP (Table 2).

Flow cytometric analysis

Flow cytometric analysis revealed MMP-2 expression in PBMC of ATL. The average proportion of MMP-2-positive cells in PBMC

Table 2 Comparison of MMP-2 and M1-MMP expression between skin lesions and lymph nodes in patients with ATLL.

Case no.	Site of involvement	MMP-2(ATL cells)	MT1-MMP(ATL cells)	MT1-MMP(fibroblasts)
1	Skin	2+	—	+
2	Skin	3+	—	+
3	Skin	2+	—	+
4	Skin	2+	—	+
5	Skin	3+	+	+
6	Skin	2+	—	+
7	Skin	2+	—	+
8	Skin	3+	—	+
9	Skin	3+	±	+
10	Lymph node	—	—	—
11	Lymph node	+	—	—
12	Lymph node	+	—	—
13	Lymph node	±	—	—
14	Lymph node	—	—	—

Nine skin lesions and five LN lesions were embedded and labelled as indicated in Materials and Methods and then immunostained for MMP-2 and MT1-MMP.

—, < 5% of neoplastic cells or fibroblasts stained, +, >5% to < 20% stained, 2+ > 20% to < 50% stained, 3+, > 50% stained.

from patients with skin lesions (24.2%, range, 9.1 to 54.8%) was not significantly different from that of cases without skin lesions (25.6%, 14.5 to 45.0%).

DISCUSSION

There is little or no information on the mechanisms responsible for ATLL cell invasion into various organs. Recent studies have identified some molecules, mainly adhesion molecules and chemokines, as candidates for ATL cell migration. These include integrin leukocyte function-associated antigen-1 (LFA-1)^{13,16,17}, MMP-9⁹, vascular endothelial growth factor (VEGF)¹⁸, CCR7/EBI1¹⁹, CCR4²⁰, MIP1- α and MIP-1 β ²¹. However, almost all the above previous studies were conducted using cell lines and/or PBMC. In the present study, we used frozen sections of LN, skin, and PB. As a first step, we used cDNA microarrays to compare each lesion to identify novel genetic alterations. In each sample, hierarchical clustering was nearly perfectly segregated according to the tissue. Comparison between skin and PB, and between LN and PB, showed definite clustering of each lesion. From the genes that showed higher expression levels in the skin relative to PB, we selected MMP-2. MMP-2 was not among the 10 most highly expressed genes in the LN compared with PB.

Matrix metalloproteinases (MMPs) are a family of enzymes that degrade various components of the extracellular matrix (ECM)^{22,23}. The 72-kDa gelatinase A (MMP-2) is the most widely distributed of all the MMPs²⁴ and is expressed constitutively on a number of cells, including endothelial and epithelial cells. MMPs play an important role in pericellular basement membrane turnover by degrading the main components of the basement membrane (Stetler-Stevenson *et al.*, 1996). They are also important in tumor invasion and metastasis and in the dissemination of hematological malignancies, such as non-Hodgkin lymphoma and acute myelogenous leukaemia (AML)^{14,15}. With regard to skin lesions, MMP-2 is reported to be involved in tumor invasion in melanoma^{25,26}, Bowen disease, invasive cutaneous carcinoma²⁷, basal cell carcinoma²⁸ and Mycosis fungoides²⁹.

The results of the present study indicate that MMP-2 is also important in the skin invasion of ATLL cells. Immunohistochemical analysis of MMP-2 revealed a stronger immunostaining in ATLL cells in skin lesions than in LN. Based on these findings, two possible scenarios deserve particular attention. The first possibility is that expression of MMP-2 is directly responsible for ATLL cell migration to the skin. Previous studies suggested that HTLV-1-infected T cells undergo multi-step changes during carcinogenesis to form aggressive leukemic cells³⁰.

Furthermore, the HTLV-I Tax protein, a 40-kDa transcriptional transactivator of the HTLV-I gene that plays a critical role in cellular transformation in various in vitro models, including T cells, is reported to transactivate MMP-9⁹. Based on our results, we suggest that migration of leukemic cells to the skin could occur upon induction of MMP-2 expression in such cells. The representative molecule, reported until now, for this scenario is cutaneous lympho-associated Ag (CLA), which has been found to be highly expressed in ATLL cells from PB of patients with skin lesions¹³. This molecule is a ligand for E-selectin and considered to account for adherence of ATLL cells to the endothelium in the skin.

An alternative explanation for the difference in immunostaining pattern between skin lesions and LN is that the strong expression of MMP-2 in ATLL cells is induced after ATLL cell homing to the skin. To examine this possibility, we analyzed PBMC of ATLL patients with or without skin involvement by flow cytometry. Our results showed that ATLL cells highly expressed MMP-2, but there was no significant difference in the proportion of MMP-2-positive cells between the two groups. These results indicate that the second scenario is more likely. We also examined the expression of MT1-MMP, an activator of pro-MMP-2³¹. MT1-MMP was expressed in fibroblasts in the skin stroma, in contrast to the LN where fibroblasts, including those positive for MT1-MMP, were rarely observed. These findings suggest possible activation of MMP on fibroblasts in the skin lesion, which is in agreement with the results of a previous study³². In this regard, other reports suggested that MMP-2 and MT1-MMP alter, in an autocrine manner, the proliferation of normal somatic and cancer cells, such as hepatic stellate cells³³ and breast carcinoma³⁴. In our study, the positive staining for MT1-MMP in lymphoma cells, even though such staining was noted in only some cases, may reflect such a mechanism.

In conclusion, the results of the present study indicated that MMP-2 does not account for migration of ATLL cells to the skin, but once these cells migrate to the skin, this molecule seems to play an important role in proliferation of ATLL cells and/or their invasion of the skin tissue.

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