

in our previous NSCLC cohort (8, 13), suggesting that EML4-ALK-positive NSCLC is a distinct subclass of lung cancer. Given that a selective inhibitor of the kinase activity of ALK rapidly induces cell death in EML4-ALK-positive cancer cells both *in vitro* (8, 9) and *in vivo*,⁸ determination of the presence or absence of EML4-ALK in a given tumor may in the future inform the choice of treatment strategy for NSCLC. The demonstration of the existence of multiple isoforms of EML4-ALK transcripts will necessitate optimization of the detection systems so that all isoforms are detected with a high accuracy and sensitivity.

⁸ M. Soda et al., submitted for publication.

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Note Added in Proof

During our revision process, a novel EML4-ALK fusion variant was reported by Koivunen et al. (*Clin Cancer Res* 2008;14:4275–83). They have designated it as variant 4, which is different from our variant 4 in the present study.

Disclosure of Potential Conflicts of Interest

K. Takeuchi is a consultant providing advisory services to Dako for their antibodies.

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EML4-ALK Fusion Is Linked to Histological Characteristics in a Subset of Lung Cancers

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Introduction: Very recently, we have found a novel fusion product between the echinoderm microtubule-associated protein-like4 (EML4) and the anaplastic lymphoma kinase (ALK) in non-small cell lung cancers (NSCLCs). Tumors featuring EML4-ALK fusion constitute one subtype of NSCLC that might be highly sensitive to ALK inhibitors. Herein, we present results of a first large scale study of EML4-ALK fusion in lung cancers.

Methods: Using reverse transcription-polymerase chain reaction for EML4-ALK fusion mRNA, we investigated 149 lung adenocarcinomas, 48 squamous cell carcinomas, 3 large-cell neuroendocrine carcinomas, and 21 small-cell carcinomas. For EML4-ALK-positive cancers, we further investigated the presence of ALK fusion proteins by immunohistochemistry.

Results: Five of 149 adenocarcinomas (3.4%) showed EML4-ALK fusion mRNA, this being totally lacking in carcinomas of other types (0/72). In all the fusion-positive cases, ALK fusion protein could be detected in the cytoplasm immunohistochemically. The five fusion cases featured two EML4-ALK variant 1 fusions and three variant 2 fusions. Histologically, both variant 1 cases were mixed type adenocarcinomas, showing papillary with bronchioloalveolar components. Interestingly, all three variant 2 cases were acinar adenocarcinomas, the link being statistically significant ($p = 0.00018$). None of the five fusion-positive cases demonstrated any mutations of EGFR or KRAS, pointing to a mutually exclusive relationship ($p = 0.014$). There was no association with smoking habits.

Conclusions: In the present first investigation of EML4-ALK fusion in a large study of lung cancers (5/221), we found an interesting histotype-genotype relationship. Furthermore, we could detect the fusion protein by immunohistochemistry, pointing to possible clinical applications.

Keywords: Lung cancer, EML4-ALK, RT-PCR, Immunohistochemistry, Histology.

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Lung cancer is the leading cause of cancer death in men and women worldwide. Identification of activating mutations of the epidermal growth factor receptor (EGFR) is one of the most intriguing recent discoveries in the field of lung cancer research.^{1,2} EGFR mutations are present in one subtype of lung adenocarcinoma, and tumors with this mutation have been shown to be highly sensitive to gefitinib (Iressa). The subtype is prevalent in women, and in patients of Japanese and other Asian ethnicity, especially in nonsmokers.^{1,3,4} With the move to personalized cancer therapy, we need to understand oncologic biology at the molecular level in individual lesions to be able to treat cancers with specific molecular-targeting strategies.

Very recently, we have found a novel transforming fusion gene resulting from linkage between the echinoderm microtubule-associated protein-like4 (EML4) and the anaplastic lymphoma kinase (ALK) genes in non-small cell lung cancers (NSCLCs).⁵ Tumors featuring EML4-ALK fusion constitute one subtype of NSCLC which might be highly sensitive to ALK inhibitors. The fusion gene is formed by a small inversion within chromosome 2p. EML4 on chromosome 2p21 belongs to the family of echinoderm microtubule-associated protein-like proteins, localized in the cytoplasm, and is necessary for correct microtubule formation.^{6,7} ALK on chromosome 2p23 codes for a receptor tyrosine kinase and was first identified as a fusion partner of nucleophosmin (NPM) in anaplastic large-cell lymphomas (ALCLs) with a t(2;5) chromosome rearrangement.^{8,9} NPM is an RNA-binding protein that transports ribonucleoproteins between the nucleus and cytoplasm and contributes a nuclear localization signal to the NPM-ALK fusion protein.¹⁰ Other chromosome translocations involving the ALK locus have been identified in ALCLs^{11,12} as well as in inflammatory myofibroblastic tumors (IMTs).¹³ The fusion point of ALK is conserved among all these chimeric tyrosine kinases including EML4-ALK, resulting in fusion of the entire intracellular kinase domain of ALK to the different partners.¹⁴

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Disclosure: The authors declare no conflict of interest.

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Herein, we present a first large scale study of EML4-ALK fusion in lung cancers, including SCLCs. Furthermore, we detail clinicopathologic and genetic features of fusion-positive lung cancers.

PATIENTS AND METHODS

Clinical Samples and RNA Extraction

This study was conducted with clinical samples from 149 lung adenocarcinomas, 48 squamous cell carcinomas, 3 large cell neuroendocrine carcinomas, and 21 SCLCs. Many of these samples were previously examined and reported.¹⁵⁻²¹ For example, most adenocarcinomas were examined as to their mRNA levels of PTEN¹⁹ or HOXB2,²¹ and some adenocarcinomas were examined as to their let-7 microRNA levels.²⁰ All were collected with ethical committee approval and informed consent from patients undergoing surgery at the Cancer Institute Hospital, Tokyo, Japan, between May 1995 and August 2004. Histologic diagnosis was according to World Health Organization classifications²² as well as to differentiation-grading criteria for adenocarcinomas of the Japanese Lung Cancer Society.^{23,24} All lesions were grossly dissected and snap-frozen in liquid nitrogen within 20 minutes of removal and stored at -80°C until total RNA extraction and purification using an RNeasy Mini Kit (QIAGEN, Valencia, CA). RNA quality and absence of genomic DNA contamination were checked by formaldehyde agarose gel electrophoresis.

Reverse Transcription-Polymerase Chain Reaction and Sequencing Analysis

Total RNAs were reverse transcribed with random primers and SuperScript III reverse transcription (Invitrogen, Carlsbad, CA). To detect fusion transcripts derived from EML4 and ALK, reverse transcription-polymerase chain reaction (RT-PCR) experiments were carried out with primers Fusion-RT-S (5'-GTGCAGTGTTTAGCATTCTTGGGG-3') and Fusion-RT-AS (5'-TCTTGCCAGCAAAGCAGTAGTTGG-3'). We used PCR primers 5'-GTCAGTGGTGGACCTGACCT-3' and 5'-TGAGCTTGACAAAGTGGTCG for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. For PCR of the fusion transcripts, after initial denaturation at 94°C for 10 minutes, 32 cycles each consisting of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and strand elongation at 72°C for 1 minute were performed, followed by a final elongation at 72°C for 10 minutes. For GAPDH, amplification was performed for 35 cycles with denaturation for 1 minute at 94°C , primer annealing for 30 seconds at 58°C , and elongation for 30 seconds at 72°C . PCR was performed using AmpliTaq Gold (Applied Biosystems, Foster City, CA) and amplified fragments were subjected to direct sequence analysis.

Immunohistochemical Analysis

A representative tissue block from each lesion was selected, and $4\text{-}\mu\text{m}$ tissue sections were routinely deparaffinized in xylene and rehydrated through graded ethanols. Immunohistochemical staining was performed using the EnVision + DAB system (DAKO, Carpinteria, CA) and a mouse monoclonal anti-ALK antibody (ALK1, DAKO, 1:20).

DNA Extraction and Mutation Analysis of EGFR and KRAS

Of 149 patients with adenocarcinomas, both EGFR and KRAS data were available for 62 and EGFR data alone for a further 12. DNA extraction and mutation analysis of EGFR and KRAS were performed as described previously.¹⁹

Analysis of Clinicopathologic Parameters

Survival data were analyzed by the log-rank test using cancer death-specific survival data. We analyzed statistical correlations for the other clinicopathologic features using the Student *t* test, Fisher exact test, or χ^2 test as appropriate. The two-sided significance level was set at $p < 0.05$.

RESULTS

Using RT-PCR for EML4-ALK fusion mRNA, we investigated the presence of the EML4-ALK translocation in 221 lung cancers (Table 1). Five of 149 adenocarcinomas (3.4%) featured EML4-ALK fusion mRNA, whereas other types of carcinoma were all negative (0/72) (Figure 1). Of the five fusion-positive cases, two had EML4-ALK variant 1 and three had variant 2.⁵ The fusions were confirmed by direct sequencing.

Histologically, both the variant 1 cases were mixed type adenocarcinomas, papillary with bronchioloalveolar components (Figure 2A). Interestingly, all three variant 2 cases were acinar adenocarcinomas, moderately or poorly differentiated (Figure 2B). The link between variant 2 and acinar morphology was statistically significant ($p = 0.00018$, Fisher exact test).

Immunohistochemically, all the five fusion-positive cases showed ALK fusion protein in the cytoplasm (Figure 3) in line with the absence of any nuclear localization signal in the EML4 gene. We cannot rule out the possibility of detecting endogenous ALK protein.

Table 2 summarizes details for clinicopathologic and genetic features of the fusion-positive lung cancers. Genetically, all lacked mutations of EGFR or KRAS ($p = 0.014$ as

TABLE 1. EML4-ALK Fusion and Histology

Histology	Total	EML4-ALK(+)	EML4-ALK(-)
Adenocarcinoma	149	5 (3.4%)	144 (97%)
Subtype			
Adenocarcinoma with mixed subtype	89	2 ^a (2.3%)	87 (98%)
Papillary adenocarcinoma	35	0 (0%)	35 (100%)
Acinar adenocarcinoma	18	3 ^b (17%)	15 (83%)
Solid adenocarcinoma with mucin	4	0 (0%)	4 (100%)
Bronchioloalveolar carcinoma	3	0 (0%)	3 (100%)
Squamous cell carcinoma	48	0 (0%)	48 (100%)
Large cell neuroendocrine carcinoma	3	0 (0%)	3 (100%)
Small cell carcinoma	21	0 (0%)	21 (100%)

^a Variant 1; Fisher exact test, $p = 0.66$ (Adenocarcinoma with mixed subtype vs. the other adenocarcinomas).

^b Variant 2; Fisher exact test, $p = 0.00018$ (Acinar adenocarcinoma vs. the other adenocarcinomas).

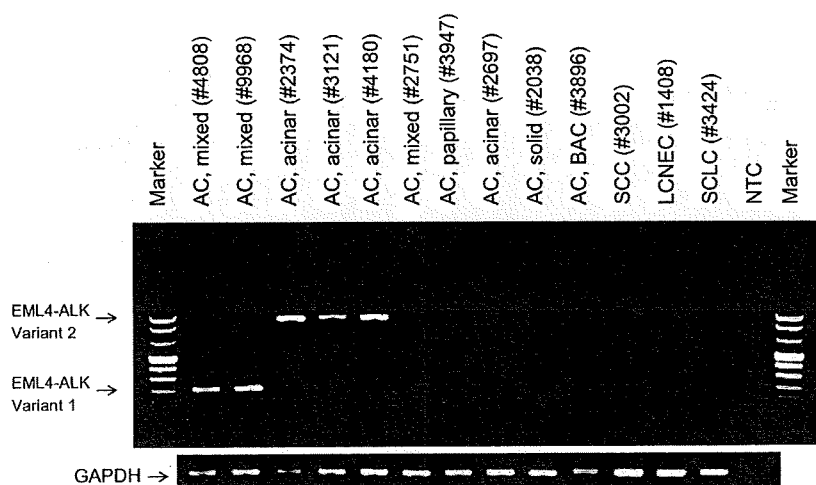


FIGURE 1. RT-PCR for EML4-ALK fusion mRNA. All the 5 fusion-positive cases and fusion negative cases of all the histologic subtypes examined are shown. RT-PCR results for GAPDH mRNA are also included as an internal control. AC, adenocarcinoma; mixed, adenocarcinoma with mixed subtype; papillary, papillary adenocarcinoma; acinar, acinar adenocarcinoma; solid, solid adenocarcinoma with mucin; BAC, bronchioloalveolar carcinoma; SCC, squamous cell carcinoma; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; NTC, no template control.

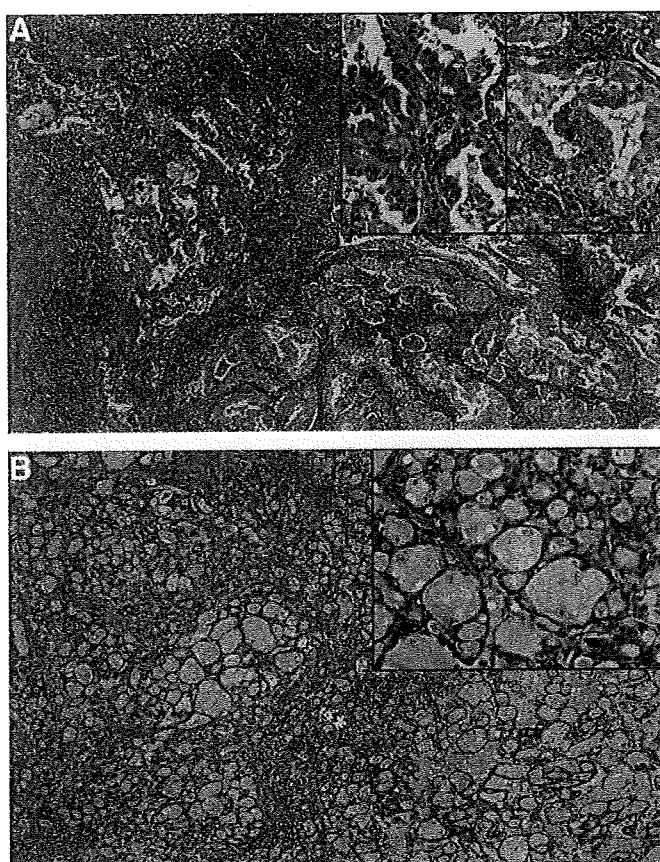


FIGURE 2. Representative examples of histologic features. Both the 2 variant 1 cases were mixed type adenocarcinomas, with papillary and bronchioloalveolar components (A). All 3 variant 2 cases were acinar adenocarcinomas (B).

compared with expectation). Patients with EML4-ALK fusion-positive tumors were younger than those without by 4 years, though this was not statistically significant. One patient was 43 years old and died 4 months after surgery (variant 2, acinar adenocarcinoma, poorly differentiated, clinical stage-IV, cerebellar metastasis). The other four patients were 58 to

66 years old and are alive now. There was no association between EML4-ALK fusion and smoking habits, although the sample numbers were small ($p = 0.77$).

We analyzed the survival data statistically with the log rank test, but there was no prognostic significance of EML4-ALK fusion ($p = 0.84$).

DISCUSSION

In the present first large scale study of a novel EML4-ALK fusion in 221 lung cancers including 21 SCLCs, 5 of 149 adenocarcinomas (3.4%) proved positive for fusion mRNA and fusion protein. Interesting histotype-genotype relationships were observed. Although both variant 1 cases were papillary adenocarcinomas with bronchioloalveolar components, all the three variant 2 cases were of acinar type. Furthermore, none of these lesions had mutations in EGFR or KRAS, pointing to a mutually exclusive relationship.

The ALK gene encodes a transmembrane receptor tyrosine kinase that belongs to the insulin receptor superfamily and is most similar to leukocyte tyrosine kinase.²⁵ Postnatal ALK expression is normally restricted to a few scattered cells in the nervous system,²⁶ but chromosomal translocations involving the ALK are characteristic of ALCLs and IMTs. An increasing number of translocation patterns have been identified and other neoplasms with similar changes have been identified, such as large B-cell lymphomas.²⁷ Very recently, we described a novel subpopulation of NSCLCs with ALK translocations,⁵ and it is very likely that other examples will be identified by further searches. ALK-positive ACLCs predominantly affect younger patients and, if treated with chemotherapy, have a more favorable prognosis than their negative counterparts.²⁸ Similarly ALK-positive IMTs primarily affect younger patients.¹³ In this study, ALK-positive lung adenocarcinomas were also found in younger patients when compared with ALK-negative tumors. Especially, one of the five patients was 43 years old, very young for lung adenocarcinomas. Although one example is insufficient for discussion, ALK-positive adenocarcinomas might include younger patients. Although survival data analysis demonstrated no significant difference between ALK-positive

FIGURE 3. Representative examples of immunohistochemical features. Both adenocarcinomas with mixed subtype (A) with the variant 1 EML4-ALK fusion and acinar adenocarcinomas (B) with the variant 2 show ALK fusion protein in their cytoplasm.

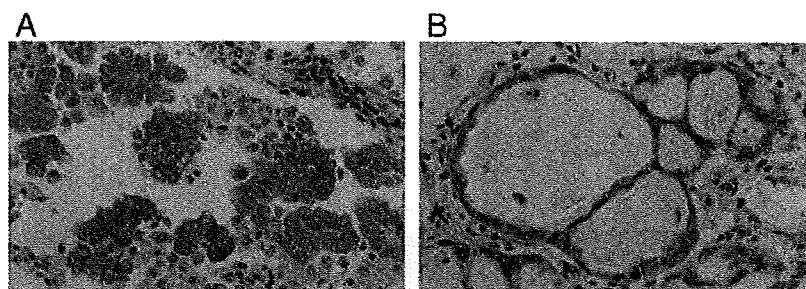


TABLE 2. Relationship between EML4-ALK Fusion and Clinicopathologic and Genetic Features in Lung Adenocarcinomas

Variables category	No. samples (%)	EML4-ALK fusion		p
		(+) (n = 5)	(-) (n = 144)	
Age (yr; mean ± SD)	149	59.4 ± 9.7	63.4 ± 8.7	0.31 ^a
Sex				0.87 ^b
Male	80 (54%)	2 (40%)	78 (54%)	
Female	69 (46%)	3 (60%)	66 (46%)	
Smoking habit				0.77 ^b
Never	65 (44%)	3 (60%)	62 (43%)	
Smoker	84 (56%)	2 (40%)	82 (57%)	
Tumor size				0.40 ^b
<30mm	77 (52%)	4 (80%)	73 (51%)	
30 mm ≤	72 (48%)	1 (20%)	71 (49%)	
Differentiation				0.73 ^c
Well	48 (32%)	1 (20%)	47 (33%)	
Moderate	62 (42%)	2 (40%)	60 (42%)	
Poor	39 (26%)	2 (40%)	37 (26%)	
EGFR				0.034 ^b
Mutation (+)	41 (55%)	0 (0%)	41 (59%)	
Mutation (-)	33 (45%)	5 (100%)	28 (41%)	
KRAS				0.92 ^b
Mutation (+)	7 (11%)	0 (0%)	7 (12%)	
Mutation (-)	55 (89%)	5 (100%)	50 (88%)	
EGFR or KRAS				0.014 ^b
Mutation (+)	38 (61%)	0 (0%)	38 (67%)	
Mutation (-)	24 (39%)	5 (100%)	19 (33%)	
p-Stage				0.73 ^b
I	63 (43%)	2 (40%)	61 (43%)	
II-IV	85 (57%)	3 (60%)	82 (57%)	

Percentages may not total 100, because of rounding.

^a Student *t* test.

^b Fisher exact test.

^c Yates χ^2 test.

and negative adenocarcinomas, this might be due to the small number of positive cases. Whatever is the cause, for ALK-positive tumors, molecular targeted therapies including ALK inhibitors may be used.

ALK1 antibody, used in the immunohistochemical analysis, detects the cytoplasmic region of the ALK protein and also detects the full-length endogenous ALK protein. When we detect the positive staining of ALK1, three possibilities are considerable: (i) EML4-ALK fusion protein, (ii) endogenous full-length ALK protein, or (iii) ALK fusion protein with another partner. The five EML4-ALK fusion

cases immunostained positive for ALK with variable intensity. Endogenous full-length ALK protein might, however, be also detected by immunohistochemistry. Therefore, EML4-ALK fusion should be confirmed by RT-PCR practically, although the immunohistochemistry can be used for the screening purpose.

In conclusion, we here found a minor subpopulation of lung adenocarcinomas featuring EML4-ALK fusion with evidence of histotype-genotype relationships. Furthermore, we could detect the fusion protein by immunohistochemistry, pointing to clinical applications.

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Glutathione peroxidase 3 is a candidate mechanism of anticancer drug resistance of ovarian clear cell adenocarcinoma

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Abstract. Ovarian clear cell adenocarcinoma has low sensitivity to platinum drugs. The molecular-biological mechanism of the low sensitivity has not been clarified. The objective of this study was to identify candidate genes associated with low sensitivity of clear cell adenocarcinoma to platinum drugs. Exhaustive gene profiling of 4 ovarian clear cell adenocarcinoma cell lines, KK, OVMANA, OVSAYO, and RMG-1 and 4 ovarian serous adenocarcinoma cell lines, KF, HRA, SHIN-3 and KOC-2S was performed by DNA microarray. Obtained candidate genes were suppressed by RNA interference and changes in the cisplatin sensitivity of clear cell adenocarcinoma cells were observed. Six genes including the *glutathione peroxidase 3 (GPX3)* gene were identified to be highly expressed in clear cell adenocarcinoma by DNA microarray analysis. GPX3 suppression by RNA interference increased cisplatin sensitivity 3.3-4.2-fold in 3 of the 4 clear cell adenocarcinoma cell lines. GPX3 was identified to be a gene highly expressed in clear cell adenocarcinoma. Since GPX3 suppression increased the cisplatin sensitivity of clear cell adenocarcinoma cells, *GPX3* may be a candidate gene associated with the low cisplatin sensitivity of clear cell adenocarcinoma.

Introduction

The highest number of patients die of epithelial ovarian cancer in the gynecology field (1). Platinum-based combination chemotherapy and debulking surgery has recently improved the prognosis of progressive epithelial ovarian cancer, but

clear cell adenocarcinoma is an exception and its prognosis remains poor. The important factor of poor prognosis of clear cell adenocarcinoma is its low sensitivity to known anticancer drugs, particularly platinum drugs. In our investigation of clinical cases, only 11% of patients responded to platinum-based chemotherapy for clear cell adenocarcinoma, which was markedly lower than the response rate in serous adenocarcinoma (73%) (2). Goff *et al* also reported that 70% of patients with stage III clear cell adenocarcinoma were resistant to platinum-based chemotherapy (3).

Regarding biological behavior of clear cell adenocarcinoma, Itamochi *et al* reported low proliferation activity (4,5). They showed that in an *in vitro* study, the doubling time of clear cell adenocarcinoma cells was 2 times or longer than that of serous adenocarcinoma cells (4), the ratios of clear cell adenocarcinoma cells in the G₂M, S and proliferation phases were low (4), and the ratio of Ki-67-positive cells was low in clinical cases of clear cell adenocarcinoma (5) and suggested that low proliferation activity of clear cell adenocarcinoma is a cause of the low sensitivity to platinum drugs. However, the molecular-biological mechanism of the low platinum sensitivity of clear cell adenocarcinoma has not been clarified.

The objective of this study was to identify candidate genes associated with cisplatin resistance of clear cell adenocarcinoma. Candidate genes associated with cisplatin resistance were exhaustively investigated in several cell lines of ovarian clear cell adenocarcinoma using DNA microarray. Obtained gene candidates were suppressed by RNA interference and changes in the cisplatin sensitivity of clear cell adenocarcinoma cells were observed.

Materials and methods

Cell cultures. Eight ovarian cancer cell lines were used in this study; four were of clear cell adenocarcinoma including KK (6), OVMANA (7), OVSAYO (7), and RMG-1 (8), and the other four were of serous adenocarcinoma including KF (9), HRA (10), SHIN-3 (11) and KOC-2S (12). All of the cells were maintained in Eagle's MEM supplemented with 10% fetal bovine serum (FBS) and were grown in Ham's F12 medium with 10% FBS.

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RNA preparation and DNA microarray analysis. Total RNA was extracted from ovarian cancer cells by the acid guanidinium method and was subjected to a synthesis of double-stranded cDNA with oligo-dT primer, which was then used to prepare biotin-labeled cRNA with the use of the GeneChip labeling system (Affymetrix, Santa Clara, CA, USA). The resultant cRNA was then hybridized to GeneChip HGU95Av2 microarray (Affymetrix) revealing the expression intensities of 12,625 probe sets in each sample. Detection of hybridization signals and the statistical analyses of the digitized data were performed with a GMS 418 array scanner (Affymetrix) and Gene Spring 3.2.2 software (Silicon Genetics, Redwood, CA, USA), respectively. The fluorescence intensity for each gene was normalized relative to the median fluorescence value for all human genes with a 'Present' or 'Marginal' call (Microarray Suite; Affymetrix) in each hybridization. In the hierarchical clustering analysis, similarity was measured by the Pearson's correlation with a separation ratio of 1.0. The details of the genes shown in the figures are available upon request.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. To verify the data obtained from microarrays, we carried out real-time RT-PCR analysis. Portions of unamplified cDNA were subjected to PCR with SYBR-Green PCR Core Reagents (PE Applied Biosystems, Foster City, CA, USA). The incorporation of the SYBR-Green dye into the PCR products was monitored in real-time with an ABI PRISM 7700 sequence detection system (PE Applied Biosystems), thereby allowing determination of the threshold cycle (Ct) at which exponential amplification of PCR products begins. The Ct values for cDNAs corresponded to the GAPDH gene and target transcripts relative to that of GAPDH mRNA. The oligonucleotide primers for PCR were as follows: GAPDH cDNA, 5'-CGCGGGGCTCTCAGAACATCAT-3' and 5'-CCAGCCCCAGCGTCAAAGGTG-3'; glutathione peroxidase 3 (GPX 3) cDNA, 5'-AGCAGTATGCTGGCAAATATGTCC-3' and 5'-CAGACCGAATGGTGAAGCTCTTC-3'.

Selection of short hairpin RNA stable cell lines. The DNA oligonucleotides, encoding short hairpin RNA (shRNA) targeting the GPX3 (forward; CACCGGGAGAGTTTGCAC TATTAACGTGTGCTGTCCGTTAATGGTGCAAGCTCT TCCTTTTT, reverse; GCATAAAAAGGAAGAGCTTGCA CCATTAACGGACAGCACACGTTAATAGTGCAAACCTC TCCC) were synthesized, annealed, and cloned into the *Bsp*MI site of the vector piGENE PURhU6 (13), which contains a human U6 promoter and a puromycin resistance gene. The shRNA expression plasmid (piGENE PURhU6/shGPX3) and the control plasmid (piGENE PURhU6) were transfected into four ovarian clear cell carcinoma cell lines by the standard calcium phosphate precipitation method (14). The cells were selected with the concentration of 1 μ g/ml puromycin (Calbiochem, Darmstadt, Germany). Resistant clones were obtained after four weeks. The cells were subsequently maintained in the presence of 1 μ g/ml puromycin.

Semiquantitative RT-PCR analysis. Using a semiquantitative RT-PCR method, we assessed transcription levels of GPX3

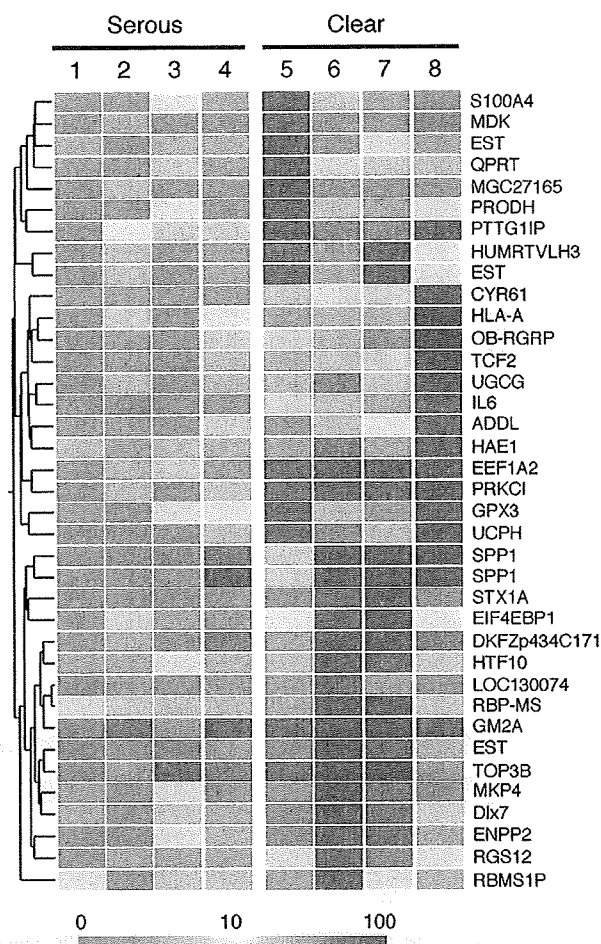


Figure 1. Identification of clear cell adenocarcinoma-specific genes. Hierarchical clustering of 34 probe sets were performed on the basis of their expression profiles in serous adenocarcinoma cell lines [HRA (1), KF (2), KOC-2S (3) and SHIN-3 (4)] and clear cell adenocarcinoma cell lines [KK (5), OVMANA (6), OVSAYO (7) and RMG1 (8)]. Each column represents a separate cell line and each row a single probe set on the microarray. Expression level of each probe set is shown color-coded as indicated by the scale at the bottom. Note that two distinct probe sets are assigned to the *PPI* gene on GeneChip HGU95Av2 array.

and GAPDH in the transfectants. Total RNAs were extracted from the transfectants by the acid guanidinium method and reverse-transcribed using Reverse Transcription System (Promega, Madison, WI). Each RT-PCR reaction consisted of 25 cycles of 30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C. Amplification of GAPDH revealed similar signal strengths in all samples, as a control for the integrity of each RNA template. PCR products were electrophoresed in 1.5% agarose gels. Primers used for amplification were described above.

Colorimetric assay. The sensitivity of the transfectants to cisplatin (Bristol-Myers Squibb Co., Ltd., Tokyo, Japan) was investigated by colorimetric assay using Cell Proliferation kit II (XTT) (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany). The transfectants were exposed to cisplatin at concentrations of 1-128 μ M for 24 h. The viable cell count measured by colorimetric assay was presented as a percent ratio to the count of the control untreated with cisplatin. A

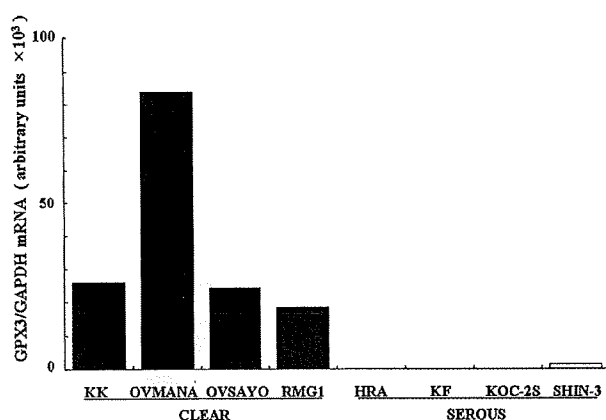


Figure 2. Quantitation of *GPX3* transcripts in ovarian cancer cell lines. Complementary DNA prepared from the ovarian cancer cells was subjected to real-time RT-PCR with primers specific for *GPX3* or *GAPDH* genes. The ratio of the abundance of the *GPX3* transcript to that of *GAPDH* mRNA was calculated as 2^n , where n is the Ct value for *GAPDH* cDNA minus the Ct value of the *GPX3* cDNA.

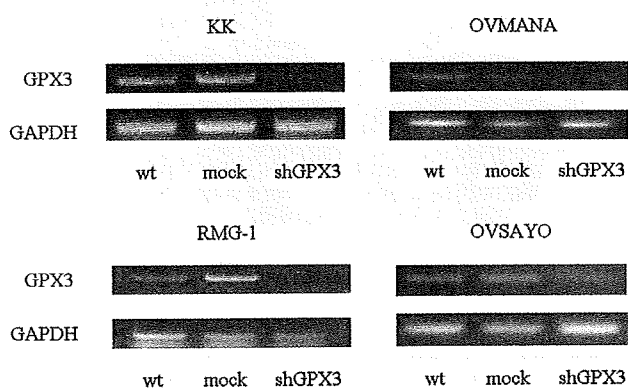


Figure 3. Expression of *GPX3* in clear cell adenocarcinoma transfectants. *GPX3* expression by the semiquantitative RT-PCR method was decreased in cells transfected with the shRNA expression plasmid in all 4 clear cell adenocarcinoma cell lines compared with the parent and control cell lines. The integrity of each RNA template was controlled through amplification of *GAPDH*.

dose-response curve was prepared and the 50% growth inhibitory concentration (IC_{50}) was obtained for cisplatin.

Results

DNA microarray analysis. Expression intensities of >12,000 human probe sets were examined in a total of 8 samples. To identify genes whose expression was specific to the clear cell adenocarcinoma subtype, we first calculated the mean expression levels of each gene in both clear cell adenocarcinoma and serous adenocarcinoma group. With the use of GeneSpring software, we then searched for genes whose expression profiles were similar, with a minimal correlation of 0.99, to that of a hypothetical 'clear cell adenocarcinoma-specific gene' with a mean expression level of 0.0 arbitrary unit (U) in the serous adenocarcinoma and of 200.0 U in the clear cell adenocarcinoma. From the resulting genes, we then

Table I. IC_{50} value (μM) and sensitivity index for cisplatin in the 4 types of clear cell adenocarcinoma transfectants.

	IC_{50} (μM)	Sensitive index
KK/mock	25.0±0.5	-
KK/shGPX3	7.5±0.9	3.3
OVMANA/mock	54.9±2.0	-
OVMANA/shGPX3	13.9±0.5	4.0
RMG-1/mock	54.7±4.4	-
RMG-1/shGPX3	12.9±0.3	4.2
OVSAYO/mock	26.6±0.4	-
OVSAYO/shGPX3	24.5±2.5	1.1

selected those whose expression level was ≥ 60.0 U in at least one of the clear cell adenocarcinoma group. A total of 34 probe sets (corresponding to 33 human genes) were finally identified to be specific to the clear cell adenocarcinoma cell lines, including those for secreted phosphoprotein 1 (SPP1), eukaryotic translation elongation factor 1 α 2 (EEF1A2), hereditary angioedema (HAE) 1, pituitary tumor-transforming gene 1 (PTTG1IP), UDP-glucose ceramide glucosyltransferase (UGCG) and *GPX3*.

Expression profiles of these clear cell adenocarcinoma-specific genes were shown as a dendrogram, or 'gene tree,' in which genes with similar expression profiles among the samples were clustered near each other (Fig. 1).

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. To confirm the group-specific expression of these genes, we measured their mRNA level by the quantitative real-time RT-PCR method. As shown in Fig. 2, the relative expression level of *GPX3* to *GAPDH* was, for instance, highly induced in the clear cell adenocarcinoma cell lines, but negligible in the serous adenocarcinoma cell lines.

Expression of *GPX3* in clear cell adenocarcinoma transfectants. As shown in Fig. 3, *GPX3* expression by the semiquantitative RT-PCR method decreased in cells transfected with the shRNA expression plasmid in all 4 clear cell carcinoma cell lines compared with the parent and control lines. The integrity of each RNA template was controlled through *GAPDH* amplification.

Cisplatin sensitivity. The dose-response curves of the 4 types of clear cell adenocarcinoma transfectants to cisplatin are shown in Fig. 4 and the IC_{50} values (μM) in Table I. The ratio of the IC_{50} value in cells transfected with the shRNA expression plasmid to that in the control cells was defined as the sensitivity index. In KK, OVMANA, and RMG-1, the sensitivity index was 3.3, 4.0 and 4.2, respectively, showing an increase in cisplatin sensitivity by the suppression of *GPX3* expression. In OVSAYO, the sensitivity index was 1.1, showing no definite change.

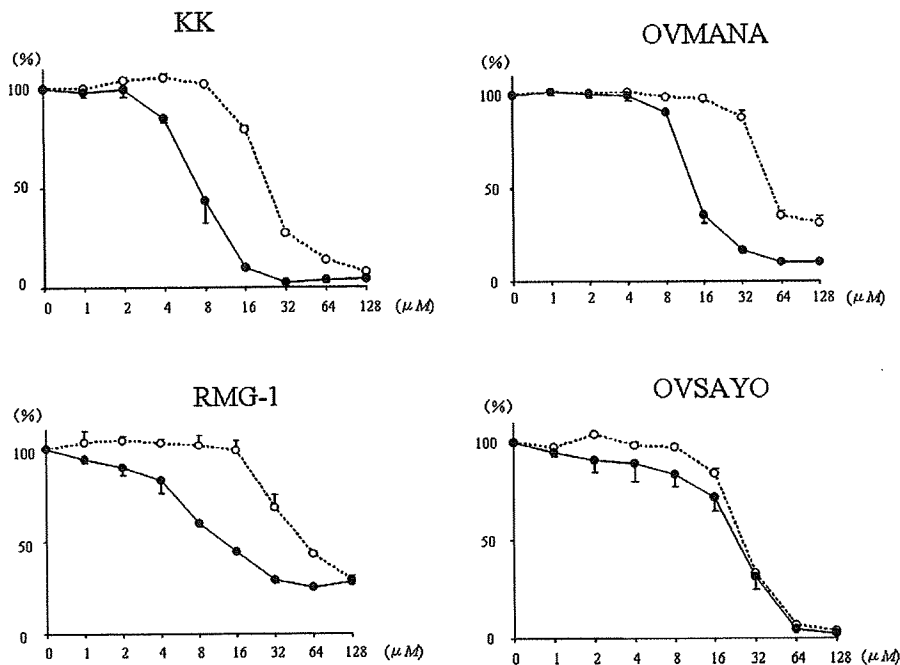


Figure 4. Dose-response curves of the 4 types of clear cell adenocarcinoma transfectants to cisplatin. Open circle, control cells; solid circle, cells transfected with the shRNA expression plasmid. The average of three independent experiments is shown and error bars indicate SD.

Discussion

Among the 33 genes that were up-regulated in the clear cell adenocarcinoma cell lines, *SPP1* has been reported to be involved in bone metabolism, *EEF1A2* in the repair of DNA damage in lymphoid cells, *HAE1* in hereditary angioedema (as the responsible gene), *PTTG1IP* in the development of pituitary tumor and *UGCG* in the biosynthesis of glycosphingolipids.

GPX3 is an oxygen radical-metabolizing enzyme. Since GPX3 functions in the detoxification mechanisms of various substances (15,16), it was interesting to find *GPX3* in the specific genes to clear cell adenocarcinoma which is a chemoresistant malignancy. Studying clinical specimens of ovarian cancer, Hough *et al.* (17) reported that *GPX3* was up-regulated in clear cell adenocarcinoma, similar to the results of our study. Thus, analyses of both cell lines and fresh clinical specimens have indicated that the up-regulation of the *GPX3* gene may be one of the molecular characteristics of clear cell adenocarcinoma.

GPX3 suppression by RNA interference definitely increased cisplatin sensitivity in 3 of the 4 clear cell adenocarcinoma cell lines. This experiment showed that GPX3 is involved in cisplatin sensitivity, suggesting that low cisplatin sensitivity is due to high GPX3 expression in clear cell adenocarcinoma, which in many cases highly expresses GPX3 (17).

For the anticancer drug resistance mechanism of cancer cells, promotion of excretion of anticancer drugs from cells, promotion of DNA repair and inhibition of apoptosis are generally considered. Previous study reports of anticancer drug resistance of clear cell adenocarcinoma, particularly cisplatin resistance, are discussed below.

Multidrug resistance-associated protein (MRP) and P-glycoprotein (P-gp) excrete anticancer drugs from cells in a ATP-dependent manner and decrease intracellular accumulation of anticancer drugs and are closely associated with multidrug resistance of various cancers. However, according to Itamochi *et al.*, MRP or P-gp expression was not related to cisplatin sensitivity in clear cell adenocarcinoma either *in vitro* or clinical cases (4).

There is almost no previous data on DNA repair system in clear cell adenocarcinoma. Only Reed *et al.* reported that ERCC1 and XPB associated with DNA repair were highly expressed in clear cell adenocarcinoma (18). However, an increase in DNA repair in clear cell adenocarcinoma has not been directly demonstrated.

Tsuchiya *et al.* found a new candidate gene associated with anticancer drug resistance of clear cell adenocarcinoma (19). They nominated hepatocyte nuclear factor-1 β (HNF-1 β) for the candidate gene based on DNA microarray analysis of 4 clear cell adenocarcinoma cell lines. They suggested that HNF-1 β is a gene involved in the low sensitivity, based on the findings that HNF-1 β was highly expressed in many patients with clear cell adenocarcinoma and inhibition of HNF-1 β expression induced apoptosis of clear cell adenocarcinoma cells *in vitro*. Although association of HNF-1 β gene with low cisplatin sensitivity was not directly demonstrated, this is an interesting study.

In summary, we identified *GPX3* as a gene highly expressed in clear cell adenocarcinoma by DNA microarray and real-time RT-PCR. GPX3 suppression by RNA interference increased the cisplatin sensitivity of clear cell adenocarcinoma cells. *GPX3* was suggested to be a candidate gene associated with low cisplatin sensitivity of clear cell adenocarcinoma.

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Autocrine and/or paracrine growth of aggressive ATLL cells caused by HGF and c-Met

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Abstract. Adult T-cell leukemia/lymphoma (ATLL) is a neoplasia characterized by the massive invasion of various organs by tumor cells. Previously, we found that expression of the gene for c-Met, a receptor tyrosine kinase for hepatocyte growth factor (HGF), was specific to the acute type among 41 patients with ATLL by microarray. First in the present study, we analyzed the survival of the patients in relation to expression of c-Met and HGF in ATLL cells. Expression of the former but not the latter was associated with poor prognosis. Then, we analyzed the growth of ATLL cells caused by HGF and c-Met. c-Met was expressed in 0/7 chronic ATLLs, 12/14 acute ATLLs, 1/1 IL-2-independent ATLL cell line and 1/7 IL-2-dependent ATLL cell lines as assessed by flow cytometry. HGF induced the proliferation of primary cells from most acute cases examined as well as the c-Met-positive KK1 cell line in contrast to c-Met-negative cells. HGF induced autophosphorylation of c-Met in c-Met-positive cells from an acute case and KK1 cells. The plasma level of HGF was elevated in acute as compared to chronic cases. The levels of HGF and/or IL-6 which induces the production of HGF by stromal cells, were elevated in the supernatant of short-term cultured cells from certain patients with acute or chronic disease. Finally, infiltrated ATLL cells

and adjacent stromal cells in liver were shown to be positive for c-Met/HGF and HGF, respectively, in acute cases. Autocrine and/or paracrine growth caused by HGF and c-Met was suggested in aggressive ATLL cells secreting HGF and/or IL-6, respectively.

Introduction

Adult T-cell leukemia/lymphoma (ATLL) is a distinct peripheral T-lymphocytic malignancy associated with human T-cell lymphotropic virus type-I (HTLV-1) (1-3). The diverse clinical features and prognosis of this disease have led to its subclassification; acute, lymphoma, chronic, and smoldering types (4). Patients with indolent ATLL, i.e. the chronic or smoldering type, have been treated as a subtype of chronic lymphoid leukemia with a watchful-waiting policy until disease progression (5-7). Aggressive ATLL generally has a very poor prognosis as compared to aggressive B-cell lymphoma and peripheral T-cell lymphoma excluding ATLL because of the multidrug-resistance of malignant cells, a large tumor burden with multi-organ failure, hypercalcemia and/or frequent infectious complications due to a profound T-cell immunodeficiency (5-7).

Hepatocyte growth factor (HGF), also known as scatter factor, was identified as a chemoattractant for a variety of cells. HGF is produced by cells of mesenchymal origin, but not by epithelial cells and has a pleiotropic function, such as liver regeneration. It also has mitogenic, morphogenic, and motogenic effects on epithelial cells, as well as endothelial cells (8). The receptor for HGF is encoded by the met proto-oncogene (c-Met). The c-Met protein is a tyrosine kinase cell surface receptor and consists of an extracellular α - and a transmembrane β chain. The β chain contains the tyrosine kinase domain as well as the site for tyrosine autophosphorylation. Ligation of HGF causes autophosphorylation of c-Met, followed by a variety of signaling cascades (9). Although normal HGF/c-Met signaling is involved in many aspects of embryogenesis, abnormal HGF/c-Met signaling has been implicated in tumor development and progression

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(10,11). In particular, HGF/c-Met signaling has been shown to play a significant role in promoting tumor cell invasion and metastasis. Furthermore, HGF and/or c-Met expression/over-expression has been documented in a wide variety of human tumors (10,11). c-Met is predominantly expressed in epithelial cells but has been detected in various hematopoietic cells as well (12). Furthermore, lymphoid malignancies, such as multiple myeloma and several B cell lymphomas, were found to express c-Met, suggesting that c-Met is involved in the pathogenesis of these diseases (13,14).

We have reported frequent hepatic involvement and the relationship between liver invasion and poor prognosis in ATLL, and the relationship was associated with c-Met expression on ATLL cells (15,16). Recently, using microarray-based gene expression profiling, we performed a comprehensive genomic analysis of ATLL in order to investigate the mechanism of progression from chronic to acute disease, and found that c-Met expression was shown to be specific to acute type ATLL and the plasma concentration of HGF was increased in some individuals with acute or chronic type ATLL (17).

To clarify the interaction of c-Met/HGF in the multi-step leukemogenesis and tissue-invasiveness of ATLL, we investigated the possible autocrine/paracrine loop using ATLL cell lines, and primary leukemic cells and liver autopsy specimens from patients with the disease.

Materials and methods

Clinical and microarray data for analyzing the relationship of prognosis and c-Met/HGF expression in ATLL. Isolated CD4⁺ leukemic cells from cases of the chronic (n=19) or acute (n=22) ATLL had been subjected to profiling of gene expression with oligonucleotide microarrays containing >44,000 probe sets including those for c-Met and HGF in our previous study (17). Collected clinical data for each patient were correlated with the expression data in this study.

Cell lines. Eight HTLV-I-positive T-cell lines were used. Six of the 7 cell lines established by us, SO4, ST1, KK1, KOB, LMY-1 and LMY2 excluding OMT, were each derived from a primary ATLL clone confirmed by Southern blotting for HTLV-I-integrated sites. The origin of HUT102 is unknown. HUT102, an interleukin (IL)-2-independent cell line, was grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, penicillin G (50 units/ml), and streptomycin (50 µg/ml) in a humidified incubator containing 5% CO₂ in air. The other 7 cell lines were IL-2-dependent, and were grown in a medium identical to that for HUT102 with 200 Japan reference units (JRUs)/ml recombinant IL-2 (provided by Takeda Chemical Industries, Osaka, Japan).

Patient samples for analyzing the growth of ATLL cells caused by HGF and c-Met. We assessed 21 patients with ATLL, 14 and 7 with acute and chronic types, respectively, having >90% ATLL cells phenotypically confirmed by more than two parameters among CD2, CD3, CD4, CD5 and CD25 in peripheral blood mononuclear cells (PBMNCs). Four out of the 21 patients (3 acute types and 1 chronic type) were included in our previous study (17). The

diagnosis of ATLL was based on clinical features, hematological findings including cytologically or histologically proven mature T-cell leukemia/lymphoma, and serum anti-HTLV-I antibodies (16,17). Integration of the monoclonal HTLV-I provirus into the genomic DNA of malignant cells was confirmed by Southern blot hybridization in all cases. PBMNC and plasma from patients with ATLL were obtained by density gradient separation from peripheral blood, before chemotherapy, with informed consent. PBMNCs and plasma were also obtained from healthy individuals.

Detection of c-Met protein on the cell surface by flow cytometric analysis. The expression of c-Met on the cell surface was analyzed by flow cytometry as described (17). Briefly, 3-5x10⁵ cells were washed twice with PBS containing 2% FBS (FBS/PBS). The cells were incubated at 4°C with a mouse anti-human c-Met MoAb (Do-24; Upstate Biotechnology, Lake Placid, NY) for 60 min. After being washed twice with FBS/PBS, they were incubated with FITC-labeled anti-mouse IgG MoAb (PharMingen), washed twice with FBS/PBS, and suspended in FBS/PBS. Thereafter, the cells were incubated with murine mAbs to human CD4 and CD25, respectively conjugated with phycoerythrin (PE) and perCP. They were analyzed by FACScan using CellQuest software (Becton-Dickinson), and positivity for c-Met was evaluated in CD4 and CD25 double positive ATLL cells.

Detection of autophosphorylation of c-Met protein. Cell lines and primary ATLL cells were incubated in the presence or absence of human recombinant HGF (Sigma). To analyze the autophosphorylation of c-Met in response to HGF treatment, cellular lysates were immunoprecipitated with c-Met-specific antibody on 10% SDS-PAGE gels, electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA), and then analyzed for immunoreactivity with a mouse anti-human c-Met polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or antiphosphotyrosine antibody (anti-PY, 4G10), and horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Life Science, Inc., Arlington Heights, IL) with an enhanced chemiluminescence detection system (Amersham Life Science, Inc.).

Proliferation assay. For assays of cell proliferation, IL-2-deprived KK-1 cells and primary ATLL cells were cultured at a density of 5x10⁵/ml and 1x10⁶/ml viable cells assessed by trypan blue staining, respectively, in 96-well flat-bottomed microtitre plates in 200 µl of RPMI-1640 medium containing 10% FBS, 2 mmol/l L-glutamine and antibiotics, with or without human recombinant HGF (Sigma), anti-human HGF Ab (R&D) or anti-human HGF R(c-Met) Ab (R&D) for 48 h, and were then mixed with MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]. Cell proliferation was measured with a CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). All samples were analyzed in triplicate.

Cytokine assay. MNCs from patients with ATLL, normal individuals, or cell lines were suspended in RPMI-1640

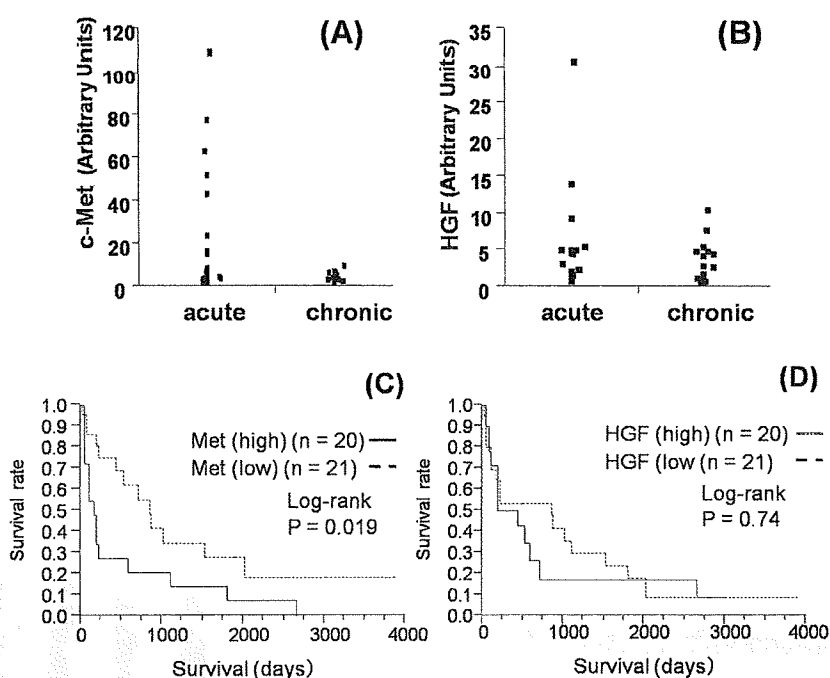


Figure 1. Expression level of c-Met but not HGF was associated with clinical subtypes of ATLL and prognosis. (A) Expression levels of c-Met and HGF by microarrays were compared between 22 acute cases and 19 chronic cases of ATLL. Leukemic CD4 positive cells from acute ATLL expressed significantly more c-Met than those from chronic ATLL (25.0 unit vs. 16.4 unit, $p=0.021$ by Mann-Whitney test). (B) In contrast, the difference was not significant for HGF (23.5 unit vs. 18.1, $p=0.15$ by Mann-Whitney test). (C) Overall survival of 41 ATLL patients in relation to c-Met expression status analyzed by microarray. (D) Overall survival of 41 ATLL patients in relation to HGF expression status analyzed by microarray.

medium containing 10% fetal bovine serum (M.A. Bio-products, Walkersville, MD) and 200 JRM/ml IL-2 at a concentration of 5×10^5 /ml and 2×10^6 /ml viable cells assessed by trypan blue staining, respectively. After 72 h of incubation, supernatant fluid was collected for assays. We examined the concentrations of HGF and IL-6 in the plasma and the supernatant fluid samples by enzyme-linked immunosorbent assay (Quantikine; R&D).

Immunohistochemistry. Using a formalin-fixed, paraffin-embedded section of liver from autopsy specimens, we performed immunohistochemical analysis. The antibodies used were a rabbit polyclonal antibody against c-Met (SC-10, Santa Cruz Biotechnology) at 1:20 dilution and a rabbit polyclonal antibody against HGF (SC-7949, Santa Cruz Biotechnology) at 1:20 dilution (18). Tissue blocks were sectioned at 5-micron thickness and were put on coated slide glass. After deparaffinization, the sections were pretreated in pH 6.0 citrate buffer by microwave for 10 min for antigen retrieval. The tissues were incubated with 3% H_2O_2 to block unspecific reaction for 5 min. After washing with PBS, the tissue were incubated with the first antibody for 1 h in room temperature, then, washed with PBS, and were incubated with DAKO EnVision system for 30 min. The stains were visualized with DAB.

Results

Expression level of c-Met but not HGF was associated with clinical subtypes of ATLL and prognosis (Fig. 1). Expression

levels of c-Met and HGF by oligonucleotide microarrays were compared between 22 acute cases and 19 chronic cases of ATLL. Leukemic CD4 positive cells from acute ATLL expressed significantly more c-Met than those from chronic ATLL. In contrast, the difference was not significant for HGF. Also, high expression of c-Met but not HGF was associated with worse overall survival.

Expression of c-Met protein in leukemic cells from ATLL patients. To examine the expression of c-Met on primary ATLL cells, FCM-based analyses were performed using PBMCs freshly isolated from ATLL patients as described previously (17). We observed the expression of c-Met on CD4 and CD25 double positive ATLL cells from 12 of 14 patients with acute-type ATLL, but none of 7 patients with chronic-type ATLL (Table I). c-Met was expressed in HUT102 and KK1, which were IL-2 independent and dependent cell lines, respectively.

Autophosphorylation of c-Met was induced in HTLV-I-positive T-cell lines and primary ATLL cells by HGF treatment. To examine whether the c-Met expressed in HTLV-I-positive ATLL cell lines and primary ATLL cells is functional, we analyzed the autophosphorylation of c-Met in response to HGF treatment. A signal of Mr 140,000 representing autophosphorylation of c-Met was detected in c-Met-positive KK-1 as described, and primary ATLL cells for the first time, when they were incubated in the presence of HGF (Fig. 2) (16). In those cells, the autophosphorylation of c-Met was not observed in the absence of HGF treatment. In contrast,

Table I. Expression of c-Met, growth response to HGF, and cytokine assay in primary ATLL cells and ATLL cell lines.

	ATLL cell line positive/tested cases	Primary ATLL cells (Chronic type) positive/tested cases	Primary ATLL cells (Acute type) positive/tested cases
Expression of c-Met	2/8	0/7	12/14
Growth response to HGF	1/2	0/7	9/11
HGF concentration (plasma)	N.A.	0/6	8/8
HGF concentration (culture ^a)	2/5	2/5	6/12
IL-6 concentration (culture ^a)	2/2	4/4	5/5

^aSupernatant of short-term cultured ATLL cells. N.A., not available.

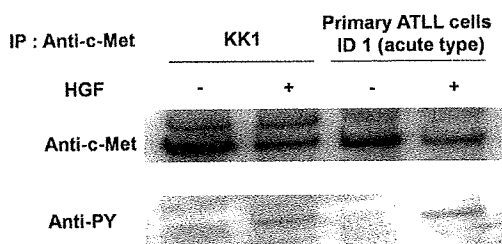


Figure 2. c-Met expressed in an HTLV-1-positive cell line and in primary ATLL cells is autophosphorylated in response to HGF. c-Met-positive KK-1 cells, and primary leukemic cells from a patient with acute ATLL were incubated in the presence (+) or absence (-) of HGF, lysed, immunoprecipitated with anti-c-Met antibody, and blotted with the same antibody or phosphotyrosine (anti-PY) antibody.

no signal representing c-Met or phosphotyrosine could be detected in c-Met-negative ATLL cell lines and primary ATLL cells (data not shown).

HGF induces ATLL cell proliferation. We next measured cell proliferation in primary ATLL cells and ATLL cell lines after stimulation with HGF. As shown in Fig. 3A, KK1, an IL-2-dependent ATLL cell line, showed a proliferative response to HGF in a dose-dependent manner after being deprived of IL-2 in contrast to c-Met negative cell lines. The response was at least partially blocked by anti-HGF or anti-c-Met antibodies (Fig. 3B). Similarly, HGF induced a proliferative response in c-Met-positive ATLL cells from 9 of the 11 acute cases with c-Met expression but not in c-Met-negative ATLL cells from 7 chronic cases (Fig. 3C).

Cytokine assay. The plasma levels of HGF in patients with ATLL are shown in Fig. 4A. The level was most elevated in acute type, and moderate in chronic type ATLL as compared to healthy individuals. Next, we analyzed HGF levels in the supernatants of short-term cultured primary ATLL cells and ATLL cell lines (Fig. 4B). In some of the patients with acute or chronic type ATLL, the cytokine level was relatively elevated as compared to healthy controls. In two (KK1 and ST1) out of 5 IL-2 dependent ATLL cell lines, the level was also relatively high.

To further analyze the mechanism of the increase in plasma HGF levels in patients with acute ATLL, cells not

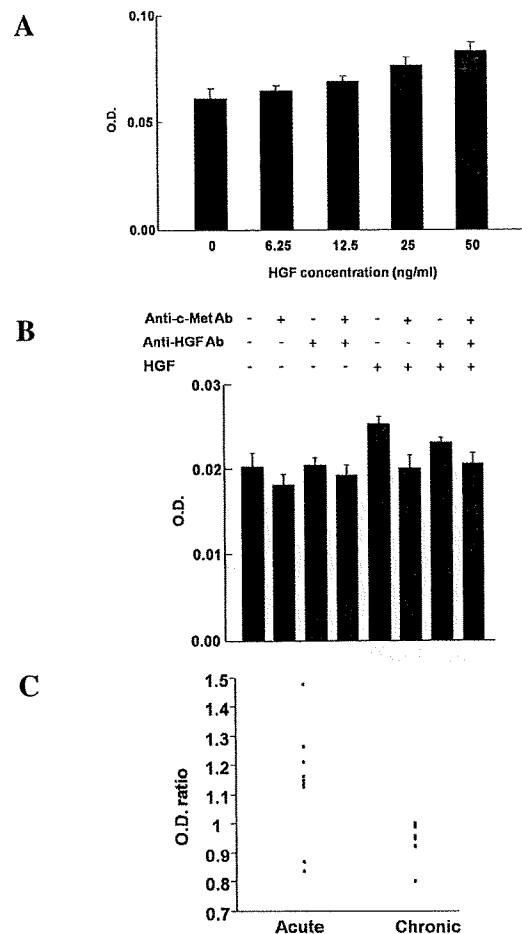


Figure 3. HGF induces proliferation of c-MET-positive cells. (A) The proliferation of c-Met-positive KK1, an IL-2 dependent ATLL cell line, was evaluated in a manner dependent on the dose of HGF after IL-2 deprivation by MTS assay. Data are expressed in absorbance unit and are means \pm SD of triplicates. (B) The proliferation of KK1 was evaluated in the absence or presence of HGF (50 ng/ml), alone or together with anti-HGF or anti-c-Met antibodies by MTS assay. Data are expressed in absorbance unit and are means \pm SD of triplicates. (C) The proliferation of c-Met-positive ATLL cells from acute cases and that of c-Met-negative ATLL cells from chronic cases were evaluated in the presence of HGF (50 ng/ml) by MTS assay. Data are expressed as ratio of the absorbance unit with that in the absence of HGF of triplicates in each case. The difference between acute and chronic cases were significant (1.13 vs. 0.94, Mann-Whitney $p=0.016$). HGF induced a proliferative response (ratio >1.06) in c-Met-positive ATLL cells from 9 of the 11 acute cases but not in the c-Met-negative ATLL cells from 7 chronic cases.

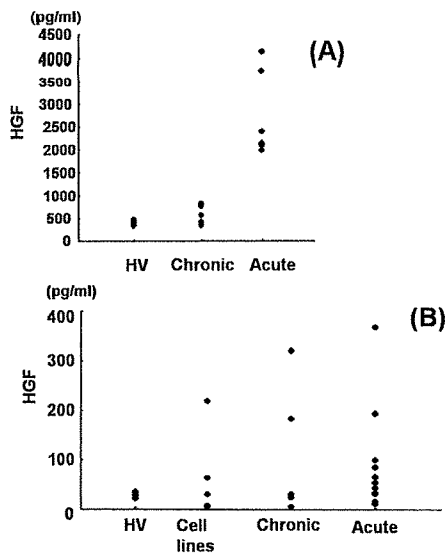


Figure 4. High level of HGF in plasma from ATLL patients and/or supernatant of short-term cultured cells from ATLL patients and HTLV-1-positive cell lines. MNCs from patients with ATLL, healthy volunteers (HV), or cell lines were cultured with IL-2. After 72 h of incubation, supernatant fluid as well as plasma was collected for assays. The concentrations of HGF in the plasma (A) and the supernatant fluid (B) samples were measured by enzyme-linked immunosorbent assay.

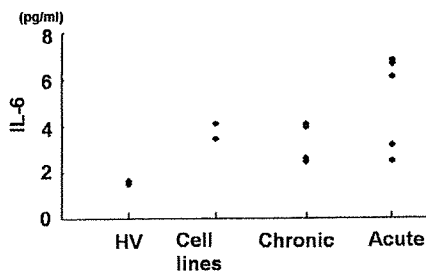


Figure 5. High level of IL-6 in supernatant of short-term cultured cells from ATLL patients and HTLV-1-positive cell lines. MNCs from patients with ATLL, healthy volunteers (HV), or cell lines were cultured with IL-2. After 72 h of incubation, supernatant fluid was collected for assays. The concentrations of IL-6 in the supernatant fluid samples were measured by enzyme-linked immunosorbent assay.

producing the cytokine *in vitro* were measured as to the concentration of IL-6, which had been reported to induce HGF production in stromal cells, in supernatant of short-term cultured ATLL cell lines and primary ATLL cells (19). The level was most elevated in acute types including cases with ATLL cells not secreting HGF, and moderate in chronic types and the cell lines as compared to healthy individuals (Fig. 5).

Immunohistochemistry. To evaluate the relationship between tissue-invasiveness and c-Met/HGF expression in ATLL, liver autopsy specimens from 2 patients suffering with liver dysfunction were analyzed. Immunostaining revealed most and some infiltrated atypical lymphocytes were c-Met-positive cells in each one case, respectively (Fig. 6). Hepatocytes

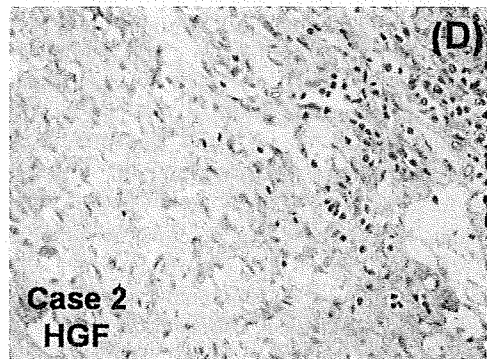
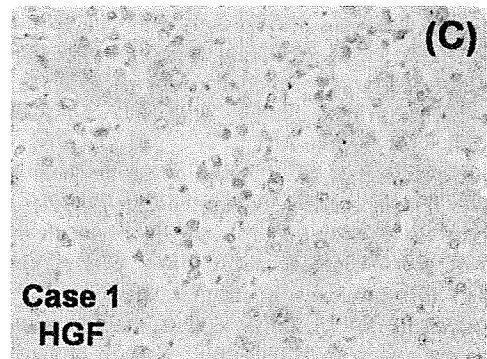
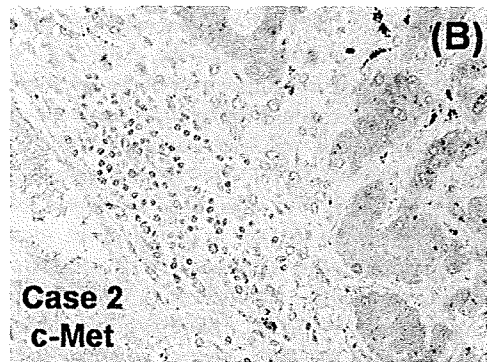
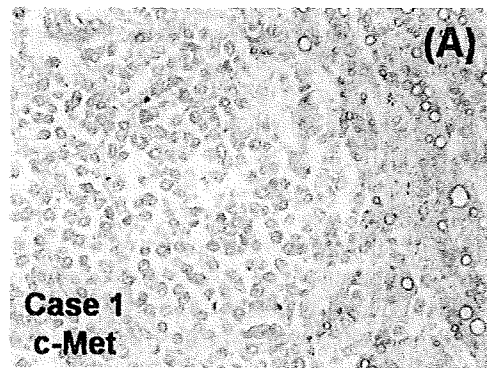


Figure 6. Immunohistochemical staining of c-Met and HGF in invasive ATLL, and adjacent normal hepatocytes and stromal fibroblasts from patients suffering from liver dysfunction. (A and C) Case 1 showed expression of c-Met in most of infiltrating large atypical lymphocytes and that of HGF in some of the atypical cells. Hepatocytes were positive for c-Met and negative for HGF, respectively. (B and D) Case 2 showed expression of c-Met in some large atypical lymphocytes. Weak expression of HGF was shown in the cytoplasm of stromal fibroblasts but was rare in ATLL cells.

were stained weak with anti-c-Met antibody as described previously (20). In the case with c-Met expression in most atypical lymphocytes, some of the cells were also positive for HGF. In the other case, HGF was positive in most adjacent stromal fibroblasts but rare in atypical lymphocytes.

Discussion

HGF/c-Met signaling has been shown to play a significant role in promoting tumor cell invasion and metastasis in a wide variety of human tumors (10,11,13,14). We previously found that the expression level of c-Met was higher in leukemic cells of acute type than chronic type ATLL by microarray-based gene expression profiling. Furthermore, the plasma concentration of HGF was increased in most acute and some chronic cases although the expression was not significantly detected in leukemic cells by microarray and PCR (17). We also previously reported that c-Met expression in ATLL was associated with liver-invasiveness of the disease (16). Here, we studied HGF/c-Met interaction using ATLL cell lines, primary leukemic cells and liver autopsy specimens from patients with the disease to elucidate the mechanism of multi-step carcinogenesis and tissue-invasiveness.

We first compared the significance of c-Met and HGF on the clinical features of ATLL. Expression of c-Met but not HGF was increased in acute cases as compared to chronic cases and associated with poor prognosis. In contrast to HGF expression in leukemic cells, plasma HGF concentration was higher in acute cases. Collectively, both c-Met expression in leukemic cells and concentrations of HGF in plasma were increased in most patients with acute ATLL, but rare in patients with chronic ATLL.

Overexpression of HGF and/or c-Met has been reported in various human cancers. Some tumor cell-derived factors, such as IL-1, IL-6 and TNF- α , are involved in the overexpression of HGF in stromal fibroblasts (18,21). Thus, such growth factors produced in stromal cells interact with the receptors expressed on tumor cells (paracrine pattern). In addition, malignant tumor cells also frequently produce growth factors and their receptors (autocrine pattern). Therefore, the HGF/c-Met pathway plays an important role during tumor progression in a paracrine pattern and/or autocrine pattern (22,23). The levels of HGF, and/or IL-6 were elevated in the supernatant of short-term cultured ATLL cells from some patients with acute or chronic disease as well as the cell lines KK1 and ST1. Notably, the IL-6 level in the supernatant was elevated in some cases without an elevation of HGF in the supernatant. These results suggest autocrine and/or paracrine growth stimulated by HGF and c-Met in acute ATLL cells secreting HGF and/or IL-6 which induce production of HGF by stroma cells, respectively. Plasma concentrations of IL-6 in the acute cases were significantly higher than those in the chronic cases of ATLL in our previous study (24). ATLL cells as well as HTLV-1 infected cells from HTLV-1 carriers and patients with HTLV-1 associated myelopathy secrete not only IL-6 but other HGF-inducers such as IL-1 β and TNF- α , through which the production of HGF in stromal cells could be up-regulated (25-27).

The expression of c-Met in c-Met-positive ATLL cells from an acute case was functional as well as in KK1 by

detecting autophosphorylation of the protein after HGF treatment. Furthermore, we showed for the first time that HGF induced the proliferation of primary ATLL cells from most c-Met-positive acute cases examined as well as the KK1 cell line in contrast to c-Met-negative cells. This effect was at least partially blocked by antibodies to HGF and c-Met in KK1. These results strongly suggest the c-Met/HGF interaction to be an important event during the acute transformation of ATLL from the chronic phase. Although the HGF concentration was elevated in supernatant of KK1 cells and plasma of the acute case, autophosphorylation of c-Met was not detected in those cells without HGF treatment possibly because the concentration was not high enough to induce autophosphorylation. Interestingly, liver dysfunction associated with ATLL invasion was detected in three of the 12 c-Met positive cases in contrast to none of the 9 negative cases in this series. In two of the cases, infiltrated ATLL cells and adjacent stromal cells in liver were shown to be positive for c-Met/HGF and HGF, respectively, suggesting the autocrine and paracrine loop. The invasion by ATLL cells of organs such as the liver could expose the cells to hypoxia and induce c-Met expression as hypothesized in invasive and metastatic cancers (10,16,28). Also, HGF concentration could be elevated at invasive lesion secreted by ATLL cells or adjacent stromal cells (18,23).

Given that the JAK-STAT signaling pathway is activated in the leukemic cells of patients with advanced ATLL (29), it may be relevant that binding sites for STAT1/STAT3 are present in the promoter regions of c-Met. The mechanism of overexpression of c-Met includes amplification of the gene (30). However, we did not detect a significant difference in DNA content at the c-Met locus between chronic and acute ATLL in our previous study (17). It is thus possible that JAK-STAT signaling contributes to the transcriptional activation of c-Met.

IL-2/IL-2R, IL-15/IL-15R and chemokine I-309 loop have been reported to induce autocrine and/or paracrine growth of aggressive ATLL cells (31-33). However, plasma levels of IL-2 and IL-15 were low in those studies. In contrast, HGF level in some acute cases were more than 4 ng/ml, which could induce some proliferation in KK1 and primary ATLL cells. Despite low plasma concentration of HGF, interestingly, the level in supernatant of short-term cultured ATLL cells from some chronic types was also elevated as acute ATLL. Although c-Met was not expressed in chronic types, the secretion of HGF by the cells after IL-2 stimulation might reflect the early phase of the transition to acute ATLL. There have been reports on several targets for the treatment of ATLL (34-37). The c-Met pathway is among drug targets in cancer progression and provides at least three avenues of selective anticancer development: antagonism of ligand/receptor interaction, inhibition of TK catalytic activity, and blockade of intracellular signaling. Human clinical trials in two of the three areas are now underway (10,11,22,23). Our study revealed that blocking the pathway might be effective against aggressive ATLL as well as solid cancers.

In conclusion, we provide the first evidence of autocrine and/or paracrine growth of primary leukemic cells and tissue-invasive cells stimulated by HGF and c-Met in aggressive ATLL secreting HGF and/or IL-6, respectively. In addition,

the c-Met/HGF system may represent a suitable target for the treatment of ATLL.

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Adhesion-dependent growth of primary adult T cell leukemia cells with down-regulation of HTLV-I p40Tax protein: a novel in vitro model of the growth of acute ATL cells

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Abstract In order to better understand the biology of adult T cell leukemia (ATL), we aimed to establish a novel method, which allows the primary growth of ATL cells using a co-culture system with murine bone marrow-derived stromal cells, MS-5. ATL cells grew in close contact with MS-5 layers and formed so-called “cobblestone areas” (CAs) without the addition of IL-2. In clinical

samples, eight of ten (80.0%) cases of acute or lymphoma type ATL cells formed CAs. The frequency of CA forming cells in ATL cells ranged from 0.03 to 1.04%. The morphology, immunophenotyping, and DNA analysis indicated that cells composing CA were compatible with ATL cells, and clonally identical to primary CD4-positive ATL cells. Furthermore, in ATL cells composing CA, the expression of p40Tax was down-regulated in transcriptional and translational level, while that of HTLV-I basic leucine zipper factor (HBZ) gene was comparable to the level of primary ATL cells, resembling expression pattern of proviral genes in in vivo ATL cells. By microarray analysis, several genes which coded products involved in cell–cell interaction, and cellular survival and proliferation, were differentially expressed in ATL cells composing CA compared with primary samples. In conclusion, our co-culture system allows for the first time the growth of primary ATL cells in vitro, and might be useful as an in vitro assay for biological and clinical studies to develop molecular targeting drugs against ATL.

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Keywords Adult T cell leukemia · Microenvironment · In vitro co-culture system · Cell adhesion

1 Introduction

Adult T cell leukemia (ATL) is a lymphoma of mature CD4-positive T cells with frequent leukemic manifestation. Patients with ATL show variable clinical manifestations and a diverse prognosis, being classified into four subtypes; smoldering, chronic, lymphoma, and acute [1, 2]. The disease is also molecularly characterized by monoclonal integration of the provirus of human T-lymphotropic virus type 1 (HTLV-1), which has been

accepted as an etiologic agent of ATL [3, 4]. However, since only a small proportion of HTLV-I carriers develop ATL [5], and the latent period from the initial infection of HTLV-1 until the onset of this disease is over 40–60 years [2], it is believed that, in addition to HTLV-1 viral proteins, as yet undetermined multi-step accumulations of leukemogenic changes in cellular genes and dysregulation of transcription by epigenetic changes may have great roles in providing a growth advantage to the malignant clone and in the evolution to the fully aggressive phase of this neoplastic disease [6].

A few culture systems have allowed primary ATL cells to grow in vitro [7–9], although they showed only transient and cytokine-dependent proliferation. Furthermore, in these reports, it was demonstrated that once placed under in vitro culture conditions, especially when stimulated with interleukin-2 (IL-2), ATL cells began to abundantly express HTLV-1 viral protein p40Tax, a protein encoded by the pX regulatory region of the HTLV-1 genome [10]. The interactions of Tax with a number of cellular molecules, transcriptional factors or modulators of cellular functions, have been reported, resulting in the trans-activation or -repression of many specific cellular genes involved in the control of cell growth or apoptosis [11–13], and it is considered to contribute to the initial immortalization and transformation of HTLV-1-infected T lymphocytes. However, ATL cells do not produce any distinct amount of Tax proteins in vivo [3], although the expression of the *tax* gene has been detected using highly sensitive RT-PCR or RT-PCR in situ hybridization in several previous reports [14, 15]. These findings suggest that in vitro growth in cultures supported with IL-2 does not necessarily reflect the growth mechanism of ATL cells in vivo.

To address this issue, we aimed at establishing a novel method to allow the primary growth of ATL cells using a co-culture system with a stromal cell layer to provide various factors to support proliferation of target cells. Previously, using long-term cultures of stromal cells obtained from lymph nodes (LNs) of ATL patients, we observed that a number of cytokines were produced which might contribute to several clinical manifestations [16]. Thus, we have speculated that there might be some important interactions between the lymphohematopoietic microenvironment of LN or bone marrow (BM) and ATL cells. It has been demonstrated that by co-culturing with the murine BM-derived stromal cell line MS-5, it is possible to support the long-term proliferation of primitive hematopoietic progenitors, B-cell progenitor cells, and acute lymphoblastic leukemia (ALL) blasts [17, 18]. Thus, the system may be assumed to have properties by which human neoplastic lymphoid cells are able to grow without any exogenous cytokine.

Fig. 1 Characterization of cobblestone areas (CAs) formed by primary ATL cells using a co-culture system of ATL cells with a murine stroma cell line, MS-5. **a** Scheme of co-culture experiments: ATL cells from patients were cultured and observed as described in "Sect. 2". **b** Phase-contrast micrographs ($\times 200$) indicate time-course changes in CAs of primary ATL cells. ATL cells from a patient (UPN007) were cultured and observed on days 3, 7, 14, and 28, as indicated, respectively. **c** Photographs indicate representative results obtained from UPN 1; May-Giemsa staining ($\times 600$), immunostaining for CD4, and PCNA ($\times 600$), respectively, left to right. **d** Integration of HTLV-1 proviral DNA; Clonality of primary ATL cells and ATL cells composing CA of UPN001 and UPN005 was examined by SBH using whole HTLV-I probes²⁶. Restriction enzyme (*EcoRI* or *PstI*) was indicated at the bottom of the figure. Lanes 1 and 15: marker, 2: ST-1, 3: MS-5 co-cultured with MT-2, 4: MS-5 co-cultured with HUT-102, 5 and 11: primary ATL cells of UPN001, 6 and 12: ATL cells in CA of UPN001, 7 and 13: primary ATL cells of UPN005, 8 and 14: ATL cells in CA of UPN005, 9: HUT-102, 10: MT-2. **e** HTLV-1 proviral load was quantified using real-time PCR method to test involvement of HTLV-1 genome to MS-5 stromal layer²⁷. Light and dark gray bars indicate the results of the proviral load in co-cultured MS-5 and original HTLV-1 related cell lines (MT-2 and HUT-102), respectively. **f** Comparison of capacity of CA formation in co-culture experiments with different types of stromal layer. 1×10^5 ATL cells from UPN001, 003, 005, and 010 were co-cultured with stromal layer of MS-5 (filled diamond), HESS-5 (filled square), and HUVEC (filled triangle), in triplicate. Culture dishes were observed daily, and the numbers of CAs were counted at days 3, 5, 7, 10, 14, and 21. Values represent the means plus or minus standard error of the mean

In the present study, primary ATL cells were co-cultured with MS-5 cells and the usefulness of this co-culture system was demonstrated also for ATL cells. The significance of this novel stroma cell-dependent in vitro clonal culture system as a new model of primary ATL cell growth will be discussed.

2 Materials and methods

2.1 Cell lines and primary ATL cells

Four human HTLV-1-infected T cell lines established from normal lymphocytes (MT-2 and HUT-102) and one IL-2-independent (OMT) and three IL-2-dependent (ST-1, KOB, and KK-1) ATL cell lines established from clinical cases were used [19]. With informed consent according to the Helsinki Declaration, clinical samples of ATL cells were obtained from 20 patients, and normal CD4⁺ T lymphocytes were obtained from peripheral blood of two healthy volunteers. The diagnosis and subtypes of ATL were defined as described by Shimoyama et al. [20]. Mononuclear cells (MNCs) were isolated from samples by Ficoll Paque gradient centrifugation (Amersham Pharmacia Biotech, NJ). ATL cells or normal CD4⁺ T lymphocytes were enriched by labeling with magnetic bead-conjugated anti-CD4 monoclonal antibody (CD4 MicroBead; Miltenyi Biotec, Auburn, CA, USA), and then purified through a miniMACS magnetic cell separation column (Miltenyi Biotec).