

- cells is a strong independent prognostic factor in acute myeloid leukemia, even in the patients with normal karyotype. *Leukemia*. 2003;17:1538–43.
7. Taguchi J, Miyazaki Y, Tsutsumi C, Sawayama Y, Ando K, Tsushima H, et al. Expression of the myeloperoxidase gene in AC133 positive leukemia cells relates to the prognosis of acute myeloid leukemia. *Leuk Res*. 2006;30:1105–12.
 8. Sawayama Y, Miyazaki Y, Ando K, Horio K, Tsutsumi C, Tsushima H, et al. Expression of myeloperoxidase enhances the chemosensitivity of leukemia cells through the generation of reactive oxygen species and the nitration of protein. *Leukemia*. 2008;22:956–64.
 9. Schnittger S, Schoch C, Kern W, Mecucci C, Tschulik C, Martelli MF, et al. Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. *Blood*. 2005;106:3733–9.
 10. Boissel N, Renneville A, Biggio V, Philippe N, Thomas X, Cayuela JM, et al. Prevalence clinical profile, and prognosis of NPM mutations in AML with normal karyotype. *Blood*. 2005;106:3618–20.
 11. Yanada M, Matsuo K, Suzuki T, Kiyoi H, Naoe T. Prognostic significance of FLT3 internal tandem duplication and tyrosine kinase domain mutations for acute myeloid leukemia: a meta-analysis. *Leukemia*. 2005;19:1345–9.
 12. Preudhomme C, Sagot C, Boissel N, Cayuela JM, Tigaud I, de Botton S, et al. Favorable prognostic significance of *CEBPA* mutations in patients with de novo acute myeloid leukemia: a study from the Acute Leukemia French Association (ALFA). *Blood*. 2002;100:2717–23.
 13. Gombart AF, Hofmann WK, Kawano S, Takeuchi S, Krug U, Kwok SH, et al. Mutations in the gene encoding the transcription factor CCAAT/enhancer binding protein alpha in myelodysplastic syndromes and acute myeloid leukemias. *Blood*. 2002;99:1332–40.
 14. Schlenk RF, Döhner K, Krauter J, Fröhling S, Corbacioglu A, Bullinger L, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N Engl J Med*. 2008;358:1909–18.
 15. Wouters BJ, Löwenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R. Double *CEBPA* mutations, but not single *CEBPA* mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. *Blood*. 2009;113:3088–91.
 16. Dufour A, Schneider F, Metzeler KH, Hoster E, Schneider S, Zellmeier E, et al. Acute myeloid leukemia with biallelic *CEBPA* gene mutations and normal karyotype represents a distinct genetic entity associated with a favorable clinical outcome. *J Clin Oncol*. 2010;28:570–7.
 17. Miyawaki S, Kobayashi T, Tanimoto M, Kuriyama K, Murakami H, Yoshida M, et al. Comparison of leucopenia between cytarabine and behenoyl cytarabine in JALSG AML-89 consolidation therapy. The Japan Adult Leukemia Study Group. *Int J Hematol*. 1999;70:56–7.
 18. Miyawaki S, Tanimoto M, Kobayashi T, Minami S, Tamura J, Omoto E, Kuriyama K, et al. No beneficial effect from addition of etoposide to daunorubicin, cytarabine, and 6-mercaptopurine in individualized induction therapy of adult acute myeloid leukemia: the JALSG-AML92 study. Japan Adult Leukemia Study Group. *Int J Hematol*. 1999;70:97–104.
 19. Ohtake S, Miyawaki S, Kiyoi H, Miyazaki Y, Okumura H, Matsuda S, Nagai T, et al. Randomized trial of response-oriented individualized versus fixed-schedule induction chemotherapy with idarubicin and cytarabine in adult acute myeloid leukemia: the JALSG AML95 study. *Int J Hematol*. 2010;91:276–83.
 20. Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, et al. Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. *Blood*. 1999;93:3074–80.
 21. Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med*. 2005;352:254–66.
 22. Kaplan E, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc*. 1958;53:457–62.
 23. Yao C, Qin Z, Works KN, Austin GE, Young AN. CEBP and C-Myb sites are important for the functional activity of the human myeloperoxidase upstream enhancer. *Biochem Biophys Res Commun*. 2008;371:309–14.
 24. Reckzeh K, Cammenga J. Molecular mechanisms underlying deregulation of C/EBP α in acute myeloid leukemia. *Int J Hematol*. 2010;91:557–68.
 25. Kato N, Kitamura J, Doki N, et al. Two types of C/EBP α mutations play distinct but collaborative roles in leukemogenesis: lessons from clinical data and BMT models. *Blood*. 2011;117:221–33.
 26. Austin GE, Zhao WG, Regmi A, Lu JP, Braun J. Identification of an upstream enhancer containing an AML1 site in the human myeloperoxidase (MPO) gene. *Leuk Res*. 1998;22:1037–48.
 27. Erickson PF, Robinson M, Owens G, Drabkin HA. The ETO portion of acute myeloid leukemia t(8;21) fusion transcript encodes a highly evolutionarily conserved, putative transcription factor. *Cancer Res*. 1994;54:1782–6.
 28. Meyers S, Lenny N, Hiebert SW. The t(8;21) fusion protein interferes with AML-1B-dependent transcriptional activation. *Mol Cell Biol*. 1995;15:1974–82.
 29. Khoury H, Dalal BI, Nantel SH, Horsman DE, Lavoie JC, Shepherd JD, et al. Correlation between karyotype and quantitative immunophenotype in acute myelogenous leukemia with t(8;21). *Modern Pathol*. 2004;17:1211–6.
 30. Shimada H, Ichikawa H, Ohki M. Potential involvement of the AML1-MTG8 fusion protein in the granulocytic maturation characteristic of the t(8;21) acute myelogenous leukemia revealed by microarray analysis. *Leukemia*. 2002;16:874–85.

Clinical Cancer Research



Pulmonary Inflammatory Myofibroblastic Tumor Expressing a Novel Fusion, PPFIBP1 –ALK: Reappraisal of Anti-ALK Immunohistochemistry as a Tool for Novel ALK Fusion Identification

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Pulmonary Inflammatory Myofibroblastic Tumor Expressing a Novel Fusion, PPFIBP1-ALK: Reappraisal of Anti-ALK Immunohistochemistry as a Tool for Novel ALK Fusion Identification

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Abstract

Purpose: The anaplastic lymphoma kinase (ALK) inhibitor crizotinib has been used in patients with lung cancer or inflammatory myofibroblastic tumor (IMT), both types harboring ALK fusions. However, detection of some ALK fusions is problematic with conventional anti-ALK immunohistochemistry because of their low expression. By using sensitive immunohistochemistry, therefore, we reassessed "ALK-negative" IMT cases defined with conventional immunohistochemistry (approximately 50% of all examined cases).

Experimental Design: Two cases of ALK-negative IMT defined with conventional anti-ALK immunohistochemistry were further analyzed with sensitive immunohistochemistry [the intercalated antibody-enhanced polymer (iAEP) method].

Results: The two "ALK-negative" IMTs were found positive for anti-ALK immunohistochemistry with the iAEP method. 5'-rapid amplification of cDNA ends identified a novel partner of ALK fusion, protein-tyrosine phosphatase, receptor-type, F polypeptide-interacting protein-binding protein 1 (PPFIBP1) in one case. The presence of PPFIBP1-ALK fusion was confirmed with reverse transcriptase PCR, genomic PCR, and FISH. We confirmed the transforming activities of PPFIBP1-ALK with a focus formation assay and an *in vivo* tumorigenicity assay by using 3T3 fibroblasts infected with a recombinant retrovirus encoding PPFIBP1-ALK. Surprisingly, the fusion was also detected by FISH in the other case.

Conclusions: Sensitive immunohistochemical methods such as iAEP will broaden the potential value of immunohistochemistry. The current ALK positivity rate in IMT should be reassessed with a more highly sensitive method such as iAEP to accurately identify those patients who might benefit from ALK-inhibitor therapies. Novel ALK fusions are being identified in various tumors in addition to IMT, and thus a reassessment of other "ALK-negative" cancers may be required in the forthcoming era of ALK-inhibitor therapy. *Clin Cancer Res*; 17(10); 3341-8. ©2011 AACR.

Introduction

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that was discovered in anaplastic large cell lymphoma (ALCL) in the form of a fusion protein, NPM-ALK (1, 2). In addition to ALCL (fused to NPM, TPM3, TPM4, ATIC, TFG, CLTC, MSN, MYH9, or ALO17; refs. 1-10), ALK

has further been found to generate fusions in inflammatory myofibroblastic tumor (IMT; TPM3, TPM4, CLTC, CARS, RANBP2, ATIC, or SEC31L1; refs. 10-15), ALK-positive large B-cell lymphoma (CLTC, NPM, SEC31L1, or SQSTM1; 16-19), lung cancer (EML4 or KIF5B; refs. 20, 21), and ALK-positive histiocytosis (TPM3; ref. 22). Besides, some ALK fusions have been reported without showing histopathologic evidence: TPM4-ALK in esophageal squamous cell carcinoma (23, 24), TFG-ALK in lung adenocarcinoma (25), and EML4-ALK in colon and breast carcinomas (26). The wild-type ALK is mainly expressed in the developing nervous system, and is usually not expressed in other normal tissues (27). A fusion protein formation with a partner through chromosomal translocations is the most common mechanism of ALK overexpression and ALK kinase domain activation. These features render ALK fusion oncokinase an ideal molecular target.

Recently, the ALK inhibitor crizotinib has been used in patients with lung cancer or IMT, both types harboring ALK fusions (28, 29). The compound showed a 57% response rate in lung cancers (28), and a strong response for several months in IMT (29). Crizotinib and other ALK inhibitors

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Translational Relevance

Anaplastic lymphoma kinase (ALK) inhibitors have become one of the most promising groups of molecularly targeted drugs. Therefore, ALK is no longer a mere research target or simply a diagnostic marker, but is directly linked to the therapeutic benefit of patients harboring the fusions.

Pathologic diagnoses for ALK fusion-positive tumors have been made reliably with anti-ALK immunohistochemistry. Since the discovery of EML4-ALK, however, an unexpected problem in anti-ALK immunohistochemistry has become apparent, that is, the inability to detect a low level of EML4-ALK expression. To overcome this, we developed the intercalated antibody-enhanced polymer immunohistochemistry, which successfully detected EML4-ALK.

In other words, this indicates that unknown ALK fusions, particularly those expressed at a low level, may wait to be discovered in "ALK-negative" tumors defined with conventional immunohistochemistry. In the forthcoming era of ALK-inhibitor therapy, "ALK-negative" tumors should be reassessed with a high sensitive immunohistochemistry and, if positive, be further examined with appropriate molecular method(s).

have thus become one of the most promising groups of molecularly targeted drugs. Therefore, the sensitive and accurate identification of ALK fusion in tumors has also become clinically relevant, because it is no longer a mere research target or simply a diagnostic marker, but is directly linked to the therapeutic benefit of patients harboring the fusions.

Identification of such ALK fusions, especially within ALCL, has been prompted by the immunohistochemical staining pattern with antibodies to ALK. In ALCL, the most common ALK fusion is NPM-ALK (comprising approximately 80% of all cases), and its immunohistochemical staining pattern is both nuclear and cytoplasmic. NPM has a nuclear localization signal in the C-terminal region, and therefore the heterodimers of wild-type NPM with NPM-ALK fusion protein are transported to the nucleus whereas NPM-ALK homodimers remain within the cytoplasm (30). In contrast, other fusions do not localize in the nucleus and do not show a nuclear staining pattern in anti-ALK immunohistochemistry. Interestingly, each ALK fusion usually has its own characteristic anti-ALK immunohistochemical staining pattern, because the subcellular localization of ALK fusions is dependent on the corresponding fusion partners. Anti-ALK immunohistochemistry has thus become a highly useful tool for both research and diagnostic purposes.

Since the discovery of EML4-ALK fusion in lung cancer (20), however, an unexpected problem in anti-ALK immunohistochemistry has become apparent, that is, the inability to detect a low level of fusion expression. To overcome this, we developed the intercalated antibody-enhanced

polymer (iAEP) method, which moderately raises sensitivity in the immunohistochemical detection system (21). With this very simple method, anti-ALK immunohistochemistry has become a potent weapon in the diagnosis of EML4-ALK-positive lung cancer (21, 31-33). Other researchers used an anti-ALK rabbit monoclonal antibody, which is usually more sensitive than mouse monoclonal antibody, which can stain EML4-ALK (34). However, most EML4-ALK-positive lung cancer tissues do not stain well with conventional anti-ALK immunohistochemical methods because of the low message/protein level of EML4-ALK (21, 35). The expression level of a fusion gene depends on the promoter activity of the 5'-side gene, and that of EML4 is likely to be lower than that of the other ALK fusion partner genes, which may explain why EML4-ALK had not been discovered until 12 years after the development of the first anti-ALK antibody became available for immunohistochemistry (36). In other words, a tumor that immunostains for ALK only by a sensitive immunohistochemistry method may harbor a novel ALK fusion. Interestingly, in this study, we detected 2 IMT cases positive for ALK immunohistochemistry only when stained by iAEP method (21), and successfully identified a novel fusion gene, protein-tyrosine phosphatase, receptor-type, F polypeptide-interacting protein-binding protein 1 (PPFIBP1)-ALK.

Materials and Methods

Materials

Pathologic specimens from 2 pulmonary IMT cases, originally diagnosed as fibrous histiocytoma (1988: case 1, 45-year-old male; 1998: case 2, 34-year-old female), were reassessed morphologically and immunohistochemically. Surgically removed tumor specimens were routinely fixed in 20% neutralized formalin and embedded in paraffin for conventional histopathologic examination. For case 2, total RNA was extracted from the corresponding snap-frozen specimen and purified with the use of an RNeasy Mini kit (Qiagen). The study was approved by the institutional review board of the Japanese Foundation for Cancer Research.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue was sliced at a thickness of 4 μ m, and the sections were placed on silane-coated slides. For antigen retrieval, the slides were heated for 40 min at 97°C in Target Retrieval Solution (pH 9.0; Dako). For the conventional staining procedure, the slides were incubated at room temperature with Protein Block Serum-free Ready-to-Use solution (Dako) for 10 minutes and then with primary antibodies against ALK (5A4), smooth muscle actin, muscle-specific actin (HHF35), CD34, cytokeratins (AE1/AE3), S100, or desmin for 30 minutes. The immune complexes were then detected with dextran polymer reagent (EnVision + DAB system; Dako) and an AutoStainer instrument (Dako). The iAEP method was also used for the sensitive detection of ALK, as described previously (21).

Isolation of PPFIBP1-ALK fusion

To obtain cDNA fragments corresponding to a novel *ALK* fusion gene, we used a 5'-RACE method with the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions, with a minor modification: the ALK2458R primer (5'-GTAGTTGGGGTTGTAGTCGGT-CATGATGGT-3') was used as the gene-specific reverse primer.

From the oligo(dT)-primed cDNA obtained from case 2 RNA, a 471bp cDNA fragment containing the fusion point was specifically amplified with the primers PPFIBP1-592F (5'-AGAGACACAGAGGGGCTGATT-3') and ALK3078RR (5'-ATCCAGTTCGTCCTGTTTCAGAGC-3').

PCR analysis of genomic DNA for *PPFIBP1-ALK* in case 2 was carried out with a pair of primers flanking the putative fusion point, PPFIBP1-607F (5'-CTGATTCAGGAGATCA-ATGATTTGAGGT-3') and Fusion-RT-AS (5'-TCTTGCCAGCAAAGCAGTAGTTGG-3').

From the cDNA, a full-length cDNA for *PPFIBP1-ALK* was amplified by PCR with the PA-w-cDNA-in-S primer (5'-TATCTGGGTTGGAATTTGCCCTG-3') and the KA-w-cDNA-in-AS primer (5'-TGAGTGTGCGACCGAGCTCAGG-3') and PrimeSTAR HS DNA polymerase (TakaraBio).

FISH

FISH analysis of gene fusion was carried out with bacterial artificial chromosome (BAC) clone-derived DNA probes for *ALK* and *PPFIBP1*. Unstained sections (4 μ m thick) were subjected to hybridization with an *ALK*-split probe set (Abbott) or BAC clone-derived probes for *ALK* (RP11-984I21, RP11-62B19) and *PPFIBP1*

(RP11-1060J15). Hybridized slides were then stained with DAPI and examined with the fluorescence microscope BX51 (Olympus).

Transformation assay for ALK fusion proteins

Analysis of the transforming activity of *PPFIBP1-ALK* was carried out as described previously (20, 37, 38). Briefly, the pMXS-based expression plasmid for *PPFIBP1-ALK*, *EML4-ALK* variant 1, or *NPM-ALK* was used to generate recombinant ecotropic retrovirus, followed by individual infection of mouse 3T3 fibroblasts (39). Formation of the transformed foci was evaluated after culturing the cells for 14 days. The same set of 3T3 cells was subcutaneously injected into nu/nu mice, and tumor formation was examined after 20 days. The animal experiments were approved by the animal ethics committee of Jichi Medical University.

Results

Morphology and immunophenotype of PPFIBP1-ALK-positive IMT

Histopathologic analysis of the 2 IMT cases revealed a marked proliferation of cells composed of somewhat histiocytoid spindle cells showing a fascicular or storiform pattern. The tumor cells were uniform and had pale eosinophilic cytoplasm and an oval vesicular nucleus, within which a small nucleolus was centrally located. Mild inflammatory infiltrate containing lymphocytes, plasma cells, foamy histiocytes, and multinucleated giant cells was observed (Fig. 1A and 1D). The immunophenotype of the 2 cases was negative for smooth muscle actin,

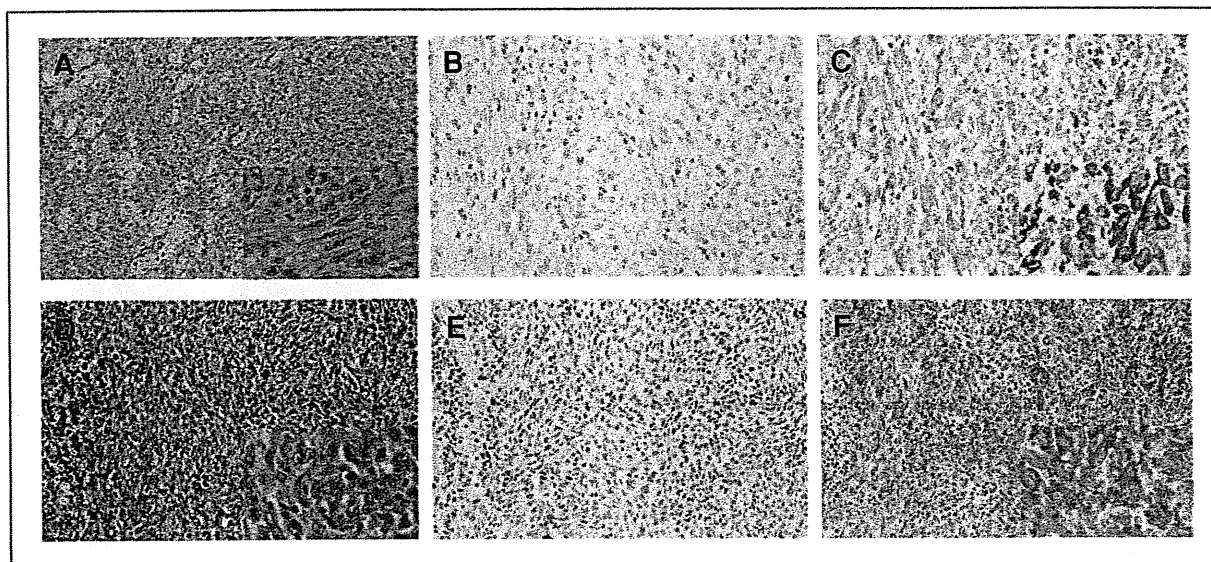


Figure 1. Histopathology of PPFIBP1-ALK-positive IMT. Diffuse proliferation of histiocytoid spindle cells showing a fascicular or storiform pattern. The tumor cells were uniform and had pale eosinophilic cytoplasm and an oval vesicular nucleus, within which a small nucleolus was centrally located. Mild inflammatory infiltrate containing lymphocytes, plasma cells, and foamy histiocytes is observed (A and D). The tumor cells were negative for ALK with conventional anti-ALK immunohistochemistry (B and E), but were clearly positive for ALK when the iAEP method was used. The staining pattern is diffuse cytoplasmic (C and F). Case 1 (A-C), Case 2 (D-F).

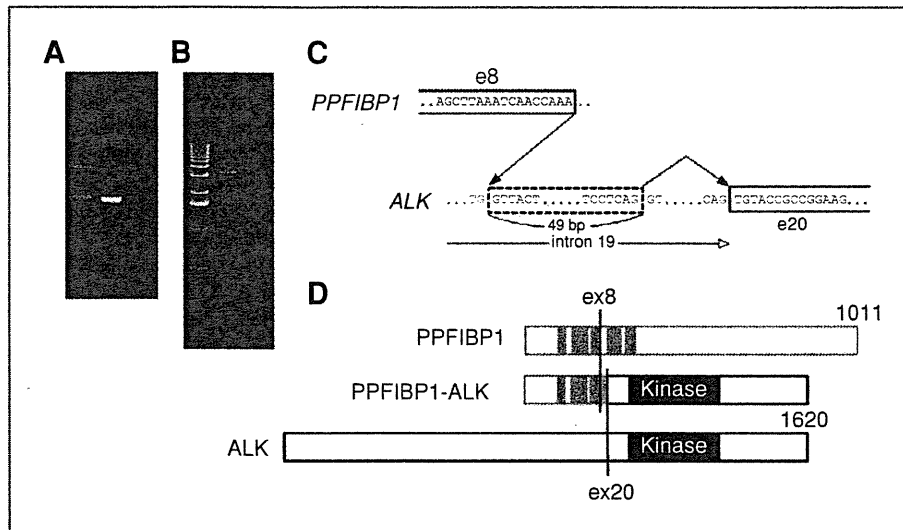


Figure 2. Identification of PPFIBP1-ALK: a PCR product of 471 bp covering the fusion point of PPFIBP1-ALK cDNA was specifically amplified from the tumor cells of case 2. The left lane contains DNA size standards (100 bp ladder). The right lane represents no template control (A). A PCR product of approximately 3 kbp covering the genomic fusion point of PPFIBP1-ALK was specifically amplified from the tumor cells of case 2. The left lane contains DNA size standards (1 kbp ladder). The right lane represents no template control (B). In our 5'-RACE products, exon 8 of PPFIBP1 cDNA was fused to a 49 bp sequence in intron 19 of ALK, followed by exon 20 of ALK (C). PPFIBP1 contains 5 coiled-coil domains. A chromosome translocation, t(2;10)(p23;p11), generates a fusion protein in which the top 3 coiled-coil domains of PPFIBP1 and the intracellular region of ALK (containing the tyrosine kinase domain) are conserved. Numbers indicate amino acid positions of each protein (D).

HHF35, CD34, AE1/AE3, and S100. Desmin was focally positive in case 1, but was negative in case 2.

Identification of PPFIBP1-ALK as a novel ALK fusion gene

We conducted anti-ALK immunohistochemistry on 2 morphologically typical pulmonary IMT cases, originally diagnosed as fibrous histiocytoma. Immunostaining for ALK with the conventional polymer method led to the revised diagnosis of "ALK-negative" IMT (Fig. 1B and E). In the present study, anti-ALK immunohistochemistry with the iAEP method, however, showed a diffuse positive cytoplasmic staining (Fig. 1C and F), indicating the possibility of ALK fusion to a novel partner gene, the expression level of which is modest. To address this issue, in case 2 we conducted 5'-RACE assay for the isolation of an upstream cDNA to the ALK kinase domain cDNA, for which snap-frozen material was available.

Interestingly, we isolated a cDNA fragment containing exon 8 of PPFIBP1 followed by a 49 bp-sequence within intron 19 of ALK and coupled to exon 20 of ALK (Fig. 2), suggesting the presence of a novel fusion between PPFIBP1 and ALK genes. Because insertion of the intronic 49 bp allows an in-frame fusion between the 2 genes, this rearrangement likely produces a novel fusion-type tyrosine kinase. To confirm the genomic rearrangement responsible for the PPFIBP1-ALK fusion, a genomic PCR assay (Fig. 2B) and both ALK split and PPFIBP1-ALK fusion FISH assays (Fig. 3) were carried out. All results were consistent with the presence of t(2;12)(p23;p11) leading to the generation of PPFIBP1-ALK. Owing to the limited material available in

case 1, only the FISH analyses were carried out. Surprisingly, these results also indicate the presence of PPFIBP1-ALK (Fig. 3, Supplementary Fig. 2A-C).

Transforming activities of PPFIBP1-ALK

To prove that the t(2;12)(p23;p11) rearrangement leads to the production of PPFIBP1-ALK kinase, in case 2 we attempted to amplify from the cDNA a full-length cDNA encoding the protein. By using a sense primer at the 5'-untranslated region of PPFIBP1 mRNA (GenBank accession no. NM_003622) and an antisense primer at

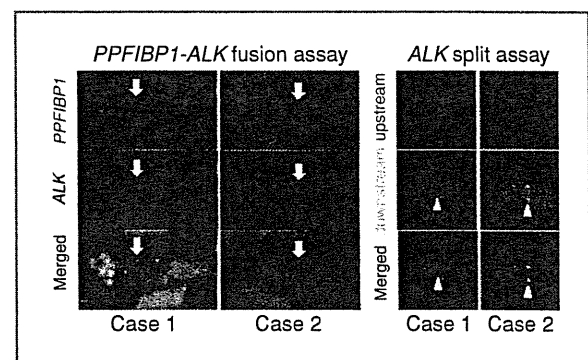


Figure 3. FISH analyses for PPFIBP1-ALK: sections of tumors positive for PPFIBP1-ALK were subjected to FISH analyses. In PPFIBP1-ALK fusion assays (left) the fusion genes are indicated by arrows. In ALK split assays (right) the 3'-sides of ALK are indicated by arrowheads. The color of fluorescence for the BAC clones and the case numbers in each hybridization are indicated. Nuclei are stained blue with DAPI.

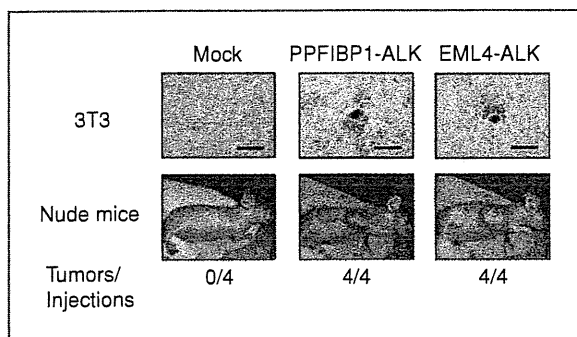


Figure 4. Transforming potential of PPFIBP1-ALK. Top, mouse 3T3 fibroblasts were infected with retroviruses encoding PPFIBP1-ALK or EML4-ALK or with the corresponding empty virus (Mock). The cells were photographed after 14 days of culture. Scale bars, 400 μ m. Bottom, Nude mice were injected subcutaneously with the corresponding 3T3 cells, and tumor formation was examined after 14 days. The number of tumors formed per 4 injections is indicated at the bottom.

the 3'-untranslated region of *ALK* mRNA (GenBank accession no. NM_004304), a full-length *PPFIBP1-ALK* cDNA of 2488 bp was successfully amplified, which should have produced a fusion kinase of 811 amino acids with a predicted molecular weight of 90,740 Da (Supplementary Fig. 1).

To examine the transforming potential of PPFIBP1-ALK, a recombinant ecotropic retrovirus was generated to express PPFIBP1-ALK, which was used to infect mouse 3T3 fibroblasts. As shown in Figure 4, PPFIBP1-ALK produced hundreds of transformed foci over 14 days of culture, which was comparable with the observation with EML4-ALK. Furthermore, subcutaneous injection of the infected 3T3 cells into the shoulder of nude mice revealed that those expressing either PPFIBP1-ALK or EML4-ALK formed large tumors *in vivo*.

Discussion

Since their discovery in 1994, appropriate diagnosis of ALK fusion-positive tumors with conventional anti-ALK immunohistochemistry methods has been accepted. However, EML4-ALK in lung adenocarcinoma, identified in 2007, did not stain positive for ALK with conventional immunohistochemistry methods (21, 35). We developed a sensitive immunohistochemistry method, the iAEP method, and successfully stained EML4-ALK with ordinary anti-ALK mouse monoclonal antibodies (21, 31-33). Such observation further indicates a possibility that staining cancer specimens with sensitive immunohistochemical methods (such as iAEP) may detect novel ALK fusions in the "ALK-negative" tumors defined by conventional anti-ALK immunohistochemistry methods. On the basis of this hypothesis, we have identified a novel ALK fusion in "ALK-negative" IMT.

Caution is needed in practical settings. For example, rhabdomyosarcoma, especially of the alveolar type, often expresses wild-type ALK at a detectable level with conventional anti-ALK immunohistochemistry (40). Moreover, in

our experience, a small portion of small cell carcinoma and large cell endocrine carcinoma of the lung, and some sarcomas, may be positive for ALK by iAEP immunohistochemistry, expressing wild-type ALK. Therefore, in order to specifically detect ALK fusions with sensitive anti-ALK immunohistochemistry, a confirmatory test by using FISH, RT-PCR, or similar is usually required. If a tumor is positive for a confirmatory test and the suspected partner gene is not a reported one, 5'-RACE or inverse reverse transcriptase PCR methods can be used for the identification of the suspected partner. Even if overexpressed, wild-type ALK may not be oncogenic (20, 21, 37, 38), although some investigators have suggested that wild-type ALK overexpression above a certain threshold level drives the growth of neuroblastoma (41). Further investigation will be required to clarify if wild-type ALK overexpression is a target for ALK inhibitor therapy.

IMT is a rare mesenchymal tumor that has been referred to as inflammatory pseudotumor, plasma cell granuloma, fibroxanthoma, fibrous histiocytoma, pseudosarcomatous myofibroblastic tumor, and invasive fibrous tumor of the tracheobronchial tree (42). It occurs in the soft tissues as well as in the viscera and the lung, and is more likely to occur in children and young adults. Histologically, IMT is composed of a variable admixture of bland, spindle-shaped myofibroblast-like cells and an inflammatory component of lymphocytes, eosinophils, plasma cells, and macrophages. Recent genetic studies have elucidated clonal chromosomal abnormality involving 2p23, at which ALK is located, in a subset of IMT. The expression of ALK fusion proteins is detected by anti-ALK immunohistochemistry in approximately 50% of IMT cases (42), in which various ALK fusion genes have been reported (Table 1). Collectively, these lines of evidence support ALK-positive IMT being a distinct neoplastic entity. However, the other 50% of IMT cases are negative for anti-ALK immunohistochemistry, and thus in terms of pathogenesis it remains unknown whether these ALK-negative IMTs should be included in the same entity or not. In fact, 1 ALK-negative IMT case did not respond to crizotinib therapy (29). However, we have detected a novel ALK-fusion in "ALK-negative" IMT that subsequently proved positive for ALK with the iAEP immunohistochemistry method. Therefore, unexpectedly lowly expressed ALK fusions may explain the pathogenesis of a portion of "ALK-negative" IMT cases. PPFIBP1-ALK represents such an ALK fusion, although we do not yet know what proportion of "ALK-negative" IMTs can be attributed to this novel subtype. "ALK-negative" IMT warrants screening with the iAEP method to detect this fusion or other, unrecognized, ALK fusions.

PPFIBP1 codes liprin beta 1 (also called PTPRF-interacting protein-binding protein 1). This 114 kDa protein is a member of the leukocyte common antigen-related (LAR) transmembrane tyrosine phosphatase-interacting protein family that may regulate LAR protein properties via interaction with another member of the family, liprin alpha1 (43). Liprin beta 1 expresses in intestinal lymphatic endothelial cells *in vitro* and lymphatic vasculature *in vivo*,

Table 1. ALK fusion partners in well-documented IMT cases

Partner	Locus	Age	Sex	Site	Year, First author
TPM3	1p23	30	F	Lung	2000, Lawrence
		23	F	Abdomen	2000, Lawrence
		4	M	Lung	2006, Yamamoto
		29	F	Ileum	2006, Milne
		4	M	Lung	2007, Kinoshita
TPM4	19p13	1	M	Abdomen	2000, Lawrence
		6	M	Mesentery	2003, Hisaoka
		25	M	Prostate	2003, Hisaoka
		5	M	Mesentery	2006, Yamamoto
		5	F	Urinary bladder	2006, Yamamoto
CLTC	17q23	3	F	Neck	2001, Bridge
		37	M	Pelvis	2001, Bridge
		2	M	Thoracic cavity	2006, Yamamoto
		6	M	Mesentery	2006, Yamamoto
		0	F	Mediastinum	2007, Patel
CARS	11p15	0	M	Abdomen	2002, Cools
		10	M	Neck	2003, Debelenko
RANBP2	2q13	7	M	Abdomen	2003, Ma
		0	M	Abdomen	2003, Ma
		2	M	Abdomen	2007, Patel
		34	M	Liver	2008, Chen
		44	M	Abdomen	2010, Butrynski
ATIC	2q35	46	M	Urinary bladder	2003, Debiec-Rychter
SEC31L1	4q21	23	M	Abdomen	2006, Panagopoulos
PPFIBP1	12p11	45	M	Lung	Present case 1
		34	F	Lung	Present case 2

and plays an important role in the maintenance of lymphatic vessel integrity in *Xenopus* tadpoles (44). PPFIBP1 has 5 coiled-coil domains in exons 5 through 12, and the upper 3 domains are conserved in fusion form with ALK (Fig. 2D). The coiled-coil domain is shared in all ALK fusion partners (except for NPM, MSN, and SQSTM1), with which the ALK fusion proteins homodimerize leading to constitutive activation of ALK kinase domains (8, 19). As expected, in the present study, the oncogenicity of PPFIBP1-ALK was clearly confirmed with an *in vitro* focus formation assay and an *in vivo* tumorigenicity assay.

The difference in subcellular localization has contributed to the discovery/identification of various ALK fusions. Likewise, the difference in the expression level found is here proved important in the accurate detection of fusion proteins. Sensitive immunohistochemical methods such as iAEP will broaden the potential value of immunohistochemistry, which is a simple and long-established histopathologic technique in the fields of research and diagnosis. The ALK positivity rate (approximately 50%) in IMT should be reassessed with these more sensitive methods, possibly leading to the identification of novel ALK fusions and more candidates for ALK inhibitor therapy. A novel ALK fusion, VCL-ALK, has recently been identified in renal cancers (45, 46). In addition to IMT,

therefore, a reassessment of diverse "ALK-negative" human cancers may be required in the forthcoming era of ALK inhibitor therapy.

Disclosure of Potential Conflicts of Interest

K. Takeuchi, scientific advisor for developing an anti-ALK iAEP immunohistochemistry kit (ALK Detection Kit, Nichirei Bioscience, Japan) and in charge of pathology screening for ALK fusions using the immunohistochemistry kit and an original probe set for ALK split FISH assay in a clinical trial of an ALK inhibitor (AF802, Chugai, Japan). The other authors disclosed no potential conflicts of interest.

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References

- Morris SW, Kirstein MN, Valentine MB, Dittmer KG, Shapiro DN, Saltman DL, et al. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. *Science* 1994; 263:1281-4.
- Shiota M, Fujimoto J, Semba T, Satoh H, Yamamoto T, Mori S. Hyperphosphorylation of a novel 80 kDa protein-tyrosine kinase similar to Ltk in a human Ki-1 lymphoma cell line, AMS3. *Oncogene* 1994;9:1567-74.
- Lamant L, Dastugue N, Pulford K, Delsol G, Mariame B. A new fusion gene TPM3-ALK in anaplastic large cell lymphoma created by a (1;2)(q25;p23) translocation. *Blood* 1999;93:3088-95.
- Meech SJ, McGavran L, Odom LF, Liang X, Meltesen L, Gump J, et al. Unusual childhood extramedullary hematologic malignancy with natural killer cell properties that contains tropomyosin 4-anaplastic lymphoma kinase gene fusion. *Blood* 2001;98:1209-16.
- Colleoni GW, Bridge JA, Garicochea B, Liu J, Filippa DA, Ladanyi M. ATIC-ALK: A novel variant ALK gene fusion in anaplastic large cell lymphoma resulting from the recurrent cryptic chromosomal inversion, inv(2)(p23q35). *Am J Pathol* 2000;156:781-9.
- Hernández L, Pinyol M, Hernández S, Beà S, Pulford K, Rosenwald A, et al. TRK-fused gene (TFG) is a new partner of ALK in anaplastic large cell lymphoma producing two structurally different TFG-ALK translocations. *Blood* 1999;94:3265-8.
- Touriol C, Greenland C, Lamant L, Pulford K, Bernard F, Rousset T, et al. Further demonstration of the diversity of chromosomal changes involving 2p23 in ALK-positive lymphoma: 2 cases expressing ALK kinase fused to CLTCL (clathrin chain polypeptide-like). *Blood* 2000;95:3204-7.
- Tort F, Pinyol M, Pulford K, Roncador G, Hernandez L, Nayach I, et al. Molecular characterization of a new ALK translocation involving moesin (MSN-ALK) in anaplastic large cell lymphoma. *Lab Invest* 2001;81:419-26.
- Lamant L, Gascoyne RD, Duplantier MM, Armstrong F, Raghav A, Chhanabhai M, et al. Non-muscle myosin heavy chain (MYH9): a new partner fused to ALK in anaplastic large cell lymphoma. *Genes Chromosomes Cancer* 2003;37:427-32.
- Cools J, Wlodarska I, Somers R, Mentens N, Pedetour F, Maes B, et al. Identification of novel fusion partners of ALK, the anaplastic lymphoma kinase, in anaplastic large-cell lymphoma and inflammatory myofibroblastic tumor. *Genes Chromosomes Cancer* 2002;34:354-62.
- Lawrence B, Perez-Atayde A, Hibbard MK, Rubin BP, Dal Cin P, Pinkus JL, et al. TPM3-ALK and TPM4-ALK oncogenes in inflammatory myofibroblastic tumors. *Am J Pathol* 2000;157:377-84.
- Bridge JA, Kanamori M, Ma Z, Pickering D, Hill DA, Lydiatt W, et al. Fusion of the ALK gene to the clathrin heavy chain gene, CLTC, in inflammatory myofibroblastic tumor. *Am J Pathol* 2001;159:411-5.
- Ma Z, Hill DA, Collins MH, Morris SW, Sumegi J, Zhou M, et al. Fusion of ALK to the Ran-binding protein 2 (RANBP2) gene in inflammatory myofibroblastic tumor. *Genes Chromosomes Cancer* 2003;37:98-105.
- Debiec-Rychter M, Marynen P, Hagemeijer A, Pauwels P. ALK-ATIC fusion in urinary bladder inflammatory myofibroblastic tumor. *Genes Chromosomes Cancer* 2003;38:187-90.
- Panagopoulos I, Nilsson T, Domanski HA, Isaksson M, Lindblom P, Mertens F, et al. Fusion of the SEC31L1 and ALK genes in an inflammatory myofibroblastic tumor. *Int J Cancer* 2006;118:1181-6.
- Delsol G, Lamant L, Mariamé B, Pulford K, Dastugue N, Brousset P, et al. A new subtype of large B-cell lymphoma expressing the ALK kinase and lacking the 2; 5 translocation. *Blood* 1997;89:1483-90.
- Gascoyne RD, Lamant L, Martin-Subero JI, Lestou VS, Harris NL, Müller-Hermelink HK, et al. ALK-positive diffuse large B-cell lymphoma is associated with Clathrin-ALK rearrangements: report of 6 cases. *Blood* 2003;102:2568-73.
- Van Roosbroeck K, Cools J, Dierickx D, Thomas J, Vandenberghe P, Stul M, et al. ALK-positive large B-cell lymphomas with cryptic SEC31A-ALK and NPM1-ALK fusions. *Haematologica* 2010;95:509-13.
- Takeuchi K, Soda M, Togashi Y, Ota Y, Sekiguchi Y, Hatano S, et al. Identification of a novel fusion, SQSTM1-ALK, in ALK-positive large B-cell lymphoma. *Haematologica* 2010.
- Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature* 2007;448:561-6.
- Takeuchi K, Choi YL, Togashi Y, Soda M, Hatano S, Inamura K, et al. KIF5B-ALK, a novel fusion oncogene identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. *Clin Cancer Res* 2009;15:3143-9.
- Chan JK, Lamant L, Algar E, Delsol G, Tsang WY, Lee KC, et al. ALK+ histiocytosis: a novel type of systemic histiocytic proliferative disorder of early infancy. *Blood* 2008;112:2965-8.
- Du XL, Hu H, Lin DC, Xia SH, Shen XM, Zhang Y, et al. Proteomic profiling of proteins dysregulated in Chinese esophageal squamous cell carcinoma. *J Mol Med* 2007;85:863-75.
- Jazii FR, Najafi Z, Malekzadeh R, Conrads TP, Ziaee AA, Abnet C, et al. Identification of squamous cell carcinoma associated proteins by proteomics and loss of beta tropomyosin expression in esophageal cancer. *World J Gastroenterol* 2006;12:7104-12.
- Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell* 2007;131:1190-203.
- Lin E, Li L, Guan Y, Soriano R, Rivers CS, Mohan S, et al. Exon array profiling detects EML4-ALK fusion in breast, colorectal, and non-small cell lung cancers. *Mol Cancer Res* 2009;7:1466-76.
- Chiarle R, Voena C, Ambrogio C, Piva R, Inghirami G. The anaplastic lymphoma kinase in the pathogenesis of cancer. *Nat Rev Cancer* 2008;8:11-23.
- Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* 2010;363:1693-703.
- Butrynski JE, D'Adamo DR, Hornick JL, Dal Cin P, Antonescu CR, Jhanwar SC, et al. Crizotinib in ALK-rearranged inflammatory myofibroblastic tumor. *N Engl J Med* 2010;363:1727-33.
- Falini B, Bigerna B, Fizzotti M, Pulford K, Pileri SA, Delsol G, et al. ALK expression defines a distinct group of T/null lymphomas ("ALK lymphomas") with a wide morphological spectrum. *Am J Pathol* 1998;153:875-86.
- Sakairi Y, Nakajima T, Yasufuku K, Ikebe D, Kageyama H, Soda M, et al. EML4-ALK fusion gene assessment using metastatic lymph node samples obtained by endobronchial ultrasound-guided transbronchial needle aspiration. *Clin Cancer Res* 2010;16:4938-45.
- Nakajima T, Kimura H, Takeuchi K, Soda M, Mano H, Yasufuku K, et al. Treatment of lung cancer with an ALK inhibitor after EML4-ALK fusion gene detection using endobronchial ultrasound-guided transbronchial needle aspiration. *J Thorac Oncol* 2010;5:2041-3.
- Jokoji R, Yamasaki T, Minami S, Komuta K, Sakamaki Y, Takeuchi K, et al. Combination of morphological feature analysis and immunohistochemistry is useful for screening of EML4-ALK-positive lung adenocarcinoma. *J Clin Pathol* 2010;63:1066-70.
- Mino-Kenudson M, Chirieac LR, Law K, Hornick JL, Lindeman N, Mark EJ, et al. A novel, highly sensitive antibody allows for the routine detection of ALK-rearranged lung adenocarcinomas by standard immunohistochemistry. *Clin Cancer Res* 2010;16:1561-71.
- Martelli MP, Sozzi G, Hernandez L, Pettrossi V, Navarro A, Conte D, et al. EML4-ALK rearrangement in non-small cell lung cancer and non-tumor lung tissues. *Am J Pathol* 2009;174:661-70.
- Shiota M, Nakamura S, Ichinohasama R, Abe M, Akagi T, Takeshita M, et al. Anaplastic large cell lymphomas expressing the novel chimeric protein p80NPM/ALK: a distinct clinicopathologic entity. *Blood* 1995;86:1954-60.
- Takeuchi K, Choi YL, Soda M, Inamura K, Togashi Y, Hatano S, et al. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. *Clin Cancer Res* 2008;14:6618-24.
- Choi YL, Takeuchi K, Soda M, Inamura K, Togashi Y, Hatano S, et al. Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. *Cancer Res* 2008;68:4971-6.

39. Onishi M, Kinoshita S, Morikawa Y, Shibuya A, Phillips J, Lanier LL, et al. Applications of retrovirus-mediated expression cloning. *Exp Hematol* 1996;24:324-9.
40. Pillay K, Govender D, Chetty R. ALK protein expression in rhabdomyosarcomas. *Histopathology* 2002;41:461-7.
41. Passoni L, Longo L, Collini P, Coluccia AM, Bozzi F, Podda M, et al. Mutation-independent anaplastic lymphoma kinase overexpression in poor prognosis neuroblastoma patients. *Cancer Res* 2009;69:7338-46.
42. Travis WD, Elisabeth B, Muller-Hermelink HK, Harris CC, editors. *Pathology and Genetics of Tumours of the Lung, Pleural, Thymus and Heart*. Lyon: IARC Press; 2004.
43. Krijavska M, Fischer-Larsen M, Moertz E, Vorm O, Tulchinsky E, Grigorian M, et al. Liprin beta 1, a member of the family of LAR transmembrane tyrosine phosphatase-interacting proteins, is a new target for the metastasis-associated protein S100A4 (Mts1). *J Biol Chem* 2002;277:5229-35.
44. Norrmén C, Vandeveld W, Ny A, Saharinen P, Gentile M, Haraldsen G, et al. Liprin (beta)1 is highly expressed in lymphatic vasculature and is important for lymphatic vessel integrity. *Blood* 2010;115:906-9.
45. Debelenko LV, Raimondi SC, Daw N, Shivakumar BR, Huang D, Nelson M, et al. Renal cell carcinoma with novel VCL-ALK fusion: new representative of ALK-associated tumor spectrum. *Mod Pathol* 2010.
46. Marino-Enriquez A, Ou WB, Weldon CB, Fletcher JA, Perez-Atayde AR. ALK rearrangement in sickle cell trait-associated renal medullary carcinoma. *Genes Chromosomes Cancer* 2011;50:146-53.

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

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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 led 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.

Key words: ALK-positive, large B-cell lymphoma, fusion.

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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),^{4,5} *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 ALKREx22-23 (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-

The online version of this article has a Supplementary Appendix.

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PCR with primers SQSTM1 565F (5'-AAACACGGA-CACTTCGGGT-3') and ALK3078RR (5'-ATCCAGTTCGTCCT-GTTCAGAGC-3').

Full-length SQSTM1-ALK cDNA was obtained from the specimen by RT-PCR with primers SQSTM1v1-F90 (5'-CTCGCTATG-GCGTCGCTCACCGTGAA-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 analyzed 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'-ACCTGAGGAGACG-GTGACC-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 pos-

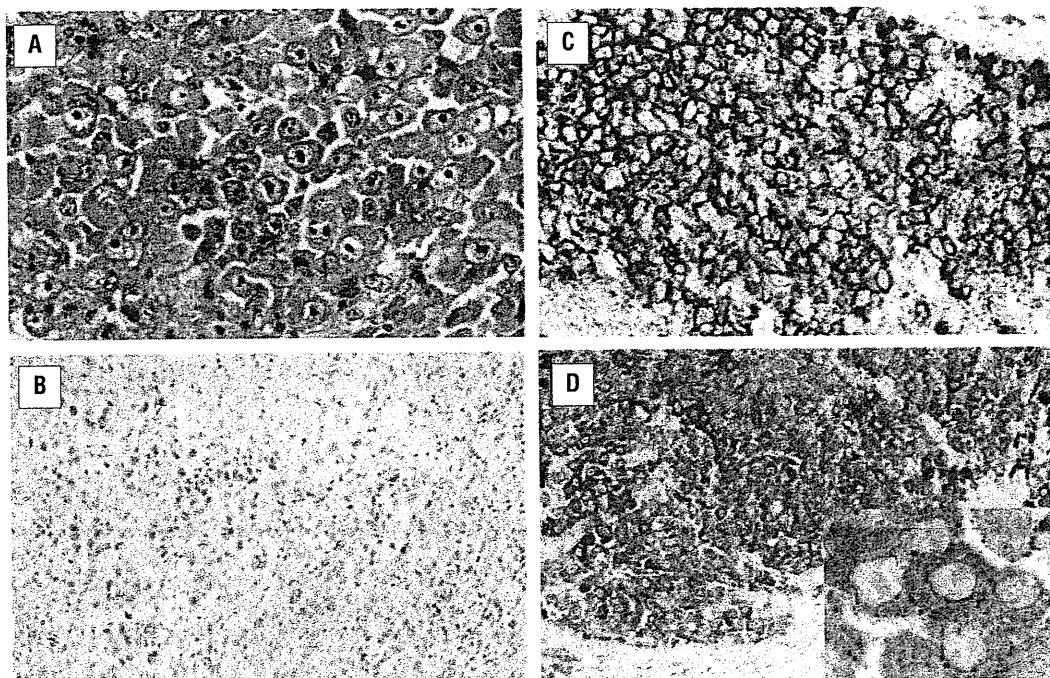


Figure 1. Histopathology of SQSTM1-ALK-positive large B-cell lymphoma. (A) The pattern of tumor infiltration was diffuse. The lymphoma cells were large with abundant cytoplasm and had round, vesicular nuclei, each containing a centrally located large nucleolus. These features may be consistent with immunoblasts or plasmablasts, but the size of tumor cells was extremely large compared with these typical cell types (Magnification 40×). (B) Some lymphoma cells expressed cytokeratin (AE1/AE3) (Magnification 20×). (C) Syndecan1/CD138 was strongly expressed (Magnification 20×). (D) In anti-ALK immunohistochemistry, a diffuse cytoplasmic staining pattern with ill-demarcated spots was clearly shown (Magnification 20×).

itive 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 cancer¹⁰ because the tumor cells were positive for some cytokeratins and immunohistochemistry for immunoglobulins was not evaluable due to background staining. Immunohistochemistry for TTF1 was negative; this 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 the *Online Supplementary Figure S1*.

SQSTM1 is an 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's 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 *SQSTM1-ALK*-positive lymphomas may be similar, and this may mean that *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

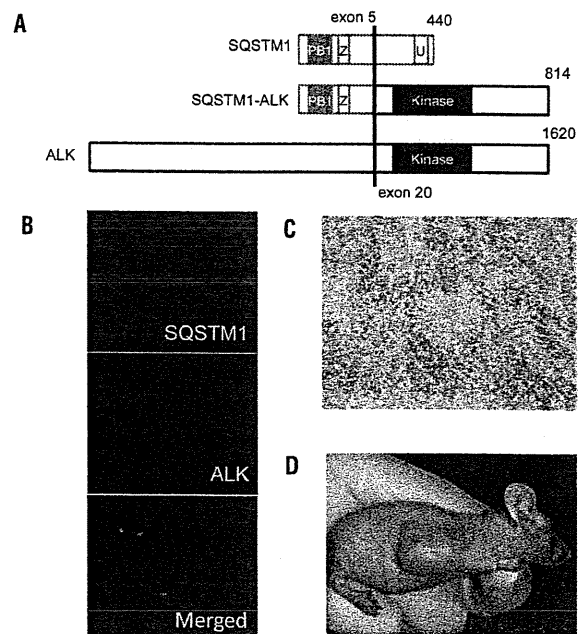


Figure 2. Discovery of *SQSTM1-ALK* fusion gene. (A) A chromosome translocation, $t(2;5)(p23.1;q35.3)$, generates a cDNA fusion in which exon 5 of *SQSTM1* is joined to the *ALK* cDNA for the intracellular region of its encoded protein (containing the tyrosine kinase domain). Numbers indicate amino acid positions of each protein. PB1: Phox and Bem1p; Z: atypical zinc finger; U: ubiquitin-associated. (B) A section of the specimen for the present case was subjected to FISH with an *SQSTM1-ALK* fusion assay. Nuclei are stained blue with DAPI. (C) Murine 3T3 fibroblasts were infected with retroviruses expressing *SQSTM1-ALK*. The cells were photographed after culture for 14 days. (D) A nude mouse was injected subcutaneously with 3T3 cells infected as in (C), and tumor formation was examined after 20 days.

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 *NPM* and *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 specu-

lated 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.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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References

- Delsol G, Lamant L, Mariane B, Pulford K, Dastugue N, Brousset P, et al. A new subtype of large B-cell lymphoma expressing the ALK kinase and lacking the 2;5 translocation. *Blood*. 1997;89(5):1483-90.
- Beltran B, Castillo J, Salas R, Quinones P, Morales D, Hurtado F, et al. ALK-positive diffuse large B-cell lymphoma: report of four cases and review of the literature. *J Hematol Oncol*. 2009;2:11.
- Laurent C, Do C, Gascoyne RD, Lamant L, Ysebaert L, Laurent G, et al. Anaplastic lymphoma kinase-positive diffuse large B-cell lymphoma: a rare clinicopathologic entity with poor prognosis. *J Clin Oncol*. 2009;27(25):4211-6.
- Gascoyne RD, Lamant L, Martin-Subero JJ, Lestou VS, Harris NL, Muller-Hermelink HK, et al. ALK-positive diffuse large B-cell lymphoma is associated with Clathrin-ALK rearrangements: report of 6 cases. *Blood*. 2003;102(7):2568-73.
- De Paepe P, Baens M, van Krieken H, Verhasselt B, Stul M, Simons A, et al. ALK activation by the CLTC-ALK fusion is a recurrent event in large B-cell lymphoma. *Blood*. 2003;102(7):2638-41.
- Chikatsu N, Kojima H, Suzukawa K, Shinagawa A, Nagasawa T, Ozawa H, et al. ALK+, CD30-, CD20- large B-cell lymphoma containing anaplastic lymphoma kinase (ALK) fused to clathrin heavy chain gene (CLTC). *Mod Pathol*. 2003;16(8):828-32.
- Onciu M, Behm FG, Downing JR, Shurtleff SA, Raimondi SC, Ma Z, et al. ALK-positive plasmablastic B-cell lymphoma with expression of the NPM-ALK fusion transcript: report of 2 cases. *Blood*. 2003;102(7):2642-4.
- Adam P, Katzenberger T, Seeberger H, Gattenlohner S, Wolf J, Steinlein C, et al. A case of a diffuse large B-cell lymphoma of plasmablastic type associated with the t(2;5)(p23;q35) chromosome translocation. *Am J Surg Pathol*. 2003;27(11):1473-6.
- Van Roosbroeck K, Cools J, Dierickx D, Thomas J, Vandenberghe P, Stul M, et al. ALK-positive large B-cell lymphomas with cryptic SEC31A-ALK and NPM1-ALK fusions. *Haematologica*. 2010;95(3):509-13.
- Takeuchi K, Choi YL, Togashi Y, Soda M, Hatano S, Inamura K, et al. KIF5B-ALK, a novel fusion oncokine identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. *Clin Cancer Res*. 2009;15(9):3143-9.
- Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature*. 2007;448(7153):561-6.
- Takeuchi K, Choi YL, Soda M, Inamura K, Togashi Y, Hatano S, et al. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. *Clin Cancer Res*. 2008;14(20):6618-24.
- Choi YL, Takeuchi K, Soda M, Inamura K, Togashi Y, Hatano S, et al. Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. *Cancer Res*. 2008;68(13):4971-6.
- Onishi M, Kinoshita S, Morikawa Y, Shibuya A, Phillips J, Lanier LL, et al. Applications of retrovirus-mediated expression cloning. *Exp Hematol*. 1996;24:324-9.
- Reichard KK, McKenna RW, Kroft SH. ALK-positive diffuse large B-cell lymphoma: report of four cases and review of the literature. *Mod Pathol*. 2007;20(3):310-9.
- Inamura K, Takeuchi K, Togashi Y, Hatano S, Ninomiya H, Motoi N, et al. EML4-ALK lung cancers are characterized by rare other mutations, a TTF-1 cell lineage, an acinar histology, and young onset. *Mod Pathol*. 2009;22(4):508-15.
- Komatsu M, Kurokawa H, Waguri S, Taguchi K, Kobayashi A, Ichimura Y, et al. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat Cell Biol*. 2010;12(3):213-23.
- Kirkin V, McEwan DG, Novak I, Dikic I. A role for ubiquitin in selective autophagy. *Mol Cell*. 2009;34(3):259-69.
- Seibenhener ML, Geetha T, Wooten MW. Sequestosome 1/p62--more than just a scaffold. *FEBS Lett*. 2007;581(2):175-9.
- Bjorkoy G, Lamark T, Johansen T. p62/SQSTM1: a missing link between protein aggregates and the autophagy machinery. *Autophagy*. 2006;2(2):138-9.
- Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem*. 2007;282(33):24131-45.
- Laurin N, Brown JE, Morissette J, Raymond V. Recurrent mutation of the gene encoding sequestosome 1 (SQSTM1/p62) in Paget disease of bone. *Am J Hum Genet*. 2002;70(6):1582-8.
- Tort F, Pinyol M, Pulford K, Roncador G, Hernandez L, Nayach I, et al. Molecular characterization of a new ALK translocation involving moesin (MSN-ALK) in anaplastic large cell lymphoma. *Lab Invest*. 2001;81(3):419-26.
- Lamark T, Perander M, Outzen H, Kristiansen K, Overvatn A, Michaelsen E, et al. Interaction codes within the family of mammalian Phox and Bem1p domain-containing proteins. *J Biol Chem*. 2003;278(36):34568-81.

MCPIP1 Ribonuclease Antagonizes Dicer and Terminates MicroRNA Biogenesis through Precursor MicroRNA Degradation

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SUMMARY

MicroRNAs (miRNAs) are versatile regulators of gene expression and undergo complex maturation processes. However, the mechanism(s) stabilizing or reducing these small RNAs remains poorly understood. Here we identify mammalian immune regulator MCPIP1 (Zc3h12a) ribonuclease as a broad suppressor of miRNA activity and biogenesis, which counteracts Dicer, a central ribonuclease in miRNA processing. MCPIP1 suppresses miRNA biosynthesis via cleavage of the terminal loops of precursor miRNAs (pre-miRNAs). MCPIP1 also carries a vertebrate-specific oligomerization domain important for pre-miRNA recognition, indicating its recent evolution. Furthermore, we observed potential antagonism between MCPIP1 and Dicer function in human cancer and found a regulatory role of MCPIP1 in the signaling axis comprising miR-155 and its target c-Maf. These results collectively suggest that the balance between processing and destroying ribonucleases modulates miRNA biogenesis and potentially affects pathological miRNA dysregulation. The presence of this abortive processing machinery and diversity of MCPIP1-related genes may imply a dynamic evolutionary transition of the RNA silencing system.

INTRODUCTION

miRNAs constitute a large family of short endogenous, noncoding RNA molecules that regulate posttranscriptional gene silencing. miRNAs operate in many important aspects of diverse biological programs, including differentiation, proliferation, and cell death, and their deregulation is associated with an expanding number of disease processes such as cancer and

cardiovascular diseases. The canonical miRNA biogenesis pathway is composed of several steps in mammalian cells (Davis and Hata, 2009; Siomi and Siomi, 2010). The primary transcripts of miRNA genes, primary miRNAs (pri-miRNAs), are transcribed and endonucleolytically cleaved to precursor miRNAs (pre-miRNAs) by the nuclear RNase III Drosha. The pre-miRNAs are exported into the cytoplasm by exportin-5 (XPO5) and further cleaved to a double-stranded miRNA duplex by another RNase III, Dicer. The mature miRNA strand is subsequently incorporated into the RNA-induced silencing complex (RISC). While it has been recently shown that multiple steps of miRNA biogenesis are regulated by several RNA binding factors such as Lin-28, hnRNP A1, and KSRP, and crosstalk with intracellular signaling networks (Suzuki and Miyazono, 2011; Suzuki et al., 2009), *cis*-regulatory elements and *trans*-acting factors responsible for stabilization or reduction of miRNAs have been largely unknown.

In mammalian systems, previous studies have established the close relationship between the immune system and the small RNA regulation. miRNA expression is dynamically regulated during the immune response (Moschos et al., 2007; O'Connell et al., 2010), and miRNA biogenesis also seems actively regulated in this setting; KSRP has been shown to regulate miRNA maturation in macrophage activation (Ruggiero et al., 2009).

In the present study, we identify the mammalian immune regulator, MCPIP1 (monocyte chemoattractant protein [MCP]-1-induced protein 1, also known as Zc3h12a [Zinc-finger CCH-type containing 12A]), as a broad suppressor of the miRNA pathway. MCPIP1 with NYN nuclease domain preferentially cleaves the terminal loops of pre-miRNAs and counteracts Dicer, leading to the inhibition of *de novo* miRNA synthesis. Inverse correlation between MCPIP1 and Dicer function was seen in human lung cancer, in which low Dicer expression is associated with poor prognosis. These results suggest that the balance of productive and abortive ribonucleases modulates miRNA biogenesis and potentially affects pathological miRNA dysregulation. The presence of this abortive processing machinery and diversity of MCPIP1-related genes also implies a dynamic evolutionary transition of the RNA silencing system.

RESULTS

Silencing of miRNA Activity by a CCCH Zinc-Finger Protein, MCPIP1

We screened regulator(s) of the miRNA pathway employing luciferase reporter constructs with the complementary sequence to several miRNAs in the 3' UTR. Based on a close relationship between small RNA and immune system (O'Connell et al., 2010), we particularly focused on the immune response-associated genes harboring a potential RNA binding domain. In this process, we found that, although the ectopic expression of short fragments of pri-miRNAs caused a 5- to 10-fold reduction of luciferase activity in this assay, human MCPIP1 (also known as Zc3h12a), one of the CCCH-type zinc-finger proteins with RNA-binding potential (Liang et al., 2008a), remarkably attenuated the RNAi activity mediated by introduction of pri-miR-122 (Figure 1A). We also confirmed similar results using various mammalian cell lines such as HeLa, HepG2, and Cos-7 cells to exclude the possibility of cell-type-specific phenomena.

miRNA silencing activity of MCPIP1 was extended to other miRNAs, such as miR-21 and miR-135b (Figures 1B and 1C) and recapitulated in the experiments using miR-135b and its target, *adenomatous polyposis coli* (*APC*) 3' UTR (Figure 1D) (Nagel et al., 2008). In addition, siRNA-mediated MCPIP1 knockdown consistently enhanced endogenous miRNA activity (Figure 1E) in HepG2 hepatocellular carcinoma cells, which express MCPIP1 at the basal level. These results suggested that MCPIP1 suppresses miRNA functions in gene silencing.

Inhibition of miRNA Biogenesis by MCPIP1

About 60 CCCH-type zinc-finger proteins have been identified so far in mouse and human (Liang et al., 2008a). MCPIP1 exerts the most potent suppressive activity to miRNAs among the MCPIP family (MCPIP1/2/3/4) and other tested CCCH-type zinc-finger proteins such as Cpsf4, Cpsf4l, Zfp36, Rc3h1, and Rc3h2 (Figure 1A), which prompted us to focus on the MCPIP1 function.

Because some CCCH proteins, including Zfp36 and Rc3h1, are involved in RNA metabolism (Liang et al., 2008a) and MCPIP1 appears to have an influence on broad miRNA activities, we examined a potential impact of MCPIP1 on the intracellular fates of miRNAs and their precursors. Quantitative RT-PCR analysis showed that MCPIP1 remarkably suppressed mature miRNA production from ectopically expressed pri-miRNAs of various miRNAs, such as miR-135b, -146a, -21, -155, -143, and -145 (Figures 1F, 1G, and S1A–S1D). Interestingly, MCPIP1 decreased the expression levels of precursor forms (pre-miRNAs) of these miRNAs, but the pri-miRNAs showed no significant change (Figures 1F, 1G, and S1A–S1D). Northern blot analyses confirmed these observations (Figures 1H, 1I, and S1E–S1H). MCPIP1 overexpression also decreased the mature miRNA levels of endogenously expressed miRNAs by 20%–30%.

Two major steps of mammalian miRNA biogenesis are executed by nuclear Drosha and cytoplasmic Dicer. We then asked in which cellular compartment MCPIP1 localizes and functions. Immunocytochemical analysis showed that MCPIP1 localized mainly in the cytoplasm as dot-like structures (Figure 2A).

GFP-tagged MCPIP1 also partly colocalized with HA-tagged GW182 or Dcp1a, suggesting that MCPIP1 localizes in GW bodies or P bodies (Figure 2B). Colocalization frequency of GFP-MCPIP1 with HA-GW182 was higher than that with HA-Dcp1a, while GFP-MCPIP1 was often adjacent to Dcp1a-positive foci. In addition, MCPIP1 strongly reduced miRNA expression and activity under pri-miRNA overexpression (Figure 1), but showed no significant effect on miRNA activity upon miRNA duplex introduction (Figures 2C and 2D) or the stability of introduced miRNA duplexes (Figures 2E and 2F).

Next, we confirmed that MCPIP1 knockdown elevated the intrinsic levels of several mature miRNAs, such as miR-21, -26a, -107, -182, -146a, -17-5p, and -135b in HepG2 cells, without concomitant changes of the corresponding pri-miRNAs (Figure 2G) and enhanced the miRNA maturation efficacy from ectopically expressed pri-miRNAs (Figures S2A–S2D). Microarray analysis of miRNA expression profile demonstrated that wide proportion of miRNAs tended to be upregulated by MCPIP1 suppression (Figure 2H). These results thus proposed that MCPIP1 acts on pre-miRNAs in the cytoplasm to inhibit de novo maturation of a broad range of miRNA species, without deteriorating the major molecular components of the miRNA pathway (Figure S2E). Immunoblot analysis also confirmed endogenous expression of Dicer and MCPIP1 in the cell lines used in this study (Figure S2F).

Cleavage of Pre-miRNAs by MCPIP1 Ribonuclease

The N terminus of MCPIP1 (residues 133–300) is well conserved among other related proteins including MCPIP2, MCPIP3, and MCPIP4 and has been previously referred to as Nedd4-BP1, bacterial YacP Nuclease (NYN) domain (Anantharaman and Aravind, 2006) (Figures 3A and S3A). The NYN domain shares a similar protein fold with two characterized nuclease domains, the PiT N-terminal (PIN) domain and FLAP nuclease domain, and is thus predicted to exert nuclease activity (Figures 3A and S3A–S3B) (Anantharaman and Aravind, 2006). In fact, it has been recently shown that MCPIP1/Zc3h12a is involved in mRNA decay of several genes as a ribonuclease (Matsushita et al., 2009).

We therefore examined whether pre-miRNAs can be cleaved by MCPIP1 through in vitro cleavage assay using the radiolabeled pre-miRNAs and FLAG-tagged MCPIP1. When the radiolabeled pre-miRNAs were incubated with immunoprecipitated FLAG-tagged MCPIP1 in vitro, the resultant products migrated as a broad and fuzzy band around 25–35 nt in length on electrophoresis, while FLAG-Dicer generated ~22 nt mature miRNA duplex (Figures 3B and 3C), demonstrating that MCPIP1 targets pre-miRNAs as a ribonuclease. We observed that both Dicer and MCPIP1 cleaved pre-miRNAs in a time- and dose-dependent manner (Figures S3C and S3D). The NYN domain of MCPIP1 has the canonical four conserved acidic residues (Asp 141, Asp 195, Asp 226, Asp 244) by analogy with other PIN domain RNases (Figure S3A) (Anantharaman and Aravind, 2006). These acidic residues have been reported to be crucial for metal ion binding and nuclease activity (Figure S3B). In accordance with these findings, mutant MCPIP1 with D141N mutation in the NYN domain, but not C306R mutation in the CCCH motif, failed to cleave pre-miRNAs in vitro (Figure 3D),

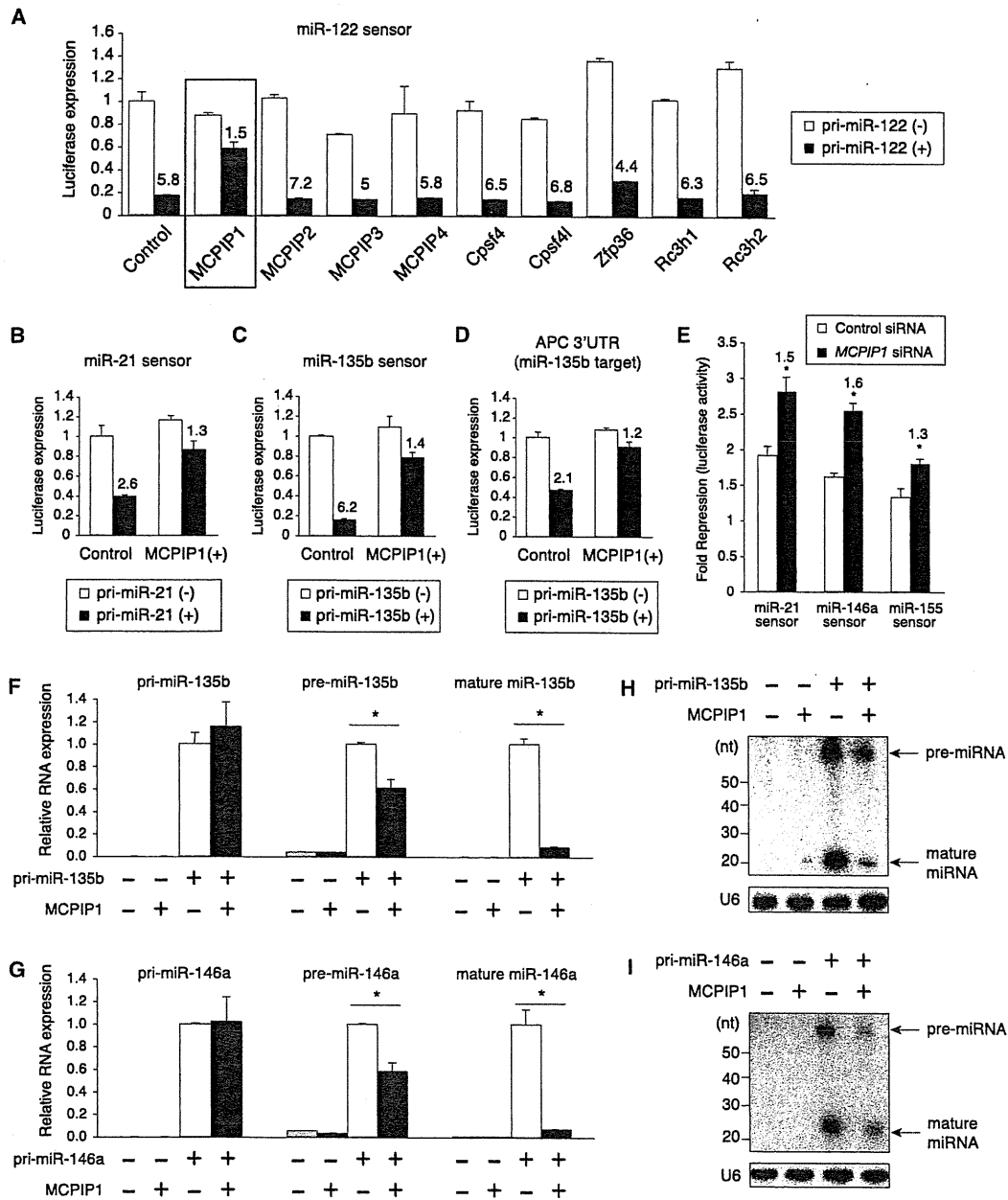


Figure 1. Suppression of miRNA Activity and Biogenesis by a CCH-Type Zinc-Finger Gene, MCPIP1

(A) Effects of CCH zinc-finger genes on miRNA activity. HEK293T cells were cotransfected with miR-122 sensor vector, pri-miR-122 expression vector, and the expression vectors of indicated genes, and applied to luciferase reporter assay at 48 hr after transfection. Numbers indicate the ratio of fold repression.

(B–D) Suppression of a range of miRNA activities by MCPIP1. miR-21 sensor (B), miR-135b sensor (C), or APC 3' UTR luciferase vector (D), and the corresponding pri-miRNA expression vectors were ectopically expressed with/without MCPIP1 plasmids in HEK293T cells and subjected to luciferase reporter assay as shown in (A). Numbers indicate the ratio of fold repression.

(E) Enhancement of endogenous miRNA activity by MCPIP1 knockdown. HepG2 cells were transfected with indicated miRNA sensor vector and control siRNA or siRNA for *MCPIP1*. miRNA activities were measured by the ratio of the luciferase expression of miRNA sensor vector to that of control sensor vector (**p* < 0.05, compared to Control siRNA; *n* = 3). Numbers indicate the ratio between control siRNA and *MCPIP1* siRNA.

(F–I) Inhibition of de novo miRNA biosynthesis by MCPIP1 overexpression. Quantitative RT-PCR (qRT-PCR) (F and G) and northern blot (H and I) analyses of RNA samples from HEK293T cells transfected with pri-miR-135b (F and H), -146a (G, I), and MCPIP1 (**p* < 0.05, compared to empty vector; *n* = 3). See also Figure S1. Error bars represent SEM.

indicating the indispensable role of NYN domain for MCPIP1 ribonuclease function. Recombinant MCPIP1 proteins further cleaved pre-miRNAs in a NYN domain-dependent manner in vitro (Figure 3E). On the other hand, a mutation in the CCCH motif (C306R), which had no significant effect on in vitro cleavage activity (Figures 3D and 3E), attenuated the miRNA suppressor activity of MCPIP1 in vivo (Figures 3F and 3G). This result suggests that the CCCH motif is additionally important for the in vivo function of MCPIP1, as investigated in Figure 7 (see below).

Functional Antagonism between MCPIP1 Ribonuclease and Dicer and Its Implication in Human Cancer

We then investigated whether MCPIP1 and its ribonuclease function can compete with Dicer function by in vitro competition analysis. As shown in Figures 4A and 4B, MCPIP1 antagonized Dicer-mediated pre-miRNA processing in vitro, depending on the intact NYN domain but not the CCCH motif, in a dose-dependent manner. Sequential in vitro cleavage analysis showed that MCPIP1-cleaved fragments were inefficient substrates for Dicer processing (Figure S4A). These results thus suggested that MCPIP1-mediated pre-miRNA cleavage could interfere with Dicer activity. In addition, absence of the degraded products in northern blot analyses suggested that MCPIP1-cleaved fragments may be unstable and subjected to a rapid subsequent degradation in vivo (Figure 1G, 1I, and S1E–S1H).

While individual miRNAs can behave as tumor suppressors or oncogenes (Suzuki and Miyazono, 2010), global miRNA downregulation has been shown to be a general trait of human cancers in several reports (Kumar et al., 2007; Lu et al., 2005; Ozen et al., 2008). Several mechanisms including downregulation of miRNA processing factors, such as Dicer and Drosha, and mutations of TRBP and XPO5 have been reported (Karube et al., 2005; Martello et al., 2010; Melo et al., 2010). Recent reports also revealed that certain oncogenesis-related molecules, such as p53, Smad, and estrogen receptor, are coupled with miRNA biogenesis (Suzuki and Miyazono, 2011). As evidence demonstrating the clinical relevance of these phenomena, reduced expression of Dicer is associated with poor survival in lung, ovarian, and breast cancer patients (Karube et al., 2005; Martello et al., 2010; Merritt et al., 2008).

Based on these observations, we investigated potential functional crosstalk between MCPIP1 and Dicer using several public gene expression datasets containing molecular and associated clinical details. We extracted gene sets associated negatively or positively with Dicer expression status (“Dicer High/Low Downregulated or Upregulated genes”) from several human cancer cohorts, and performed gene set enrichment analysis (GSEA) (Subramanian et al., 2005) to examine whether these gene sets are overexpressed or underexpressed along the expression status of MCPIP1. During these trials, we found a potential antagonistic relationship between MCPIP1 and Dicer in human lung cancer. In the dataset of lung adenocarcinoma patients (Bild et al., 2006), GSEA demonstrated that “Dicer High/Low Downregulated genes” and “Dicer High/Low Upregulated genes” are enriched in patients with high MCPIP1 expression (“MCPIP1 High Case”) and low MCPIP1 expression (“MCPIP1 Low Case”), respectively (Figure 4C). GSEA using the

sets of potential miRNA target genes also demonstrated that a large proportion of miRNA target gene sets are upregulated in the MCPIP1-High group against the MCPIP1-Low group, while the opposite phenomenon was seen in the comparison between Dicer-High and -Low group (Figure 4D). Furthermore, we observed that high MCPIP1 levels are associated with poor survival in lung adenocarcinoma patients (Figure 4E), in an opposite manner to the association between low Dicer levels and poor prognosis (Karube et al., 2005; Merritt et al., 2008). Similar results were also obtained in another dataset of lung adenocarcinoma patients (Shedden et al., 2008) and a dataset of lung squamous cell carcinoma patients (Larsen et al., 2007) (Figures S4B–S4E). Together with in vitro antagonism, these findings demonstrate an inverse correlation between MCPIP1 expression and Dicer function in human cancer and suggest that MCPIP1 could drive opposite effects against Dicer on the transcriptome.

Modulation of miR-155/c-Maf axis by MCPIP1

We next investigated the impact of MCPIP1 on the function of endogenous miRNAs. MCPIP1 expression dynamically changes during the inflammatory response (Liang et al., 2008a; Liang et al., 2008b; Matsushita et al., 2009), and the importance of MCPIP1 in the immune system has recently emerged from a manifestation of profound autoimmune phenotype in mice lacking MCPIP1 (Matsushita et al., 2009). MCPIP1 has been shown to promote the mRNA decay of several inflammatory genes such as IL-6 as an endoRNase (Matsushita et al., 2009). The severity of autoimmune phenotype in *MCPIP1*^{-/-} mice suggests the existence of additional targets of MCPIP1 other than IL-6 (Matsushita et al., 2009), and it might thus be partly explained by miRNA deregulation. In support of this notion, it has been shown that transgenic mice overexpressing several miRNAs, such as miR-17-92 and miR-155, exhibit autoimmune and/or lymphoproliferative diseases (Costinean et al., 2009; Xiao et al., 2008). Interestingly, increased and decreased expression of Th2 cytokine, IL-4, has been observed in miR-155^{-/-} and *MCPIP1*^{-/-} T cells, respectively (Matsushita et al., 2009; Rodriguez et al., 2007).

We then examined the potential involvement of MCPIP1 in regulation of miR-155 function in Jurkat T cells. In accordance with our previous results, MCPIP1 knockdown, in fact, caused miR-155 upregulation in Jurkat T cells (Figure 5A). MCPIP1 knockdown further suppressed the expression of the miR-155 target c-Maf and the induction of its transcriptional target IL-4 by phorbol myristate acetate (PMA) and ionomycin in a miR-155-dependent fashion (Rodriguez et al., 2007) (Figures 5B–5D), while IL-6 showed no significant effect on this axis (Figure S5A). These results are consistent with increased and decreased expression of IL-4 in miR-155^{-/-} and *MCPIP1*^{-/-} T cells (Matsushita et al., 2009; Rodriguez et al., 2007), reinforcing the physiological relevance of MCPIP1-mediated miRNA regulation. In addition, we observed that MCPIP1 is induced by lipopolysaccharide (LPS) and involved in the restriction of miR-155 upregulation under innate immune response and miR-16 downregulation during macrophage differentiation, while Dicer expression is not affected by LPS (Li et al., 2010) (Figures 5E, S5B, and S5C). These findings underscore active MCPIP1 involvement in miRNA biogenesis, together with the inverse

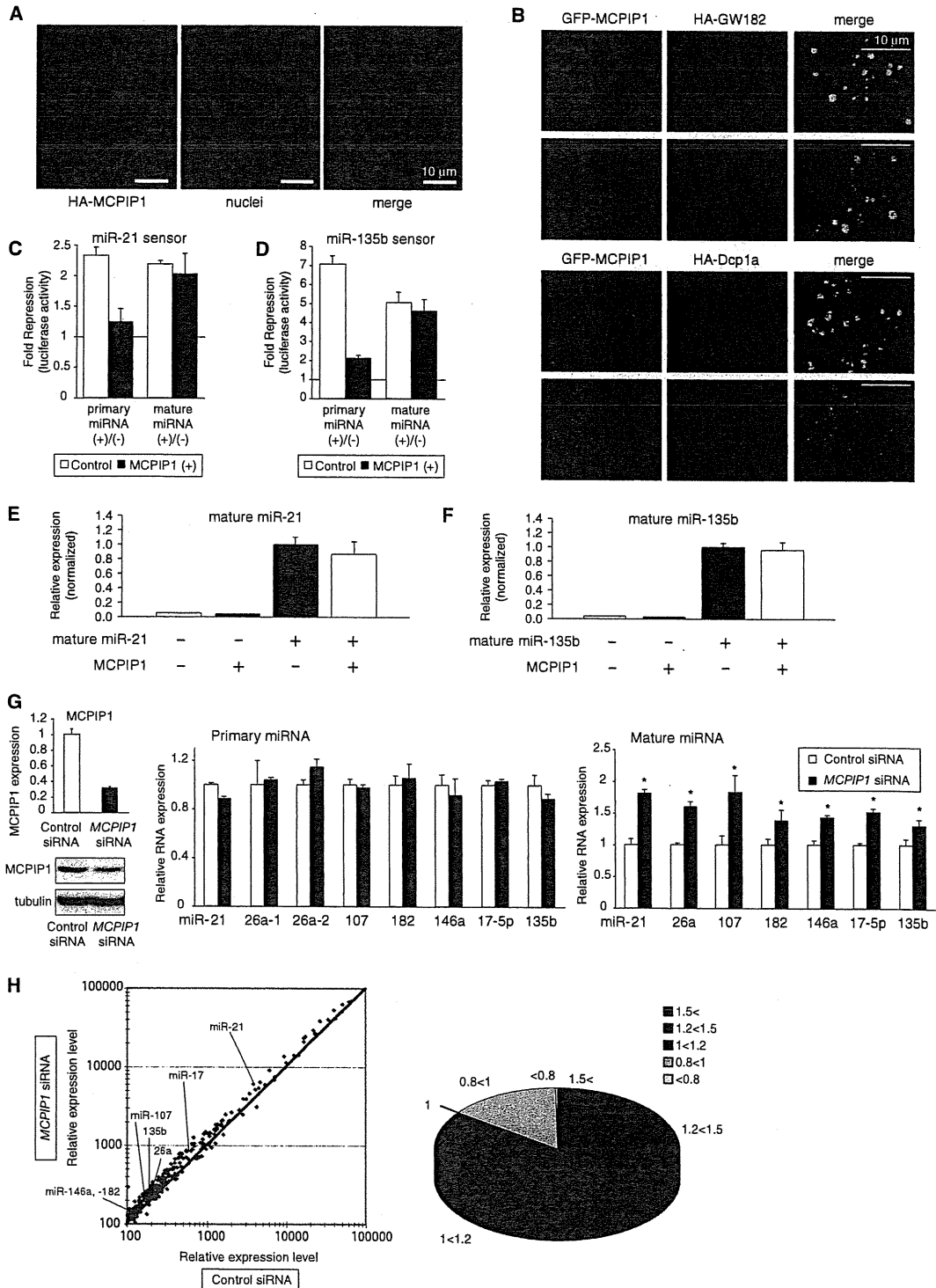


Figure 2. Cytoplasmic Localization of MCPIP1 and Enhancement of miRNA Maturation by MCPIP1 Depletion
(A) Immunocytochemical analysis of overexpressed HA-MCPIP1. Scale bar: 10 μ m.
(B) Localization of GFP-MCPIP1, HA-GW182 and HA-Dcp1a.

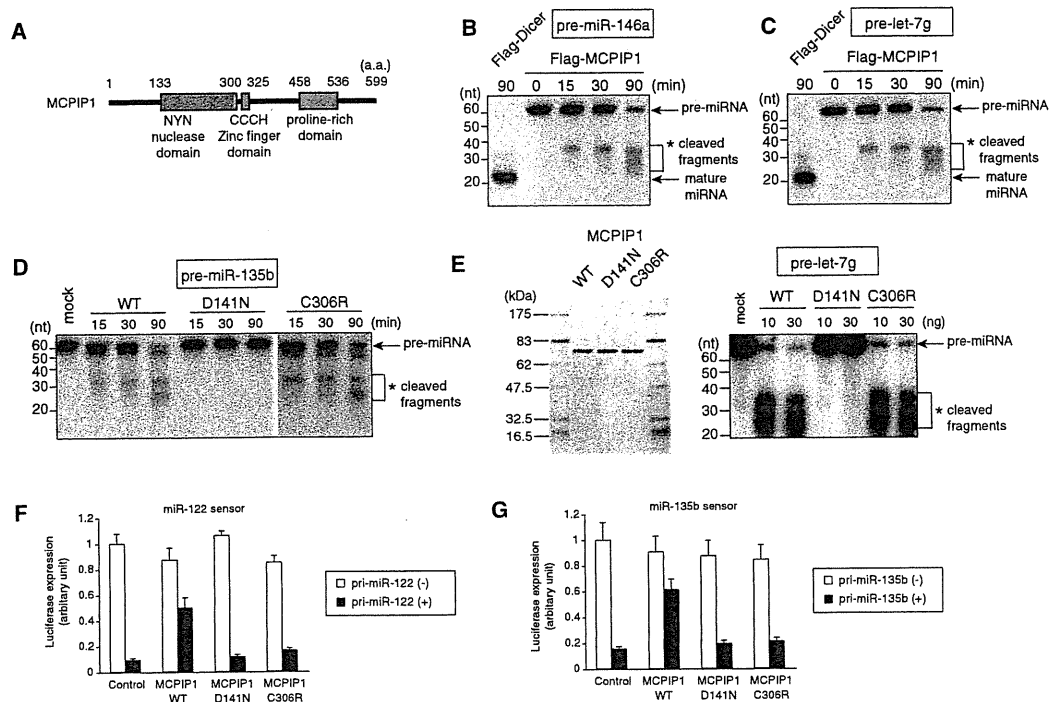


Figure 3. Cleavage of Pre-miRNAs by MCPIP1 Ribonuclease

(A) Domain organization of MCPIP1. NYN, Nedda4-BP1, bacterial YacP Nuclease domain; CCCH, CCCH zinc-finger domain; PRD, proline-rich domain. See also Figure S3.

(B and C) In vitro cleavage of pre-miRNAs by MCPIP1. Radiolabeled pre-miR-146a (B) or pre-let-7g (C) were incubated with immunoprecipitated FLAG-Dicer or FLAG-MCPIP1 from HEK293T cells for indicated periods. The RNA was then extracted and analyzed by denaturing PAGE and autoradiography. 'nt' denotes nucleotides.

(D and E) Requirement of an intact NYN domain for in vitro RNase activity of MCPIP1. Radiolabeled pre-miRNAs were incubated with immunoprecipitated FLAG-MCPIP1 proteins (wild-type [WT], D141N, and C306R) (D) or recombinant MCPIP1 proteins (E).

(F and G) Effects of mutations in the NYN domain and CCCH domain on in vivo miRNA silencing activities. miR-122 sensor (F) or miR-135b sensor (G) and the corresponding pri-miRNA were coexpressed with/without MCPIP1 into HEK293T cells, and subjected to luciferase reporter assay ($n = 3$). Error bars represent SEM.

correlation between MCPIP1 and Dicer function in human cancer (Figure 4).

Targeting of the Terminal Loops of Pre-miRNAs by MCPIP1 Ribonuclease

The results shown in Figures 3 and 4 demonstrate that MCPIP1 cleaves pre-miRNAs and antagonizes Dicer function. We further analyzed the mode of MCPIP1 action on pre-miRNAs by direct cloning of pre-miRNA fragments cleaved by MCPIP1. Various 5' and 3' miRNA strands accompanied with additional bases

from the terminal loop were recovered as the cleaved fragments of pre-miR-146a, pre-let-7g, pre-miR-135b, pre-miR-143 and pre-miR-16-1 (Figures 6A, 6B, and S6A–S6C), indicating that the primary cleavage sites of MCPIP1 were dispersed in the terminal loop of pre-miRNAs. A comparison with the predicted secondary structures of pre-miRNAs (Zuker, 2003) shows that MCPIP1 preferentially cleaves the unpaired regions around the terminal loops as an endoribonuclease (Figure 6C). Furthermore, addition of Lin-28b, an RNA-binding protein interacting with the terminal-loop of pre-let-7 (Heo et al., 2008), abolished the

(C and D) Differential effects of MCPIP1 on miRNA activities under introduction of pri-miRNAs and miRNA duplexes. miRNA sensor vectors (C: miR-21, D: miR-135b) and the corresponding pri-miRNA expression vectors or synthetic miRNA duplexes were cotransfected with/without MCPIP1 plasmids into HEK293T cells, and applied to luciferase reporter assay.

(E and F) Effect of MCPIP1 on the stability of introduced miRNAs duplexes. MCPIP1 plasmid was cotransfected with synthetic miRNA duplexes (10 nM) (E: miR-21, F: miR-135b) in HEK293T cells. At 48 hr posttransfection, miRNA expression levels were analyzed with qRT-PCR.

(G) Upregulation of mature miRNA levels, but not pri-miRNA levels, of several miRNAs by MCPIP1 depletion. The levels of MCPIP1, pri-miRNAs, and mature miRNAs were compared by qRT-PCR analyses in HepG2 cells transfected with control siRNA or siRNA for MCPIP1 ($*p < 0.05$, compared to Control siRNA; $n = 3$).

(H) Global effects of MCPIP1 knockdown on endogenous miRNA expression, analyzed by miRNA microarray analysis in HepG2 cells. The proportion of miRNAs at different fold change levels is shown in the right panel. See also Figure S2. Error bars represent SEM.