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Identification of Genes Associated with the Progression of Adult T Cell Leukemia (ATL)

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Patients with adult T-cell leukemia/lymphoma (ATL) exhibit a variety of clinical features, and this disease is therefore clinically subclassified into acute, lymphomatous, chronic, and smoldering types. Acute ATL is a typical leukemic form of ATL with rapid progression, and chronic ATL is a less aggressive clinical form allowing long-term survival even without chemotherapy. In the present study, we used fresh peripheral blood mononuclear cells (PBMC) from both types of ATL patients to identify molecules that may contribute to the difference between acute and chronic ATL. Isolated mRNAs expressed differentially between the two types of ATL include a T-cell differentiation antigen (MAL), a lymphoid-specific member of the G-protein-coupled receptor family (EBI-1/CCR7), a novel human homologue to a subunit (MNLL) of the bovine ubiquinone oxidoreductase complex, and a human fibrinogen-like protein (hpT49). We found that the former three are upregulated in acute ATL and the last is down-regulated in both chronic and acute ATL. We speculate that dysregulation of the genes may account for the malignant features of ATL cells, in terms of growth, energy metabolism, and motility.

Key words: ATL — MAL — EBI-1/CCR7 — hMNLL — hpT49

Adult T cell leukemia (ATL) is a lymphoproliferative disorder of helper/inducer T cell origin, caused by human T lymphotrophic virus type 1 infection (HTLV-1).[1–3] ATL is prevalent in areas endemic for HTLV-1, including southwestern Japan and the Caribbean basin.[4] HTLV-1 infection mainly occurs early in life via the breast milk of carrier mothers,[5] and approximately 4–5% of HTLV-1 carriers are thought to develop ATL after long latency periods, usually 40 to 50 years.[6] The leukemic cells of ATL always harbor in their genome the HTLV-1 provirus, which is often defective, and show a common characteristic morphological appearance. Patients with ATL, however, exhibit a variety of clinical features, and this disease is therefore clinically subclassified into acute, lymphomatous, chronic, and smoldering types.[7] The acute ATL progresses rapidly and the median survival after diagnosis is approximately six months. The resistance of ATL to conventional chemotherapy is mainly due to hypercalcemia, and leukemic cell infiltration into various organs. In contrast, chronic ATL has a less aggressive clinical course and allows long-term survival even without chemotherapy.

Little is known about the underlying molecular mechanisms of the clinical course of ATL. Recently, mutations or deletions in certain tumor suppressor genes, such as those encoding *p53*[8, 9] and cyclin-dependent kinase inhibitors, p15 and p16,[10, 11] have been reported in acute ATL cells. Thus, a functional loss of these tumor suppressor gene products is likely to play a role in determining the malignant growth potential in acute ATL cells. However, it has been proposed that multiple steps or multi-factorial events are required for the development of ATL[12]; therefore other unidentified molecules also seem to contribute to this malignant cell transformation.

In an attempt to identify molecules differentially expressed in acute and chronic ATLs, we analyzed mRNAs of peripheral blood mononuclear cells (PBMC) of acute and chronic ATL patients by differential display.[13, 14] Consequently, we identified three up-regulated genes in acute ATL cells, encoding a T-cell differentiation antigen, MAL,[15, 16] a lymphoid-specific member of G-protein-coupled receptor family, EBI-1/CCR7,[17, 18] and a novel human homologue to a subunit (MNLL) ubiquinone oxidoreductase complex.[19] Another gene, *hpT49*, which encodes a fibrinogen-like protein,[20, 21] was found to be down-regulated in acute ATL.

MATERIALS AND METHODS

Patients Peripheral blood mononuclear cells were obtained from three patients with acute ATL, five with chronic ATL, and three normal subjects. The diagnoses of acute and chronic ATL were based on the criteria of Shimoyama.[7] Informed consent was obtained from all subjects prior to blood sample collection.

Cell lines Cell lines used in this study included two

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human T-cell lines, Jurkat and MOLT4; a human erythroleukemia cell line, K562; a human myelomonocytic cell line, U937; three HTLV-1 infected cell lines, MT2, MT4 and HUT102; an IL-2 dependent T-cell line, SO4[22]; and a subline of Jurkat stably transformed with the pMAXRneo-1 plasmid containing *Tax* gene driven by the metallothionine promoter.[23] The JPX-9 was kindly provided by K. Sugamura, Tohoku University, Sendai. Cells were cultured in RPMI1640 medium with 10% fetal bovine serum. To culture SO4 cells, the culture medium was supplemented with 1 U/ml of IL-2 (a gift from Takeda Pharmaceutical Co., Tokyo).

Differential display screening Total RNA was extracted from cells with ISOGEN (Nippon Gene, Tokyo) and chloroform, and was precipitated in isopropanol. Differential display was performed using a commercial kit (RNAmap Kit A and B, GenHunter, Brookline, MA) according to the instructions provided by the manufacturer with slight modifications. Briefly, total cellular RNA (50 μg) was treated with 10 U of RNase-free DNase I (TaKaRa, Tokyo) at 37°C for 30 min in a buffer containing 10 m*M* Tris-Cl, 1.5 m*M* $MgCl_2$, 50 m*M* KCl, and 10 U of RNase inhibitor (Promega, Madison, WI). The DNase I-treated RNA (0.2 μg) was reverse-transcribed using each of four different, degenerate, anchored oligo-dT primer sets (T_{12}MN: T_{12}MG, T_{12}MA, T_{12}MT, and T_{12}MC, where M is a mixture of G, A and C). Reverse transcription was performed at 37°C for 60 min in a total volume of 20 μl containing 20 μM dNTP, 200 U of MMLV-Superscript-II, 1× RT buffer (50 m*M* Tris-HCl, 75 m*M* KCl, and 3 m*M* $MgCl_2$) (GIBCO BRL, Gaithersburg, MD), 10 m*M* DTT, and 10 U of RNase inhibitor (Promega). After inactivation of the reverse transcriptase at 95°C for 5 min, 2 μl of the reaction mixture was subjected to polymerase chain reaction (PCR) in 20 μl of PCR buffer (10 m*M* Tris pH 8.4, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, and 0.001% gelatin) containing 4 pmol of one of the arbitrary 10-mers (AP1–AP10) as the 5′-primer, 4 pmol of T_{12}MA, T_{12}MC, T_{12}MG, or T_{12}MT as the 3′-primer, 2 μM dNTP mixture, 1 U of *Taq* polymerase (Promega) and 37 kBq of [α-^{32}P]dCTP (Amersham, Buckinghamshire, UK). Parameters for the 40-cycle PCR were as follows: denaturation at 94°C for 30 s, annealing at 42°C for 2 min, and extension at 72°C for 30 s. Radiolabeled products were analyzed by electrophoresis on 6% sequencing gels. The dried gel was exposed to Kodak XAR film (Eastman Kodak, Rochester, NY). According to the resulting autoradiogram, positive bands were excised from the dried gel, boiled in 100 μl of dH_2O for 15 min, concentrated by Microcon 30 ultrafiltration (Amicon, Beverly, MA), and suspended in 10 μl of dH_2O. Four microliters of recovered cDNA was re-amplified by PCR in a 20 μl reaction volume using primer sets and conditions similar to those used in the first PCR reaction, but the dNTP concentration was 20 μmol/liter and no radioactive nucleotide was used. The re-amplified samples were cloned into the PCRII vector using the TA cloning system (Invitrogen, San Diego, CA).

DNA sequencing The DNA sequencing reaction of the cloned cDNA was carried out using the delta *Taq* fluorescent dye-primer cycle sequencing kit (Amersham) and either T7 or M13 primers. The resultant sequences were analyzed on an automated sequencer (Model SQ-5500, Hitachi Co., Tokyo), and homology search was performed by the use of Genetyx-Mac version 28.0.

Northern blot analysis Ten micrograms of total cellular RNA was resolved by electrophoresis on a 1% MOPS-formaldehyde gel and transferred to a Hybond-N membrane (Amersham). The PCR products differentially amplified in the display were used as probes. The cDNA probe and the β-actin probe were labeled with [α-^{32}P]dCTP using the Megaprime kit (Amersham) according to the manufacturer's protocol. Hybridization was performed in a buffer containing 50% formamide at 42°C overnight. The membranes were washed four times at room temperature in 2× standard sodium citrate (SSC) (0.3 mol/liter NaCl, and 30 mmol/liter sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS), and then 3 times at 65°C in 0.1× SSC containing 0.5% SDS. The washed membrane was autoradiographed at −80°C overnight.

Rapid amplification of cDNA ends (RACE) 5′-RACE was performed to amplify sequences between a defined internal site and unknown sequences at the 5′-end of the mRNA using a 5′-RACE system kit (GIBCO BRL). The first strand cDNA was synthesized from 1 μg of total RNA using the gene-specific primer (GSP)-1 5′-CCAGGTAACTTCTTCACTGG-3′. The sample RNA and GSP-1 were incubated at 70°C for 10 min and then added to the 1× PCR buffer (20 m*M* Tris pH 8.4, and 50 m*M* KCl) with 2.5 m*M* $MgCl_2$, 1 m*M* dNTP, 10 m*M* DTT, and 200 U of SuperScript II reverse transcriptase. The mixture was incubated at 42°C for 50 min and at 70°C for 15 min. After the removal of the mRNA template by treatment with RNase Mix (mixture of RNase H), a homopolymeric tail was added to the 3′-end of the cDNA by incubation at 37°C for 10 min with terminal deoxynucleotidyl transferase (TdT) in buffer containing 10 m*M* Tris-HCl pH 8.4, 25 m*M* KCl, 1.5 m*M* $MgCl_2$, and 0.2 m*M* dCTP. In the next step, 5′-end primary PCR amplification was performed using a nested gene specific primer, GSP-2 5′-GCCGTTCATCACTCTTTCG-3′, and the 5′-RACE Abridged Anchor Primer 5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′. The parameters for PCR were at 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, at 55°C for 1 min, and 72°C for 2 min, followed by a final 72°C extension step for 5 min. Successive nested PCR was performed with the gene specific primer, GSP-3 5′-GGGACAAGAACATGAACCCA-3′ as the 3′-primer, and the Universal Amplification Primer 5′-CUACUA-

Table I. Clinical Features of ATL Patients

Case No.	Diag	WBC (/μl)	Neutro (%)	Lympho (%)	Abnormal lymph (%)	Mono (%)	CD3 (%)	CD4 (%)	CD8 (%)	CD25 (%)
1	chronic	9 300	47	6	32	4	81.6	76.6	11.9	55.1
2	chronic	21 300	30	20	43	6	87.3	92.2	2.6	89.2
3	chronic	13 300	48	11	36	3	86.0	75.5	10.2	71.2
4	chronic	12 200	28	15	21	8	59.3	78.0	5.8	—
5	chronic	13 400	29	50	15	6	89.8	76.6	7.8	79.1
6	acute	27 800	24	15	59	2	96.3	95.2	12.4	38.3
7	acute	32 600	29	6	60	5	94.9	90.8	3.8	14.8
8	acute	23 600	26	9	64	1	53.3	88.9	2.1	81.5

CUACUAGGCCACGCGTCGACTAGTAC-3′ as the 5′-primer. The nested PCR conditions were similar to those of the primary PCR reaction, except that a diluted primary PCR product and a different primer were used. The resultant product was cloned into the PCRII vector using the TA cloning system (Invitrogen) and sequenced.

RESULTS

Differential display of mRNA of chronic and acute ATL Differential displays developed from the PBMC of six ATL patients (cases 1, 2, 3, 6, 7, and 8) exhibited four PCR products differentially amplified in the chronic and acute types of ATL. As shown in Table I, the proportion of $CD4^+$ T cells in each of the eight patients was significantly high (range, 70 to 90%). Abnormal cells with characteristic segmented nuclei were detected in the peripheral blood of all of the patients. As shown in Fig. 1, three products (C316, C416, and G811) were amplified preferentially in samples of PBMC from patients with acute ATL. In contrast, the remaining product (G319) was produced in only chronic ATL samples. Table II shows the primer sets for each product. PCR of C316 and C416 was performed with AP-3 (5′-AGGTGACCGT-3′), AP-4 (5′-GGTACTCCAC-3′) as the 5′-primer, and T_{12}MC as the 3′-primer, respectively. PCR of G811 and G319 was performed with AP-8 (5′-CTACTACTAG-3′), AP-3 as the 5′-primer, and T_{12}MG as the 3′-primer, respectively.

DNA sequence analysis of products using differential display The sequences of the candidate mRNAs (C316, C416, G319, and G811) were identical or significantly homologous to already published sequences. The sequence of C316 was almost identical (98.1%) with base positions 813–1024 of the MAL protein gene, which is expressed in T cells at intermediate and late stages of differentiation.[15, 16] The C416 sequence was completely homologous to base positions 2004 to 2139 of the published sequences for Burkitt's lymphoma receptor 2 (BLR2)/Epstein-Barr virus induced gene 1 (EBI-1)/CCR7, a lymphoid-specific member of the G-protein-coupled receptor family.[17, 18] The 310

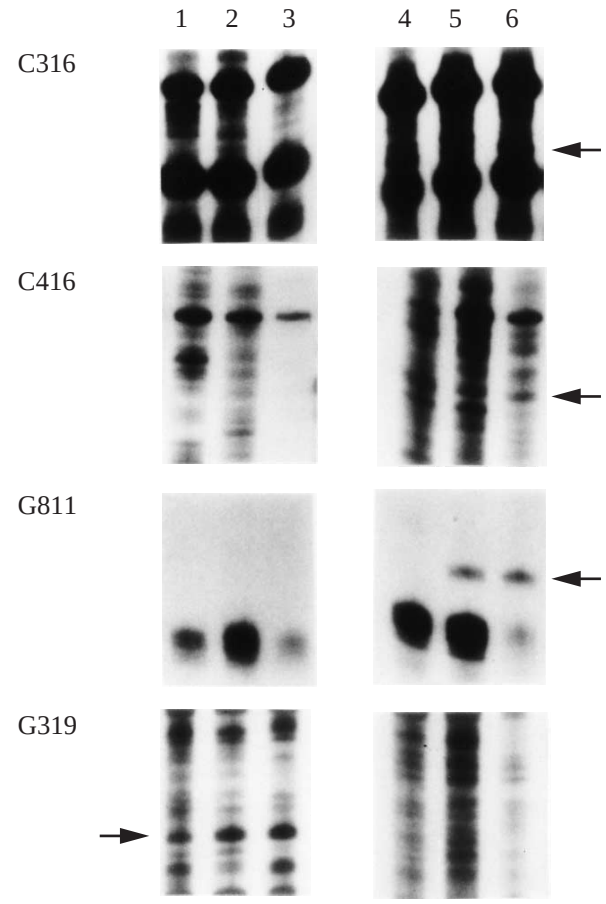

Fig. 1. Differential mRNA display of PBMC from patients with chronic and acute ATL. Total RNA was isolated from chronic ATL (lanes 1, 2, and 3; cases 1, 2, and 3, respectively) and acute ATL (lanes 4, 5, and 6; cases 6, 7, and 8, respectively), reverse-transcribed, and then subjected to PCR in the presence of [α-^{32}P]dCTP using various combinations of primers as described in "Materials and Methods." Amplified cDNAs were fractionated on a 6% sequencing gel and were autoradiographed. The bands differentially amplified in chronic and acute ATL cells (C316, C416, G811, and G319) are indicated by arrows.

Table II. Analysis of PCR Products Identified by Differential Display

mRNA	Combination primers	Size of DD[a)] product (bp)	Expression pattern	mRNA (kb)	Sequence homology
C316	T12MC-AP3	226	A[b)]>C[c)]	1.1	MAL protein
C416	T12MC-AP4	136	A>C	2.4	EBI-1/CCR7
G811	T12MG-AP8	241	A>C	0.3	MNLL (81.9%)
G319	T12MG-AP3	310	A<C	4.5, 1.5	hpT49

a) Differential display.
b) Acute type ATL.
c) Chronic type ATL.

bp sequence of G319 was identical to the 3′ untranslated region of the human fibrinogen-like protein (hpT49).[20, 21] The G811 sequence (241 bp) showed significant homology (about 80%) to the bovine Cl-MNLL mRNA for the ubiquinone oxidoreductase complex.[19] Table II summarizes the results of the analysis of the four PCR products identified by the differential display.

Expression of mRNA of genes identified by differential display The differential expression of these genes was re-evaluated by northern blotting of RNA samples from the PBMC of ATL patients and normal volunteers, using the amplified PCR products as probes (Fig. 2). As shown in Table II, the sizes of C316 and C416 mRNA were approximately 1.1 and 2.4 kb, respectively. These sizes were consistent with the reported sizes of MAL and EBI-1/CCR7, respectively.[15, 17] The size of G811 mRNA was approximately 0.3 kb; however, the size of MNLL has not been reported. G319 probe detected 4.5 and 1.5 kb bands, which were consistent with the previously reported sizes of hpT49 mRNA.[21] As the size of the open reading frame of hpT49 was about 1.5 kb, we regarded a 4.5 kb band as the functional mRNA and used it for the measurement.

To avoid any alterations caused by a sorting procedure in gene expression status *in vivo*, we used PBMC in this work. However, when PBMC, but not sorted $CD4^+$ T cells, are used, it is always necessary to take into account possible contamination with other types of cells. In regard to MAL and hpT49, both are only expressed in T cells and the possible differences in the number of T cells between the three groups were minimum (Table I, and about 75% in normal subjects (data not shown)). Compared with the expression level of MAL, the average of acute ATL's was significantly higher than that of chronic ATL or normal subject. With hpT49, the average of normal subjects was significantly higher than that of chronic or acute ATL. On the other hand, as the expressions of EBI-1/CCR7 and hMNLL are not limited to T cells, we only compared the differences between acute and chronic ATL where the largest fraction of PBMC was $CD4^+$ T cells (Table I) and found up-regulation of both genes in acute ATL. There remains the possibility that a small fraction of contaminating cells of acute ATL modified this result, so the development of antibodies against EBI-1/CCR7 product is currently under way.

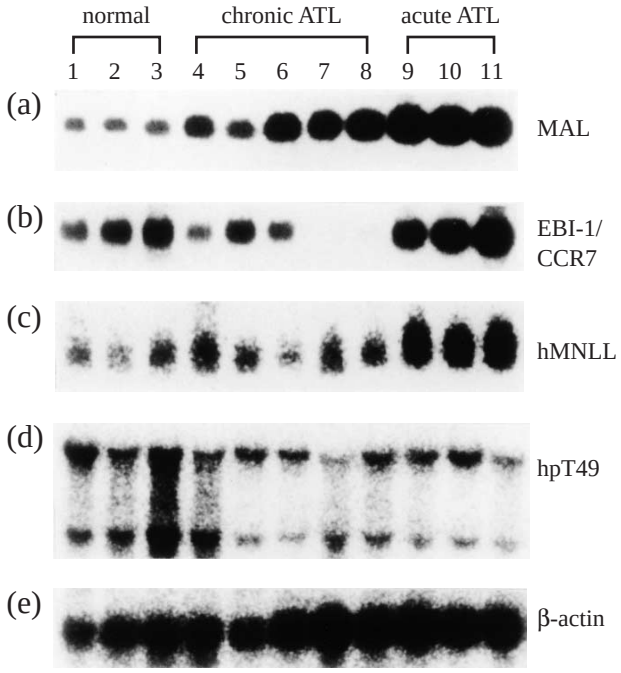

Fig. 2. Northern blot analysis for the differentially amplified mRNA in cells from patients with chronic and acute ATL and normal subjects. Total RNA (10 μg) was extracted from PBMC and was fractionated by electrophoresis through 1% agarose. After blotting onto a nylon membrane, hybridization was performed using each of the cloned cDNAs and β-actin DNA as probes. Lanes 1–3, normal subjects; lanes 4–8, patients with chronic ATL (cases 1, 2, 3, 4, and 5); lanes 9–11, patients with acute ATL (cases 6, 7, and 8). Probes: (a) C316, MAL; (b) G416, EBI-1/CCR7; (c) G811, hMNLL; (d) G319, hpT49; (e) β-actin.

Molecular cloning of hMNLL The level of homology between the G811 and bovine Cl-MNLL was about 80%. Since the sequence of G811 contained no candidate for the initiation codon, we proceeded to 5′-RACE. The complete nucleotide sequence of the resulting 303 bp *G811* gene and the deduced amino acid sequence of its protein prod-

uct are shown in Fig. 3. The cDNA seems to be nearly full length, since its size was close to that of the mRNA. The 3′ untranslated region contains a potential polyadenylation signal, AATAAA, at positions 271 to 276. Translation probably starts from the ATG sequence at nucleotides 46 to 48, which conforms with the consensus translational initiation sequence.[24] An open reading frame was identified, which terminated 174 bp downstream of this initiator codon. This open reading frame would specify a protein with an estimated molecular weight of about 7000. The deduced amino acid sequence was 81% identical to that of bovine MNLL (Fig. 3B), strongly suggesting that G811 is the human version of the *MNLL* gene (hMNLL). The human homologue showed higher serine and arginine contents than the bovine MNLL.

Expression in hematopoietic cell lines The expression of these genes in various hematopoietic cell lines was analyzed by northern blotting (Fig. 4). The MAL mRNA was highly expressed in Jurkat and to a lesser extent in MOLT4 and MT-2, but was not detected in other cell lines, including MT4, SO4, HUT102, U937, and K562.[15] Expression of EBI-1/CCR7 was preferentially detected in the HTLV-1 infected T cell lines: SO4, MT-2, MT4, and HUT102, but not in uninfected cell lines, including the T cell lines Jurkat and MOLT4. In order to ascertain whether EBI-1/CCR7 was inducible by the HTLV-1 transactivator, Tax, we examined the expression of EBI-1/CCR7 mRNA in JPX-9 cells, in which Tax could be induced by the addition of heavy-metal ions. Tax was induced by the $CdCl_2$, as confirmed by western blotting (data not shown). However, the addition of $CdCl_2$ failed to induce EBI-1/CCR7 expression in JPX-9 cells (data not shown). The hMNLL mRNA was ubiquitously expressed in all tested hematopoietic cell lines. In contrast, hpT49, which was expressed in normal lymphocytes, was not detected in any cell line except MT-2.

DISCUSSION

The molecular mechanisms that influence the clinical development of ATL, as well as those determining malig-

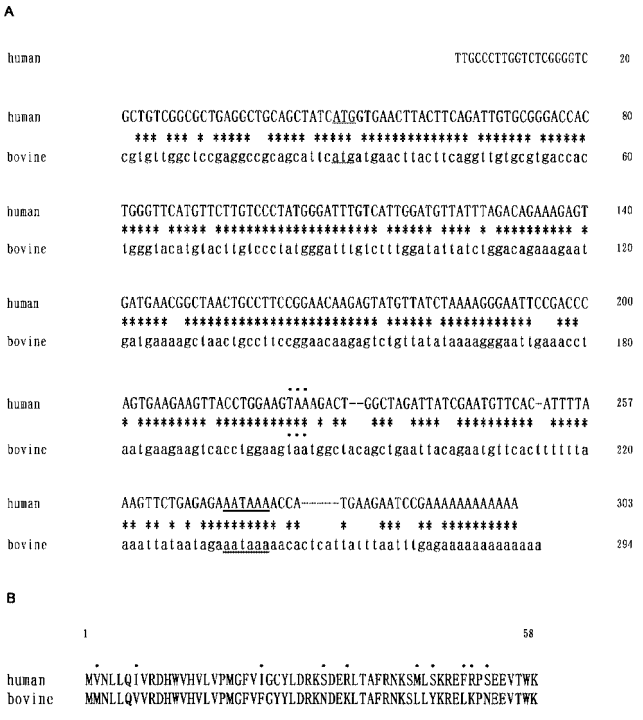

Fig. 3. A. Comparison of nucleotide sequences between human and bovine MNLL cDNA. Putative initiation codons are singly underlined. In-frame stop codons are indicated by dots. Potential polyadenylation signals AATAAA, are doubly underlined. Asterisks mark human MNLL sequences identical with those in bovine MNLL. The nucleotide sequence accession number is AB003593. B. Amino acid sequences of the human and bovine MNLL cDNA clones. Dots mark human MNLL amino acids different from those in bovine MNLL.

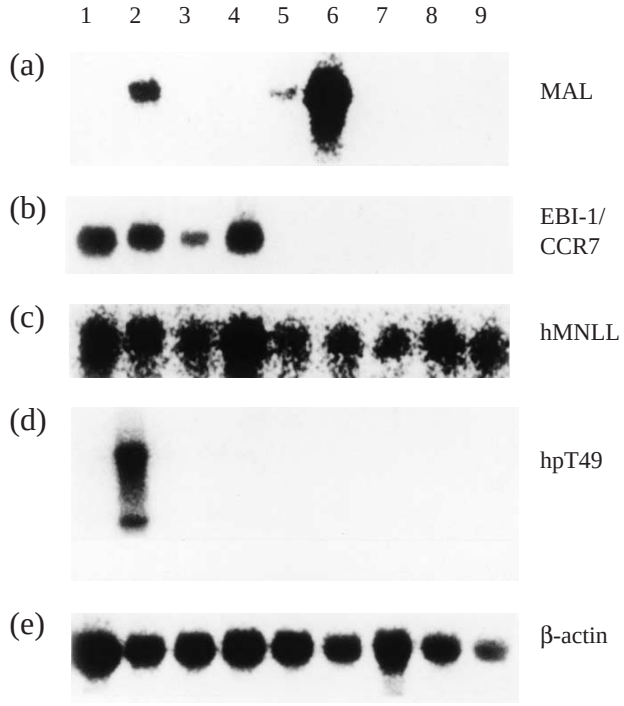

Fig. 4. Northern blot analysis of MAL, BLR-2/EBI-1, hMNLL and hpT49 in hematopoietic cell lines. Total RNA (10 μg) extracted from each cell line was hybridized with each of the ^{32}P-labeled cloned cDNAs and β-actin DNA as probes. Lane 1, SO4; lane 2, MT2; lane 3, MT4; lane 4, HUT102; lane 5, MOLT4; lane 6, Jurkat; lane 7, U937; lane 8, K562; and lane 9, HL60. Probes: (a) MAL, (b) EBI-1/CCR7, (c) hMNLL, (d) hpT49, and (e) β-actin.

nant cell transformation after HTLV-1 infection, are not fully understood. In the present study, we tried to identify the mRNA species that are differentially expressed in PBMC from patients with acute and chronic ATL and normal subjects. We found that the *MAL*, *EBI-1/CCR7*, and *hMNLL* genes were up-regulated in acute ATL cells. In contrast, the *hpT49* gene was down-regulated in these cells.

It was reported that the MAL mRNA is expressed in T cells at intermediate and late stages of differentiation,[15] thyroid epithelial cells,[25, 26] and myelin-forming cells.[27] MAL is a proteolipid that has been identified as a component of glycolipid-enriched membrane (GEM).[25–27] GEM requires protein-sorting machinery to operate as a route of transport.[28] MAL associates with glycosylphosphatidylinositol (GPI)-anchored proteins and the Src-like tyrosine kinase Lck in T lymphocytes, and cross-linking of GPI-anchored proteins triggers signaling pathways leading to Lck activation and T cell proliferation.[29, 30] Thus, the MAL molecule is closely associated with molecules for T cell activation, and it probably has roles in activation and proliferation. We found that the expression of the MAL mRNA was significantly high in the PBMC of patients with acute ATL, as compared to that of chronic ATL patients and normal subjects. It should be stressed that the chronic ATL samples contained a number of $CD4^+$ T cells comparable to that in the acute ATL samples, excluding a possible difference derived simply from using different numbers of $CD4^+$ T cells. Interestingly, the MAL mRNA was not detected in the HTLV-1 infected cell lines examined in this study, indicating that the expression of MAL is not directly related to HTLV-1 infection. It is possible that MAL is induced during the process of, or as a consequence of, malignant cell transformation *in vivo*.

The present study showed that the expression of the *EBI-1/CCR7* gene was down-regulated in chronic ATL cells and was up-regulated in some of the acute ATL cells as compared to the normal subjects. EBI-1/CCR7 is a lymphoid-specific member of the G-protein-coupled receptor family and is expressed exclusively in B- and T-lymphocyte cell lines and in lymphoid tissues.[17] The expression of EBI-1/CCR7 is induced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA), phytohemagglutinin (PHA), or anti-CD3 antibody in cultured peripheral blood lymphocytes, and is also inducible in B cells and $CD4^+$ T cells by infection with Epstein-Barr virus (EBV) and human herpes virus 6/7 (HHV 6/7),[31, 32] respectively. The ligand for EBI-1/CCR7 is ECL, a CC chemokine that presents in the thymus and lymph nodes.[33] CCR7 is a major homing receptor and an important regulatory molecule for initiating an antigen-specific immune response.[34] Therefore, the altered expression level in acute ATL cells may reflect the homing/infiltrating ability of ATL cells, rather than malignant cell transformation.

MNLL is a component of NADH: ubiquinone oxidoreductase (complex I), which is the first enzyme in the respiratory electron transport chain of mitochondria. Complex I is a membrane-bound multi-subunit assembly containing about 41 different polypeptides in bovine cells.[19] Complex I consists of sub-complexes Iα and Iβ, and MNLL is a component of sub-complex Iβ.[35] It is conceivable that hMNLL is an indispensable factor in the mitochondrial respiratory electron transport resulting in ATP synthesis. The high expression of hMNLL observed in acute ATL samples and all tested cell lines suggests that the energy metabolism in fast growing cells is up-regulated as compared to chronic ATL and normal cells.

pT49, encoding a secretory protein homologous to the fibrinogen β and γ subunits, was initially identified as a gene preferentially expressed in murine cytotoxic T lymphocytes but not in helper T lymphocytes or B lymphocytes.[21] The homology between the pT49 protein and the fibrinogen β and γ subunits is about 36%.[21] We consistently detected two bands, 1.5 and 4.5 kb, that hybridize with the hpT49 probe. In murine CTL cell lines, a single hybridizing mRNA band of 4.2 kb was detected.[21] A size of 1.5 kb is barely enough for the open reading frame of hpT49; therefore, 4.5 kb seems to correspond to the functional mRNA, which is likely to be generated by differential splicing or polyadenylation. The human version, hpT49, is expressed in fresh peripheral blood T lymphocytes, including both $CD4^+$ and $CD8^+$ cells, and its protein product is secreted as a multi-chain complex.[22] In contrast, it is not detected in most cultured cell lines, including those of lymphoid, myeloid, and epithelial cell origins.[21, 22] In this study, hpT49 (G319) was produced in only chronic ATL samples in differential display. However, hpT49 is strongly expressed in normal PBMC but down-regulated in both acute and chronic ATL in northern blotting. This discrepancy was solely derived from the source of mRNAs we used, with which hpT49 (G319) was expressed preferentially in chronic ATL regardless of the detection method; differential display or northern blotting. Since little is known about the function of hpT49 in T-lymphocytes, it is necessary to investigate the physiological role of hpT49 in resting T cells and the biological consequence of its down-regulation in ATL cells.

In this study, we used freshly isolated cells from ATL patients. It is widely accepted that studies using HTLV-I transformed cell lines have provided fruitful information about the molecular mechanism of this malignant phenotype. However, this study shows that discrepancies exist between fresh samples and cell lines, such as the expression of MAL and EBI-1/CCR7. The expression of MAL mRNA was significantly high in the PBMC of patients with acute ATL, while it was not detected in HTLV-1-infected cell lines examined in this study. The expression of EBI-1/CCR7 mRNA was down-regulated in chronic

ATL cells. Although SO4 cell line was established from a patient with chronic ATL, the expression of *EBI-1/CCR7* gene was high. Furthermore, Tax, a potent transactivator protein in many biological settings, is not expressed in clinically isolated ATL cells, and becomes expressed after several hours in culture.[36, 37] Therefore, it is possible that the gene expression of the leukemic cells of ATL patients is free from Tax transactivation, especially after malignant cell transformation, underlining the importance of an analysis using *in vivo* samples.

We successfully identified four genes with aberrantly regulated expression in ATL cells. The differential display technique we used is a powerful molecular tool that allows the identification and subsequent isolation of transcripts differentially expressed between biological samples. However, as previously reported, we encountered several drawbacks in the application of this technique to a limited amount of samples.[38] First, we found significantly high rates of false-positives, and required a large amount of mRNA to screen for the truly differentially expressed cDNAs. Second, although high rates of false-positives were produced, there are still genes that remain unidentified. The failure to isolate previously identified genes that should have been differentially expressed, such as p53, p15, and p16, clearly supports this notion. However, the limited amount of the clinical samples prevented further study. Furthermore, techniques analogous to differential display, such as representation display analysis, have similar drawbacks for clinical application, since the methods require a sufficient amount of mRNA to screen false-positives. DNA microarray technology would probably overcome these drawbacks and allow us to screen differentially expressed genes more systematically. However, the DNA microarray technique requires repeated experiments to obtain reliable results.[39] In this regard, the genes we identified here will provide useful information in evaluating the reliability of the data obtained by application of the DNA microarray technique to clinically isolated ATL cells in the near future.

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