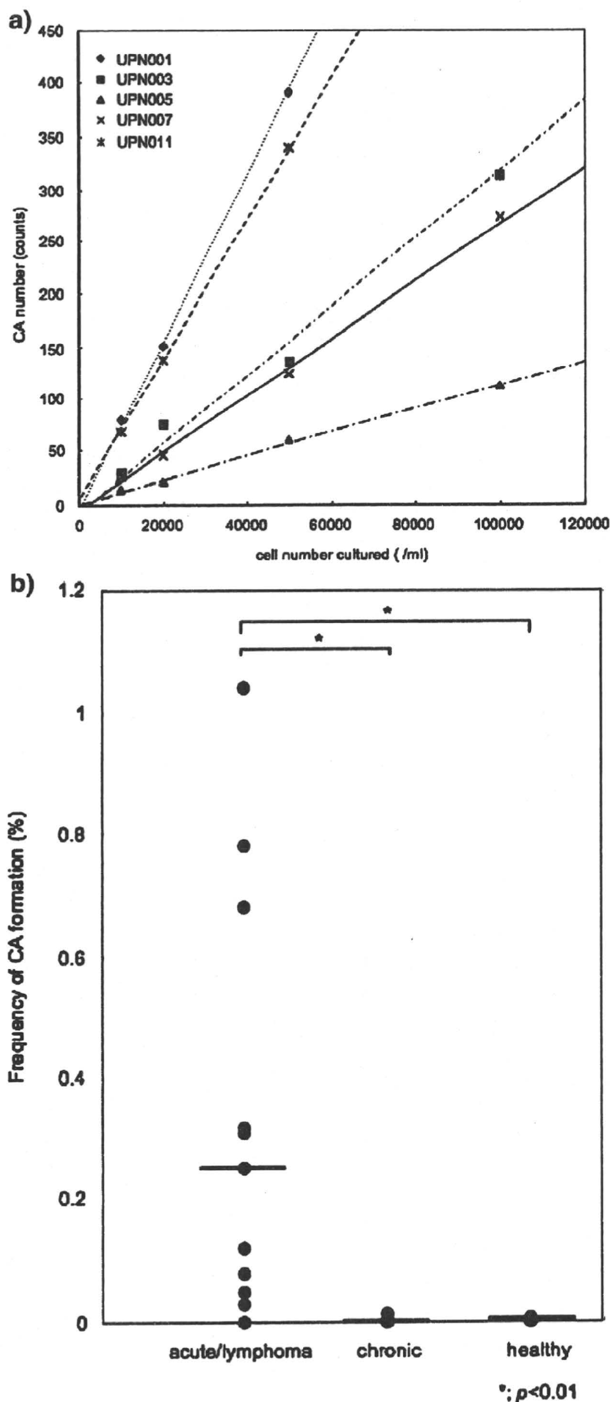


Fig. 2 Plating efficiency of ATL cells in a co-culture system with MS-5 cells. **a** The frequency of CA-forming cells (CAFC) of five cases (UPN001 (PB), 003, 005, 007, and 011) was examined with semi-solid collagen gel in the co-culture system. A clear linear relationship was obtained between the inoculated cell number and CAFCs in all cases examined. **b** The distribution plots of frequency of CA formation in different sample group; There is statistical difference in the median (*horizontal bars*) among acute/lymphoma type (0.25%), chronic type (0%), and healthy volunteers (0.04%) ($P < 0.01$)

three cases of acute ATL, the results of ISH revealed that the expression of the Tax gene of ATL cells in CA was markedly decreased when compared with that of growing cells in liquid culture with IL-2 (Fig. 3c).

Next, we quantified the expression level of HBZ gene, which was recently identified in the 3″-LTR of the complementary sequence of HTLV-1 and has been suggested as a critical gene in leukemogenesis of ATL [6, 32, 33]. As shown in Fig. 3d, the HBZ gene was highly expressed in MT-2 cells in CA, and the level of mRNA load was equivalent to those without co-culturing. With regard to *tax* gene, the expression level was significantly higher in cells in liquid culture without stroma than in cells in CA, although the difference was not so striking as observed in RNA-ISH analysis. Moreover, in the contact-inhibited condition to the stromal layer, the expression levels of these two genes were comparable to those without co-culturing.

3.4 Gene expression profiles of ATL cells composing CA

Next, we compared the gene expression profiles of sets of ATL cells composing CA matched with their corresponding $CD4^+$ primary samples from the same individuals by high-density oligonucleotide microarray analysis, to search for candidates of disease-specific molecules and signaling pathways, which contribute to the mechanism of adhesion-dependent proliferation in our co-culture system. After removal of transcriptionally silent genes from the analysis of 44,764 probe sets using Microarray Suite software, Student's t test ($P < 0.001$) was then used to extract genes, the expression level of which significantly differed between cells in CA and primary samples. Genes were considered up- or down-regulated if each value and the average fold change were 3.5 or greater in all three data sets. Finally, we could identify 110 and 98 genes significantly up- and down-regulated in ATL cells in CA compared with primary samples by selecting genes based on the reproducibility, respectively (Fig. 4a).

To validate the microarray findings, we performed RTQ-PCR for eight genes that were significantly up- or down-regulated in ATL cells in CA of three-paired array samples, and with code products correlating with adhesion molecules and cell–cell interaction or with cellular apoptosis and proliferation. All experiments were performed in triplicate. Variance among triplicates was less than 5%. Standard curves with correlation coefficiency greater than 0.970 were produced from the data for each gene, indicating the large dynamic range and accuracy of RTQ-PCR (data not shown). As shown in Table 3 and Fig. 4b, we confirmed that these genes were actually up- or down-regulated in ATL cells in CA, compared with primary

neoplastic cells as indicated in the microarray data. Up-regulated genes, *CDH11* [34], *Twist1* [35] and *Cav-1* [36], are considered to play a major role in tumor promotion, progression, survival and metastasis in several neoplasias. Furthermore, the down-regulation of several genes, *hCdc14A* [37], *CUGBP2* [38], *HBP1* [39], and *ZNFN1A1* [40], the products of which are recognized to play a role in suppressing carcinogenesis through various signaling pathways, were also confirmed in ATL cells in CA.

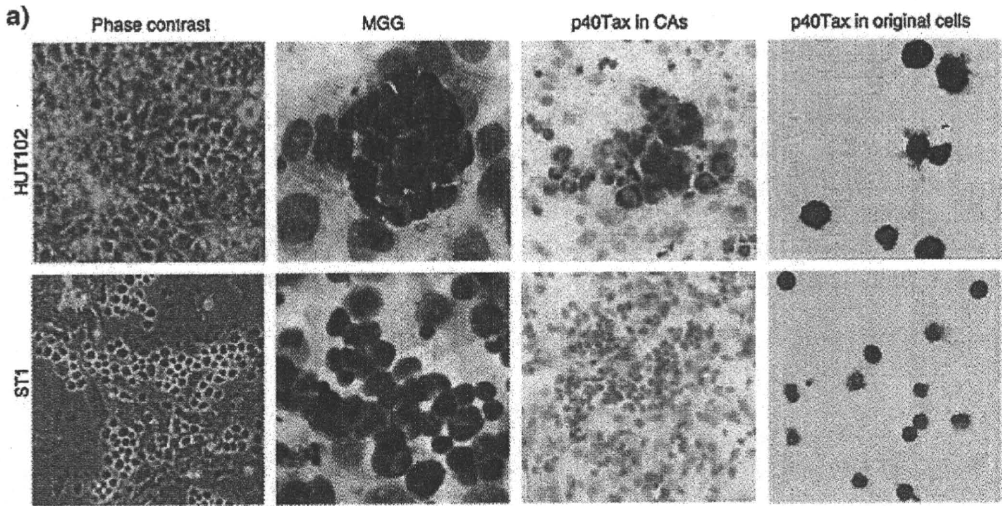
4 Discussion

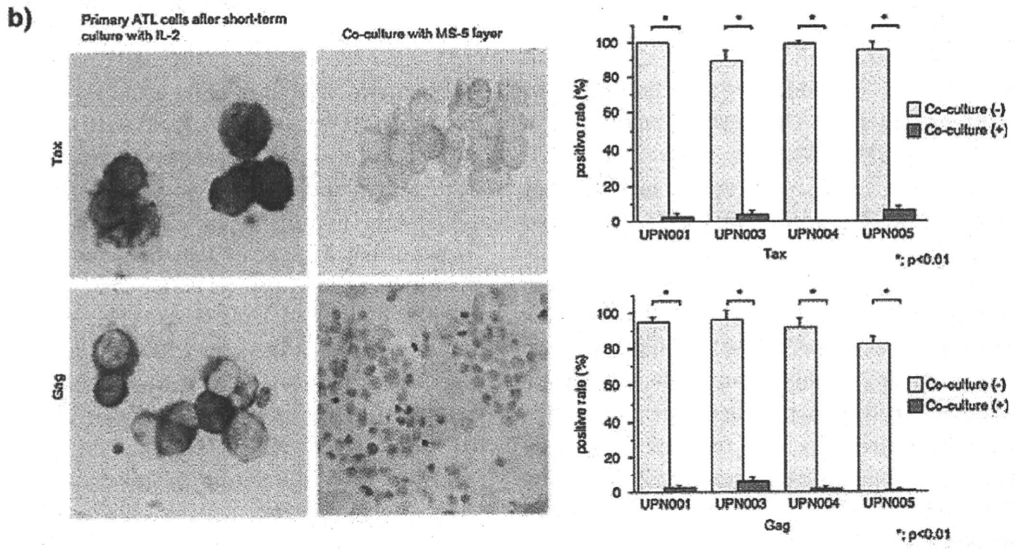
In the present study, we established a new ATL cell/murine stroma cell co-culture system with which we could observe the proliferation of primary ATL cells without any additional growth factor. In previous studies, primary ATL cells were able to grow in a liquid culture containing IL-2; however, they showed only a transient and cytokine-dependent proliferation [8–10]. On the other hand, we also observed involvement of HTLV-1 genome to patients' LN stroma cells and production of several cytokines from them [16]. In the present study, infection of HTLV-1 to MS-5 was considered to be dispensable to adhesion-dependent ATL cell growth, as shown in the negative or minimal viral load in co-cultured MS-5 cells. Furthermore, since many murine cytokines cannot affect human cells [41], and especially in this co-culture system, any soluble factor or co-cultured CM did not stimulate ATL cells growth in the contact-inhibition test, direct adhesion of target cells to the stromal layer of MS-5 layers are considered to be essential for CA formation by primary ATL cells in this co-culture system.

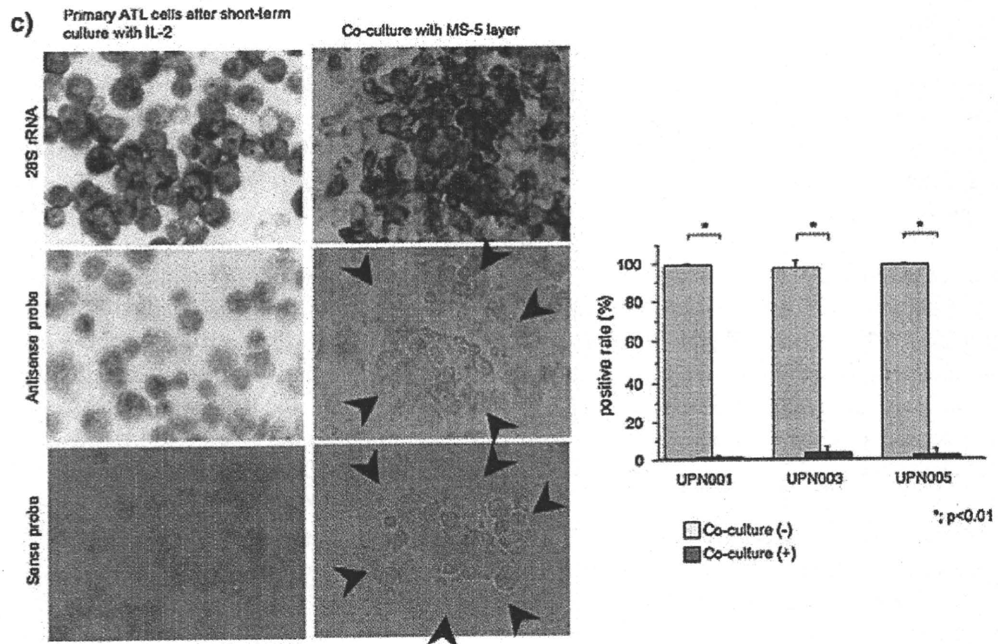
In the lymphohematopoietic microenvironment, normal and neoplastic lymphoid cells are affected by various molecules [42, 43]. Concerning lymphoid malignancies, recent reports has indicated that interactions between neoplastic cells and BM or LN stromal cells regulate growth, survival, and homing in multiple myeloma, mantle cell lymphoma, and other non-Hodgkin lymphoma [44, 45]. Whereas, the expression of various adhesion molecules and chemokine receptors on leukemic cells, also in ATL, is assumed to be essential to the pathophysiology of this disease [46–50], the significance of these molecules in the growth and survival of ATL cells is still poorly understood. Thus, extensive investigation of the molecular interaction between neoplastic cells and lymphohematopoietic microenvironment is essential to clarify not only adhesion and transmigration, but also the growth and survival mechanism of primary ATL cells in this co-culture system.

In the present study, two murine stromal cell lines, MS-5 and HESS-5 showed significant supportive capacity on leukemic CA formation comparing with HUVEC, and also indicated their differential abilities for support of growth and survival of target cells. Both of them were originally established from C3H/HeN strain mice and might share common characters concerning the interaction between hematopoietic cells and stroma layer through integrin family members [51, 52]. Furthermore, target cells could adhere and start to grow on both stromal layers in our series. Our limited studies did not reveal the mechanism of differential abilities to support the growth of ATL cells between MS-5 and HESS-5 with respect to the direct interaction to target cells for the maintenance of CA formation. Previous reports concerning successful xeno-transplantation of ATL cells into immunodeficient mice indicated that indispensable extrinsic elements might be supplied to human neoplastic cells in these in vivo animal models sharing similarity with human lymphohematopoietic microenvironment [53, 54]. In analogy, our in vitro

Fig. 3 Expression of HTLV-1-related genes in ATL cells in CA. ▶ **a** HUT-102 and ST-1 cells were examined by phase-contrast micrographs (final magnification ×400), May-Giemsa staining (final magnification ×600), and immunostaining of Tax protein on ATL cells in CA and on cells after liquid culture using anti p40Tax antibody, Lt-4 (final magnification ×400). **b** Expression of HTLV-1 viral proteins on ATL cells in CA obtained from primary ATL cells. Four patients with acute-type ATL were examined. *Color* photographs show representative results of immunostaining of Tax (*upper*) and Gag (*lower*) in primary cells after culture with IL-2 for 7 days (*left*) and ATL cells in CA (*right*) (UPN004). *Bar graphs* are a summary of results of immunostaining of Tax and Gag protein in four cases. Light and *dark gray bars* indicate the results of primary ATL cells after liquid culture with IL-2 and those of ATL cells in CA in our co-culture system, respectively. Data are presented as the mean percentage of positive cells in triplicate experiments with error bars indicating 1SD. Mann–Whitney's *U* test was performed for statistical analysis and, in every sample, the positive rate of immunostaining of p19 and p40 was significantly lower in ATL cells in CA than in primary ATL cells after liquid culture with IL-2 ($P < 0.01$). **c** *tax* mRNA expression in ATL cells in CA obtained from primary ATL cells. ATL cells obtained from three cases were analyzed by RNA-ISH method. *Color* photographs show representative results of the analysis of UPN001; primary ATL cells after liquid culture with IL-2 (*left*) and ATL cells in CA (*right*): tested with the probe to 28S ribosomal RNA as an internal control (*upper*), the antisense oligonucleotide probe to the *tax* mRNA (*middle*), and the sense oligonucleotide probe (*lower*), indicated in "Sect. 2". Areas surrounded by arrowheads are CAs. *Bar graphs* are a summary of the results of three primary samples, which were analyzed RNA-ISH for *tax* mRNA. Light and dark gray bars indicate the results of primary ATL cells after liquid culture with IL-2 and those of ATL cells in CA in our co-culture system, respectively. Data are presented as the mean percentage of positive cells in triplicate experiments with error bars indicating 1SD. Mann–Whitney's *U* test was performed for statistical analysis and, in every sample, the positive rate of *tax* mRNA expression was significantly lower in ATL cells in CA than in primary ATL cells after liquid culture with IL-2 ($P < 0.01$). **d** Results of quantification of mRNA load of HBZ and *tax* genes. The copy number of target mRNA per 50 ng total RNA was estimated from the standard curves [29]. *Light* and *dark gray* and *white bars* indicate data, which were obtained from HUT-102 and MT-2 cell lines in three different culture conditions; conventional liquid culture, co-culture with MS-5, and contact-inhibited condition to MS-5 stromal layer, respectively







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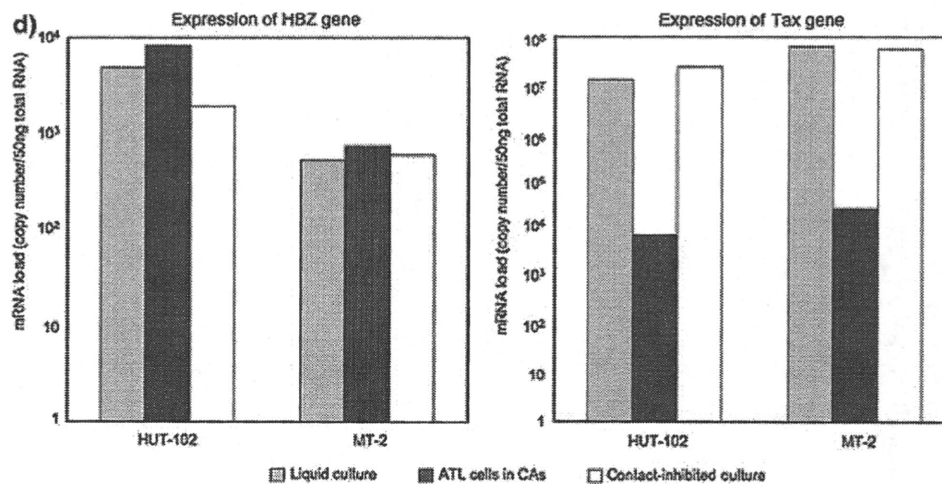

Fig. 3 continued

co-culture system is also considered to provide ATL cells with common factors with in vivo adhesion-dependent growth mechanism of ATL cells.

Although neoplastic cells of lymphoid malignancies have been considered to proliferate primarily in lymphoid tissues, there are so far few cell lines established from LN stroma and available for reproducible analysis of neoplastic cell growth. Since ATL cells frequently invade and proliferate in BM, especially in aggressive phase, this co-culture system is considered to provide components which are necessary for growth and survival of neoplastic cells in BM microenvironment. Surprisingly, we reported previously that in a semi-solid colony assay system, IL-2-dependent colony formation occurred in almost all cases of chronic or smoldering type, but rarely in acute and lymphoma types [55]. These results observed among our limited number of clinical cases indicate that in the prodromal phase of ATL, some cytokines were needed for the growth of neoplastic cells in vitro, while in a more aggressive phase, such a cytokine-dependency declines and some aberrant signal transducers might contribute to a more autonomous proliferation and enhance survival of ATL cells through anti-apoptotic process, which is considered to be closely related to stroma-dependent cell growth.

It has been demonstrated recently in several cancers, especially leukemias, that neoplastic cells are heterogeneous in terms of their capacity to grow and self renew, and that only a small proportion of cells are actually clonogenic in culture and also in vivo [56]. Many recent evidences indicate that microenvironment contributes significantly to tumorigenesis originated from such "cancer stem cells" [57]. In the present study, the leukemic CAFCs exhibited clonogenic potential by showing a linear relationship between inoculated ATL cells and CA numbers as carefully enumerated in the semi-solid collagen gel culture. Furthermore, in four of eight acute-type cases, which gave rise to primary growth, we could observe the secondary CA formation in replating experiments (data not shown). Although it remains controversial whether there exists such a hierarchy in a malignant clone of ATL, our observation supports the hypothesis that there might be a neoplastic stem cell system also in ATL, and future studies on the cellular biology of leukemic CAFCs would shed light on our knowledge of this concept, especially in the context of interaction with the microenvironment of lymphohematopoietic tissue. This is important in that any treatment modality must eradicate such clonogenic cells that are quiescent or proliferating in close contact with stromal cells to obtain a cure.

In the present study, immunostaining and RNA-ISH analysis revealed that our co-culture system allowed the growth of primary ATL cells even with a trace amount of Tax, at the level of transcription and translation. On the other hand, expression of HBZ gene in ATL cells was not affected by our co-culture system. Interestingly, this difference in expression pattern of two genes apparently resembles the behavior of ATL cells in vivo. Together with the result of mRNA load assay in the adhesion blockade system and the finding of reappearance of Tax protein in ATL cell lines which grew upward from the CAs and detached from the MS-5 monolayer into the culture medium (data not shown), it is suggested that there might be a direct interaction between ATL cells and stromal layer as a regulatory mechanism of *tax* gene expression, including epigenetic modification, in this co-culture system. Furthermore, we believe that further investigation of the actions of the *HBZ* gene in this unique in vitro culture system is important to clarify not only regulatory mechanism of *tax* gene expression [58], but also interactions between microenvironment and ATL

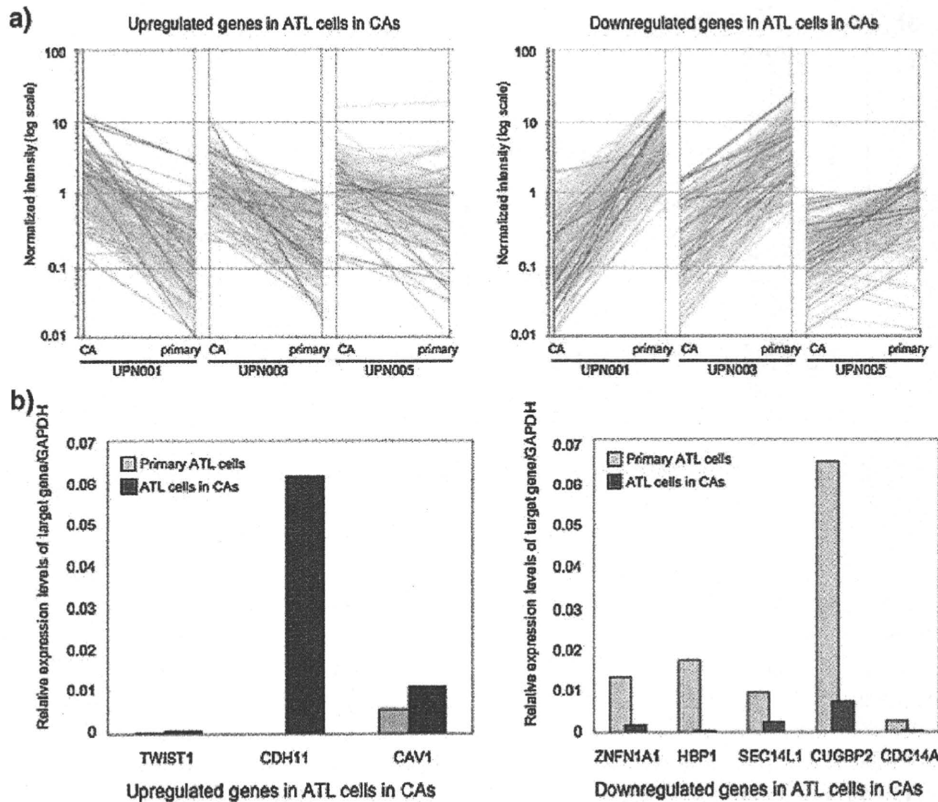

Fig. 4 Comparative studies of gene expression profiles between ATL cells in CA and corresponding primary cells from three clinical samples. For the expression data set of subjects, we first set a condition in which the expression level of a given gene should receive the "Present" call [from Microarray Suite 5.0 (Affymetrix)] in at least 60% (four samples) of the samples, aiming to remove transcriptionally silent genes from the analysis. The mean expression intensity of the internal positive control probe sets (http://www.affymetrix.com/support/technical/mask_files.affx) was set to 500U in each hybridization, and the fluorescence intensity of each test gene was normalized accordingly. **a** Diagrams of comparative profiles of three matched ATL cells in CA and their corresponding $CD4^+$ primary samples from the same individuals (UPN001, UPN003, and UPN005); up-regulated genes (*left*) and down-regulated genes (*right*) in ATL cells in CA. **b** Validation of the expression of eight selected genes from the expression profiles by RTQ-PCR. Primers and probes used in this study are shown in Table 3. Results are expressed in *graphs* of primary cells (*light gray bars*) and those of ATL cells in CA (*dark gray bars*) as fold differences between samples in their target gene expression relative to their levels of GAPDH. Standard curves were generated for each gene with tenfold serial dilution (100×10^{-6}) of cDNA from the KOB cell line and lymphocytes obtained from healthy volunteers. After confirming the precise log-linear relation of the standard curve, the final results were expressed as fold differences between samples in their target gene expression relative to their levels of GAPDH

cells for their growth and survival. This co-culture system is considered to be useful for the analysis of ATL cell growth and survival in a condition without excessive effect of Tax which was always present in the former in vitro culture systems.

Application of transcriptional profiling of ATL cells in CA revealed that there were no significant difference between ATL cells composing CA and primary ATL cells in expression level of several molecules, which play important roles in signaling pathways in ATL, including NFkB pathway. NFkB and related molecules have been shown to be constitutively activated in vivo without expression of Tax [59], although the mechanism of the constitutive activation of NFkB is still unclear. Therefore, several essential signaling pathways could also contribute to the growth of ATL cells under the adhesion-dependent and Tax-independent condition, which apparently resembles in vivo proliferation. On the other hand, interestingly also, we confirmed significant up- and down-regulation of several adhesion molecules and adaptor proteins, which play important roles in signaling pathways through cell-cell or cell-matrix interaction in ATL cells in CA. Taken together, these findings are assumed to indicate that our co-culture system might expose novel molecular mechanisms, which specifically regulate the growth and survival of clonogenic ATL cells. We believe that "outside-in" signaling pathways from interaction with the microenvironment might control such a mechanism in ATL cells. Further investigations are needed to elucidate the precise functions of these molecules.

Recently, a number of novel therapeutic agents to treat neoplastic diseases have been developed, as the growth

Table 3 Genes whose expressions were up- or down-regulated in ATL cells in CA compared with corresponding primary cells as determined by microarray analysis and validated by RTQ-PCR

Gene title	Genbank Accession ID	Description	Primers	Product size	Fold change
Upregulated genes in CAs					
CDH11	D21254	Osteoblast-cadherin	S CTGGAACCATTTTTGTGATT AS TCCACCGAAAAATAGGGTTG	343 bp	32.93
TWIST1	X99268	Twist-related protein 1, H-Twist, Twist homolog 1 (acrocephalosyndactyly 3; Saethre-Chotzen syndrome)	S GTCCGCAGTCTTACGAGGAG AS CCAGCTTGAGGGTCTGAATC	159 bp	5.026
CAV1	AU147399	Caveolin 1, caveolae protein	S CGCACACCAAGGAGATCGA AS GTGTCCCTTCTGGTTCTGCAAT	106 bp	5.301
Downregulated genes in CAs					
CDC14A	NM_003672	CDC14 cell division cycle 14 homolog A	S GCACTTACAATCTCACCATTC AS CATGTTGTAATCCCTTTCTG	58 bp	0.0748
CUGBP2	N36839	CUG triplet repeat, RNA binding protein 2	S CCTTTGAGGACTGCCATTGT AS TGAGGGGGAAAGTCCTTTTT	236 bp	0.0723
SEC14L1	AI017770	SEC14 like 1 protein	S TCCAAGAGGTCGCCACAACCAC AS AGAGACCTGCAGGGACGCAA	288 bp	0.0686
HBP1	AI689935	High mobility group (HMG) box transcription factor 1	S GCTTCCTTTGCAATGGTTCT AS CTGTGCAGCTCACATCTGTATG	243 bp	0.0646
ZNFN1A1	NM_018563	DNA-binding protein Ikaros, Zinc finger protein, subfamily 1A, 1	S CGAGTTCTCGTCGCACATAA AS ATCAAACCCCAATCAACCAA	221 bp	0.0571

Matching primers and fluorescence probes were designed for each of the genes selected by microarray data, employing PRIMER3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) using sequence data from the NCBI database

mechanism of neoplastic cells becomes clearer at the molecular level. We have previously reported that this co-culture system could be used to test the sensitivity of neoplastic progenitors of Philadelphia chromosome (Ph[1])/BCR-ABL-positive leukemia to p210 tyrosine kinase inhibitor, imatinib mesylate (Gleevec™) [18]. This observation provides evidence that our co-culture system is extremely valuable for testing human tumor cells to grow using similar signaling pathways as in vivo. IL-2-dependent T cell lines established from a bulk liquid culture were usually clonally different from primary ATL cells [60], whereas our co-culture system exhibited growth of primary ATL cells which were identical clone to clinical samples as shown in SBH analysis, indicating the superiority of our method for the study of primary ATL cells as a surrogate assay system to predict response of neoplastic progenitors to therapeutic candidate drugs.

5 Conclusion

The present study showed that our newly established ATL cell/murine stroma cell co-culture system is highly efficient for the assay of primary ATL cell growth. Further investigation should focus on the elucidation of novel disease-specific signaling pathways starting from adhesion molecules and extracellular matrix proteins in this co-culture system, which contribute to adhesion-dependent proliferation and survival of ATL cells with a resemblance of expression pattern of proviral genes to that of in vivo ATL cells. This achievement might also be useful in developing novel therapeutic agents targeting ATL progenitors.

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ORIGINAL ARTICLE

Torque teno virus DNA titre elevated in idiopathic pulmonary fibrosis with primary lung cancer

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Torque teno virus DNA titre elevated in idiopathic pulmonary fibrosis with primary lung cancer
BANDO M, TAKAHASHI M, OHNO S, HOSONO T, HIRONAKA M, OKAMOTO H, SUGIYAMA Y. *Respirology* 2008; **13**: 263–269

Background and objective: IPF is an independent risk factor for lung cancer, but the mechanism of this association has not fully been elucidated. The role of Torque teno virus (TTV) in respiratory disease is poorly understood, although it has been shown that infection with TTV is associated with the activity and prognosis of IPF. This study aimed to investigate the prevalence and titre of TTV DNA among patients with IPF and lung cancer.

Methods: The presence of TTV DNA was determined by PCR in the sera of patients with both lung cancer and IPF ($n = 22$), patients with IPF only ($n = 35$), and patients with lung cancer only ($n = 142$).

Results: TTV DNA was detectable in all patients with both IPF and lung cancer, in 94.3% of the patients with IPF only and 97.2% of the patients with lung cancer only. The TTV DNA titre in the patients with IPF and lung cancer was significantly higher than that in the patients with IPF only or lung cancer only. The percentage of TTV-positive patients with a high TTV titre in the IPF and lung cancer group was significantly higher than that in the IPF only group.

Conclusions: These findings are the first report on the association between TTV and the complication of lung cancer in IPF and suggest that TTV infection might be associated with the development of lung cancer in IPF.

Key words: chronic inflammation, IPF, latent virus, primary lung cancer, Torque teno virus.

INTRODUCTION

IPF is a chronic interstitial lung disease with a poor prognosis, the mean survival time after diagnosis being 4–5 years.[1] The main causes of death in IPF patients are respiratory failure, heart failure and lung cancer. IPF is frequently associated with lung cancer which influences the patients' management and prognosis.[2] Aubry *et al.*[3] reviewed the clinical, radiological and pathological findings in patients with primary lung cancer and IPF, and concluded that carcinoma tended to occur in older male smokers and was associated with poor prognosis. IPF is now recognized as an independent risk factor for lung cancer, but the mechanism of this is unclear. There has been increasing evidence for the association of neoplasms with antecedent chronic inflammation. Patients with IPF may have persistent activation of macrophages and lymphocytes,[4,5] which could be a natural reservoir for latent viruses such as cytomegalovirus.[6] Hepatitis C virus (HCV) and Epstein–Barr virus have been implicated in the aetiology of IPF;[7–9] however, these viruses have not been cultured from lung specimens of patients with IPF. In addition, a recent study did not find evidence for an aetiological role of Epstein–Barr virus in the development of lung cancer in IPF patients.[10]

Replicative forms of Torque teno virus (TTV) DNA have been detected in the lung tissue of a viraemic patient with IPF, suggesting an association between TTV infection and IPF.[11] TTV DNA was originally isolated in 1997 from the serum of a patient with post-transfusion hepatitis of unknown aetiology (not A to G), and this virus was named TTV after the initials of

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the first patient in whom it was discovered.[12] TTV is an unenveloped, single-stranded, circular DNA virus with a total genomic length of approximately 3.6–3.9 kb, and it infects human beings worldwide.[13] The ICTV Circoviridae Study Group proposed naming TTV as TTV that is classifiable into a novel genus Anellovirus, unassigned to any family.[14] TTV may replicate in the liver and in bone marrow cells, as circular, double-stranded TTV DNA molecules, which are the replicative intermediate form, and have been detected in these organs.[13] The link between TTV infection and a given pathophysiology remains unproven, although it has been suggested that the viral load is related to the immune status of the host. Recently, Tokita *et al.*[15] demonstrated that a high TTV viral load was independently associated with the complication of hepatocellular carcinoma and may have prognostic significance in patients with HCV-related chronic liver diseases.

The aims of the present study were to investigate the prevalence and titre of TTV DNA in patients with IPF and lung cancer, and to examine the relationship between the extent of TTV viraemia and the complication of primary lung cancer in patients with IPF.

METHODS

Patients

Between January 1991 and December 2003, 22 patients with both IPF and primary lung cancer (21 men; age 67.4 ± 8.6 years (mean ± SD), 46–82 years (range)) were admitted to the Division of Pulmonary Medicine, Jichi Medical University Hospital, and these patients were enrolled in this study. To get as many cases as possible, we searched for all patients fitting the criteria for this group from medical records from 1991, and identified 22 patients as study subjects whose serum was preserved at admission and whose consent was obtained for measurements of serum markers. One patient had two lung cancers. Patients with connective tissue disease, asbestos exposure, previous radiation therapy or metastatic disease in the lung were excluded.

Two control groups were employed. The first control group consisted of 35 patients with histologically documented usual interstitial pneumonia in the clinical setting of IPF without lung cancer (IPF only). The clinical characteristics of this group have been previously published.[11] The second control group consisted of 142 patients with primary lung cancer without evidence of IPF (lung cancer only).

The serum samples used for the measurement of TTV DNA titre were all collected before treatment at the initial admission, and the patients had never received steroids, immunosuppressants or chemotherapeutic agents. The survival status of each patient as of September 2005 was established based on hospital clinical records. The survival period was calculated based on the date of diagnosis of lung cancer. Informed written consent was obtained from each patient. The study of protocols were approved by the Committee for Human Subjects at Jichi Medical University Hospital.

Extraction of nucleic acids from serum

From 50 μL of the serum sample, nucleic acids were extracted using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany), and dissolved in 50 μL of nuclease-free distilled water. Then, nucleic acids were extracted and precipitated with ethanol. DNA species of chromosomal origin, which emerged as a cloudy precipitate immediately after the addition of ethanol, were removed. The remaining nucleic acids were collected by centrifugation and dissolved in 40 μL of nuclease-free distilled water.

Detection and quantitation of TTV DNA

Two different PCR methods (N22 PCR and untranslated region (UTR) PCR) for the detection of TTV DNA were used.[16–18] N22 PCR can detect primarily TTV of genotypes 1–6 which are classifiable in group 1, whereas UTR PCR can detect essentially all 39 TTV genotypes in groups 1–5 including TTV DNA detectable by N22 PCR.[14,19–21] In N22 PCR, nucleic acids extracted from serum were serially diluted 10-fold in distilled water containing 20 μg of glycogen (Roche Diagnostics GmbH) per millilitre, and the highest dilution in which TTV DNA was detectable by the PCR with semi-nested primers was determined. The primers for the first PCR were NG059 (sense: 5′-ACA GAC AGA GGA GAA GGC AAC ATG-3′) and NG063 (antisense: 5′-CTG GCA TTT TAC CAT TTC CAA AGT T-3′). The conditions for the first PCR were 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 45 s and extension at 72 °C for 45 s, followed by a final extension at 72 °C for 7 min. The primers for the second PCR (25 cycles: same conditions as the first PCR) were NG061 (sense: 5′-GGC AAC ATG YTR TGG ATA GAC TGG-3′ (Y = T or C, R = A or G)) and NG063. The first and second PCRs amplified 286-bp and 271-bp DNA fragments, respectively. The amplification products were subject to electrophoresis on a 2.5% NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME, USA), stained with ethidium bromide, and observed under UV light. Based on the results, semi-quantification of TTV DNA in 10^n copies per 10 μL in serum was performed.

UTR PCR was carried out in the presence of Perkin-Elmer AmpliTaq Gold (Roche Molecular Systems) and nested primers by the method described previously,[18] with slight modifications. Briefly, primers NG472 (sense: 5′-GCG TCC CGW GGG CGG GTG CCG-3′ (W = A or T)) and NG351 (antisense: 5′-GAG CCT TGC CCA TRG CCC GGC CAG-3′ (R = A or G)) were used for the first-round PCR, and primers NG473 (sense: 5′-CGG GTG CCG DAG GTG AGT TTA CAC-3′ (D = G, A or T)) and NG351 (antisense: 5′-CCC ATR GCC CGG CCA GTC CCG AGC-3′) were used for the second-round PCR; the primers were derived from the same well-conserved area in the UTR of the TTV genome as in the original method.[18] The size of the amplification

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Table 1 Patients whose serum was positive for Torque teno virus (TTV) DNA detectable by N22 PCR and UTR PCR and serum titres, by clinical group

TTV DNA	IPF only (n=35)	Lung cancer only (n=142)	IPF and lung cancer (n=22)
N22 PCR-positive	13 (37.1%)	56 (39.4%)	9 (40.9%)
$\geq 10^3$ copies/mL	3 (8.6%)	15 (10.6%)	4 (18.2%)
UTR PCR-positive	33 (94.3%)	138 (97.2%)	22 (100%)
$\geq 10^3$ copies/mL	27 (77.1%)*	122 (85.9%)	22 (100%)
UTR PCR titre (mean ± SD) ($\times 10^4$ copies/mL)	2.3 ± 4.2	2.5 ± 4.0	3.3 ± 3.6[†]

*$P < 0.02$ for the comparison between IPF only group and the IPF and lung cancer group.
[†]Significantly higher than in the IPF only group ($P < 0.05$) and lung cancer only group ($P < 0.05$).
UTR, un-translated region.

product from the first-round PCR was 91 bp and that from the second-round PCR was 71 bp. TTV DNA was quantified by real-time PCR (UTR PCR) which can detect essentially all TTV genotypes in groups 1–5, using 10 μL of the nucleic acid solution as a template, primers NG473-NG352, a doubly labelled probe (NG369-P: 5′-(Fam)-AGT CAA GGG GCA ATT CGG GCT CGG GA-(Tamra)-3′), and the LightCycler-Fast-Start DNA Master Hybridization Probes kit (Roche Diagnostics GmbH). PCR amplification was started with an initial denaturation at 95 °C for 10 min, followed by 50 cycles of denaturation at 95 °C for 10 s and annealing-extension at 62 °C for 30 s. All reactions were carried out in the LightCycler (Roche Diagnostics GmbH). The quantification limit of the system was 3–5 copies per test capillary (20 μL of reaction mixture). Intra- and inter-assay reproducibility was determined by testing on 5 different days 10 independent DNA extractions of two reference TTV-positive sera, and the %CV (coefficient of variance based on the value of the crossing point on the LightCycler) was 0.7 and 1.8, respectively. The overall variation was less than 0.53 log.

Statistical analyses

The results are presented as mean ± SD. The frequency between groups was compared using the Mann–Whitney U-test or chi-squared test. The survival rate between groups was compared by the log rank test.

RESULTS

Percentage of patients whose serum was positive for TTV DNA

The percentage of patients in each group whose serum was positive for TTV DNA by N22 PCR or UTR PCR is shown in Table 1. Among the 22 patients with IPF and lung cancer, nine (40.9%) were positive for TTV DNA detectable by N22 PCR, and all patients were positive for TTV DNA detectable by UTR PCR. Among the patients with IPF only or lung cancer only, the TTV DNA-positive rates using N22 PCR were 37.1% and 39.4%, respectively, and the TTV DNA-positive rates using UTR PCR were 94.3% and 97.2%, respectively. The percentage of UTR PCR-positive patients with a high TTV titre ($\geq 10^3$ copies/mL) in the IPF and lung cancer group was significantly higher than that in the IPF only group ($P < 0.02$). The TTV DNA titre by UTR PCR in the patients with IPF and lung cancer ($(3.3 \pm 3.6) \times 10^4$ copies/mL) was significantly higher than those in the patients with IPF only ($(2.3 \pm 4.2) \times 10^4$ copies/mL, $P < 0.05$) or lung cancer only ($(2.5 \pm 4.0) \times 10^4$ copies/mL, $P < 0.05$).

Demographic and clinical features of patients with IPF complicated with lung cancer

The demographic features and smoking habits of the patients in the three groups were compared (Table 2). The male : female ratio was 21:1 among the patients with IPF and lung cancer and 22:13 among the patients with IPF only, showing a significant difference ($P < 0.01$). The prevalence of smoking in patients with IPF and lung cancer was significantly higher than that in patients with IPF only ($P < 0.01$), and the smoking index (pack-years) was significantly higher among the patients with IPF and lung cancer than among the patients with IPF only ($P < 0.05$) (Table 2). There was no significant difference in age among the three groups.

Among the 23 cancers in the 22 patients with IPF complicated with lung cancer (including double cancer in one patient), 21 cancers (91.3%) were in peripheral lung fields and 65.2% of the cancers were in the non-fibrotic areas. The tumours consisted of adenocarcinomas ($n = 7$), squamous cell carcinomas ($n = 7$), large cell carcinomas ($n = 4$) and small cell carcinomas ($n = 3$). There were no significant differences in the TTV DNA titres by UTR PCR between the four histology groups (Table 3). The clinical stage IV was the most frequent stage, followed by stage IIIA, stage I and stage IIIB. There were also no significant differences in the TTV DNA titres among the clinical stages (Table 3). Smoking index (pack-years) was not correlated with the TTV DNA titre detectable by UTR PCR among the 22 patients (Fig. 1). Among the

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Table 2 Comparison of the sociodemographic characteristics of patients, by clinical group

Feature	IPF only (*n*=35)	Lung cancer only (*n*=142)	IPF and lung cancer (*n*=22)
Gender (male/female)	22/13*	104/38†	21/1
Age (years), mean ± SD (range)	65.1 ± 9.9 (44–81)	65.0 ± 9.9 (33–85)	67.4 ± 8.6 (46–82)
Smoking history			
Smokers (%)	60.0‡§	78.2	95.5
Pack-years, mean ± SD	30.3 ± 35.2¶	42.0 ± 34.6	62.4 ± 40.7

*$P < 0.01$ for the comparison between the IPF only group and the IPF and lung cancer group.
†$P < 0.03$ for the comparison between the lung cancer only group and the IPF and lung cancer group.
‡$P < 0.01$ for the comparison between the IPF only group and the IPF and lung cancer group.
§$P < 0.03$ for the comparison between the IPF only group and the lung cancer only group.
¶$P < 0.05$ for the comparison between the IPF only group and the IPF and lung cancer group.

Table 3 Torque teno virus (TTV) DNA titres of the patients with IPF complicated with lung cancer, by tumour cell type and stage

	n	TTV UTR PCR titre ($\times10^4$ copies/mL)
Histology		
Adenocarcinoma	7	1.7 ± 1.7
Squamous cell carcinoma	7	2.9 ± 3.0
Small cell carcinoma	3	4.6 ± 6.5
Large cell carcinoma	4	5.6 ± 4.7
Other	1	3.7
Stage		
I	4	4.5 ± 2.9
II	2	0.6
IIIA	5	4.2 ± 4.9
IIIB	3	2.0 ± 1.8
IV	8	3.2 ± 4.0

The results are presented as mean ± SD.
UTR, un-translated region.

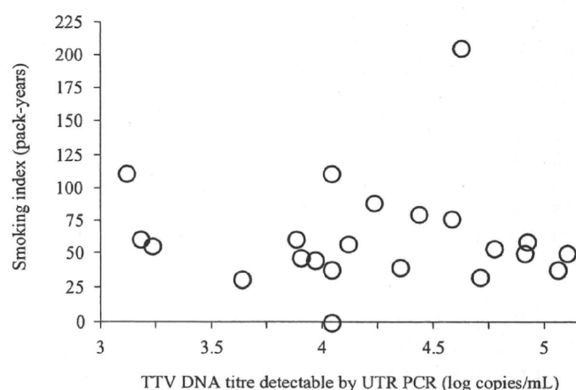

Figure 1 Correlation between the smoking index and TTV DNA titre among patients with IPF complicated with lung cancer. There was no correlation between the smoking index and the TTV DNA titre detectable by UTR PCR ($n = 22$; $P = 0.96$, $R = -0.012$). TTV, Torque teno virus; UTR, un-translated region.

patients with IPF and lung cancer, mortality was mainly due to progression of the lung cancer and not the IPF. Only one patient with IPF and lung cancer, who had undergone surgery, died of acute exacerbation of IPF. The 5-year survival rate was 38.3% and the median survival period was 16.2 months.

DISCUSSION

This study has shown that the TTV DNA titre detectable by UTR PCR in patients with IPF and lung cancer was significantly higher than that in patients with IPF only or lung cancer only. In addition, the percentage of UTR PCR-positive patients with a high TTV titre ($\geq10^3$ copies/mL) in the IPF and lung cancer group was significantly higher than that in the IPF only group. TTV is a newly discovered human virus composed of a circular, single-stranded DNA of approximately 3.6–3.9 kb,[12,13] and it most closely resembles a member (chicken anaemia virus) of the Gyrovirus genus in the Circoviridae family,[22] but it has recently been classified into a novel genus Anellovirus, unassigned to any family.[14,23] TTV is widely distributed in human populations throughout the world and produces chronic viraemia in around 90% of healthy individuals of all ages.[24] Itoh *et al.*[25] demonstrated that TTV DNA was detected by UTR PCR at a high frequency (≥93%) in all age groups. However, it is unknown whether these higher frequencies of PCR positivity are also associated with higher circulatory virus loads in plasma. The natural history and pathogenic potential of TTV are currently under intensive investigation. Biagini *et al.*[26] reported a newborn with primary infection of TTV and clinical symptoms of benign viral rhinitis. Maggi *et al.*[27] demonstrated that the average TTV load was considerably higher in children with bronchopneumonia than in children with milder acute respiratory disease. Recently, Pifferi *et al.* reported that TTV might contribute to the pathogenesis of asthma in children.[28] Infection with TTV is characterized by persistent lifelong viraemia. Infection with TTV has been shown to influence the activity and prognosis of IPF.[11] However, the pathophysiology of TTV in the respiratory tract of infected humans has

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not yet been defined. TTV may replicate in the liver and in bone marrow cells, as circular, double-stranded TTV DNA has been detected in these organs. TTV DNA has also been detected in both frozen normal lung tissue and in cancer tissue obtained by thoracoscopy from a single lung cancer patient with TTV DNA in the serum.

There are two possible explanations for the findings of the present study. One explanation is that an impaired or suppressed immune system is involved in the TTV viral load in patients with IPF complicated with lung cancer. Maggi *et al.*[29] demonstrated that TTV loads were negatively related to the percentages of circulating $CD3^+$ and $CD4^+$ T cells and were positively related to the percentage of circulating B cells, suggesting that TTV might contribute to lymphocyte imbalances and immunosuppressive effects. Furthermore, it has been reported that the TTV viral load is inversely correlated with the CD4 T-cell count among patients infected with HIV type 1,[20,30] and that it may reflect the immune status of these immunocompromised hosts. A possible relationship between an elevated TTV viral load and the level of immunocompetence of the populations studied among TTV-infected patients on maintenance haemodialysis or with diabetes mellitus has also been suggested.[31] Therefore, it is likely that an impaired or suppressed immune system is associated with the elevated TTV viral load in IPF patients with lung cancer. A second explanation is that the high TTV viraemia influences the progression of IPF and promotes the development of lung cancer. Although the precise mechanism for the increased incidence of lung cancer among IPF patients remains unclear, it seems probable that the chronic inflammatory process resulting in remodelling of the lung is an important factor in the development of lung cancer in IPF patients who are also heavy smokers. There has been increasing evidence for the association of neoplasms with antecedent chronic inflammation; for example, gastric cancer in patients with *Helicobactor pylori* infection, malignant lymphoma secondary to chronic pyothorax or colon cancer in patients with ulcerative colitis.[32,33] Recently, Tokita *et al.*[15] demonstrated that a high TTV viral load was independently associated with the development of hepatocellular carcinoma, and may have prognostic significance in patients with HCV-related chronic liver diseases. Although the underlying mechanism by which high TTV viraemia increases the risk for HCC among patients with HCV-related chronic liver disease remains unknown, Moriyama *et al.*[34] reported that the score for irregular regeneration of hepatocytes in TTV-infected cirrhotic patients with chronic hepatitis C was higher than that in non-viraemic patients, suggesting that TTV infection may influence the development of HCV-related HCC. The potential involvement of TTV in the development of lung cancer associated with IPF and its influence on the airway and alveolar epithelial cells can be studied using bio-molecular techniques.

In the present study, the smoking index was significantly higher in patients with IPF and lung cancer than in patients with IPF alone. In addition, the smoking index was not correlated with the TTV DNA titre. These results support the hypothesis that smoking is a predictive factor for cancer in IPF rather than TTV. Turner-Warwick *et al.*[35] first reported the importance of smoking habit as a risk factor for lung cancer among patients with usual interstitial pneumonia using detailed statistical analyses. Several studies have also found an association between cigarette smoking and IPF combined with lung cancer. Nagai *et al.*[36] reported that the relative risk of cigarette smoking and lung cancer development was approximately 3.5 among patients with IPF. Matsushita *et al.*[37] reported that patients with lung cancer and IPF showed a significant predominance of smokers and a significant increase in the smoking index compared with the lung cancer cases without IPF. Smoking, especially heavy smoking, is one of the most important risk factors in the development of lung cancer in patients with IPF, and IPF and smoking may serve as cofactors in the development of lung cancer. On the other hand, smoking itself was associated with an increased risk of IPF in case–control studies.[38–40] Conversely, Hubbard *et al.*[40] provided evidence that the increased prevalence of lung cancer in patients with IPF was independent of the effect of cigarette smoking in a population-based cohort study. To further explore the role of cigarette smoking in the development of lung cancer in patients with IPF, data on the increased lung cancer risk associated with IPF in smokers and non-smokers are needed.

In conclusion, high TTV viraemia was significantly associated with IPF complicated with lung cancer in our study. However, it is not clear whether high TTV viraemia plays a role in the development of IPF and lung cancer; whether it is a cofactor in the progression of lung fibrosis; whether it is a serological marker reflecting the occurrence of lung cancer in IPF patients, or whether it reflects the host's immune system. Prospective studies need to be conducted to elucidate whether a high TTV viral load is associated with the development of lung cancer and whether it has clinical significance in predicting the outcome of patients with IPF complicated with lung cancer. In this context, future studies on the role of high TTV viral loads in patients with respiratory diseases of unknown aetiology are also warranted.

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Accepted Abbreviations for *Respirology*

Abbreviation	Full Name	Units
6MWD	6 minute walk distance	m
A-a O_2 gradient	alveolar-arterial oxygen gradient	
AHI	apnoea/hypopnoea index	
AIDS	acquired immune deficiency syndrome	
ARDS	acute respiratory distress syndrome	
BAL	bronchoalveolar lavage	
bd	twice daily	
BHR	bronchial hyperresponsiveness	
BMI	body mass index	kg/m^2
BSA	bovine serum albumin	
cAMP	cyclic AMP	
cDNA	complementary DNA	
CPAP	continuous positive airway pressure	
CRP	C-reactive protein	mg/L
COPD	chronic obstructive pulmonary disease	
CT	computed tomography	
CXR	chest X-ray	
d	day	
DL_{CO}	diffusing capacity of carbon monoxide	mL/min/mm Hg
DNA	deoxyribonucleic acid	
ECG	electrocardiogram	
ELISA	enzyme-linked immunosorbent assay	
ESR	erythrocyte sedimentation rate	mm/h
FACS	fluorescence-activated cell sorter	
$FEF_{25-75\%}$	forced mid-expiratory flow	L/s
FEV_1	forced expiratory volume in 1 second	L
FEV_1%	percent of predicted forced expiratory volume in 1 second	
FEV_1%FVC	FEV_1 as percentage of forced vital capacity	%
FRC	functional residual capacity (method of measurement to be specified)	L
FVC	forced vital capacity	L
FVC%	percent of predicted forced vital capacity	
h	hour	
Hb	haemoglobin	g/L
HIV	human immunodeficiency virus	
HPLC	high performance liquid chromatography	
Hz	hertz	
Ig	immunoglobulin	
IL	interleukin	
IPF	idiopathic pulmonary fibrosis	
IU	international unit	
i.v.	intravenous	
kg	kilogram	
KPa	kilopascals	
L	litre	
LDH	lactate dehydrogenase	
LPS	lipopolysaccharide	
m	metre	
m^2	square metre	
mAb	monoclonal antibody	
MHC	major histocompatibility complex	
min	minute	
mm	millimetre	
mm Hg	millimetre of mercury	
MRI	magnetic resonance imaging	
mRNA	messenger RNA	
MW	molecular weight	
n	number in study group	
°C	degree Celsius	
OSA	obstructive sleep apnoea	
P	probability	
PaO_2	partial pressure of arterial oxygen	mm Hg
$PaCO_2$	partial pressure of arterial carbon dioxide	mm Hg
PBS	phosphate buffered saline	
PC_{20}	provocation concentration of a bronchoconstrictor agonist causing a 20% fall in FEV_1	
PCR	polymerase chain reaction	
PD_{20}	provocation dose of a bronchoconstrictor agonist causing a 20% fall in FEV_1	
PEEP	positive end expiratory pressure	kPa
PEF	peak expiratory flow	L/min
PET	positron emission tomography	
PET FDG	positron emission tomography with fluorodeoxyglucose	
RCC	red cell count	$\times 10^9$/L
RNA	ribonucleic acid	
RV	residual volume (method should be specified)	L
s	second	
SaO_2	arterial oxygen saturation	%
SD	standard deviation	
SEM	standard error of the mean	
SPECT	single photon emission computed tomography	
$T_{1/2}$	half life	
tds	thrice daily	
TLC	total lung capacity (method should be specified)	L
UV	ultraviolet	
V_A	alveolar volume	L
VATS	video-assisted thoracoscopic surgery	
V/Q	ventilation perfusion	
VC	vital capacity	L
WCC	white cell count	$\times 10^9$/L
μg	microgram	

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Identification of Novel Isoforms of the *EML4-ALK* Transforming Gene in Non–Small Cell Lung Cancer

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Abstract

The genome of a subset of non–small-cell lung cancers (NSCLC) harbors a small inversion within chromosome 2 that gives rise to a transforming fusion gene, *EML4-ALK*, which encodes an activated protein tyrosine kinase. Although breakpoints within *EML4* have been identified in introns 13 and 20, giving rise to variants 1 and 2, respectively, of *EML4-ALK*, it has remained unclear whether other isoforms of the fusion gene are present in NSCLC cells. We have now screened NSCLC specimens for other in-frame fusion cDNAs that contain both *EML4* and *ALK* sequences. Two slightly different fusion cDNAs in which exon 6 of *EML4* was joined to exon 20 of *ALK* were each identified in two individuals of the cohort. Whereas one cDNA contained only exons 1 to 6 of *EML4* (variant 3a), the other also contained an additional 33-bp sequence derived from intron 6 of *EML4* (variant 3b). The protein encoded by the latter cDNA thus contained an insertion of 11 amino acids between the EML4 and ALK sequences of that encoded by the former. Both variants 3a and 3b of EML4-ALK exhibited marked transforming activity *in vitro* as well as oncogenic activity *in vivo*. A lung cancer cell line expressing endogenous variant 3 of EML4-ALK underwent cell death on exposure to a specific inhibitor of ALK catalytic activity. These data increase the frequency of *EML4-ALK*–positive NSCLC tumors and bolster the clinical relevance of this oncogenic kinase. [Cancer Res 2008;68(13):4971–6]

Introduction

Lung cancer is the leading cause of cancer deaths in the United States, with >160,000 individuals dying of this condition in 2006 (1). The efficacy of conventional chemotherapeutic regimens with regard to improving clinical outcome in lung cancer patients is limited. Activating mutations within the epidermal growth factor receptor gene (*EGFR*) have been identified in non–small-cell lung cancer (NSCLC), the major subtype of lung cancer (2, 3), and chemical inhibitors of the kinase activity of EGFR have been found to be effective in the treatment of a subset of NSCLC patients harboring such mutations. However, these somatic mutations of *EGFR* are prevalent only among young women, nonsmokers, and Asian populations (3, 4).

We recently identified a novel transforming fusion gene, *EML4* (echinoderm microtubule–associated protein–like 4)-*ALK* (anaplastic lymphoma kinase), in a clinical specimen of lung adenocarcinoma from a 62-year-old male smoker (5). This fusion gene was formed as the result of a small inversion within the short arm of chromosome 2 that joined intron 13 of *EML4* to intron 19 of *ALK* (transcript ID ENST00000389048 in the Ensembl database[5]). The EML4-ALK protein thus contained the amino-terminal half of EML4 and the intracellular catalytic domain of ALK. Replacement of the extracellular and transmembrane domains of ALK with this region of EML4 results in constitutive dimerization of the kinase domain of ALK and a consequent increase in its catalytic activity (5).

Whereas this *EML4-ALK* fusion gene was detected in 3 of 75 individuals with NSCLC, we further identified another isoform of *EML4-ALK* in two patients of the same cohort (5). In these two individuals, intron 20 of *EML4* was disrupted and joined to intron 19 of *ALK*, with the fusion protein thus consisting of the amino-terminal two thirds of EML4 and the intracellular domain of ALK. This larger version of EML4-ALK was referred to as variant 2, with the original smaller version being termed variant 1. A total of 5 of the 75 (6.7%) patients in the cohort were thus positive for *EML4-ALK*.

Given that detection of *EML4-ALK* cDNA by the PCR would be expected to provide a highly sensitive means for diagnosis of lung cancer, and given that inhibition of the catalytic activity of EML4-ALK may be an effective approach to treatment of this disorder, we have examined whether other isoforms of EML4-ALK are associated with NSCLC. We now describe a third isoform of EML4-ALK (variant 3) that is smaller than variants 1 and 2.

Materials and Methods

PCR. This study was approved by the ethics committees of Jichi Medical University and The Cancer Institute of the Japanese Foundation for Cancer Research. Total cDNA of NSCLC specimens was synthesized with PowerScript reverse transcriptase (Clontech) and an oligo(dT) primer from total RNA purified with the use of an RNeasy Mini RNA purification kit (Qiagen). Reverse transcription-PCR (RT-PCR) to amplify the fusion point of *EML4-ALK* variant 3 mRNA was done with a QuantiTect SYBR Green kit (Qiagen) and the primers 5′-TACCAGTGCTGTCTCAATTGCAGG-3′ and 5′-TCTTGCCAGCAAAGCAGTAGTTGG-3′. A full-length cDNA for *EML4-ALK*

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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[5] http://www.ensembl.org/index.html

variant 3 was amplified from total cDNA of a NSCLC specimen (ID no. 2075) with PrimeSTAR HS DNA polymerase (Takara Bio) and the primers 5′-ACTCTGTCGGTCCGCTGAATGAAG-3′ and 5′-CCACGGTCTTAGG-GATCCCAAGG-3′; PCR was done for 35 cycles of 98°C for 10 s and 68°C for 6 min. The fusion point of *EML4-ALK* in the genome was amplified by PCR with genomic DNA of NSCLC specimens, PrimeSTAR HS DNA polymerase, and the primers 5′-GGCATAAAGATGTCATCATCAAC-CAAGG-3′ and 5′-AGCTTGCTCAGCTTGTACTCAGGG-3′. The nucleotide sequences of the *EML4-ALK* variant 3a and 3b cDNAs have been deposited in DDBJ/EMBL/GenBank under accession nos. AB374361 and AB374362, respectively.

Fluorescence *in situ* hybridization. Fluorescence *in situ* hybridization (FISH) analysis of the fusion gene was done with archival pathology specimens and with bacterial artificial chromosomes containing genomic DNA corresponding to *EML4* or *ALK* and their flanking regions as probes. In brief, surgically removed lung cancer tissue was fixed in 20% neutral buffered formalin, embedded in paraffin, and sectioned at a thickness of 3 μm. The sections were placed on glass slides and processed with a Histology FISH Accessory Kit (DakoCytomation) before hybridization with the *EML4* and *ALK* probes and examination with a fluorescence microscope (BX61, Olympus).

Transforming activity of EML4-ALK variant 3. Analyses of the function of EML4-ALK variant 3 were done as described previously (5). In brief, the cDNA for EML4-ALK variant 3a or 3b was fused with an oligonucleotide encoding the FLAG epitope tag and then inserted into the retroviral expression plasmid pMXS (6). The resulting plasmids as well as similar pMXS-based expression plasmids for EML4-ALK variant 1, variant 1 (K589M), or variant 2 were individually introduced into mouse 3T3 fibroblasts by the calcium phosphate method for a focus formation assay and assay of tumorigenicity in nu/nu mice. The same set of EML4-ALK proteins was expressed in HEK293 cells and assayed for kinase activity *in vitro* with the YFF peptide (7).

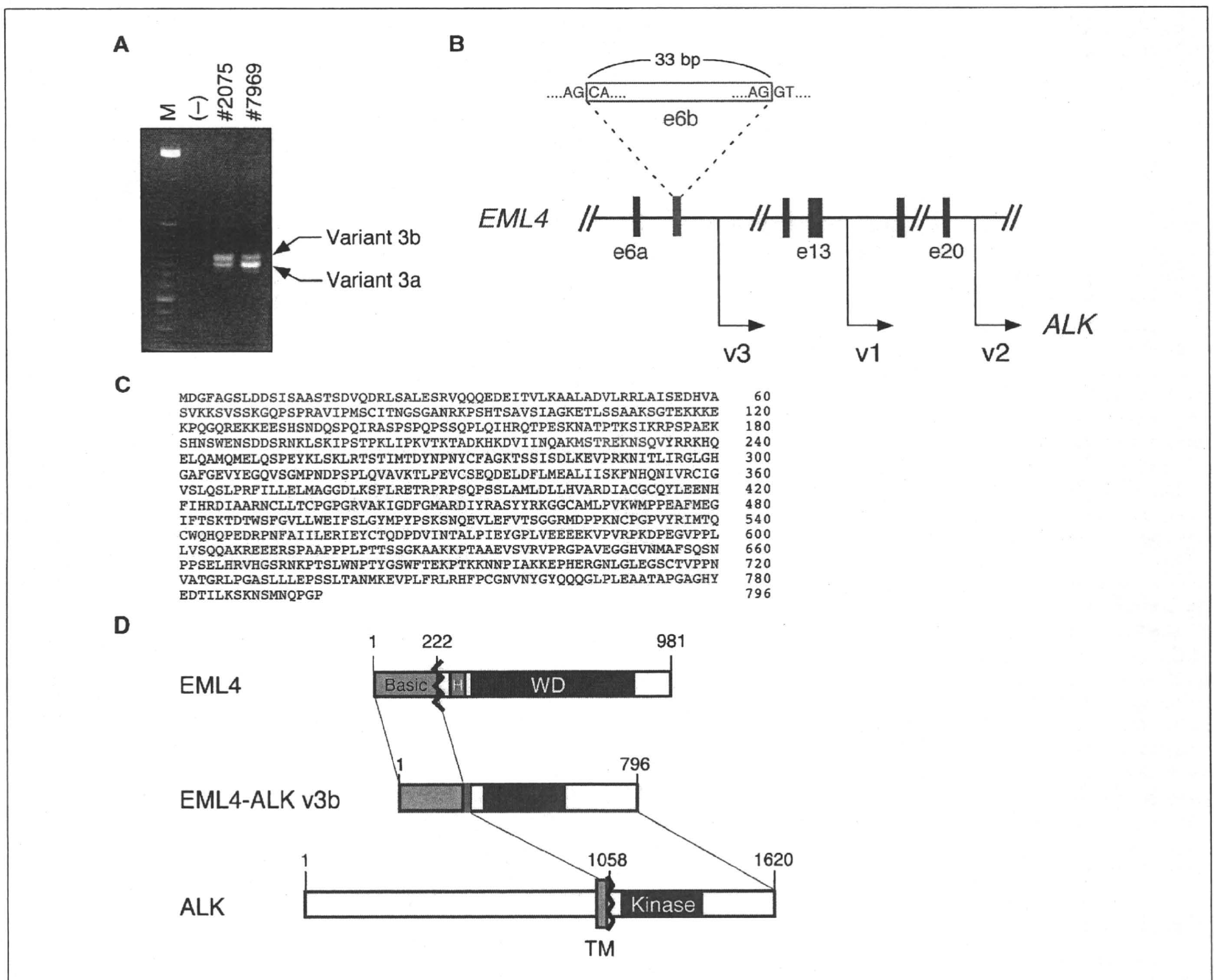

Figure 1. Identification of *EML4-ALK* variant 3. *A,* detection of fusion cDNAs linking exon 6 of *EML4* to exon 20 of *ALK* by RT-PCR analysis. Two RT-PCR products of 548 bp (corresponding to variant 3b) and 515 bp (corresponding to variant 3a) were detected by agarose gel electrophoresis with total RNA from two NSCLC specimens (tumor ID nos. 2075 and 7969). *Lane (–),* no-template control; *lane M,* size markers (50-bp ladder). *B,* genomic organization of *EML4*. Intronic sequences downstream of exons (*e*) 6, 13, and 20 of *EML4* are fused to intron 19 of *ALK* to generate variants (*v*) 3, 1, and 2 of *EML4-ALK*, respectively. Exon-intron boundary sequences as well as the size of exon 6b are indicated. *C,* predicted amino acid sequence of EML4-ALK variant 3b. *Blue, green,* and *red,* amino acids corresponding to exons 1 to 6a of *EML4*, exon 6b of *EML4*, and *ALK*, respectively. Amino acid number is indicated on the right. *D,* fusion of an amino-terminal portion of EML4 [which consists of a basic region (*Basic*), HELP domain (*H*), and WD repeats] to the intracellular region of ALK (containing the tyrosine kinase domain) generates EML4-ALK variant 3b. *Green,* the region of the fusion protein encoded by exon 6b of *EML4*. *TM,* transmembrane domain.