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ORIGINAL ARTICLE

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Chromosomal imbalances in adult T-cell leukemia revealed by comparative genomic hybridization: gains at 14q32 and 2p16-22 in cell lines

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Abstract Comparative genomic hybridization was used to identify chromosomal imbalances in eight cell lines and 12 blood samples from patients with adult T-cell leukemia/lymphoma (ATL). The chromosomes most often over-represented in the cell lines were 2p (6 cases), 7q (4 cases), and 14q (4 cases), with minimal common regions at 2p16-22, 7q21-36, and 14q32, respectively. Distinct imbalances were detected in only 7 of the clinical samples. Chromosomes 14q32 and 2p16-22 harbor *TCL1* and a transcription factor, *HTLF* (human T-cell leukemia virus enhancer factor), respectively. FISH analysis revealed that *TCL1* did not juxtapose to *TCRA*, and we detected no expression of *TCL1* in any of the ATL cell lines despite the 14q32 amplifications. Moreover, expression of *HTLF* was not elevated in the ATL cell lines bearing multiplication of 2p. These results suggest that chromosomal regions 2p16-22 and 14q32 harbor genes other than *HTLF* and *TCL1* that are involved in cellular immortalization or in the pathogenesis of ATL.

Key words ATL · CGH · 14q32 · *TCL1* · 2p · *HTLF*

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Introduction

Adult T-cell leukemia/lymphoma (ATL) is defined on the basis of clinical manifestations that include distinct morphology of abnormal lymphocytes, T-cell markers, and an association with HTLV-1 virus infection (Hinuma et al. 1982; Seiki et al. 1984). However, a lack of distinctive oncogenes in the HTLV-I genome (Seiki et al.1983) and the fact that ATL occurs in a very small proportion (0.01%–0.02%) of individuals infected with HTLV-1 (Seiki et al. 1983) imply that additional genetic changes may be required for the onset of the disease.

Various cytogenetic aberrations have been reported in ATL, such as trisomies of chromosomes 3, 7, and 21, deletions of chromosomes 6q, 3q, and 10p, and translocations involving 14q32 or 14q11 (Kamada et al. 1992). However, the karyotypes are often complex, with widespread alterations throughout various chromosomes, a feature that complicates efforts to identify consistent aberrations that might include distinct copy-number changes with potential diagnostic or prognostic significance.

Comparative genomic hybridization (CGH) is a valuable procedure for whole-genome scanning because it allows identification of chromosomal imbalances such as gains, losses, or amplifications of DNA sequences in entire tumor genomes (Kallioniemi et al. 1993). Because copy-number changes are detected by CGH only if they are present in at least 50% of the cells, this method can identify consistent clonal aberrations associated with tumor development or progression (Kallionemi et al. 1993). However, we are aware of no published reports of CGH analyses in ATL.

We performed CGH to identify genomic imbalances in peripheral blood mononuclear cells (PBMCs) from patients with acute-type ATL and in cell lines derived from ATL.

The cell lines we examined showed preferential over-representation indicative of gene multiplication and/or amplification in chromosomal regions 14q32 and 2p16-22. Chromosome 14q32 harbors *TCL1*, a gene that is sometimes juxtaposed to *TCRA* or *TCRD* in peripheral T-cell lymphocytic leukemia (PTLL) (Virgilio et al. 1993, 1994); a transcriptional factor, *HTLF* (human T-cell leukemia virus enhancer factor) lies on 2p16-22 (Li et al. 1992). To investigate the possible involvement of these two genes in the pathogenesis of ATL, we examined our panel of cells for rearrangement and/or coamplification of *TCL1* with *TCRA*, and determined the expression status of *TCL1* and *HTLF* in the same cell preparations.

Materials and methods

Samples

Characteristics of eight cell lines and PBMCs isolated from 12 clinical cases of acute-type ATL are listed in Table 1. All these patients showed more than 70% of leukemic cells typical of ATL in the peripheral blood, respectively. Details concerning five of the cell lines (HUT78, ATL-5T, SD-4, KPNT-1, and IPAT-1) have been described elsewhere (Miyamoto et al. 1987; Tatewaki et al. 1995; Takizawa et al. 1998); the other three cell lines (OMT-1, KK-1, and ST-1) were newly established by Y. Yamada (Dept. of Clinical Laboratories, Nagasaki University Hospital) and Y. Kagami (Dept. of Hematology and Chemotherapy, Aichi Cancer Center Hospital) from PBMCs of untreated ATL patients, and will be described elsewhere. Among them, six (OMT-1, KK-1, SD-4, KPNT-1, IPAT, and ST-1) required medium containing IL-2 for passage (Table 1). PBMCs from 12 patients diagnosed with acute ATL were isolated by density gradient centrifugation using Ficoll/Hypaque (Pharmacia LKB, Uppsala, Sweden). Diagnoses were made on the basis of clinical features, hematological findings, and serum anti-HTLV-1 antibodies according to the criteria described by Shimoyama et al. (1991). Clonal integration of HTLV-1 DNA into the host genome was investigated by Southern blot analysis in all cases as described (Kagami et al. 1993).

Comparative genome hybridization (CGH)

Genomic DNAs were isolated from leukemic cells and from PBMCs of healthy donors with the conventional method. CGH was performed as described elsewhere (Kallioniemi et al. 1993; Ariyama et al. 1998a). Briefly, tumor (test) DNA and normal DNA were labeled by nick-translation with Spectrum Green-dUTP (Vysis, Boston, MA, USA) and Texas Red-dUTP (NEN-Dupont, Boston, MA, USA), respectively. Equal amounts (180ng) of tumor and normal DNAs were mixed with 15μg of human Cot-1 DNA (Gibco/BRL, Gaithersburg, MD, USA), resuspended in hybridization mixture, and hybridized *in situ* to human metaphase chromosome spreads prepared from phytohemagglutinin (PHA)-stimulated lymphocytes of a normal male volunteer.

After hybridization, the slides were washed and the chromosomes counter-stained with 4′,6′-diamino-2-phenylindol (DAPI) to permit identification of the chromosomes. Fluorescent hybridization signals and DAPI-staining patterns were captured with a cool charge-coupled device (CCD) camera (Photometrics, Tucson, AZ, USA) attached to a Nikon microscope (Ecrips E600) and processed using a Quips image analysis system (Vysis). The manufacturer's software calculated the green (tumor DNA)-to-red (normal DNA) fluorescent ratio along the length of each chromosome. Averaged readings from at least six chromosomes were graphed for each chromosome and compared with the profile for the same chromosome in a reference hybridization to set the boundaries of gain and loss (Sakakura et al. 1999). Heterochromatic regions near the centromeres, and the entire Y chromosome, were excluded from analysis.

Two-color fluorescent *in situ* hybridization (FISH)

Our CGH analysis of the ATL cell lines showed preferential multiplication at 14q32, a chromosomal region harboring *TCL1*. Frequent translocations or inversions involving the *TCRA/D* loci and *TCL1*, such as t(14;14)(q11;q32.1) and inv(14)(q11;q32.1), are observed in PTLL (Virgilio et al. 1993, 1994). Therefore, to verify copy-number changes and detect possible rearrangements of *TCL1* and *TCRA* in our series of ATL samples, we performed two-color FISH, using as probes cosmids specific for *TCL1* (pLC1; 14q32) and *TCRA* (Plcos22, 14q11) (Narducci et al. 1995). Probe labeling, in situ hybridization, and signal evaluation were performed as described previously (Inazawa et al. 1994). Briefly, Plcos22 was labeled with digoxigenin-11-dUTP (Boehringer, Indianapolis, IN, USA), and PLC1 with biotin-16-dUTP (Boehringer), by nick-translation. Labeled probes were precipitated with sonicated salmon sperm DNA and dissolved in 20μl of formamide. The probe solutions were mixed with Cot 1 DNA (250ng/μl), and denatured before addition of hybridization buffer. Signals were detected by antidigoxigenin rhodamine and avidin-FITC. After being counterstained with DAPI, the slides were examined through a Nikon Eclipse epifluorescent microscope equipped with a CCD camera (Photometrics). Images were captured with Quips (Vysis) software and processed with Adobe Photoshop 3.0 software as described elsewhere (Ariyama et al. 1998b).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNAs were isolated from the ATL cell lines using TRIzol reagent (Gibco BRL). In each case, cDNA was prepared from total RNA using random primer oligonucleotides. Approximately 5μg of RNA in a final volume of 11μl was first denatured at 70°C for 10min and then cooled on ice. First-strand DNA synthesis was performed by adding 1μl

Table 1. Characteristics of ATL cell lines and clinical samples and results of chromosome imbalances detected by CGH

Sample name	Sex/Age	HTLV-1	IL-2 dependency	14q32 gain	2p16-22 gain		Copy-number abnormalities detected by CGH
HUT-78		+	−	+	+	Gains	1p33-35, 1p13, 2p16-25, 3q27-29, 4p16, 6p11.2-21.3, 6q22-27, 7q21-36, 8q24, 9q33-34, 10p15, 12q23-24, 14q32, 15q22-26, 17p11.2-13, 17q11.2-25, 19p13.3, 19q13.2-13.3, 20p13, 20q12-13.3, 22q12-13, Xq27-28
						Losses	2q24-33, 4q13-24, 7p15-22, 9p12-24, 9q13-22, 10q23-25, 13q14-21, 14q11.2-22, 16q12-21, 18q11.2-23
OMT-1		+	+	+	+	Gains	2p12-22, 3p21-26, 6p24-25, 7p21-22, 12q21, 14q31-32, 16p13.1-13.2, 17q25, 20p12-13
						Losses	9q11.2-32, 13q21-34, 2p22-25
KK-1		+	+	−	+	Gains	2p11.2-25, 2q11.2-22, 6p11.2-23
						Losses	10q24-26
ATL-5T		−	−	−	−	Gains	1q43-44, 11p14-15, 12p11.2-13, 13q32-34, 17p11.2-12, 17q11.2-25
						Losses	3q26-29, 4q34-35, 10p11.2-12
SD-4		+	+	+	+	Gains	2p16-25, 2p16-12, 2q32-33, 7q22-36, 9p21-23, 11q23-25, 12p13, 14q32, 18q11.2-23, 20p11.2-13, 20q11.2-13.3, Xp11.2-22.3, Xq12-21
						Losses	1p35-36, 3q12-21, 6q21–23, 9q13-21, 10p11.2-15, 18p11.2-11.3
KPNT-1		+	+	−	+	Gains	1q31-41, 2p11.2-23, 2q11.2-37, 8q24
						Losses	None
IPAT-1		+	+	−	+	Gains	1p34-36.1, 1q22-24, 2p22-25, 6p23-25, 9p13-24, 9q13-34, 12p11.2-12, 13q22, 17p11.2, 17q11.2-21, 19p13.1-13.2
						Losses	3p24-26, 10p11.2-15, 10q11.2-26, 13q32-34, 14q12-21, 15q24-26, 16q12.1-24
ST-1		+	+	+	−	Gains	7q21-36, 8q22-24, 14q24-32, 18q12-23, 21q21-22
						Losses	2p22-36, 2q32-37, 3q12-21, 18p11.2-11.3
G529	F/73	+		−	−	Gains	4p15-16, 7q34-36, 17p11.2-13, 17q12, 17q25, 18p11.2-11.3, 19p13.1-13.3
						Losses	None
G523	M/54	+		−	−	Gains	1p34-36, 7q12-36, 10q21, 16p12-13.1, 19p11-13.3
						Losses	4q35, 9p23-24, 15q26
G614	M/70	+		−	−	Gains	1p34-36, 1q21-41, 16p11.2-13.1, 19p13.1-13.3, 19q13.1-13.4
						Losses	Xp22.2-22.3
G616	F/43	+		−	−	Gains	5p12-15, 5q31-35, 11q24-25
						Losses	None
G540	F/47	+		+	−	Gains	1p36, 14q21-32, 16p11.2-13.3
						Losses	None
G537	F/40	+		−	−	Gains	16p12, 18p11.2-11.3, 18q22-23
						Losses	None
G531	M/34	+		−	−	Gains	Xp11.2, Xq12-28
						Losses	None
G615	F/40	+		−	−	Gains	None
						Losses	None
G603	F/64	+		−	−	Gains	None
						Losses	None
G602	M/58	+		−	−	Gains	None
						Losses	None
G643	M/53	+		−	−	Gains	None
						Losses	None
AZI	M/46	+		−	−	Gain	None
						Losses	None

(200U) of Superscript II reverse transcriptase (Gibco BRL), 200mM DTT, 40pM random primers (Boehringer), 10mM deoxynucleotide triphosphate, and 40 units of RNase block, to a final volume of 20µl in RT buffer (Han et al. 1995). *TCL1*-specific primers used for the PCR were *TCL1*-U1 (5′-AGGCCTATGACCCCCACC-3′), corresponding to the (+) strand from bp 222–239, and *TCL1*-R2 (5′-CATTCCTCCCAGACCCCA-3′), corresponding to the (−) strand from bp 511–529 of the *TCL1* cDNA. The *TCL1*-specific product corresponded to 308bp Narducci et al. (1997). *HTLF*-specific primers were *HTLF*-2F (5′-CACCAACAGGCTGGAAGAAT-3′), corresponding to the (+) strand from bp 466–485, and *HTLF*-R2 (5′-GGATCAATGCTGGTGGCTAA-3′), corresponding to the (−) strand from bp 885–904 of *HTLF* cDNA. The *HTLF*-specific product corresponded to 439bp (Li et al. 1992). Primers specific for control amplification of a housekeeping gene (β-actin) were *ACTIN*-S (5′-CCAGAGATGGCCACGGCTGCT-3′) and *ACTIN*-AS (5′-TCCTTCTGCATCCTGTCGGCT-3′). Amplifications were performed at 94°C for 30s, 60°C for 30s, and 72°C for 30s for 25 (*TCL1*), 30 (*HTLF*), or 25 cycles (β-actin). RT-PCR products were electrophoresed through a 3.0% GTG agarose, Southern-blotted onto a nylon membrane, and hybridized with ^{32}P-labeled internal oligonucleotides internal to the PCR products specific for *TCL*1 or *HTLF*.

Results

Copy-number changes detected by CGH

All the clinical samples and cell lines were successfully analyzed by CGH. In 5 of the 12 primary samples we found no copy-number changes, but in the other 7 patients and all eight cell lines, distinct copy-number changes were identified by CGH analysis (Table 1). A schematic summary is shown in Fig. 2. The most commonly gained regions in the cell lines were at 2p (6/8), 7q (4/8), and 14q (4/8), with the minimal common regions at 2p16-22, 7q21-36, and 14q32, respectively (Figs. 1,2). High-level gains (HLGs) indicative of genomic amplifications were detected at 2p16–25 in three cell lines (HUT-78, SD-4, and KK-1), and in one line each at 9q33-34 (HUT-78), 12p11.2-13 (ATL-5T), and 14q31-32 (OMT-1). However, no frequent common regions for copy-number gains or losses could be detected among the clinical samples.

FISH with *TCL1* and *TCRA*-specific cosmids

To identify the status of *TCL1* amplification and to ascertain a possible juxtaposition of *TCL1* to *TCRA*, we performed two-color FISH to metaphase and interphase chromosomes with cosmids specific for *TCL1* (PLC1) and

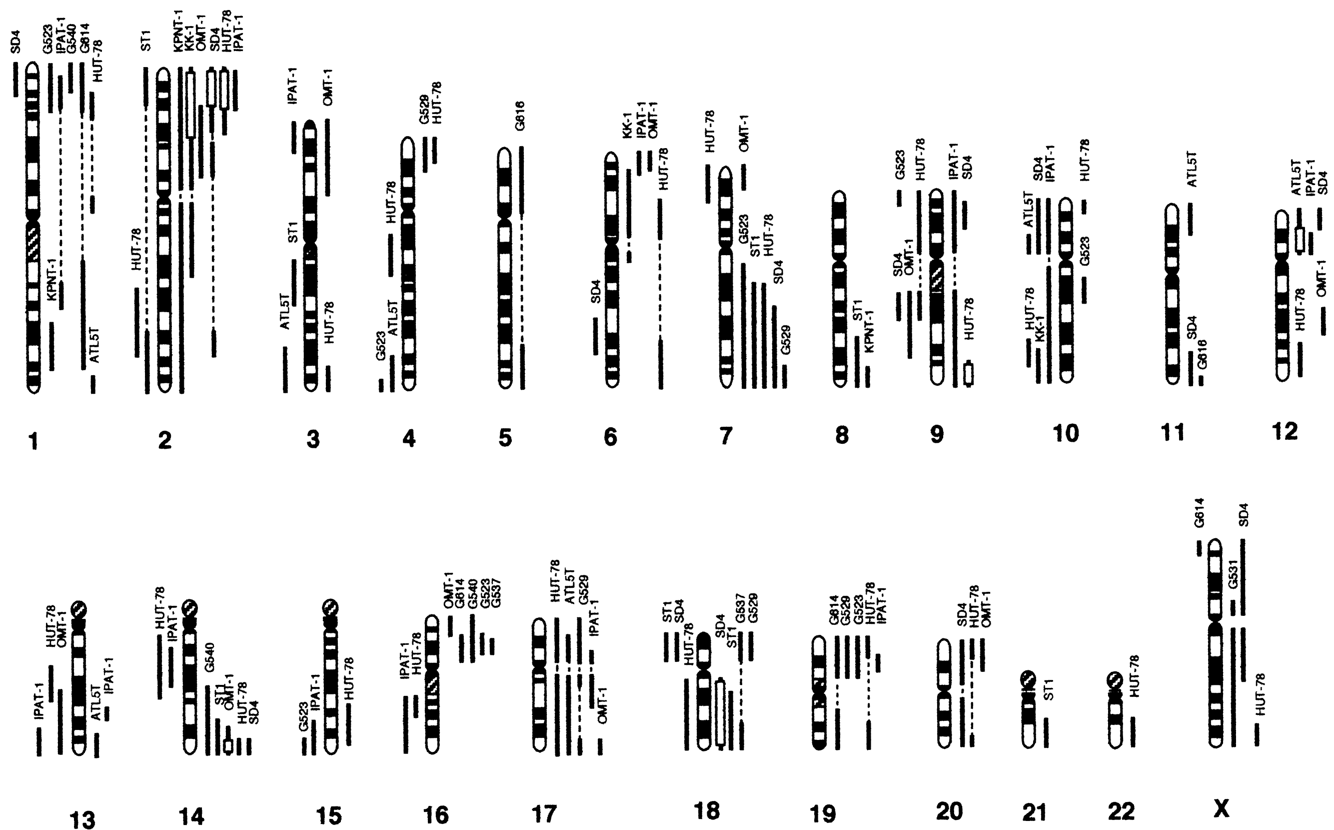

Fig. 1. Summary of comparative genomic hybridization (CGH) imbalances in eight cell lines and blood samples from seven ATL patients. Gains are represented by *vertical lines* on the *right*, and losses by *vertical lines* on the *left*, of each chromosome ideogram. Highly amplified regions are indicated by *rectangles*. *Broken lines* connect genomic aberrations present in the same sample

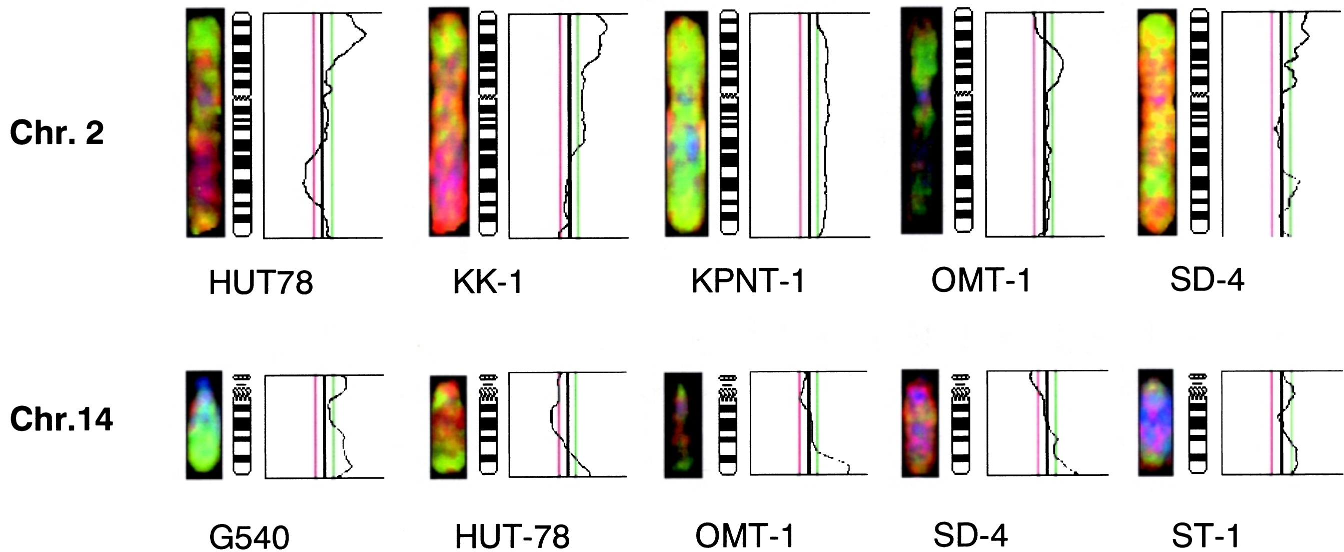

Fig. 2. Representative CGH images of chromosomes 2 and 14 and the corresponding profiles of ATL cell lines showing overrepresentations of chromosomal regions containing 2p16-22 and 14q32

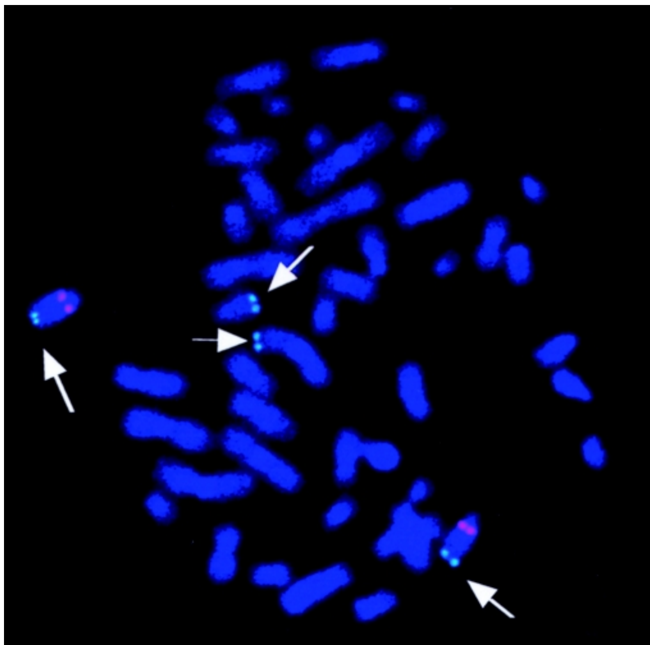

Fig. 3. Two-color FISH with cosmids specific for *TCRA* (rhodamine; *pink signals*) and *TCL1* (FITC; *green signals*) on metaphase chromosomes from OMT-1, revealing high-level amplification at 14q32 (see Fig. 3). *TCL1* did not juxtapose to *TCRA* in any cells, although the *TCL1* locus was multiplied to four copies

TCRA (PLC22) in the four ATL cell lines that had exhibited overrepresentations at 14q32 (HUT78, SD-4, ST-1, and OMT-1). In these experiments, at least 65 interphase nuclei and 20 metaphase cells were observed and scored in each cell line examined (data not shown). A typical pattern of FISH, using metaphase chromosomes of OMT-1, is shown in Fig. 3. *TCL1* was multiplied to more than three copies in each of the four cell lines, a result in accord with the results obtained by CGH. However, we observed no fusion of the signals for *TCL1* and *TCRA* that would indicate *TCL1*/*TCRA* rearrangement in either interphase or metaphase cells (Fig. 3).

Expression of *TCL1* and *HTLF*

To evaluate expression of the *TCL1* gene, we performed RT-PCR assay of mRNAs from each of the ATL cell lines, using human fetal thymocytes and a leukemic cell line (BV173) as positive controls (Virgilio et al. 1994). No *TCL1* expression was detected in any ATL cell line whether or not it had shown 14q32-multiplication, even though we employed an autoradiographic exposure of 2 days (Fig. 4a). We did however detect *TCL1* in human fetal thymocytes and in BV173, in agreement with results reported by Virgilio L. et al. (1994).

To investigate whether the *HTLF* gene was expressed in ATL cell lines with or without multiplication of 2p16-p25, the site of the *HTLF* locus, we analyzed mRNAs from the ATL cell lines, and from Jurkat and normal lymphocytes, in RT-PCR assay. *HTLF* expression was detected in six of the ATL cell lines (KK-1, HUT78, SD-4, OMT-1, KPNT-1, and ST-1) and in Jurkat and normal lymphocytes, but not in ATL-5T or IPAT-1 (Fig. 4b).

Discussion

Overrepresentation or HLG of chromosomes 7q, 14q32, and 2p16-22 were the most prominent features among the ATL-derived cells in our study. Of them, trisomy 7 has been reported to be one of the frequent chromosome aberrations of ATL in extensive cytogenetic studies (Kamada et al. 1992). Our data confirmed the previous results and suggest

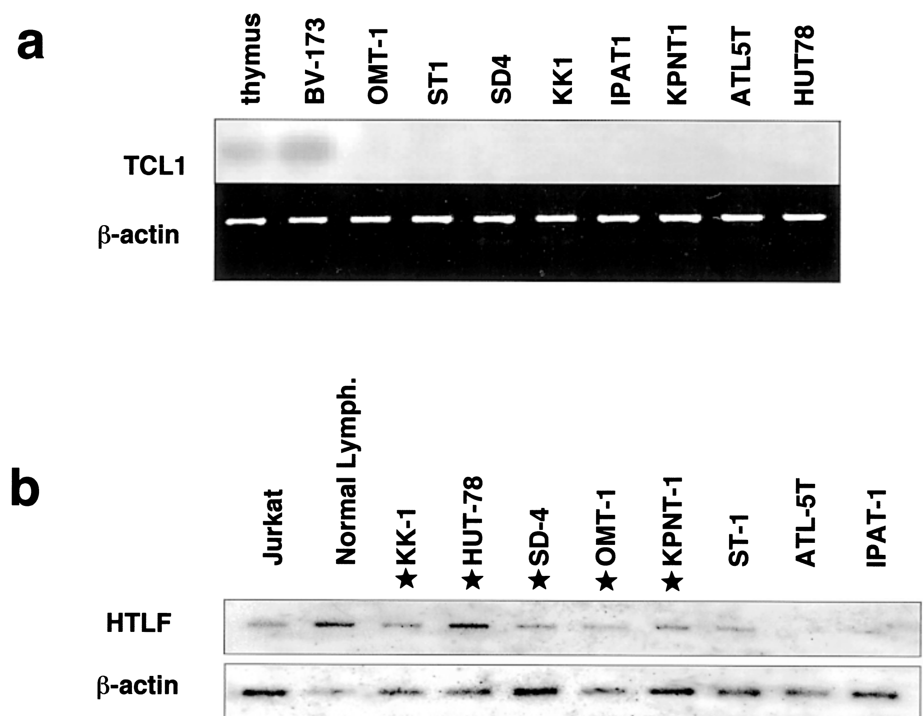

Fig. 4a,b. RT-PCR of the *TCL1* (**a**) and *HTLF* (**b**) gene products. **a** *Top*: Southern blot hybridization. *Bottom*: ethidium bromide staining of the RT-PCR performed with β-actin primers. No *TCL1* expression could be detected in ATL cell lines with 14q32 multiplication (OMT-1, SD-4, ST-1, HUT78) or without 14q32 multiplication (IPAT-1, KK-1, KPNT-1, ATL-5T). Human fetal thymocytes and BV173 were positive controls for expression of *TCL1* (Virgilio et al. 1994). Autoradiography was carried out for 2 days. **b** *HTLF* was expressed in six of the eight ATL cell lines (KK-1, HUT78, SD-4, OMT-1, KPNT-1, and ST-1), and in Jurkat and normal lymphocytes, but not in ATL-5T or IPAT-1. *Asterisk* indicates the cell lines exhibited chromosomal gains of 2p16-p22 by CGH

that the long arm of chromosome 7 might contain the gene(s) responsible for the pathogenesis of ATL by a mechanism of the gene dosage effect.

Multiplication of 14q32 seems to be important, because it was present in four of eight cell lines and also in one clinical case. The *TCL1* oncogene lies at 14q32, where it is involved in the specific cytogenetic rearrangements, such as t(14;14)(q11;q32.1) and inv(14)(q11;q32.1), that occur frequently in mature peripheral T-cell leukemias (Virgilio et al. 1993, 1994). As a consequence of these rearrangements, regulation of the *TCL1* proto-oncogene is disrupted by its juxtaposition to the enhancer element of the *TCRA* gene. This oncogenic stimulation is considered to be an early event in the development of T-cell neoplasms (Narducci et al. 1995). Recent molecular and immunocytochemical analyses have revealed that *TCL1* is overexpressed in ATL in comparison to normal T cells, and also in T-cell lines derived from patients with T-acute lymphocytic leukemia (T-ALL) (Narducci et al. 1997). More recently, the same group of investigators has isolated a new member of the *TCL1* gene family, *TCL1b*, which is localized approximately 16 kb centromeric of *TCL1* (Pekarsky et al. in press). These results have suggested that this gene might play a critical role in the pathogenesis of ATL (Narducci et al. 1997; Pekarsky et al. in press). Our FISH results, on the contrary, showed that *TCL1* did not juxtapose to *TCRA* in any of the four cell lines that exhibited multiplication at 14q32. Furthermore, *TCL1* expression was undetectable in any of the cell lines we examined, whether they exhibited multiplication at 14q32 or not. These results strongly suggest that one or more oncogenes on 14q32 other than *TCL1*, upregulated by mechanisms of amplification or gene dosage effect, might be responsible for the leukemogenesis leading to ATL.

Overrepresentation of 2p16-22 also seems to be important in the immortalization of leukocytes or the pathogenesis of ATL, because we detected it in six of our eight cell lines. *HTLF*, a cellular factor that binds to the long terminal repeat (LTR) in HTLV-I, has been mapped to human chromosome 2p16-22 (Li et al. 1992). Genomic amplification of a gene often results in its overexpression, and therefore it is plausible to assume that the multiplication of *HTLF* provides a signal responsible for development of ATL. In particular, the regulatory protein "*tax*" of HTLV-1 positively regulates the transcription of the viral genome as well as specific cellular genes, and it shows a weak oncogenic activity *in vitro* (Tanaka et al. 1990; Franchini 1995). *Tax* protein does not bind directly to the HTLV-1 LTR, but it serves as a potential transcriptional transactivator of the cellular factors that do specifically bind the LTR (Yoshida et al. 1995; Fujisawa et al. 1985). Therefore, if *HTLF* is localized within 2p22, *HTLF* is a reasonable candidate for involvement in the pathogenesis of ATL, like other cellular factors (e.g., *IL2*, *IL2R*, *IL3*, *IL6*, *CSF2*, *TNFA*, *TNFB*, *FOS*, and *NFKB*) whose expression can be induced by "*tax*" (Yoshida et al. 1994). However, our quantitative RT-PCR analysis showed that expression of *HTLF* was not preferentially elevated in our ATL cell lines despite genomic multiplication of 2p16-22, as judged by comparison to its expression in Jurkat or normal lymphocytes. Since in our experiments genomic multiplication of 2p16-22 did not necessarily result in overexpression of *HTLF*, this chromosomal region also

may harbor some other gene(s) that confer the specific biological aspects of pathogenesis in ATL. We observed preferential gains in copy number of 2p16-22 in five of the six cell lines that grew in an *IL2*-dependent manner; therefore, this chromosomal region must contain a gene(s) encoding protein(s) serving through the *IL2*-dependent signaling pathway.

Our data indicate that more detailed analyses are required to clarify whether unknown candidate oncogenes for ATL lie within chromosomal regions 14q32 and 2p16-22.

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