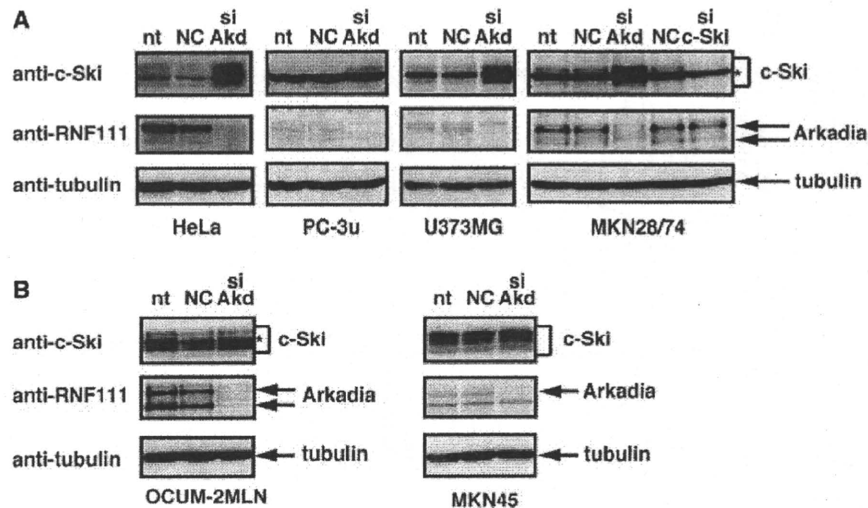

Fig. 3 Effect of knockdown of Arkadia on c-Ski protein expression. (A and B) Expression of endogenous Arkadia was knocked down by transfection of siArkadia oligonucleotide into the indicated tumour cell lines. Lysates from non-transfected cells (nt), control oligonucleotide-transfected cells (NC) and Arkadia-knocked down cells (siAkd) were subjected to SDS–PAGE followed by immunoblotting with anti-c-Ski (top panels). Bands of c-Ski were confirmed by siRNA of c-Ski in MKN28/74 cells (right two lanes in the right top panel of A). Knockdown of Arkadia was confirmed by immunoblotting with anti-RNF111 (middle panels). Asterisks denote non-specific bands.

gene amplification of c-Ski or SnoN (*29*, *33*, *34*). As shown in Fig. 2B and C, mRNA levels of c-Ski and SnoN varied markedly among the cell lines tested. Levels of c-Ski mRNA differed nearly 8-fold between EBC-1 and MKN7 (Fig. 2B, top panel), and levels of SnoN mRNA differed nearly 10-fold between KATOIII and MCF7 (Fig. 2C, top panel). Levels of expression of c-Ski/SnoN proteins were also varied markedly among these cancer cell lines. When bands of immunoblotting were quantified, the levels of expression of c-Ski protein differed 14-fold between A549 and OCUM-2M (Fig. 2B, second panel), and those of SnoN protein differed >30-fold between EBC-1 and OCUM-2MD3 (Fig. 2C, second panel).

In some cell lines, the levels of expression of c-Ski were high at the mRNA but low at the protein level (MKN7 and MKN28/74), whereas in other cell lines they were low at the mRNA level but high at the protein level (OCUM-2M, OCUM-2D, OCUM-2MLN, KATOIII, MKN45 and A375). Similar results were obtained for SnoN (Fig. 2C; A549 and MCF7).

Arkadia was thus ubiquitously expressed in various cancer cell lines at both the mRNA and protein levels, whereas in some cell lines levels of expression of c-Ski and SnoN varied at both mRNA and protein levels and levels of expression of mRNA and protein were not correlated.

Dysfunction of Arkadia in degradation of c-Ski in some cancer cell lines

Since Arkadia was expressed in all the 22 cell lines tested, we examined whether Arkadia functions as an E3 ubiquitin ligase in these cell lines. We knocked down Arkadia and determined protein expression of c-Ski. c-Ski protein accumulated in MDA-MB-231 (Fig. 1A), HeLa, PC-3u, U373MG and MKN28/74 cells upon knockdown of Arkadia (Fig. 3A), indicating that Arkadia down-regulates c-Ski in these types of cells. However, as shown in Fig. 3B, accumulation of c-Ski protein was not observed in OCUM-2MLN and MKN45 cell lines upon silencing of Arkadia. These findings suggest that Arkadia does not degrade c-Ski in some cancer cell lines. The high levels of expression of c-Ski protein in these cell lines can be attributed to dysfunction of Arkadia.

Enhancement of TGF-β target gene expression by endogenous Arkadia

We next examined mRNA expression of TGF-β target genes upon knockdown of Arkadia. HeLa cells were transfected with siRNA oligonucleotide and treated with TGF-β for the indicated periods of time. SnoN is one of the target genes of TGF-β (*42*), and, as shown in Fig. 4A, induction of SnoN mRNA was reduced (left panel) when expression of Arkadia was silenced (right panel), suggesting that endogenous Arkadia contributes to enhancement of TGF-β signalling. We also used OCUM-2MLN cells in which c-Ski protein did not accumulate upon knockdown of Arkadia. As shown in Fig. 4B, induction of target genes including *SnoN* (left top panel), *PAI-1* (left bottom panel) and *Smad7* (right bottom panel) was attenuated when Arkadia was silenced (right top panel). These findings suggest that Arkadia functions as an enhancer of TGF-β signalling in OCUM-2MLN cells, although it does not function as an E3 ubiquitin ligase for c-Ski.

Reduction of c-Ski protein expression by exogenous Arkadia

We further performed gain-of-function experiments, and examined the effects of exogenous Arkadia on c-Ski protein expression. HepG2 cells were used since the levels of expression of c-Ski and SnoN proteins

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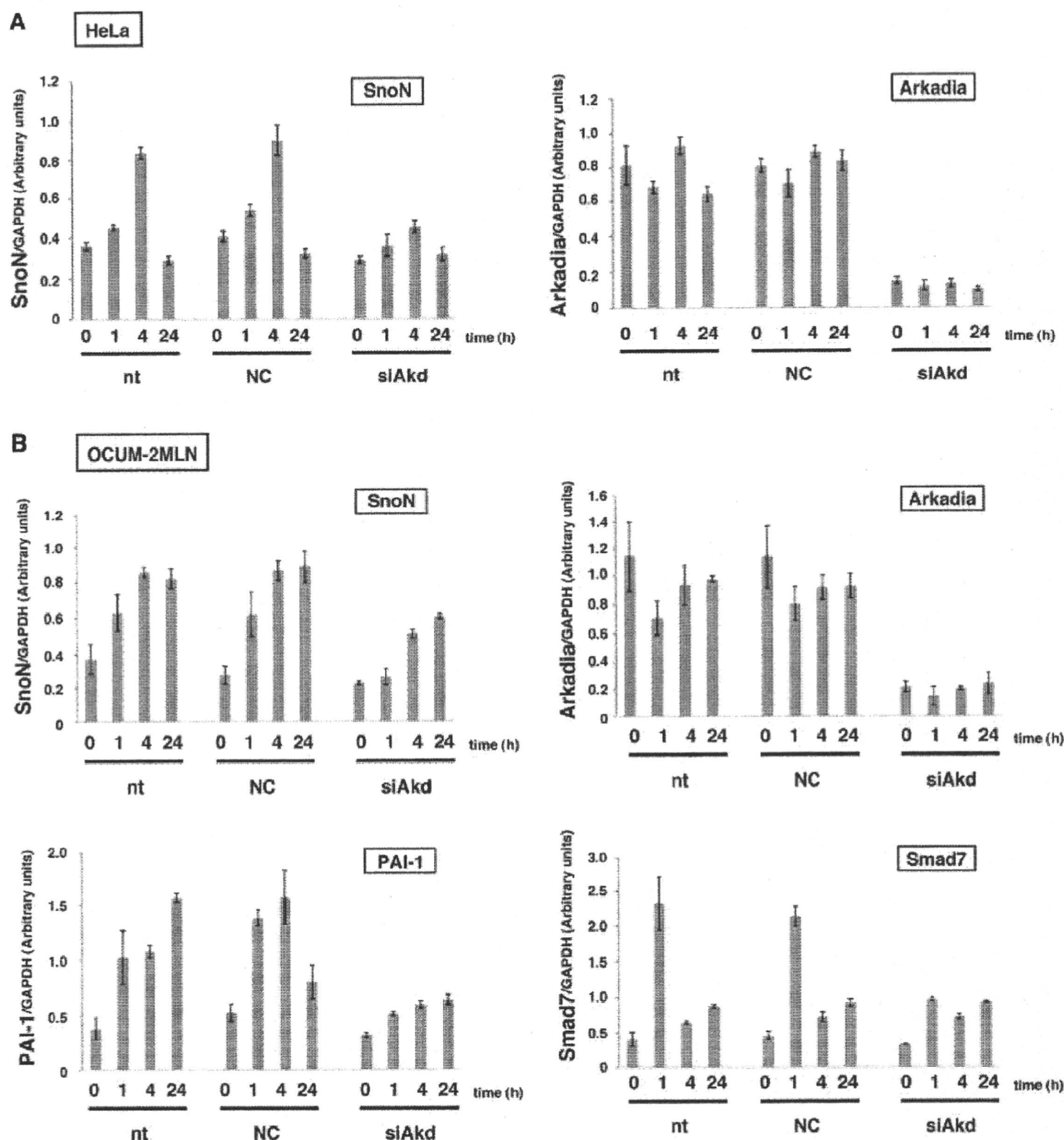

Fig. 4 Knockdown of Arkadia attenuates induction of TGF-β target genes. (**A** and **B**) HeLa cells (A) and OCUM-2MLN cells (B) were transfected with siArkadia oligonucleotide (siAkd) for silencing of endogenous Arkadia expression, or control oligonucleotide (NC), or remained untreated (nt). Cells were treated with 1 ng/ml TGF-β and harvested at the indicated time points. mRNA expression of SnoN, Arkadia, PAI-1 and Smad7 was determined by real-time RT–PCR. Vertical axis shows relative expressions of these genes normalized to human GAPDH.

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were high and that of Arkadia was low in them (Fig. 2). OCUM-2MLN cells were also used since endogenous Arkadia did not contribute to degradation of endogenous c-Ski protein in them (Fig. 3B). Wild-type Arkadia (WT) or a RING finger domain-deleted mutant of Arkadia (ΔC) was expressed in these cells using a lentivirus vector expression system. Expression of Arkadia was confirmed using anti-Arkadia antibody. As shown in Fig. 5 (top panels), expression of exogenous Arkadia was higher than that of endogenous Arkadia in control cells. In both cell lines examined, c-Ski protein was down-regulated in cells expressing Arkadia-WT but up-regulated in those expressing Arkadia-ΔC. These findings showed that exogenous Arkadia induced degradation of endogenous c-Ski in these cells through its ubiquitin ligase activity.

Inhibition of growth of HepG2 cells by Arkadia in the presence and absence of TGF-β stimulation

To examine the effects of Arkadia in cancer cells, growth assay was performed using cells that express

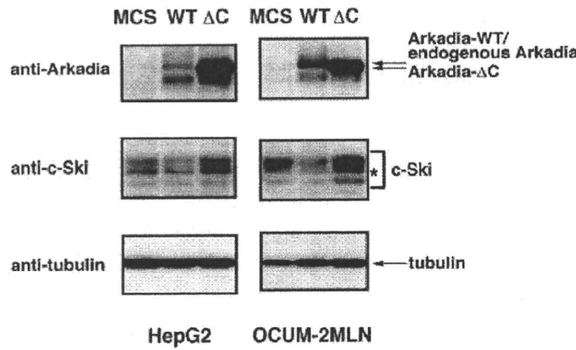

Fig. 5 Exogenous Arkadia reduces protein expression of c-Ski. HepG2 and OCUM-2MLN cells were infected with lentivirus harbouring multi-cloning site control (MCS), Arkadia-WT (WT) or Arkadia-ΔC (ΔC). Lysates from these cells were subjected to SDS–PAGE followed by immunoblotting with anti-Arkadia antibody (top panels), anti-c-Ski antibody (middle panels) and anti-tubulin antibody (bottom panels). Asterisk denotes non-specific bands.

Arkadia-WT or Arkadia-ΔC. We used HepG2 cells, since OCUM-2MLN cells do not respond to TGF-β for growth inhibition (*38*). As previously reported (*43*), growth of HepG2 cells was inhibited by treatment with TGF-β (Fig. 6A MCS). Cell growth was inhibited by expression of Arkadia-WT but not by Arkadia-ΔC in the absence of ligand. In addition, Arkadia-WT, but not Arkadia-ΔC, enhanced TGF-β-induced growth inhibition. These findings suggest that Arkadia represses HepG2 cell growth in the presence as well as the absence of TGF-β.

We then examined mRNA expression of TGF-β target genes in Arkadia-expressing HepG2 cells. Control cells (MCS), wild-type Arkadia-expressing cells (WT) and Arkadia-ΔC-expressing cells (ΔC) were treated with TGF-β for the indicated periods of time. Arkadia-WT, but not ΔC, enhanced induction of target genes of TGF-β, including *SnoN* and *Smad7* (Fig. 6B, top panels), suggesting that ectopic Arkadia enhanced TGF-β signalling through its C-terminal RING domain. Since TGF-β has been reported to

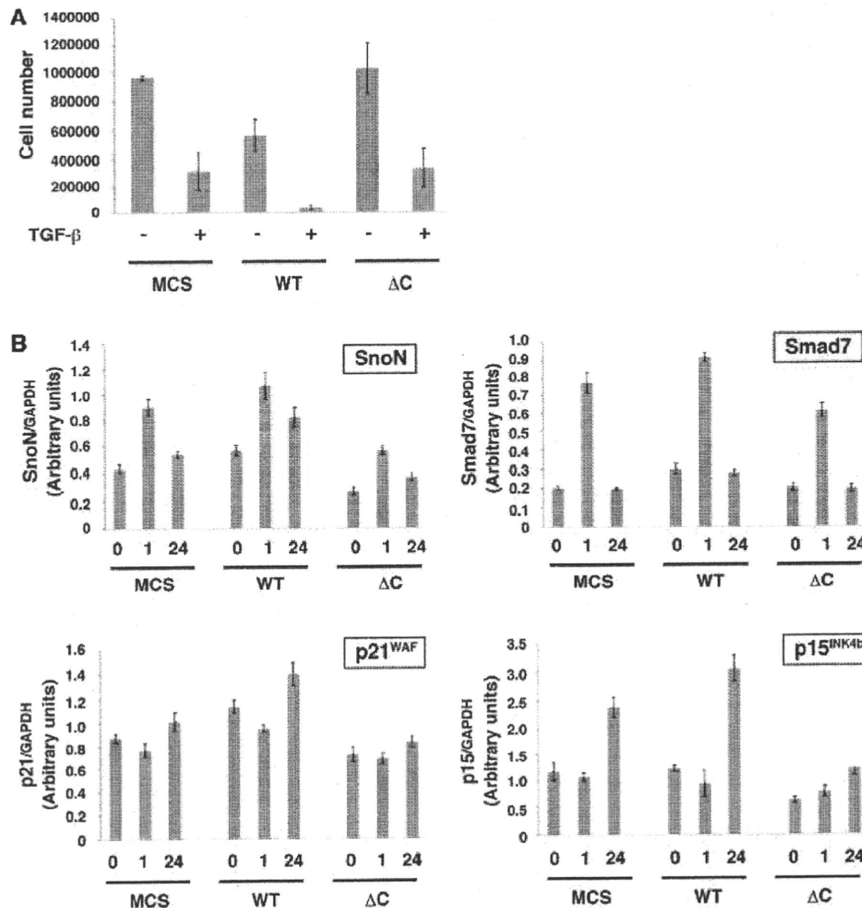

Fig. 6 Arkadia inhibits HepG2 cell growth. (A) Cell growth assay was performed as previously described (*43*). Numbers of HepG2 cells expressing Arkadia-WT (WT) or Arkadia-ΔC (ΔC) and control cells (MCS) were counted at day 4 with or without treatment with 0.5 ng/ml TGF-β. (**B**) HepG2 cells expressing Arkadia-WT (WT) or Arkadia-ΔC (ΔC) or control cells (MCS) were treated with 0.5 ng/ml TGF-β for the indicated periods of time. mRNA expressions of p21, p15, SnoN and Smad7 were determined by real-time RT–PCR. Vertical axis shows relative expressions of these genes normalized to human GAPDH.

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inhibit cell growth by regulating expression of cell cycle regulators (44), we examined the expression of $p21^{WAF}$ and $p15^{INK4b}$. As shown in Fig. 6B, expression of $p21^{WAF}$ was up-regulated in WT cells but not in ΔC cells, in the presence as well as the absence of TGF-β stimulation (left bottom panel). Expression of $p15^{INK4b}$ was also up-regulated in WT cells but not in ΔC cells in the presence of TGF-β stimulation (right bottom panel). These findings suggest that Arkadia inhibits HepG2 cell growth, at least in part through induction of $p21^{WAF}$ and $p15^{INK4b}$.

Discussion

c-Ski has been reported to undergo degradation in response to TGF-β stimulation (41). The E3 ubiquitin ligases involved in this process have yet to be fully determined. Recently, Le Scolan *et al.* (45) reported that knockdown of Arkadia abrogated TGF-β-induced degradation of c-Ski, suggesting that Arkadia is responsible for the degradation of c-Ski. In our study, however, knockdown of Arkadia in MDA-MB-231 cells failed to attenuate the decrease in c-Ski protein upon TGF-β stimulation (Fig. 1A). We also found that TGF-β-stimulation resulted in down-regulation of c-Ski in *Arkadia*$^{-/-}$ MEFs (Fig. 1B), although the down-regulation was incomplete. These findings suggest that ubiquitin ligase(s) other than Arkadia are involved in TGF-β-induced degradation of c-Ski protein, at least under some experimental conditions. The differential effects of E3 ubiquitin ligases may depend on cell type or cellular context.

Arkadia was expressed broadly in various cancer cell lines. In contrast, the levels of expression of c-Ski/SnoN protein varied markedly among these cancer cells (Fig. 2). Interestingly, in some cancer cell lines examined, levels of expression of c-Ski/SnoN at the mRNA and protein levels were not correlated. These findings suggest that c-Ski and SnoN are regulated at the post-transcriptional level in these cancer cells. The lack of correlation between expressions of c-Ski/SnoN at the mRNA and protein levels may be due in part to dysfunction of Arkadia, since Arkadia did not degrade c-Ski protein in some of these cancer cell lines. Regulation by ubiquitin ligases other than Arkadia or regulation at translational level may also account for this lack of correlation.

In OCUM-2MLN cells, Arkadia degrades neither c-Ski protein (Fig. 3B) nor SnoN protein (our unpublished data), but does enhance TGF-β signalling (Fig. 4B). These findings indicate that endogenous Arkadia enhances TGF-β signalling through ubiquitylation of substrates other than c-Ski or SnoN. In HepG2 cells, c-Ski, SnoN and Smad7 are important substrates of Arkadia in maximal enhancement of TGF-β signalling (13). It remains to be determined whether Arkadia degrades Smad7 in OCUM-2MLN cells, since Smad7 protein was not detected by immunoblotting in the present study (data not shown). Thus, the possibility cannot be excluded that substrate(s) of Arkadia other than c-Ski, SnoN, or Smad7 are involved in the negative regulation of TGF-β signalling in OCUM-2MLN cells.

The question why Arkadia does not degrade c-Ski in OCUM-2MNL cells remains to be addressed. Expression of c-Ski protein was reduced when Arkadia was ectopically expressed in OCUM-2MLN cells (Fig. 5). Endogenous c-Ski in these cells is thus sensitive to degradation by Arkadia. We detected no mutations in the RING finger domain of endogenous Arkadia in OCUM-2MLN cells (data not shown), consistent with the finding that Arkadia still enhances TGF-β signalling in these cells. It is possible that in OCUM-2MLN cells Arkadia harbors mutation(s) in its c-Ski/SnoN-interacting region. Alternatively, endogenous Arkadia in these cells may be post-translationally modified and thereby lose its effects on c-Ski/SnoN. Investigation of the mutations and intracellular modifications of Arkadia is of importance for further understanding of the regulation of TGF-β signalling in cancer cells.

Overexpression of Arkadia inhibited basal growth of HepG2 cells. We found that expression of $p21^{WAF}$ was higher in HepG2 cells overexpressing Arkadia-WT than in control cells or cells overexpressing Arkadia-ΔC in the absence of TGF-β stimulation (Fig. 6B). It remains to be determined how Arkadia enhances the expression of $p21^{WAF}$ in the absence of TGF-β stimulation. Arkadia may have substrates other than c-Ski, SnoN or Smad7 when it inhibits the basal growth of HepG2 cells, although overexpression of Arkadia may exhibit non-physiological effects.

In the present study, we have shown that endogenous as well as exogenous Arkadia positively regulates the expression of TGF-β target genes in HeLa, OCUM-2MLN and HepG2 cells. In addition, we found that Arkadia inhibits the growth of HepG2 cells in the presence of TGF-β stimulation. Arkadia may function as a tumor suppressor by inhibiting the growth of tumour cells that are sensitive to TGF-β-induced cytostasis. Examination of the roles of Arkadia in late-stage cancer will also be needed in the near future. Further analysis will reveal how Arkadia regulates the dual effects of TGF-β on tumourigenesis and cancer development.

Supplementary Data

Supplementary data are available at JB online.

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Conflict of interest
None declared.

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Successful Treatment with Pemetrexed in a Patient with Mucinous Bronchioloalveolar Carcinoma

Long-Term Response Duration with Mild Toxicity

Chiyuki Okuda, MD, Young Hak Kim, MD,* Kengo Takeuchi, MD, PhD,† Yosuke Togashi, MD,* Katsuhiro Masago, MD, PhD,* Yuichi Sakamori, MD,* Tadashi Mio, MD, PhD,* and Michiaki Mishima, MD, PhD**

A 36-year-old female former smoker presented with a productive cough. One year after visiting our hospital in February 2008, chest computed tomography (CT) revealed diffuse bilateral pulmonary nodules combined with a consolidation shadow (Figure 1*A*). A definite diagnosis could not be made by bronchofiberscopic examination; however, CT-guided needle biopsy specimens revealed bronchioloalveolar carcinoma (BAC) of mucinous subtype (Figure 2). Her clinical stage was T4N2M1a according to the seventh edition of the tumor, node, metastasis classification. Activating epidermal growth factor receptor (EGFR) gene mutations were not detected in her biopsy specimens. She received chemotherapy with gemcitabine and carboplatin as her first-line chemotherapy; however, her disease progressed after four cycles of chemotherapy. She then received further chemotherapy with docetaxel, erlotinib, paclitaxel, and irinotecan; however, neither regimen was effective, and her symptoms worsened. In May 2009, pemetrexed was approved for non-small cell lung cancer in Japan and was chosen as her sixth-line regimen and started in June 2009. The initial dose of pemetrexed was 500 mg/m^2 with vitamin B_{12} and folic acid supplementation. Chest CT after the two cycles of chemotherapy showed a radiographic response, and her symptoms also improved. The dose of pemetrexed was reduced to 400 mg/m^2 from the fourth cycle because of grade 3 liver dysfunction (Common Terminology Criteria for Adverse Events, version 3). Other adverse events were urticaria, skin hyperpigmentation, and general fatigue; however, they were all generally mild. Chest CT showed continuous improvement (Figure 1*B*, *C*), and her liver function has been stable since the dose reduction. Pemetrexed is currently being administered for its 20th cycle, and she is doing very well.

*Department of Respiratory Medicine, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto; and †Division of Pathology, The Cancer Institute, Japanese Foundation for Cancer Research, Koto-ku, Tokyo, Japan.

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Address for correspondence: Young Hak Kim, MD, Department of Respiratory Medicine, Graduate School of Medicine, Kyoto University, 54 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: ekim@kuhp.kyoto-u.ac.jp

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DISCUSSION

BAC is a distinctive form of lung adenocarcinoma and is further divided into two subtypes: mucinous and nonmucinous.[1] Although approximately 20% of adenocarcinomas have BAC features, "pure" BAC represents less than 5% of adenocarcinomas.[2] Historically, BAC was believed to be rather refractory to cytotoxic chemotherapy, and it is still debatable whether cytotoxic chemotherapy is equally effective in BAC and other types of adenocarcinoma.[2] Recently, it became widely known that the frequency of activating mutations of EGFR, the strongest predictive factor of a response to EGFR tyrosine kinase inhibitors, such as gefitinib and erlotinib, is significantly higher in BAC[3]; however, the frequency of EGFR mutations is significantly lower in the mucinous subtype than in the nonmucinous subtype.[4]

Our patient presented with BAC of the mucinous subtype, and her tumor did not express activating EGFR mutations. She received multiple lines of chemotherapy, including platinum based, docetaxel, and erlotinib; however, only pemetrexed was effective.

Pemetrexed is a multitargeted antifolate agent and has been approved as standard first-line (combination with platinum) and second-line chemotherapy for non-small cell lung cancer, and more recently, maintenance chemotherapy with pemetrexed has been under debate. Interestingly, pemetrexed is significantly more effective for nonsquamous than squamous histology.[5] One possible explanation is that the expression of thymidylate synthase, one of the molecular targets of pemetrexed, is generally higher in squamous than nonsquamous histology; however, it needs further confirmation.

We performed immunohistochemical examination to detect the echinoderm microtubule-associated protein-like 4 gene and the anaplastic lymphoma kinase gene, fusion gene, using the intercalating antibody-enhanced polymer

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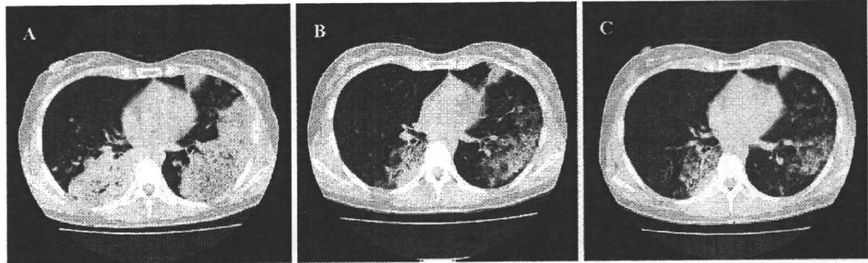

FIGURE 1. Computed tomography (CT) of the chest showed diffuse bilateral pulmonary nodules combined with consolidation shadow before pemetrexed treatment (*A*). CT after 12 cycles (*B*) and 18 cycles (*C*) showed continuous improvement of the shadow.

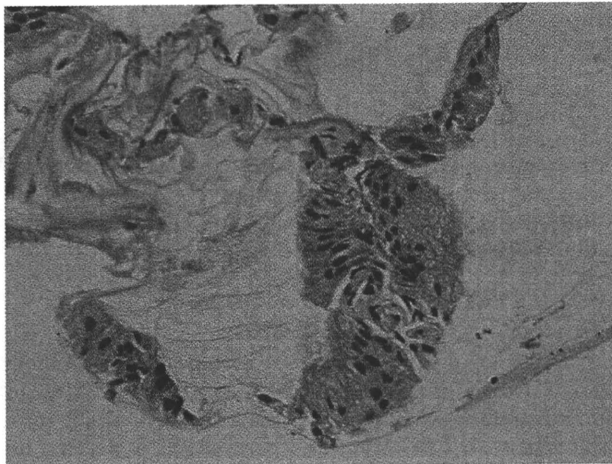

FIGURE 2. Biopsy specimen. There are a few fragments of columnar tumor cells containing mucin in the cytoplasm (hematoxylin and eosin stained).

method[6]; however, her tumor did not harbor the echinoderm microtubule-associated protein-like 4 gene and the anaplastic lymphoma kinase gene, fusion gene.

Finally, pemetrexed was safely administered for more than 1 year to our patient without deterioration of the performance status. This may also indicate the usefulness of pemetrexed as maintenance chemotherapy. Further investigations of pemetrexed are needed in patients with BAC.

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Early Release Paper

Identification of a novel fusion, SQSTM1-ALK, in ALK-positive large B-cell lymphoma

by Kengo Takeuchi, Manabu Soda, Yuki Togashi, Yasunori Ota, Yasunobu Sekiguchi, Satoko Hatano, Reimi Asaka, Masaaki Noguchi, and Hiroyuki Mano

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Identification of a novel fusion, SQSTM1-ALK, in ALK-positive large B-cell lymphoma

Running title: SQSTM1-ALK-positive large B-cell lymphoma

Kengo Takeuchi,[1] Manabu Soda,[2] Yuki Togashi,[1] Yasunori Ota,[3] Yasunobu Sekiguchi,[4] Satoko Hatano,[1] Reimi Asaka,[1] Masaaki Noguchi,[4] and Hiroyuki Mano[2,5]

[1]Pathology Project for Molecular Targets, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo 135-8550, Japan; [2]Division of Functional Genomics, Jichi Medical University, Tochigi 329-0498, Japan; [3]Department of Pathology, Toranomon Hospital, Tokyo 105-8470, Japan; [4]Department of Hematology, Urayasu Hospital, Juntendo University, 279-0021 Chiba, Japan, and [5]CREST, Japan Science and Technology Agency, Saitama 332-0012, Japan

Correspondence

Kengo Takeuchi, M.D., Ph.D. Pathology Project for Molecular Targets, Cancer Institute, Japanese Foundation for Cancer Research. 3-8-31 Ariake, Koto, Tokyo 135-8550, Japan.
Phone: international +81.3.35200111. Fax: international +81.3.35700558.
E-mail: kentakeuchi-tky@umin.net

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Abstract

ALK-positive large B-cell lymphoma is a rare subtype of lymphoma, and most cases follow an aggressive clinical course with a poor prognosis. We examined an ALK-positive large B-cell lymphoma case showing an anti-ALK immunohistochemistry pattern distinct from those of 2 known ALK fusions, CLTC-ALK and NPM-ALK, for the presence of a novel ALK fusion; this lead to the identification of SQSTM1-ALK. SQSTM1 is an ubiquitin binding protein that is associated with oxidative stress, cell signaling, and autophagy. We showed transforming activities of SQSTM1-ALK with a focus formation assay and an in vivo tumorigenicity assay using 3T3 fibroblasts infected with a recombinant retrovirus encoding SQSTM1-ALK. ALK-inhibitor therapies are promising for treating ALK-positive large B-cell lymphoma, especially for refractory cases. SQSTM1-ALK may be a rare fusion, but our data provide novel biological insights and serve as a key for the accurate diagnosis of this rare lymphoma.

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Introduction

Anaplastic lymphoma kinase-positive large B-cell lymphoma (ALK+LBCL) is a rare subtype of lymphoma that was first described in 1997.(1) Approximately 50 cases have been reported to date,(2) with most cases (60%) following an aggressive clinical course.(3) In well-characterized cases, 3 genes have been reported as a fusion partner of *ALK*: *clathrin* (*CLTC-ALK*),(4-6) *nucleophosmin* (*NPM-ALK*),(7-8) and *SEC31A* (*SEC31A-ALK*).(9) In this paper, we report a case of ALK+LBCL that harbored a novel ALK fusion partner, sequestosome1 (SQSTM1).

Design and Methods

Materials

Biopsied specimens were fixed in 20% neutralized formalin and embedded in paraffin for conventional histopathological examination. We extracted DNA and total RNA from the snap-frozen specimens and subsequently purified the samples. Written informed consent was obtained from the patient. The study was approved by the Institutional Review Board of the Japanese Foundation for Cancer Research.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue was used. For antigen retrieval, we heated the slides for 40 min at 97°C in Target Retrieval Solution (pH 9.0; Dako), and subsequently detected the immune complexes with a dextran polymer reagent (EnVision+DAB system, Dako) and an AutoStainer instrument (Dako).

Isolation of *ALK* fusion cDNA

To obtain cDNA fragments corresponding to novel ALK fusion genes, we used an inverse reverse transcription-polymerase chain reaction (RT-PCR) method slightly modified from one previously reported.(10) Double-stranded cDNA was synthesized from 2 μg of total RNA with 1 pM of the primer ALKREVex22-23 (5′-TGGTTGAATTTGCTGATGATC-3′) and a cDNA Synthesis System (Roche), and was self-ligated by incubation overnight with T4 DNA ligase (TaKaRa Bio). We subjected the resulting circular cDNA to PCR (35 cycles of 94°C for 15 sec, 62°C for 30 sec, and 72°C for 1 min) with primers ALKREV3T (5′-CTGATGGAGGAGGTCTTGCC-3′) and ALKFWDex20-21

(5′-ATTCGGGGTCTGGGCCAT-3′) in a final volume of 20 μl. We subjected 1 μl of the 1:100 diluted reaction products to a second PCR step (the same settings as above), with primers ALKREV4T (5′-GGTTGTAGTCGGTCATGATGGTC-3′) and ALKFWDex21-22 (5′-AGTGGCTGTGAAGACGCTGC-3′) in a final volume of 20 μl. The resulting products were purified by gel extraction and directly sequenced in both directions with primers ALKFWDex20-21 and ALKREV4T.

The fusion point of *SQSTM1-ALK* cDNA was amplified by RT-PCR with primers SQSTM1 565F (5′-AAACACGGACACTTCGGGT-3′) and ALK3078RR (5′-ATCCAGTTCGTCCTGTTCAGAGC-3′).

Full-length *SQSTM1-ALK* cDNA was obtained from the specimen by RT-PCR with primers SQSTM1v1-F90 (5′-CTCGCTATGGCGTCGCTCACCGTGAA-3′) and KA-W-cDNA-out-AS (5′-CCACGGTCTTAGGGATCCCAAGG-3′).

Fluorescence in situ hybridization (FISH)

We performed FISH analysis of the gene fusion for unstained slides (4 μm thick) with bacterial artificial chromosome (BAC) clone-derived DNA probes for *ALK* (RP11-984I21, RP11-62B19) and *SQSTM1* (RP11-55M16).

Transformation assay for ALK fusion protein

We performed analysis of the transforming activity of SQSTM1-ALK as described previously.(11-13) Briefly, cDNA for SQSTM1-ALK was inserted into the retroviral expression plasmid pMXS.(14) The resulting plasmid and similar pMXS-based expression plasmids for EML4-ALK variant 1 or NPM-ALK were used to generate recombinant ecotropic retroviruses, which were then used to infect mouse 3T3 fibroblasts. We evaluated formation of transformed foci after culturing the cells for 14 days. We subcutaneously injected the same set of 3T3 cells into nu/nu mice and examined tumor formation after 20 days.

PCR for *IGH* gene rearrangement

Genomic PCR was used for amplification of the rearranged *IGH* gene using the primers FR2A 5′-TGG(A/G)TCCG(A/C)CAG(C/G)C(C/T)(C/T)CNGG-3′ and LJH 5′-ACCTGAGGAGACGGTGACC-3′. Several clones were sequenced after subcloning the PCR product into pGEM-T-Easy Vector (Promega).

Results and Discussion

Case presentation

A 67-year-old man was admitted with a tumor in the left side of his neck. A systemic workup revealed swelling of cervical, mediastinal, and hilar lymph nodes. Blood counts were within normal ranges. Lactose dehydrogenase was slightly elevated (223 IU/L) in peripheral blood with high IgG (2,425 mg/dL), normal IgA (157 mg/dL) and low IgM (32 mg/dL) levels.

Histopathological examination of the biopsied specimen from the cervical lymph node showed a diffuse infiltrate of tumor cells with a round, vesicular nucleus containing a centrally located large nucleolus. The cytoplasm was abundant (Figure 1A). These features may be consistent with immunoblasts or plasmablasts, but the size of tumor cells was large compared with typical immunoblasts and plasmablasts. Immunophenotypically, the tumor cells were negative for CD3, CD4, CD5, CD10, CD20, CD57, CD79a, and most cytokeratins (CK5/6, CK8, CK19, CK20); focally positive for CD30 and cytokeratins (AE1/AE3, CAM5.2, CK7, CK18) (Figure 1B); weakly positive for PAX5; and positive for CD138 (Figure 1C), EMA, and ALK (Figure 1D). The positivity of focal cytokeratin, which has been reported in a small proportion of ALK+LBCL cases,(15) and the cytomorphology of this case may have led to a misdiagnosis of undifferentiated metastatic carcinoma. The presence of *ALK* translocation was demonstrated by an ALK split FISH assay, which was performed at a commercial laboratory (data not shown). The tumor cells were positive for PAX5, which is suggestive of ALK+LBCL. However, we carefully excluded a possibility of metastasis of ALK-positive lung cancer(10) because the tumor cell were positive for some cytokeratins and immunohistochemistry for immunoglobulins was not evaluable due to background staining. Immunohistochemistry for TTF1 was negative, which is usually positive in ALK-positive lung cancers.(16) In addition, PCR and sequencing analyses revealed that *IGH* was monoclonally rearranged and somatically hypermutated (data not shown).

The patient was diagnosed as having ALK+LBCL and achieved complete remission after 6 cycles of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) treatment. Four months later, however, he relapsed.

Identification of SQSTM1-ALK

The 2 major ALK fusions in ALK+LBCL are CLTC-ALK and NPM-ALK, and they show a coarse granular cytoplasmic pattern and a nuclear and cytoplasmic pattern in anti-ALK immunohistochemistry, respectively. In the present case, anti-ALK immunohistochemistry showed a diffuse cytoplasmic staining pattern with ill-demarcated spots (Figure 1D), which was different from either of the former 2 patterns. Therefore, we carried out inverse RT-PCR to examine the presence of a novel fusion of *ALK*. We indeed isolated a cDNA containing the exon 5 of *SQSTM1* in-frame fused to the exon 20 of *ALK* (Figure 2A). A separate RT-PCR assay amplified the fusion point of *SQSTM1-ALK* cDNA (data not shown). To confirm the chromosome rearrangement, we performed *SQSTM1-ALK* fusion FISH. This result was consistent with the presence of a t(2;5)(p23.1;q35.3) leading to the generation of *SQSTM1-ALK* (Figure 2B). The complete sequences of SQSTM1-ALK are shown in Supplementary Figure 1.

SQSTM1 is a ubiquitin binding protein that is associated with oxidative stress, cell signaling, and autophagy.(17-20) Autophagosomal membrane protein LC3/Atg8 binds SQSTM1 and makes SQSTM1-containing protein aggregate to the autophagosome.(21) Mutations within *SQSTM1* are identified in patients with Paget disease of bone.(22)

SQSTM1 is located very near *NPM*, which is on 5q35.1. Therefore, the cytogenetic findings of the NPM-ALK-positive and the SOSTM1-ALK-positive lymphomas may be similar, because of which SQSTM1-ALK occurrence in lymphoma may be underestimated. As mentioned, however, NPM-ALK and SQSTM1-ALK differ in terms of the anti-ALK immunostaining pattern. NPM has a nuclear transport signal, while SQSTM1 does not. Therefore, NPM-ALK shows a nuclear and cytoplasmic staining pattern while SQSTM1-ALK shows only a cytoplasmic staining pattern. ALK is a representative “promiscuous” molecule because of its various fusion partners. The subcellular localization of ALK fusions depends on the fusion partners. The anti-ALK immunohistochemical staining pattern is, therefore, a simple and useful means to identify the possible partner in a tested case, and in fact, has prompted the identification of many ALK fusion partners, including the present case.

Transforming activities of SQSTM1-ALK

We generated a recombinant retrovirus encoding SQSTM1-ALK and used it to infect cultured 3T3 fibroblasts. Infection with the virus, but not with an empty virus, resulted in the formation of multiple transformed foci in vitro (Figure 2C). As control experiments for formation, EML4-ALK (variant 1) and NPM-ALK similarly produced transformed foci (data not shown). The same 3T3 cells were injected into nude mice for an in vivo tumorigenicity assay. As expected, 3T3 cells expressing SQSTM1-ALK developed subcutaneous tumors at all injection sites within an observation period of 20 days (Figure 2D), confirming the transforming potential of the novel fusion kinase, SQSTM1-ALK.

All ALK fusion partners identified so far except moesin (MSN) have a coiled-coil domain(s) in their sequences, and the domain is conserved in its fusion form. The coiled-coil domain allows the protein to homodimerize. The tyrosine kinase domain of the ALK fusions is constitutively phosphorylated and activated through homodimerization via the coiled-coil domain. It has been speculated that the binding properties of MSN to cell membrane proteins lead to the dimerization of MSN-ALK proteins, enabling the constitutive phosphorylation of the chimeric MSN-ALK protein. (23) SQSTM1 does not harbor a coiled-coil domain and does not bind to membrane proteins. Instead, it has the Phox and Bem1p (PB1) domain in its N-terminus and forms heteromeric and homomeric complexes mediated by this domain. (24) Therefore, SQSTM1-ALK probably homodimerizes through the PB1 domain, leading to constitutive activation of the ALK kinase domain.

In conclusion, we reported a novel ALK fusion, SQSTM1-ALK, and its oncogenicity. ALK+LBCL is an aggressive lymphoma with poor prognosis;(3) ALK inhibitors are promising therapeutic agents for this condition. SQSTM1-ALK may be a rare fusion, but our data provide novel biological insights and may serve as a key to the accurate diagnosis of this rare lymphoma.

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Authorship and Disclosures

KT, MN, and HM conceived the study, collected and analyzed the data, and drafted the paper; KT and YO contributed to the pathology diagnosis; YS and MN contributed patient care; and MS, YT, YO, SH, and RA performed special studies and analyzed the data.

The authors reported no potential conflicts of interest.

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