

progression from TMD to AMKL in a subset of patients. Firstly, in MGS cells, the two individual mutations were not as potent as a combination of the two in conferring IL-3 independent growth of Ba/F3 cells (and p-STAT5 levels). This might infer that one of these mutations was present during the initial TMD phase and that it needed a second mutation, leading to a stronger JAK3 activation (higher p-STAT5), to precipitate AMKL. Secondly, the mutation detected in TMD (*JAK^{687T}*) confers IL3 independent growth only weakly, whereas all mutations in AMKL show a much stronger effect. Of course, the analysis of additional JAK3 mutations in TMD/AMKL patients will need to be conducted to substantiate or refute this claim. In particular, the analysis of sequential samples from individual TMD and AMKL patients could be very important to determine this.

JAK3 is predominantly expressed in haematopoietic cells and is specifically associated with the common γ chain (γ_c)-containing receptors including interleukin 2 (IL-2), IL-4, IL-7, IL-9, IL-15 and IL-21 (Miyazaki *et al*, 1994). Loss-of-function mutations of the γ_c chain or JAK3 result in SCID (Russell *et al*, 1995; Leonard, 2000). Targeting JAK3, therefore, would theoretically offer ideal immune suppression when it is needed without causing any effects outside of these cell populations (Borie *et al*, 2004). Recently, a more specific, potent and orally active inhibitor of JAK3 was developed. This JAK3 inhibitor, CP-690,550, produces sufficient immune suppression by itself to prevent organ transplant rejection, without inducing many of the side effects observed with current therapies (Changelian *et al*, 2003). In this study, we showed that treatment with the JAK3 inhibitor WHI-P131 (JAK3 inhibitor I) or WHI-P154 (JAK3 inhibitor II) resulted in significantly decreased growth and viability of cells expressing activating JAK3 mutants, although these compounds were less specific and potent than CP-690,550. These results provide proof-of-principle evidence that JAK3 inhibitors should have therapeutic effects for TMD and DS-AMKL patients carrying an activating JAK3 mutation.

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Supplementary material

The following supplementary material is available for this article online:

Fig S1. Growth curves of Ba/F3 cells expressing JAK3 mutants in the presence and absence of JAK3 inhibitors. Wild Ba/F3 cells with added IL-3 (A and B) and Ba/F3 cells expressing JAK3^{Q501H} and R657Q (C and D), JAK3^{A572V} (E and F), JAK3^{V674A} (G and H), or JAK3^{I87T} (I and J) without added IL-3 were treated with increasing concentrations of JAK3 inhibitor I (A, C, E, G and I) or JAK3 inhibitor II (B, D, F, H and J). Values represent mean \pm SD. The experiments were repeated twice, and both data sets were essentially identical.

Table S1. Primer sequences used in the PCR amplification of cDNA and subsequent sequencing.

The material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2141.2008.07081.x>

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Multiplex Reverse Transcription-PCR Screening for *EML4-ALK* Fusion Transcripts

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Abstract Purpose: *EML4-ALK* is a fusion-type protein tyrosine kinase that is generated by *inv(2)(p21p23)* in the genome of non-small cell lung cancer (NSCLC). To allow sensitive detection of *EML4-ALK* fusion transcripts, we have now developed a multiplex reverse transcription-PCR (RT-PCR) system that captures all in-frame fusions between the two genes.

Experimental Design: Primers were designed to detect all possible in-frame fusions of *EML4* to exon 20 of *ALK*, and a single-tube multiplex RT-PCR assay was done with total RNA from 656 solid tumors of the lung ($n = 364$) and 10 other organs.

Results: From consecutive lung adenocarcinoma cases ($n = 253$), we identified 11 specimens (4.35%) positive for fusion transcripts, 9 of which were positive for the previously identified variants 1, 2, and 3. The remaining two specimens harbored novel transcript isoforms in which exon 14 (variant 4) or exon 2 (variant 5) of *EML4* was connected to exon 20 of *ALK*. No fusion transcripts were detected for other types of lung cancer ($n = 111$) or for tumors from 10 other organs ($n = 292$). Genomic rearrangements responsible for the fusion events in NSCLC cells were confirmed by genomic PCR analysis and fluorescence *in situ* hybridization. The novel isoforms of *EML4-ALK* manifested marked oncogenic activity, and they yielded a pattern of cytoplasmic staining with fine granular foci in immunohistochemical analysis of NSCLC specimens.

Conclusions: These data reinforce the importance of accurate diagnosis of *EML4-ALK*-positive tumors for the optimization of treatment strategies.

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The nucleotide sequences of the *EML4-ALK* variant 4, 5a, and 5b cDNAs have been deposited in DDBJ/EMBL/Genbank under the accession numbers AB374363, AB374364, and AB374365, respectively.

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Chromosome rearrangement is a major mechanism giving rise to transforming potential in human cancers, especially in hematologic malignancies (1). A balanced translocation between chromosomes 9 and 22, for instance, generates an activated protein tyrosine kinase, BCR-ABL, that plays an essential role in the pathogenesis of chronic myeloid leukemia (2). The gene for another protein tyrosine kinase, ALK, is fused to those for NPM1 or other partner proteins in anaplastic lymphoma and soft tissue tumors, resulting in an increase in the kinase activity of ALK (3).

Mitelman et al. have suggested that chromosome translocations, in addition to being common in hematologic malignancies, are not rare in epithelial tumors (4, 5). These researchers also proposed that the genetic mechanisms underlying oncogenesis might not differ fundamentally between hematologic and epithelial malignancies, and that the current apparent difference in the frequency of chromosomal translocations between these two types of cancer is likely to disappear with the advent of new and more powerful investigative tools.

Consistent with this notion, recurrent chromosome rearrangements involving genes for ETS transcriptional factors have been identified in many cases of prostate cancer and may contribute to the hypersensitivity of prostate cancer cells to androgens (6, 7). In addition, we recently discovered another

Translational Relevance

EML4-ALK is a fusion-type protein-tyrosine kinase generated through a recurrent chromosome rearrangement, *inv(2)(p21p23)*, observed in non-small cell lung cancer (NSCLC). Because both *EML4* and *ALK* genes are mapped to the short arm of chromosome 2 in opposite orientations, PCR with primer sets flanking the fusion points of the two genes would not produce any specific products from cells without *inv(2)(p21p23)*. Reverse transcription (RT)-PCR for the fusion point would, therefore, become a highly sensitive and accurate means to detect tumors positive for *EML4-ALK*. Such analyses may detect small amounts of cancer cells in sputa from individuals with NSCLC at early clinical stages. Because several isoforms have been already reported for *EML4-ALK*, it is mandatory to detect all isoforms of the fusion kinase in a sensitive and reliable way. Toward this goal, we here developed a single-tube multiplex RT-PCR screening system to capture all possible isoforms of *EML4-ALK*. Examination of various tumor samples ($n = 656$) with our multiplex RT-PCR has indeed identified 11 specimens positive for the variants of *EML4-ALK* only among lung adenocarcinoma ($n = 253$). Our system, thus, paves a way for a sensitive molecular detection of this intractable disorder at early curable stages.

recurrent chromosome translocation in non-small cell lung cancer (NSCLC; ref. 8), a major cause of cancer deaths in humans. A small inversion within the short arm of chromosome 2, *inv(2)(p21p23)*, was found to be present in <10% of NSCLC cases and to give rise to a novel fusion-type tyrosine kinase, *EML4-ALK*, that exhibited marked transforming activity *in vitro* (8). Transgenic mice that specifically express *EML4-ALK* in lung epithelial cells were also found to develop hundreds of adenocarcinoma nodules in both lungs at only a few weeks after birth, and such nodules disappeared rapidly in response to oral administration of a specific inhibitor of the catalytic activity of *ALK*.⁵ These data thus indicate that *EML4-ALK* plays a pivotal role in malignant transformation in lung cancer, and they suggest that chemical compounds that inhibit the tyrosine kinase activity of *EML4-ALK* may provide an effective treatment for *EML4-ALK*-positive lung cancer. The selection of suitable drugs for individuals with lung cancer will thus require accurate determination of the absence or presence of the *EML4-ALK* fusion gene in biopsy specimens.

Given that *EML4* and *ALK* map in opposite orientations within the short arm of chromosome 2, reverse transcription-PCR (RT-PCR) analysis with primers designed to amplify the fusion points of *EML4-ALK* transcripts would not be expected to yield specific products from normal cells or cancer cells without *inv(2)(p21p23)*. Such analysis should thus provide a highly reliable and sensitive means to detect *EML4-ALK* in clinical specimens. Given that sputum has been shown to be a suitable specimen for such molecular diagnosis of *EML4-ALK* positivity (8), detection of *EML4-ALK*-positive cells by RT-PCR analysis of sputa may be effective for the identification of lung

cancer at early clinical stages. The accurate diagnosis of *EML4-ALK*-positive tumors, however, will require that all isoforms of *EML4-ALK* are detected.

The fusion of intron 13 or 20 of *EML4* to intron 19 of *ALK* gives rise to variant 1 or 2 of *EML4-ALK*, respectively (8). We have recently discovered another isoform (variant 3) of *EML4-ALK* in which intron 6 of *EML4* is ligated to intron 19 of *ALK* (9). Theoretically, in addition to such fusion of exons 6, 13, and 20 of *EML4*, an in-frame fusion to exon 20 of *ALK* can occur with exons 2, 18, or 21 of *EML4*. Given that the amino-terminal coiled-coil domain of *EML4* is responsible for the dimerization and constitutive activation of *EML4-ALK* (8) and that exon 2 of *EML4* encodes the entire coiled-coil domain, all of these possible fusion genes would encode *EML4-ALK* proteins containing the coiled-coil domain and therefore likely produce oncogenic *EML4-ALK* kinases.

To establish a highly sensitive and accurate PCR-based screening system for *EML4-ALK*-positive cancer, we have now developed a high-throughput multiplex RT-PCR assay for the detection of all potential *EML4-ALK* in-frame fusion transcripts. Among a consecutive series of lung adenocarcinoma specimens ($n = 253$) as well as other solid tumor samples ($n = 403$), we have now identified a total of 11 lung adenocarcinoma specimens positive for *EML4-ALK*, two of which harbor previously unidentified fusion mRNAs.

Materials and Methods

Clinical samples and RNA extraction. This study was done with clinical samples from 253 lung adenocarcinomas, 90 other NSCLCs (71 squamous cell carcinomas, 7 adenosquamous carcinomas, 7 large cell carcinomas, 2 pleomorphic carcinomas, and 3 large cell endocrine carcinomas), 21 small cell lung carcinomas, 50 breast carcinomas, 46 renal cell carcinomas, 48 colon carcinomas, 13 prostate carcinomas, 29 urothelial carcinomas, 33 gastric carcinomas, 10 uterine carcinomas, 9 hepatocellular carcinomas, 8 pancreatic carcinomas, and 46 malignant fibrous histiocytomas. All specimens were collected with the approval of the ethical committee at the Cancer Institute Hospital (Tokyo, Japan) and with the informed consent of individuals undergoing surgery from May 1995 to July 2003. The NSCLC cases were consecutive and spanned a period of 19 mo. Histologic diagnosis of NSCLC was made according to the WHO classification (10). All lesions were grossly dissected, rapidly frozen in liquid nitrogen, and stored at -80°C until RNA extraction with an RNeasy Mini Kit (Qiagen). RNA quality and the absence of contamination with genomic DNA were verified by formaldehyde-agarose gel electrophoresis.

Multiplex RT-PCR analysis and nucleotide sequencing. Total RNA was subjected to RT with random primers and SuperScript III reverse transcriptase (Invitrogen). For detection of *EML4-ALK* fusion cDNAs, multiplex PCR analysis was done with AmpliTaq Gold DNA polymerase (Applied Biosystems), the forward primers *EML4* 72F (5'-GTCAGCTCTTGAGTCCAGGAT-3') and *Fusion-RT-S* (5'-GTCCAGTGTTTAGCATTCTGGGG-3'), and the reverse primer *ALK* 3078RR (5'-ATCCAGTTCGTCGTTTCAGAGC-3'). The *GAPDH* cDNA was amplified by PCR with the primers 5'-GTCAGTGGTGACCT-3' and 5'-TGAGCTTGACAAAGTCGTC-3'. For amplification of *EML4-ALK* fusion cDNAs, the samples were incubated at 94°C for 10 min and then subjected to 35 cycles of denaturation at 94°C for 1 min, annealing at 64°C for 1 min, and polymerization at 72°C for 1 min. For amplification of *GAPDH* cDNA, the samples were subjected to 35 cycles of 94°C for 1 min, 58°C for 30 s, and 72°C for 30 s. Virtual gel electrophoresis of multiplex RT-PCR products was done with a 2100 Bioanalyzer (Agilent Technologies).

⁵ M. Soda et al., submitted for publication.

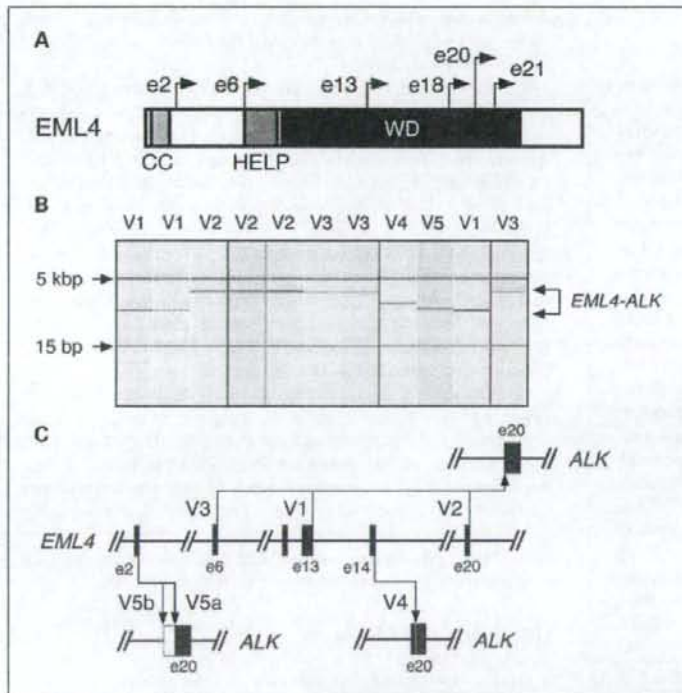


Fig. 1. Identification of *EML4-ALK* variants 4 and 5. **A**, schematic representation of the structure of *EML4*. The corresponding positions of exons (*e*) that can theoretically be fused in-frame to exon 20 of *ALK* are indicated by arrows, with known fusion points being denoted in red. CC, coiled-coil domain; HELP, hydrophobic EMAP (echinoderm microtubule-associated protein)-like protein domain; WD, WD repeats. **B**, virtual gel electrophoresis of multiplex RT-PCR products derived from lung adenocarcinoma specimens. Seven samples (blue) were known to harbor *EML4-ALK* variants (V 1, 2, or 3), whereas four samples were newly detected by multiplex RT-PCR. Two of the latter four specimens yielded PCR products corresponding to the newly identified variants 4 and 5. The positions of the fusion products of *EML4-ALK* are indicated on the right, and those of DNA size standards (5 kbp and 15 bp) are shown on the left. **C**, fusions between exons of *EML4* and *ALK*. Fusion of exons 6, 13, or 20 of *EML4* to exon 20 of *ALK* gives rise to variants 3, 1, and 2 of *EML4-ALK*, respectively. In addition, nucleotide sequencing of the PCR products shown in **B** revealed that exon 14 or 2 of *EML4* was fused to exon 20 of *ALK* in the cDNAs for *EML4-ALK* variants 4 and 5, respectively.

The primers used for direct amplification of the fusion points of individual cDNAs were 5'-AGGAGAGAAGCTCAGCGACTACC-3' and 5'-TCCACGCTCAAAAAGTCCCAAGTCC-3' for variant 4 and 5'-GCITTCGCCGCAAGATGGACGG-3' and 5'-AGCTTGCTCAGCTTG-TACTCAGGG-3' for variant 5. Full-length cDNAs for *EML4-ALK* variants were amplified with PrimeSTAR DNA polymerase (Takara Bio) and the primers 5'-ACTCTGCTCCGCTCCGCTGAATGAAG-3' and 5'-CCACGGTCTTAGGGATCCCAAGG-3'.

Fluorescence in situ hybridization analysis. Surgically resected lung cancer tissue was fixed in 20% formalin, embedded in paraffin, sectioned at a thickness of 4 μm, and placed on glass slides. The unstained sections were processed with a Histology FISH Accessory Kit (Dako), subjected to hybridization with fluorescently labeled bacterial artificial chromosome clone probes for *EML4* and *ALK* (GSP Laboratory) or for genomic regions upstream and downstream of the *ALK* break point (Dako), stained with 4,6-diamidino-2-phenylindole, and examined with a fluorescence microscope (BX51; Olympus).

Immunohistochemical analysis. Unstained paraffin-embedded sections were depleted of paraffin with xylene, rehydrated with a graded series of ethanol solutions, and then subjected to heat-induced antigen retrieval with Target Retrieval Solution pH 9.0 (Dako) before immunohistochemical staining with a mouse monoclonal antibody to ALK (ALK1, Dako) at a dilution of 1:20. Immune complexes were detected with the use of an EnVision+DAB system (Dako) with minor modifications.⁶

Transforming potential of *EML4-ALK* proteins. Protein analysis of *EML4-ALK* variants was done as described previously (8). In brief, the *EML4-ALK* variant 4, 5a, or 5b cDNAs were fused with an oligonucleotide encoding the FLAG epitope tag and inserted into the retroviral expression plasmid pMXS (11). The resulting plasmids and similar

pMXS-based expression plasmids for *EML4-ALK* variant 1, variant 1(K589M), variant 2, variant 3a, and variant 3b were individually introduced into HEK293 cells. Lysates of the transfected cells were subjected to immunoprecipitation with antibodies to FLAG, and the resulting precipitates were subjected either to immunoblot analysis with the same antibodies or to an *in vitro* kinase assay with the YFF peptide (12). Mouse 3T3 fibroblasts were also infected with recombinant retroviruses for each of the *EML4-ALK* variants or wild-type ALK and were then cultured for 12 d for a focus formation assay. The same set of 3T3 cells was injected s.c. into nu/nu mice, and tumor formation was examined after 20 d.

Results

Multiplex RT-PCR screening for *EML4-ALK* fusion transcripts in lung adenocarcinoma. As described above, exons 2, 6, 13, 18, 20, and 21 of *EML4* may participate in an in-frame fusion to exon 20 of *ALK* (Fig. 1A). To identify all possible *EML4-ALK* fusion cDNAs in a single-tube experiment, we designed a mixture of two sense primers (one targeted to exon 2 and the other to exon 13 of *EML4*) and a single antisense primer (targeted to exon 20 of *ALK*) and did multiplex RT-PCR with these primers and total cDNA preparations from tumor specimens. The exon 2 primer for *EML4* would be expected to generate a PCR product of 458 bp with the exon 2 (*EML4*)-exon 20 (*ALK*) fusion cDNA or of 917 bp with the exon 6-exon 20 fusion cDNA (variant 3). In addition, the exon 13 primer for *EML4* would be expected to generate PCR products of 432, 999, 1,185, or 1,284 bp with the exon 13-exon 20 (variant 1), exon 18-exon 20, exon 20-exon 20 (variant 2), and exon 21-exon 20 fusion cDNAs, respectively.

⁶ K. Takeuchi et al., manuscript in preparation.

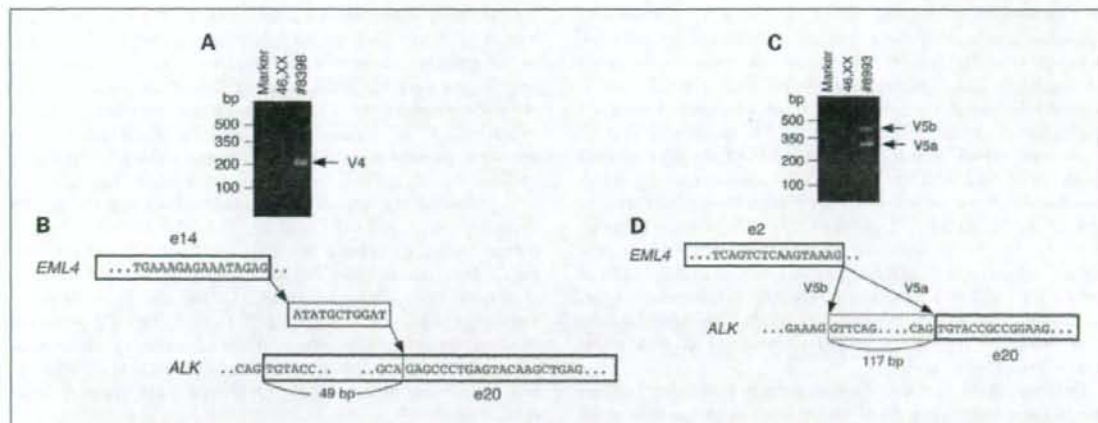


Fig. 2. Structure of *EML4-ALK* variant 4 and 5 cDNAs. **A.** RT-PCR amplification of the fusion point of *EML4-ALK* variant 4 mRNA in NSCLC specimen ID no. 8398 as well as in peripheral blood mononuclear cells of a female volunteer (46,XX). A PCR product of 203 bp corresponding to *EML4-ALK* variant 4 was specifically amplified from the tumor cells. The left lane contains DNA size standards (50-bp ladder). **B.** Nucleotide sequencing of the PCR product in **A** revealed that exon 14 of *EML4* (blue) was connected to an 11-bp cDNA fragment of unknown identity (black), which was ligated in turn to the nucleotide at position 50 of exon 20 of *ALK* (red). **C.** RT-PCR amplification of the fusion point of *EML4-ALK* variant 5 mRNA in NSCLC specimen ID no. 8993 as well as in peripheral blood mononuclear cells of a female volunteer (46,XX). Two specific products of 415 and 298 bp were obtained, corresponding to variants 5b and 5a, respectively. The left lane contains DNA size standards (50-bp ladder). **D.** Nucleotide sequencing of the PCR products in **C** revealed that exon 2 of *EML4* was fused either to exon 20 of *ALK*, generating the variant 5a cDNA, or to a position 117 bp upstream of exon 20 of *ALK*, generating the variant 5b cDNA.

Virtual gel electrophoresis of the multiplex RT-PCR products (Fig. 1B) revealed that 11 samples (4.35%) were positive for *EML4-ALK* cDNA among a consecutive series of 253 lung adenocarcinoma specimens, including those examined in our previous studies (8, 9, 13). All of the specimens previously shown to harbor *EML4-ALK* (two cases with variant 1, three with variant 2, and two with variant 3) were faithfully detected with our multiplex RT-PCR system. No specific PCR products were obtained for other types of lung cancer ($n = 111$) or other solid tumors ($n = 292$). Nucleotide sequencing of the PCR products for the newly identified positive cases revealed that one specimen was positive for variant 1 and another for variant 3 of *EML4-ALK*, but that the remaining two specimens harbored previously unidentified variants (Fig. 1B and C). Exon 14 of *EML4* was ligated to a position within exon 20 of *ALK* in the product from tumor ID no. 8398 (designated variant 4), whereas exon 2 of *EML4* was ligated to exon 20 of *ALK* in the product from tumor ID no. 8993 (designated variant 5).

Structure of *EML4-ALK* variant 4 cDNA. To verify the presence of novel *EML4-ALK* variants in the cancer cells, we first did direct RT-PCR analysis for the cDNA of tumor ID no. 8398 with a new set of primers encompassing the putative fusion point of variant 4. This analysis showed the presence of the fusion cDNA (Fig. 2A). Nucleotide sequencing of the PCR product revealed that exon 14 of *EML4* was fused to an unknown sequence of 11 bp, which in turn was connected to the nucleotide at position 50 of exon 20 of *ALK* (Fig. 2B). (We failed to detect a region of the human genome (build 36) homologous to the 11-bp connecting sequence in a BLAST search.⁷) Although exon 14 of *EML4* is not expected to produce an in-frame fusion to exon 20 of *ALK*, insertion of

the unknown 11-bp sequence and its ligation to a position within the *ALK* exon allows an in-frame connection between the two genes. Fusion cDNAs in which the point of connection is located within, rather than at the 5' terminus of, exon 20 of *ALK* have also been described for *MSN-ALK* (14) and *MYH9-ALK* (15).

We further examined whether a full-length cDNA encoding such an unexpected *EML4-ALK* variant could be isolated from the cancer cells. For this purpose, we designed a sense primer targeted to the 5' untranslated region of *EML4* cDNA as well as an antisense primer targeted to the 3' untranslated region of *ALK* cDNA. Direct RT-PCR analysis with this primer set yielded a single PCR product of ~3.4 kbp with total cDNA of tumor ID no. 8398 (Supplementary Fig. S1A). Complete nucleotide sequencing of the PCR product revealed that the cDNA contained an open reading frame for 1,097 amino acids comprising residues 1 to 547 of human *EML4*, residues 1,075 to 1,620 of human *ALK*, and 4 amino acids of unknown origin between these two sequences (Supplementary Fig. S1B). The isolation of a full-length cDNA containing the 11-bp insert indicated that the variant 4 protein was likely expressed in the cancer cells.

Structure of *EML4-ALK* variant 5 cDNAs. We similarly investigated the presence of variant 5 mRNA in the cells of tumor ID no. 8993. Direct RT-PCR analysis to amplify the fusion point of this variant cDNA yielded two independent products of 298 and 415 bp (Fig. 2C). Nucleotide sequencing of each product revealed that the former contained exon 2 of *EML4* and exon 20 of *ALK*, as expected, whereas in the latter, exon 2 of *EML4* was connected to a position within intron 19 of *ALK* located 117 bp upstream of exon 20 (Fig. 2D). These fusion constructs were designated variants 5a and 5b, respectively.

Although no mRNAs or expressed sequence tags in the nucleotide sequence database were found to contain the

⁷ <http://www.ncbi.nlm.nih.gov/genome/seq/blastgen/blastgen.cgi?taxid=9606>

117-bp sequence of intron 19 of *ALK*, the human genome sequence surrounding the 5' terminus of this 117-bp sequence is AG-GT (Fig. 2D), which conforms to the consensus sequence for a splicing acceptor site. To show that such a cryptic exon is indeed involved in the production of an oncogenic kinase, we attempted to detect full-length cDNAs for variants 5a and 5b from total cDNA of tumor ID no. 8993. A doublet of PCR products of ~2.0 kbp was obtained (Supplementary Fig. S1A), and nucleotide sequencing of these products revealed that they indeed encode EML4-*ALK* variant 5a and 5b proteins (Supplementary Fig. S1C). Genomic PCR and fluorescence *in situ* hybridization (FISH) analyses further revealed that the cells of tumor ID no. 8993 harbor a single EML4-*ALK* fusion gene, suggesting that variant 5a and 5b mRNAs are generated by alternative splicing of the primary transcript of this single fusion gene (see below).

Detection of the EML4-*ALK* fusion genes by FISH. To confirm the rearrangements involving the *ALK* locus in the specimens harboring variants 4 and 5 of EML4-*ALK* cDNA, we did FISH analysis with tissue sections. We first designed a FISH-based "fusion assay" for EML4 and *ALK* genes. Bacterial artificial chromosome fragments encompassing the entire genes were fluorescently labeled green and red, respectively. An overlapping signal for both probes was readily identified in a merged image for the tumor cells harboring variants 4 or 5 of EML4-*ALK* (Fig. 3A). To confirm further the breakage of the *ALK* locus, we did an "ALK split assay" with bacterial artificial chromosome fragments encompassing the 5' or 3' regions of the locus and labeled green and red, respectively. In this assay, the normal *ALK* locus would be expected to yield an overlapping signal, whereas a pair of separate green and red signals would indicate genomic breakage within *ALK*. As expected, a proportion of cells of tumor ID no. 8398 or no. 8993 in the histologic sections generated one overlapping signal and one pair of split signals (Fig. 3B), suggesting that these tumor cells each have at least one normal and at least one rearranged *ALK* locus.

These data, together with genomic PCR analysis (data not shown), thus indicated that the cells of each of these tumors harbor one normal chromosome 2 and a chromosome 2 with an inv(2)(p21p23) rearrangement. The other EML4-*ALK* cDNA-positive specimens (variants 1 to 3) in this cohort showed a similar FISH labeling profile, consistent with the presence of the corresponding EML4-*ALK* rearrangements (data not shown).

Detection of EML4-*ALK* proteins in situ. To detect EML4-*ALK* proteins in the cancer cells, we did immunohistochemical analysis with the ALK1 monoclonal antibody to ALK (16). The cytoplasm of tumor cells harboring EML4-*ALK* variant 1 (ID no. 9034), variant 4 (ID no. 8398), or variant 5 (ID no. 8993) manifested a diffuse pattern of immunoreactivity with fine granular concentrations (Fig. 3C). No normal pulmonary epithelial cells or lymphocytes in the sections of these specimens reacted with the antibody.

Transforming activity of EML4-*ALK* variants. We prepared expression plasmids for FLAG epitope-tagged EML4-*ALK* variants 1, 2, 3a, 3b, 4, 5a, and 5b, the predicted molecular sizes of which are 118,356; 146,913; 87,613; 88,874; 122,541; 71,046; and 74,867 Da, respectively. Each of these proteins, as well as a kinase-inactive mutant of EML4-*ALK* variant 1 (8), was expressed independently in HEK293 cells, immunoprecipitated, and subjected to immunoblot analysis with antibodies to FLAG. Each cDNA generated an EML4-*ALK* protein of the expected molecular size (Fig. 4A). The same immunoprecipitates were subjected to an *in vitro* kinase assay with the synthetic peptide YFF (12). Each variant protein (with the exception of the kinase-inactive mutant of variant 1) was shown to possess protein tyrosine kinase activity, with that of variants 3a, 3b, and 5b being most prominent (Fig. 4A).

To examine the transforming potential of the EML4-*ALK* variants, we transfected mouse 3T3 fibroblasts with the corresponding expression plasmids and then cultured the cells for 12 days. Transformed foci were readily detected for the cells expressing the variants of EML4-*ALK* but not for cells overexpressing wild-type *ALK* (Fig. 4B). Furthermore, s.c. injection of the transfected 3T3 cells into the shoulder of nude mice revealed that those expressing the various EML4-*ALK* isoforms, but not those overexpressing wild-type *ALK*, formed large tumors *in vivo* (Fig. 4B).

Discussion

We have done multiplex RT-PCR analysis to detect all possible isoforms of EML4-*ALK* transcripts in NSCLC cells, and unexpectedly identified two novel subtypes of the fusion event. This finding was supported by detection of the corresponding fusion genes by genomic PCR and FISH

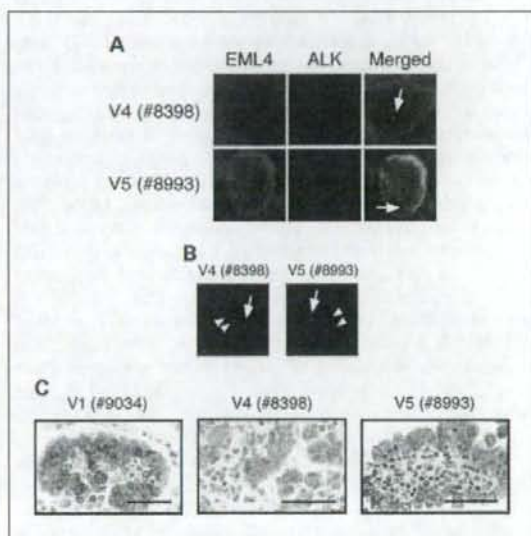


Fig. 3. FISH and immunohistochemical analyses of NSCLC specimens. **A**, FISH analysis of representative cancer cells in sections of lung adenocarcinoma harboring EML4-*ALK* variant 4 (ID no. 8398) or variant 5 (ID no. 8993). Each section was subjected to hybridization with differentially labeled probes for EML4 (left) or for ALK (center). A fusion signal (arrow) and a pair of green (EML4) and red (ALK) signals are present in each merged image (right). **B**, The same clinical specimens as in **A** were subjected to FISH analysis with differentially labeled probes for the 5' (green) or 3' (red) regions of the *ALK* locus. A pair of split signals (arrowheads) and an overlapping signal (arrow) indicate the rearranged and normal *ALK* loci, respectively. **C**, Immunohistochemical analysis of NSCLC specimens positive for EML4-*ALK* variants 1 (ID no. 9034), 4 (ID no. 8398), or 5 (ID no. 8993) with a monoclonal antibody to ALK. A pattern of diffuse staining with fine granular foci was apparent in the cytoplasm of all three tumors. Scale bars, 50 μ m.

analyses and by that of the encoded proteins by immunohistochemical analysis in the NSCLC cells. Together with the previously isolated variants (8, 9), we have to date identified a total of seven distinct isoforms of EML4-ALK (variants 1, 2, 3a, 3b, 4, 5a, and 5b). Given that each of these isoforms possesses marked transforming activity, they all likely play an important role in the development of NSCLC. Our failure to detect *EML4-ALK* cDNA in the other solid tumors ($n = 313$) examined suggests that *EML4-ALK* may be an oncogene specific to NSCLC, especially to lung adenocarcinoma.

In our multiplex RT-PCR analysis, a sense primer targeted to exon 2 of *EML4* was designed to detect fusion events involving exon 2 or 6 of *EML4*, and PCR products of the expected sizes were indeed obtained with NSCLC specimens positive for such fusion events (variants 5 and 3, respectively). The other sense primer was targeted to exon 13 of *EML4* and was designed to detect fusion events involving exon 13, 18, 20, or 21 of *EML4*. Given that we were able to readily amplify a specific product of 1185 bp corresponding to the fusion event involving exon 20 of *EML4* (variant 2), it is likely that all possible fusions giving rise to PCR products up to this size would have been detected in our cohort. It should be noted, however, that a possible fusion between exon 21 of *EML4* and exon 20 of *ALK* would be expected to generate a PCR product of 1,284 bp. Although the size difference between the 1,185- and 1,284-bp products is small (99 bp), it is still possible that our multiplex RT-PCR analysis failed to efficiently amplify the longer product and that there may be as-yet-undetected fusion events for *EML4-ALK* in our cohort.

All EML4-ALK isoforms manifested a similar subcellular distribution profile despite marked differences in the size and domain structure of the EML4 portions of these chimeric

proteins. In addition, the intracellular signaling systems activated by EML4-ALK may be shared among variants 1 to 5 (Supplementary Fig. S2). The EML4 portion of variant 5 comprises only the coiled-coil domain. This domain of EML4 may therefore play an essential role not only in the dimerization and activation of EML4-ALK isoforms (8) but also in tethering EML4-ALK to specific subcellular components. The pattern of subcellular immunostaining for EML4-ALK (cytoplasmic staining with fine granular foci) was distinct from that for other ALK fusion proteins associated with other malignancies (17, 18), suggesting that the subcellular localization of ALK fusion kinases varies substantially. The first such fusion kinase to be identified, NPM-ALK, preferentially phosphorylates STAT3, which is thought to participate in mitogenic signaling by NPM-ALK (19–21). Five ALK fusion kinases (NPM-ALK, TFG-ALK, ATIC-ALK, TPM3-ALK, and CLTC-ALK) were shown to differ markedly in their abilities to transform 3T3 fibroblasts, to phosphorylate STAT3 and AKT, and to activate phosphoinositide 3-kinase (17). Furthermore, a proteomics approach to identify tyrosine-phosphorylated proteins failed to detect marked phosphorylation of STAT3 in NSCLC specimens positive for EML4-ALK (22). It is therefore likely that each ALK fusion kinase exerts its effects through fusion-specific (although possibly partially overlapping) downstream pathways. In addition, we detected slight differences in catalytic and transforming activities among the variants of EML4-ALK (Fig. 4). These differences are likely due to the different portions of EML4 present in the different variants, which may affect dimerization affinity or the recruitment of substrates.

In addition to *EML4-ALK*, NSCLC cells harbor other potent oncogenes such as mutant versions of *EGFR* or *KRAS*. These three oncogenes, however, were found to be mutually exclusive

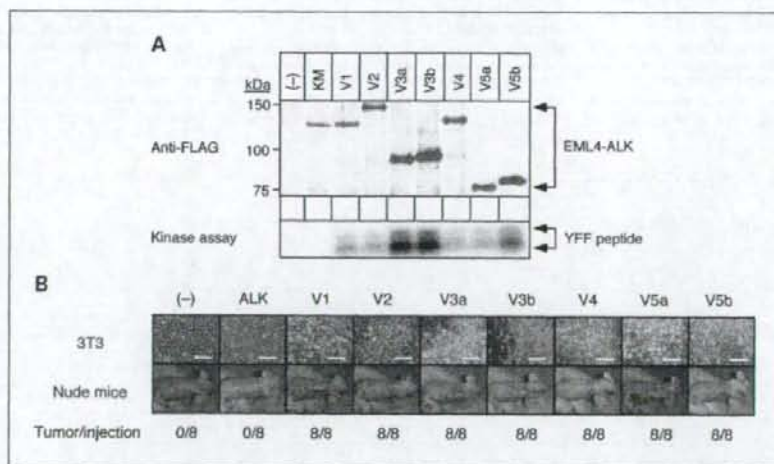


Fig. 4. Transforming potential of EML4-ALK variants. **A**, HEK293 cells expressing FLAG-tagged variant 1, 2, 3a, 3b, 4, 5a, or 5b of EML4-ALK were lysed and subjected to immunoprecipitation with antibodies to FLAG. The resulting precipitates were then either subjected to immunoblot analysis with antibodies to FLAG (*top*) or assayed for kinase activity with the synthetic YFF peptide (*bottom*). Cells transfected with the empty vector (-) or with a vector for a kinase-inactive mutant (KM) of EML4-ALK variant 1 were also analyzed. The positions of molecular size standards (kDa) and of EML4-ALK proteins are indicated on the left and right of the top panel, respectively. **B**, mouse 3T3 fibroblasts were transfected with expression plasmids for wild-type ALK or FLAG-tagged EML4-ALK variants, or with the empty plasmid (-), and were photographed after culture for 12 d (*top*). Scale bars, 200 μ m. Alternatively, the transfected cells were injected s.c. into the shoulder of nu/nu mice and tumor formation was examined after 20 d (*bottom*). The number of tumors formed per eight injections is indicated at the bottom.

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.

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 bronchioalveolar 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.

(*J Thorac Oncol*. 2008;3: 13-17)

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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The first two authors contributed equally to this work.

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'-TGAGCTTGACAAAGTGGTTCG 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- μ 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).

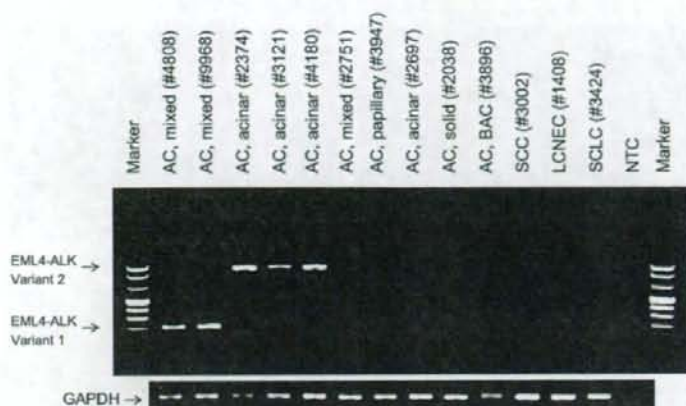


FIGURE 1. RT-PCR for EML4-ALK fusion mRNA. All the 5 fusion-positive cases and fusion negative cases of all the histologic subtypes examined are shown. RT-PCR results for GAPDH mRNA are also included as an internal control. AC, adenocarcinoma; mixed, adenocarcinoma with mixed subtype; papillary, papillary 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.

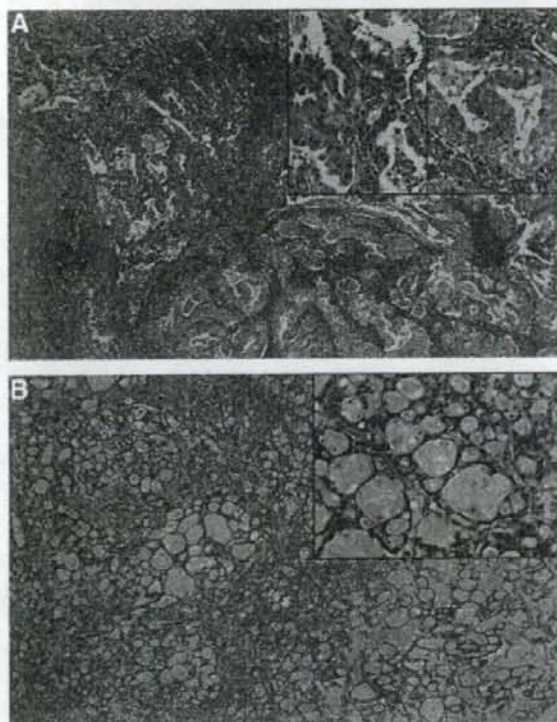


FIGURE 2. Representative examples of histologic features. Both the 2 variant 1 cases were mixed type adenocarcinomas, with papillary and bronchioloalveolar components (A). All 3 variant 2 cases were acinar adenocarcinomas (B).

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.

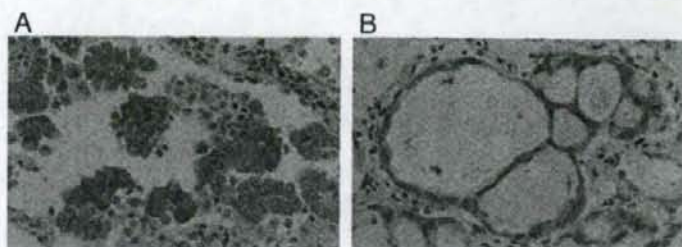


TABLE 2. Relationship between EML4-ALK Fusion and Clinicopathologic and Genetic Features in Lung Adenocarcinomas

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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Key words: ovarian cancer, clear cell carcinoma, glutathione peroxidase 3, cisplatin, drug resistance

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'-CGCGGGGCTCTCAGAATCATCAT-3' and 5'-CCAGCCCCAGCGTCAAAGGTG-3'; glutathione peroxidase 3 (GPX 3) cDNA, 5'-AGCAGTATGCTGGCAA ATATGTCC-3' and 5'-CAGACCGAATGGTGAAGCTC TTC-3'.

Selection of short hairpin RNA stable cell lines. The DNA oligonucleotides, encoding short hairpin RNA (shRNA) targeting the GPX3 (forward; CACCGGGAGAGTTGAC TATTAACGTGTGCTGTCCGTTAATGGTGCAAGCTCT TCCTTTTT, reverse; GCATAAAAAGGAAGAGCTTGCA CCATTAACGGACAGCACAGTAAATAGTGCAAACTC 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

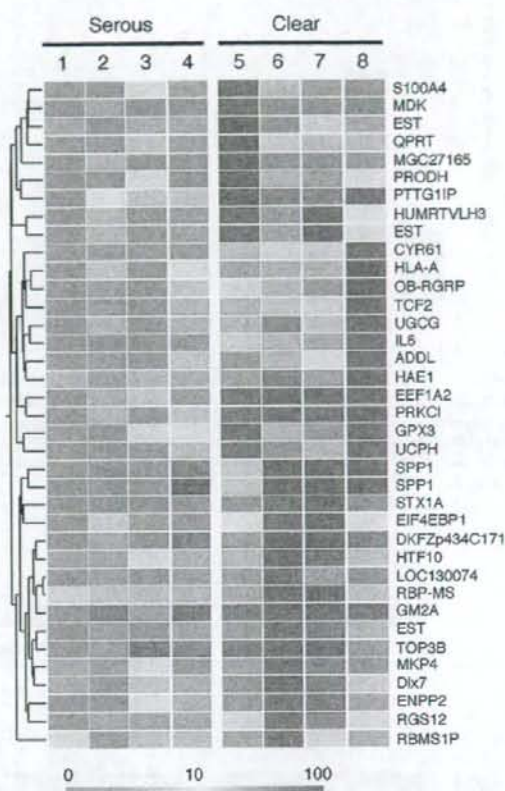


Figure 1. Identification of clear cell adenocarcinoma-specific genes. Hierarchical clustering of 34 probe sets were performed on the basis of their expression profiles in serous adenocarcinoma cell lines [HRA (1), KF (2), KOC-2S (3) and SHIN-3 (4)] and clear cell adenocarcinoma cell lines [KK (5), OVMANA (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

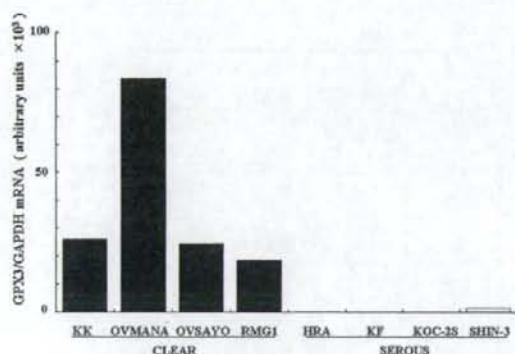


Figure 2. Quantitation of *GPX3* transcripts in ovarian cancer cell lines. Complementary DNA prepared from the ovarian cancer cells was subjected to real-time RT-PCR with primers specific for *GPX3* or *GAPDH* genes. The ratio of the abundance of the *GPX3* transcript to that of *GAPDH* mRNA was calculated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

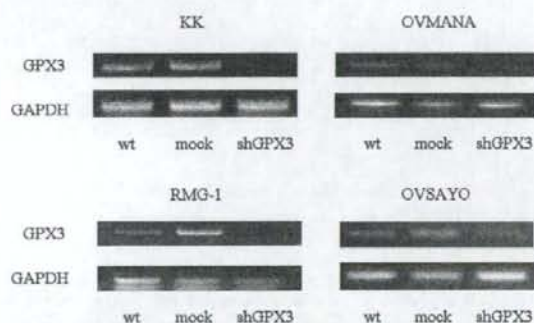


Figure 3. Expression of *GPX3* in clear cell adenocarcinoma transfectants. *GPX3* expression by the semiquantitative RT-PCR method was decreased in cells transfected with the shRNA expression plasmid in all 4 clear cell adenocarcinoma cell lines compared with the parent and control cell lines. The integrity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

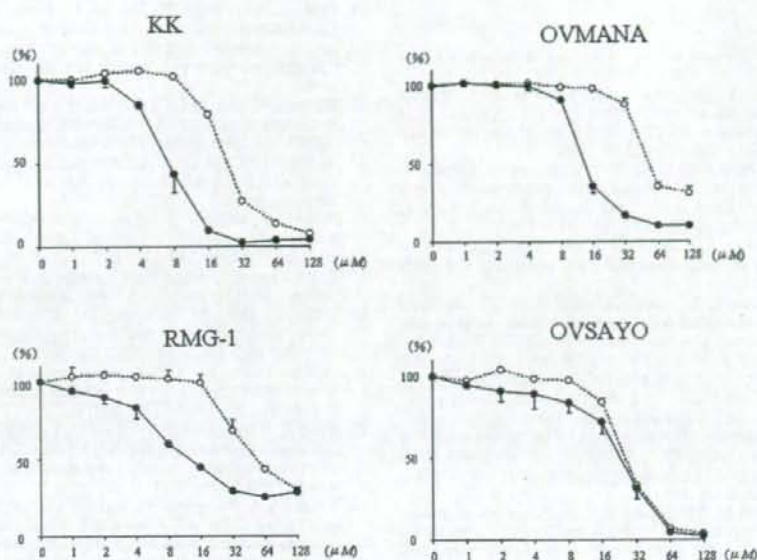


Figure 4. Dose-response curves of the 4 types of clear cell adenocarcinoma transfectants to cisplatin. Open circle, control cells; solid circle, cells transfected with the shRNA expression plasmid. The average of three independent experiments is shown and error bars indicate SD.

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-1B (HNF-1B) for the candidate gene based on DNA microarray analysis of 4 clear cell adenocarcinoma cell lines. They suggested that *HNF-1B* is a gene involved in the low sensitivity, based on the findings that HNF-1B was highly expressed in many patients with clear cell adenocarcinoma and inhibition of HNF-1B expression induced apoptosis of clear cell adenocarcinoma cells *in vitro*. Although association of *HNF-1B* 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. Ligand 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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