expression of *MET* in the leukemic cells (Figure 5a). The increased concentration of HGF might therefore confer a growth advantage on ATL cells after they upregulate the expression of MET. Given that the JAK-STAT signaling pathway is activated in the leukemic cells of patients with advanced ATL (Migone *et al.*, 1995), it may be relevant that binding sites for STAT1 or STAT3 are present in the promoter regions of five (including *MET*) of the six acute stage-specific genes identified in the present study (Figure 4a). We did not detect a significant difference in DNA content (in our data set) for the *MET* locus between chronic and acute stages of ATL. It is thus possible that JAK-STAT signaling contributes to transcriptional activation of *MET*.

Given that our data are derived from purified ATL cells, they can be further used to characterize ATL in various ways. For instance, we attempted to isolate genes whose expression was linked to the presence of hypercalcemia in the study patients (data not shown); such genes included that for parathyroid hormone-like hormone (GenBank accession no. BC005961), which has been shown to be responsible for many instances of humoral hypercalcemia in individuals with cancer including ATL (Broadus *et al.*, 1988; Motokura *et al.*, 1988).

We have demonstrated the existence of an HGF-MET paracrine loop specific to the acute stage of ATL. Given that ligation of MET by HGF promoted the proliferation of ATL cells, activation of the HGF-MET signaling pathway is a candidate molecular mechanism for stage progression in ATL. Furthermore, our observation that this mitogenic effect was blocked by antibodies to HGF provides potential new targets for ATL therapy.

Materials and methods

Expression profiling

All clinical specimens were obtained with written informed consent, and the study was approved by the ethics committees of both Jichi Medical University and Nagasaki University. The diagnosis of ATL in all cases was based on clinical features, immunophenotypes of leukemic cells, and the monoclonal integration of HTLV-1 proviral DNA into the genome of leukemic cells (Shimoyama, 1991). MNCs isolated from PB were labeled with magnetic bead-conjugated mouse monoclonal antibodies to CD4 (CD4 MicroBeads, Miltenyi Biotec, Auburn, CA, USA). For PHA stimulation, purified $CD4^+$ cells from healthy individuals were incubated for 48 h in Rosewell's Park Memorial Institute media (RPMI) 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and PHA-P (8 μg/ml) (Sigma, St Louis, MO, USA).

Column fractionation of MNCs, RNA preparation, and hybridization with HGU133A & B microarrays (Affymetrix, Santa Clara, CA, USA) were performed as described previously (Choi *et al.*, 2004). The mean expression intensity of the internal positive control probe sets (http://www.affymetrix.com/support/technical/mask_files.affx) was set to 500 U in each hybridization, and the fluorescence intensity of each test gene was normalized accordingly. All HGU133A & B microarray data are available at the Gene Expression Omnibus web site (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE1466.

Student's *t*-test with the Benjamini and Hochberg false discovery rate option was performed with GeneSpring 7.0 software (Silicon Genetics). Effect size was defined as an absolute difference in mean expression intensity between a given pair of classes (Dhanasekaran *et al.*, 2001). Education of and prediction by our ANN were performed with NeuralWorks Professional II Plus v.5.3 software (NeuralWare, Carnegie, PA, USA) as described previously (O'Neill and Song, 2003).

Analysis of CNA

Genomic DNA was obtained from purified $CD4^+$ ATL cells ($n=24$) and from MNCs of patient ID6 with the use of a QIAmp DNA Mini Kit (Qiagen, Valencia, CA, USA). Each DNA sample (250 ng) was digested with *Hind*III, ligated to Adaptor-Hind (Affymetrix), amplified by PCR, and subjected to hybridization with Mapping 50K Hind 240 arrays (Affymetrix). Chromosome copy number at each SNP site was inferred from hybridization signal intensity on the arrays with the use of CNAG software (http://www.genome.umin.jp) (Nannya *et al.*, 2005). For a normal reference, we used array data of PB MNCs isolated from four healthy volunteers. Assessment of copy number for all SNP sites is demonstrated in Supplementary Table 6. The raw data of Mapping 50K Hind 240 arrays is available upon request. Statistical analysis of copy number was performed with GeneSpring 7.0. Alterations in the amount of genomic DNA were confirmed by quantitative real-time PCR with an ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Foster City, CA, USA). The oligonucleotide primers were 5′-AGCATGTCCACAAATGGCCTTTGG-3′ and 5′-CAGTTTTCCTGTCATGGGAAAGGG-3′ for a region of chromosome 6, and 5′-CTGACCTGCCGTCTA GAAAAACCT-3′ and 5′-CAGGAAATGAGCTTGACAA AGTGG-3′ for the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene.

MET expression

ATL cells were stained with rabbit polyclonal antibodies to MET (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with mouse monoclonal antibodies to CD3 and to CD4 (both from BD Biosciences, San Diego, CA, USA) and were then subjected to flow cytometry with a FACScan instrument (BD Biosciences). The concentration of HGF in plasma was determined with a Quantikine ELISA kit for human HGF (R&D Systems, Minneapolis, MN, USA).

For quantitative RT–PCR analysis of *MET* expression, portions of nonamplified cDNA were subjected to PCR with a QuantiTect SYBR Green PCR kit (Qiagen). The oligonucleotide primers for PCR were 5′-GTCAGTGGTGGACCTG ACCT-3′ and 5′-TGAGCTTGACAAAGTGGTCG-3′ for *GAPDH* cDNA, and 5′-ACTTGTTGCAAGGGAGAAGAC TCC-3′ and 5′-AGCGTTCACATGGACATAGTGCTC-3′ for *MET* cDNA.

KK-1 cell experiments

KK-1 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and recombinant human interleukin-2 (10 ng/ml) (Sigma). For immunoblot analysis, cells were cultured for 48 h without FBS and interleukin-2 and then incubated for 10 min with recombinant human HGF (50 ng/ml) (Sigma) either alone or together with rabbit polyclonal antibodies to HGF (10 μg/ml) (Montesano *et al.*, 1991). The

cells were then lysed and subjected to immunoblot analysis with mouse monoclonal antibodies to phosphotyrosine (4G10, Upstate Biotechnology, Charlottesville, VA, USA) or with rabbit polyclonal antibodies to MET (#05–237, Upstate Biotechnology) as described previously (Yamashita *et al.*, 2001). For assay of cell proliferation, serum-deprived KK-1 cells were cultured for 24 h at a density of 5×10^5/ml with HGF (50 ng/ml) either alone or together with antibodies to HGF (10 μg/ml) and were then mixed with MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt]. Cell proliferation was measured with a CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA).

Abbreviations

ATL, Adult T-cell leukemia; PCR, polymerase chain reaction; PHA, phytohemagglutinin; ANN, artificial neural network; CNA, copy number alterations; HGF, hepatocyte growth factor.

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References

Alon U, Barkai N, Notterman DA, Gish K, Ybarra S, Mack D *et al.* (1999). Broad patterns of gene expression revealed by clustering analysis of tumor and normal colon tissues probed by oligonucleotide arrays. *Proc Natl Acad Sci USA* **96**: 6745–6750.

Ariyama Y, Mori T, Shinomiya T, Sakabe T, Fukuda Y, Kanamaru A *et al.* (1999). Chromosomal imbalances in adult T-cell leukemia revealed by comparative genomic hybridization: gains at 14q32 and 2p16–22 in cell lines. *J Hum Genet* **44**: 357–363.

Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmiecik TE, Vande Woude GF *et al.* (1991). Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science* **251**: 802–804.

Broadus AE, Mangin M, Ikeda K, Insogna KL, Weir EC, Burtis WJ *et al.* (1988). Humoral hypercalcemia of cancer. Identification of a novel parathyroid hormone-like peptide. *N Engl J Med* **319**: 556–563.

Cesarman E, Chadburn A, Inghirami G, Gaidano G, Knowles DM. (1992). Structural and functional analysis of oncogenes and tumor suppressor genes in adult T-cell leukemia/lymphoma shows frequent p53 mutations. *Blood* **80**: 3205–3216.

Choi YL, Makishima H, Ohashi J, Yamashita Y, Ohki R, Koinuma K *et al.* (2004). DNA microarray analysis of natural killer cell-type lymphoproliferative disease of granular lymphocytes with purified CD3(−)CD56(+) fractions. *Leukemia* **18**: 556–565.

Dhanasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, Kurachi K *et al.* (2001). Delineation of prognostic biomarkers in prostate cancer. *Nature* **412**: 822–826.

Edlich RF, Arnette JA, Williams FM. (2000). Global epidemic of human T-cell lymphotropic virus type-I (HTLV-I). *J Emerg Med* **18**: 109–119.

Funakoshi H, Nakamura T. (2003). Hepatocyte growth factor: from diagnosis to clinical applications. *Clin Chim Acta* **327**: 1–23.

Hatta Y, Hirama T, Miller CW, Yamada Y, Tomonaga M, Koeffler HP. (1995). Homozygous deletions of the p15 (MTS2) and p16 (CDKN2/MTS1) genes in adult T-cell leukemia. *Blood* **85**: 2699–2704.

Imaizumi Y, Murota H, Kanda S, Hishikawa Y, Koji T, Taguchi T *et al.* (2003). Expression of the c-Met proto-oncogene and its possible involvement in liver invasion in adult T-cell leukemia. *Clin Cancer Res* **9**: 181–187.

Lin M, Wei LJ, Sellers WR, Lieberfarb M, Wong WH, Li C. (2004). dChipSNP: significance curve and clustering of SNP-array-based loss-of-heterozygosity data. *Bioinformatics* **20**: 1233–1240.

Lockwood WW, Chari R, Chi B, Lam WL. (2005). Recent advances in array comparative genomic hybridization technologies and their applications in human genetics. *Eur J Hum Genet* **14**: 139–148.

Matsumoto K, Okazaki H, Nakamura T. (1992). Up-regulation of hepatocyte growth factor gene expression by interleukin-1 in human skin fibroblasts. *Biochem Biophys Res Commun* **188**: 235–243.

Matsuoka M. (2003). Human T-cell leukemia virus type I and adult T-cell leukemia. *Oncogene* **22**: 5131–5140.

Migone TS, Lin JX, Cereseto A, Mulloy JC, O'Shea JJ, Franchini G *et al.* (1995). Constitutively activated Jak-STAT pathway in T cells transformed with HTLV-I. *Science* **269**: 79–81.

Montesano R, Matsumoto K, Nakamura T, Orci L. (1991). Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* **67**: 901–908.

Motokura T, Fukumoto S, Takahashi S, Watanabe T, Matsumoto T, Igarashi T *et al.* (1988). Expression of parathyroid hormone-related protein in a human T cell lymphotrophic virus type I-infected T cell line. *Biochem Biophys Res Commun* **154**: 1182–1188.

Nannya Y, Sanada M, Nakazaki K, Hosoya N, Wang L, Hangaishi A *et al.* (2005). A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. *Cancer Res* **65**: 6071–6079.

Nosaka K, Maeda M, Tamiya S, Sakai T, Mitsuya H, Matsuoka M. (2000). Increasing methylation of the CDKN2A gene is associated with the progression of adult T-cell leukemia. *Cancer Res* **60**: 1043–1048.

O'Neill MC, Song L. (2003). Neural network analysis of lymphoma microarray data: prognosis and diagnosis near-perfect. *BMC Bioinformatics* **4**: 13.

Oshiro A, Tagawa H, Ohshima K, Karube K, Uike N, Tashiro Y *et al.* (2006). Identification of subtype-specific genomic alterations in aggressive adult T-cell leukemia/lymphoma. *Blood* **107**: 4500–4507.

Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. (1980). Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci USA* **77**: 7415–7419.

Reiner A, Yekutieli D, Benjamini Y. (2003). Identifying differentially expressed genes using false discovery rate controlling procedures. *Bioinformatics* **19**: 368–375.

Sasaki H, Nishikata I, Shiraga T, Akamatsu E, Fukami T, Hidaka T *et al.* (2005). Overexpression of a cell adhesion molecule, TSLC1, as a possible molecular marker for acute-type adult T-cell leukemia. *Blood* **105**: 1204–1213.

Shimoyama M. (1991). Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984–1987). *Br J Haematol* **79**: 428–437.

Tajima K. (1990). The 4th nation-wide study of adult T-cell leukemia/lymphoma (ATL) in Japan: estimates of risk of ATL and its geographical and clinical features. The T- and B-cell Malignancy Study Group. *Int J Cancer* **45**: 237–243.

Tamiya S, Etoh K, Suzushima H, Takatsuki K, Matsuoka M. (1998). Mutation of CD95 (Fas/Apo-1) gene in adult T-cell leukemia cells. *Blood* **91**: 3935–3942.

Tamura M, Arakaki N, Tsubouchi H, Takada H, Daikuhara Y. (1993). Enhancement of human hepatocyte growth factor production by interleukin-1 alpha and -1 beta and tumor necrosis factor-alpha by fibroblasts in culture. *J Biol Chem* **268**: 8140–8145.

Tsukasaki K, Krebs J, Nagai K, Tomonaga M, Koeffler HP, Bartram CR *et al.* (2001). Comparative genomic hybridization analysis in adult T-cell leukemia/lymphoma: correlation with clinical course. *Blood* **97**: 3875–3881.

Tsukasaki K, Tanosaki S, DeVos S, Hofmann WK, Wachsman W, Gombart AF *et al.* (2004). Identifying progression-associated genes in adult T-cell leukemia/lymphoma by using oligonucleotide microarrays. *Int J Cancer* **109**: 875–881.

Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. (1977). Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood* **50**: 481–492.

Wano Y, Hattori T, Matsuoka M, Takatsuki K, Chua AO, Gubler U *et al.* (1987). Interleukin 1 gene expression in adult T cell leukemia. *J Clin Invest* **80**: 911–916.

Yamada Y, Hatta Y, Murata K, Sugawara K, Ikeda S, Mine M *et al.* (1997). Deletions of p15 and/or p16 genes as a poor-prognosis factor in adult T-cell leukemia. *J Clin Oncol* **15**: 1778–1785.

Yamada Y, Ohmoto Y, Hata T, Yamamura M, Murata K, Tsukasaki K *et al.* (1996). Features of the cytokines secreted by adult T cell leukemia (ATL) cells. *Leuk Lymphoma* **21**: 443–447.

Yamashita Y, Kajigaya S, Yoshida K, Ueno S, Ota J, Ohmine K *et al.* (2001). Sak serine/threonine kinase acts as an effector of Tec tyrosine kinase. *J Biol Chem* **276**: 39012–39020.

Yoshida M, Miyoshi I, Hinuma Y. (1982). Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc Natl Acad Sci USA* **79**: 2031–2035.

Zhao X, Weir BA, LaFramboise T, Lin M, Beroukhim R, Garraway L *et al.* (2005). Homozygous deletions and chromosome amplifications in human lung carcinomas revealed by single nucleotide polymorphism array analysis. *Cancer Res* **65**: 5561–5570.

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ERRATUM

Imatinib enhances human melanoma cell susceptibility to TRAIL-induced cell death: relationship to Bcl-2 family and caspase activation

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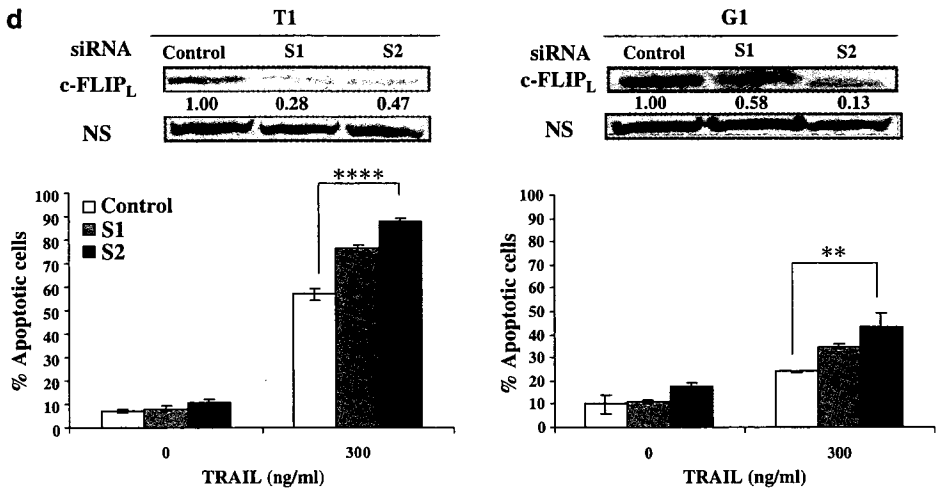
Due to a typesetting error, an incorrect version of figure 6d was published. The correct version of the figure is given here.



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Transforming activity of purinergic receptor P2Y, G-protein coupled, 2 revealed by retroviral expression screening

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Abstract

Colorectal cancer (CRC) is one of the leading causes of cancer death in humans. In order to identify novel cancer-promoting genes in CRC, we here constructed a retroviral cDNA expression library from a CRC cell line RKO, and used it for a focus formation assay with mouse 3T3 fibroblasts, leading to the identification of 42 independent cDNAs. One of such cDNAs turned out to encode purinergic receptor P2Y, G-protein coupled, 2 (P2RY2). The oncogenic potential of P2RY2 was confirmed *in vitro* with the focus formation assay as well as soft agar-growth assay, and also *in vivo* with a tumorigenicity assay in nude mice. While our *P2RY2* cDNA encodes a protein with two amino-acid substitutions compared to the reported one, we have confirmed that the wild-type P2RY2 has a strong transforming potential as well. These results indicate an unexpected role of P2RY2 in the carcinogenesis of human cancers.

Keywords: Purinergic receptor; Oncogene; Colorectal carcinoma; cDNA library; Retrovirus

Colorectal cancer (CRC) is a common malignancy accounting for ~10% of all cancer deaths in United States [1]. In 1990, Feason and Vogelstein postulated a "multistep process" model for the development of CRC [2]. They demonstrated that germline and/or somatic gene mutations are required for malignant transformation of colonic epithelial cells, and that it is the accumulation of multigenetic mutations rather than their sequence that determines the biological behavior of the tumor. Although vast efforts have been used to isolate candidates for such oncogenes involved in CRC, we still have few therapeutic targets to treat this intractable disorder.

The focus formation assay with 3T3 or RAT1 fibroblasts has been extensively used to screen for transforming genes in various carcinomas [3]. In such screening, genomic DNA is isolated from cancer specimens and used to transfect 3T3 fibroblasts to obtain transformed cell foci. It should be noted, however, that, since expression of any genes in these experiments is driven by their own promoters/enhancers, oncogenes in CRC can exert their effects in 3T3 cells only when the promoter/enhancer regions of such genes are active in fibroblasts, which is not always guaranteed.

To ensure the sufficient expression of cDNAs in 3T3 cells, their transcription should be regulated by an exogenous promoter fragment. We have therefore constructed a retroviral cDNA expression library from a CRC cell line, which was used to infect 3T3 cells. In the preparation of cDNA library, we further took advantage of the SMART polymerase chain reaction (PCR) system (Clontech, Palo

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Alto, CA), which preferentially amplifies full-length cDNAs. A focus formation assay with the library resulted in an identification of a transforming purinergic receptor P2Y, G-protein coupled, 2 (P2RY2) cDNA.

Materials and methods

Cell lines and culture. RKO, NIH 3T3, and BOSC23 packaging cell lines [4] were obtained from American Type Culture Collection, and maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and 2 mM L-glutamine.

Construction of retroviral cDNA expression library. Total RNA was extracted from RKO cells with the use of an RNeasy Mini column and RNase-free DNase (both from Qiagen, Valencia, CA), and a retrovirus library was constructed as described previously [5,6]. Briefly, the first-strand cDNA was synthesized by PowerScript reverse transcriptase, SMART IIA oligonucleotide, and CDS primer IIA (all from Clontech). Full-length cDNAs were then amplified for 15 cycles with 5′ PCR primer IIA according to the instructions of the SMART PCR cDNA synthesis kit (Clontech) except a substitution of LA *Taq* polymerase (Takara Bio, Shiga, Japan) for Advantage 2 DNA polymerase provided with the kit. Resultant cDNAs were blunt-ended by T4 DNA polymerase and ligated to the BstXI adaptor (Invitrogen). Unbound adaptors were removed through the cDNA size fractionation column (Invitrogen), and the cDNAs were finally ligated to the pMXS retroviral plasmid (a kind gift of Dr. T. Kitamura at Institute of Medical Science, University of Tokyo) [7]. The pMXS-cDNA plasmids were introduced into ElectroMax DH10B cells (Invitrogen) with electroporation. A cDNA to encode wild-type P2RY2 was generated based on our P2RY2 cDNA with the use of QuickChange Multi Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA).

Focus formation assay. BOSC23 cells (1.8×10^6) were seeded into a 6-cm culture dish, cultured for 24 h, and then transfected with 2 μg of retroviral plasmids mixed with 0.5 μg of pGP plasmid (Takara Bio), 0.5 μg of pE-eco plasmid (Takara Bio), and 18 μl of Lipofectamine reagent (Invitrogen). Two days after the transfection, polybrene (Sigma, St. Louis, MI) was added to the culture supernatant at a concentration of 4 μg/ml, and the supernatant was subsequently used to infect 3T3 cells for 48 h. For the focus formation assay, culture medium of 3T3 cells was then changed to DMEM-F12 supplemented with 5% calf serum and 2 mM L-glutamine. Transformed foci were picked up after 3 weeks of culture. Genomic DNA was extracted from each transformed focus, and was subjected to PCR with 5′ PCR primer IIA (Clontech) and LA Taq polymerase for 40 cycles of 98 °C for 20 s and 68 °C for 6 min. Amplified genome fragments were purified after gel electrophoresis and ligated to pGEM-T Easy vector (Promega) for nucleotide sequencing.

Tumorigenicity assay in nude mice. 3T3 cells (2×10^6) were infected with a retrovirus encoding P2RY2, resuspended in 500 μl of phosphate-buffered saline, and injected into each shoulder of nu/nu Balb-c mouse (6 weeks old). Tumor formation was assessed after 3 weeks.

Anchorage-independent growth in soft agar. 3T3 cells (2×10^6) were infected with a retrovirus encoding P2RY2 or v-Ras, resuspended in the culture medium supplemented with 0.4% agar [Sea Plaque GTG agarose (Cambrex, East Rutherford, NJ)], and seeded onto a base layer of complete medium supplemented with 0.5% agar. Cell growth was assessed after culture for 2 weeks.

Results

Screening with focus formation assay

From mRNA of RKO cells, full-length cDNAs were selectively amplified and ligated to a retroviral vector pMXS. We could obtain a total of 2.5×10^6 colony forming units of independent plasmid clones. Thirty-nine clones were randomly isolated from the library and examined for the incorporated cDNAs. Thirty-seven (94.8%) out of the 39 clones contained inserts with an average length of 1.86 kbp.

By introducing the plasmid DNA into a packaging cell line, we generated a recombinant ecotropic retrovirus library that was subsequently used to infect mouse 3T3 fibroblasts. Infection experiments were repeated for a total of six times. After 3 weeks of culture, 50 transformed foci were observed. No foci could be found among the cells infected with an empty virus, while numerous foci were easily identified in the cells infected with a virus expressing v-Ras oncoprotein.

Each focus was isolated, expanded independently, and used to prepare genomic DNA. We then tried to recover retroviral inserts from such genomic DNA by PCR

Table 1
RKO cDNAs isolated from 3T3 transformants

Clone ID #	Gene symbol	GenBank No.	Presence of full ORF
1	*EIF5A*	NM_001970	Yes
2	*SQLE*	NM_003129	Yes
3	*APEH*	NM_001640	Yes
4	*C7orf20*	NM_015949	Yes
5	*WDR34*	NM_052844	ND
6	*ADRM1*	NM_007002	Yes
7	*C7orf20*	NM_015949	ND
8	*BSG*	NM_198589	Yes
9	*RRAS2*	NM_012250	Yes
10	*SNX3*	NM_152827	Yes
11	*RPUSD1*	NM_058192	Yes
12	*MRPS34*	NM_023936	Yes
13	*KIAA0192*	NM_014730	No
14	*H2AFZ*	NM_002106	Yes
15	*FLJ37562*	NM_152409	Yes
16	*ESRRA*	NM_004451	ND
17	*RPS2*	NM_002952	Yes
18	*YWHAB*	NM_139323	Yes
19	*DEGS1*	NM_003676	Yes
20	*VKORC1*	NM_024006	Yes
21	*PITX1*	NM_002653	No
22	*P2RY2*	NM_002564	Yes
23	*CD320*	NM_016579	ND
24	*SNX3*	NM_152828	Yes
25	*MKNK2*	NM_017572	Yes
26	*FLOT1*	NM_005803	ND
27	*KIAA0196*	NM_014846	No
28	*ARHGEF1*	NM_199002	Yes
29	*SCLY*	NM_016510	Yes
30	*ASH2L*	NM_004674	Yes
31	*IFRD2*	NM_006764	Yes
32	*PTGES2*	NM_025072	Yes
33	*SLC43A1*	NM_003627	Yes
34	*AKR7A2*	NM_003689	ND
35	*CD81*	NM_004356	ND
36	*ASB6*	NM_017873	Yes
37	*PRO1855*	NM_018509	ND
38	*PLEC1*	NM_201384	No
39	*HSPA14*	NM_016299	Yes
40	*IARS2*	NM_018060	Yes
41	*GPC1*	NM_002081	Yes
42	*TGFB1*	NM_000660	ND

ORF, open-reading frame; ND, not determined.

amplification with the primer used originally to amplify the cDNAs in the construction of the library. In most cases, two to three DNA fragments were recovered from each genome, implying a multiple retroviral infection on the recipient 3T3 cells.

We obtained a total of 78 cDNA fragments by PCR, each of which was ligated into a cloning vector, and subjected to nucleotide sequencing from both ends. Screening of the 78 cDNA sequences against the public nucleotide sequence databases revealed that the 78 fragments correspond to 42 independent genes (Table 1).

Identification of mutated P2RY2

To confirm the transforming potential of the isolated cDNAs, each cDNA clone was ligated to pMXS, and corresponding retrovirus was used to re-infect 3T3 cells. Focus formation assays were conducted for 22 independent genes, discovering a reproducible transforming activity for clone ID #9 corresponding to *RRAS2* (GenBank Accession No., NM_012250), clone ID #28 corresponding to *ARHGEF1* (GenBank Accession No., NM_0199002), and clone ID #22 corresponding to *P2RY2* (GenBank Accession No., NM_002564) (Fig. 1, top). We focus on *P2RY2* in this report.

The entire coding region of our ID #22 cDNA was sequenced, revealing two non-synonymous nucleotide changes compared to the published *P2RY2* cDNA sequence. One such change was that the "C" at nucleotide position 470 in the reported sequence is replaced with a "T" in our sequence, leading to the proline to leucine change at amino-acid position 46 (Fig. 2).

The other change was at nucleotide position 1269 of the reported *P2RY2* cDNA sequence. Substitution of a "C" at this position induces replacement of an arginine residue with a serine at amino-acid position 312 (Fig. 2). To our best knowledge, neither oncogenic activity nor non-synonymous mutations at these sites have been reported.

```
44  C   V   P   G   L
    TGC GTG CCT GGG CTG
            ↓
    TGC GTG CTT GGG CTG
44  C   V   L   G   L

310 G   Q   R   L   V
    GGG CAG AGG CTC GTA
            ↓
    GGG CAG AGC CTC GTA
310 G   Q   S   L   V
```

Fig. 2. Identification of mutated *P2RY2* cDNA. The nucleotide sequence of our *P2RY2* cDNA was aligned with the previously determined nucleotide sequence of the *P2RY2* cDNA (NM_002564). A single nucleotide change (C to T) at the second position of codon 46 results in a change in the encoded amino acid from proline to leucine. Another nucleotide substitution (G to C) at the third position of codon 312 induces an amino-acid change from arginine to serine.

Confirmation of transforming activity of P2RY2

To confirm the transforming activity of P2RY2(P46L/R312S), we examined its effect on anchorage-independent growth of 3T3 cells in soft agar. Whereas cells infected with an empty virus did not grow in the agar, those infected with a virus expressing P2RY2(P46L/R312S) formed multiple foci in repeated experiments (Fig. 1, middle). In addition, 3T3 cells expressing v-Ras readily grew in the agar.

The transforming activity of P2RY2(P46L/R312S) was also tested by the tumor-formation assay with athymic nude mice. 3T3 cells infected with the empty virus or retrovirus expressing P2RY2(P46L/R312S), wild-type P2RY2 or v-Ras were inoculated subcutaneously into nude mice.

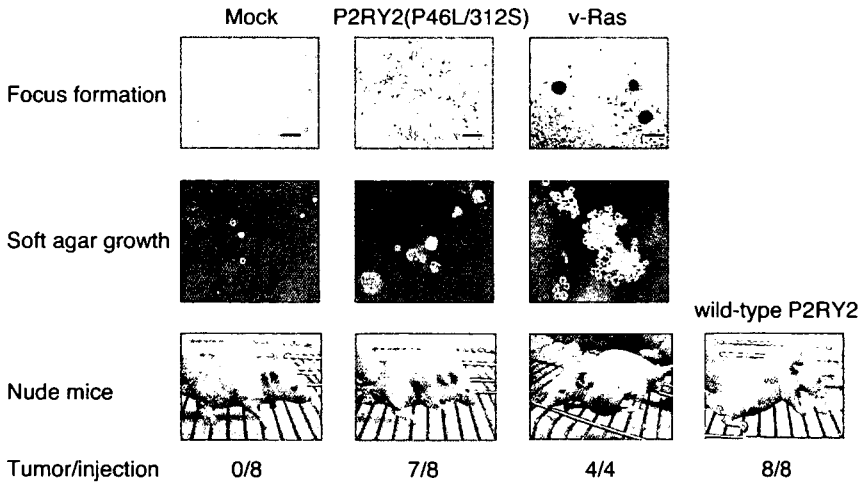

Fig. 1. Transforming activity of P2RY2. Mouse 3T3 cells were infected with empty retrovirus (Mock) or with retrovirus encoding P2RY2(P46L/R312S) or v-Ras, and cultured for 8 days (top). Scale bar, 1 mm. Each cell fraction was also seeded into soft agar and incubated for 10 days (middle). The same cell fractions and 3T3 cells expressing the wild-type P2RY2 were injected into the shoulder of nu/nu mice and tumor formation was examined after 3 weeks (bottom). The frequency of tumor formation determined is indicated.

As shown in the bottom panel of Fig. 1, tumor formation was readily observed for the cells expressing P2RY2(P46L/R312S), wild-type P2RY2, and v-Ras. These results clearly revealed an unexpected transforming potential of P2RY2 regardless of the amino-acid substitutions identified in our clone.

Discussion

In this study, we have constructed a retroviral cDNA expression library for a CRC cell line RKO. Since 95% (37/39 clones) of the viral plasmids carried cDNA inserts and since the overall clone number was >2 millions, our library should cover nearly all transcriptome in RKO cells.

Purinergic receptors may be subdivided to two families: a P2X family of ligand-gated ion-channel receptors and a P2Y family of G protein-coupled purinoreceptors [8]. Currently, seven or eight members have been reported for the P2X or P2Y subtype, respectively. It is widely recognized that purinergic receptor system may be linked to a wide array of cellular processes, including immune responses, inflammation, pain, platelet aggregation, endothelial-mediated vasodilatation, cell proliferation, and death [9,10].

Interestingly, contrasting effects of the P2 receptors have been documented in some tumor models. Activation of P2 receptors may lead to cell growth in various cancer cells [11,12]. In response to nucleotide activation, P2Y receptors can activate phospholipase C, mobilize intracellular calcium, and alter cAMP levels. On the contrary, P2 receptors may provide signals for anti-proliferation and apoptosis [13,14]. Indeed, ATP, at high concentrations, induces apoptosis through ligation of P2X7 and P2Y1 receptors [15]. Conversely, however, a low concentration of ATP stimulates proliferation. These data suggest that a plethora of functions can be provided by P2 receptors probably in a dependent manner to the receptor subtype, cellular context, stimuli or the concentration of stimuli.

Our data clearly indicate the transforming potential of P2RY2 at least in the mouse fibroblast cell line, suggesting the possible oncogenic activity of P2RY2 in human cancers. Interestingly, Rapaport et al. demonstrated that addition of ATP or ADP in the culture medium modulates the cell cycle of cell lines [16]. Therefore, regulation of P2RY2 or its ligands may provide a novel strategy to develop means to treat cancers.

Acknowledgments

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References

[1] A. Jemal, R. Siegel, E. Ward, T. Murray, J. Xu, C. Smigal, M.J. Thun, Cancer statistics, 2006, CA Cancer J. Clin. 56 (2006) 106–130.
[2] E.R. Fearon, B. Vogelstein, A genetic model for colorectal tumorigenesis, Cell 61 (1990) 759–767.
[3] S.A. Aaronson, Growth factors and cancer, Science 254 (1991) 1146–1153.
[4] W.S. Pear, G.P. Nolan, M.L. Scott, D. Baltimore, Production of high-titer helper-free retroviruses by transient transfection, Proc. Natl. Acad. Sci. USA 90 (1993) 8392–8396.
[5] Y.L. Choi, R. Kaneda, T. Wada, S. Fujiwara, M. Soda, H. Watanabe, K. Kurashina, H. Hatanaka, M. Enomoto, S. Takada, Y. Yamashita, H. Mano, Identification of a constitutively active mutant of JAK3 by retroviral expression screening, Leuk. Res. 31 (2007) 203–209.
[6] S. Fujiwara, Y. Yamashita, Y.L. Choi, T. Wada, R. Kaneda, S. Takada, Y. Maruyama, K. Ozawa, H. Mano, Transforming activity of the lymphotoxin-beta receptor revealed by expression screening, Biochem. Biophys. Res. Commun. 338 (2005) 1256–1262.
[7] M. Onishi, S. Kinoshita, Y. Morikawa, A. Shibuya, J. Phillips, L.L. Lanier, D.M. Gorman, G.P. Nolan, A. Miyajima, T. Kitamura, Applications of retrovirus-mediated expression cloning, Exp. Hematol. 24 (1996) 324–329.
[8] G. Burnstock, Introduction: P2 receptors, Curr. Top. Med. Chem. 4 (2004) 793–803.
[9] K. van Kolen, H. Slegers, Atypical PKCzeta is involved in RhoA-dependent mitogenic signaling by the P2Y(12) receptor in C6 cells, FEBS J. 273 (2006) 1843–1854.
[10] L.A. Sellers, J. Simon, T.S. Lundahl, D.J. Cousens, P.P. Humphrey, E.A. Barnard, Adenosine nucleotides acting at the human P2Y1 receptor stimulate mitogen-activated protein kinases and induce apoptosis, J. Biol. Chem. 276 (2001) 16379–16390.
[11] C.J. Dixon, W.B. Bowler, P. Fleetwood, A.F. Ginty, J.A. Gallagher, J.A. Carron, Extracellular nucleotides stimulate proliferation in MCF-7 breast cancer cells via P2-purinoceptors, Br. J. Cancer 75 (1997) 34–39.
[12] A.V. Greig, C. Linge, V. Healy, P. Lim, E. Clayton, M.H. Rustin, D.A. McGrouther, G. Burnstock, Expression of purinergic receptors in non-melanoma skin cancers and their functional roles in A431 cells, J. Invest. Dermatol. 121 (2003) 315–327.
[13] M. Hopfner, K. Maaser, B. Barthel, B. von Lampe, C. Hanski, E.O. Riecken, M. Zeitz, H. Scherubl, Growth inhibition and apoptosis induced by P2Y2 receptors in human colorectal carcinoma cells: involvement of intracellular calcium and cyclic adenosine monophosphate, Int. J. Colorectal Dis. 16 (2001) 154–166.
[14] K. Maaser, M. Hopfner, H. Kap, A.P. Sutter, B. Barthel, B. von Lampe, M. Zeitz, H. Scherubl, Extracellular nucleotides inhibit growth of human oesophageal cancer cells via P2Y(2)-receptors, Br. J. Cancer 86 (2002) 636–644.
[15] R. Coutinho-Silva, L. Stahl, K.K. Cheung, N.E. de Campos, C. de Oliveira Souza, D.M. Ojcius, G. Burnstock, P2X and P2Y purinergic receptors on human intestinal epithelial carcinoma cells: effects of extracellular nucleotides on apoptosis and cell proliferation, Am. J. Physiol. Gastrointest. Liver Physiol. 288 (2005) G1024–G1035.
[16] E. Rapaport, Treatment of human tumor cells with ADP or ATP yields arrest of growth in the S phase of the cell cycle, J. Cell. Physiol. 114 (1983) 279–283.

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ORIGINAL ARTICLE: RESEARCH

Transforming activity of purinergic receptor P2Y, G protein coupled, 8 revealed by retroviral expression screening

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Abstract

Biphenotypic acute leukemia (BAL) is a relatively rare subtype of acute leukemia characterized by the presence of both myeloid and lymphoid cell surface antigens. We have now screened for transforming genes in BAL blasts with the use of the focus formation assay with a retroviral cDNA expression library constructed from malignant blasts isolated from a BAL patient. Some of the retroviral inserts recovered from transformed foci were found to encode wild-type purinergic receptor P2Y, G protein coupled, 8 (P2RY8). The oncogenic potential of P2RY8 was confirmed with the in vitro focus formation assay as well as with an in vivo tumorigenicity assay in nude mice. A variety of luciferase-based reporter assays revealed that P2RY8 increased both the trans-activation activities of CREB and Elk-1 as well as the transcriptional activities of the serum response element and enhancer-promoter fragments of the c-Fos and c-Myc genes. Quantitation of *P2RY8* mRNA in $CD34^+$ cells of bone marrow showed that *P2RY8* expression is frequently increased in leukemia patients, especially in those with refractory disease. Our data thus reveal an abundant expression of P2RY8 in leukemic cells and its unexpected role in the pathogenesis of acute leukemia.

Keywords: *Purinergic receptor, P2RY8, biphenotypic acute leukemia, retroviral expression library*

Introduction

Malignant blasts of acute leukemia are classified as of the myeloid or lymphoid lineage on the basis of immunophenotypic features and the presence or absence of gene rearrangement within the T cell receptor or immunoglobulin gene loci. In relatively rare instances, however, such blasts coexpress cell surface antigens of both lineages [1,2], with the underlying condition being referred to as biphenotypic acute leukemia (BAL) according to the revised World Health Organization classification [3]. A scoring system for the diagnosis of BAL has also been proposed by the European Group for the Immunological Characterization of Leukemias [4].

The clinical outcome of BAL is generally poor [5], and it remains controversial as to whether BAL should be treated by regimens for acute myelocytic leukemia (AML) or acute lymphocytic leukemia (ALL). The unfavorable prognosis for BAL may be related to the associated Ph1 positivity, chromosome rearrangements affecting 11q23, and overexpression of P-glycoprotein [1,6]. However, little is yet understood of the molecular pathogenesis of this intractable disorder. Elucidation of oncogenic events in BAL might provide insight into possible new treatment strategies.

The focus formation assay with 3T3 or Rat1 fibroblasts is a functional screening system for transforming genes [7] and has been extensively applied to

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various human cancers. However, the conventional format for this assay may not allow expression of all transforming genes in the target cells. Given that the genomic DNA of clinical specimens is introduced directly into the recipient cells, the expression of the exogenous genes is controlled by their own promoters or enhancers. Oncogenes are thus able to exert their effects in the recipient cells only if these regulatory regions are active in fibroblasts, which is not always the case.

Regulation of the transcription of test cDNAs by a promoter known to function efficiently in fibroblasts would be expected to ensure sufficient expression of the encoded proteins in the focus formation assay. We have now constructed a retroviral cDNA expression library from a $CD34^+$ cell fraction isolated from a patient with BAL, and we have tested this library in the focus formation assay with 3T3 cells. For library construction, we took advantage of a polymerase chain reaction (PCR) system that preferentially amplifies full-length cDNAs.

With this assay system, we found that purinergic receptor P2Y, G protein coupled, 8 (P2RY8) possesses transforming activity. We could further demonstrate the relatively abundant expression of *P2RY8* message in leukemic cells. P2RY8 belongs to the G protein–coupled purinergic receptor family, members of which preferentially bind to, and are activated by, adenosine and uridine nucleotides [8,9]. Although the *P2RY8* gene was proved to be disrupted in individuals with X-linked mental retardation [10], its expression profile has been rarely documented in humans. Our data thus reveals an unexpected role of P2RY8 in human leukemia.

Materials and Methods

Clinical specimens and cell lines

Fresh clinical specimens were obtained from individuals with leukemia and healthy volunteers, with informed consent, and the study was approved by the institutional review board of Jichi Medical University. Mononuclear cells were isolated from bone marrow aspirates by centrifugation, labeled with CD34 MicroBeads (Miltenyi Biotec, Gladbach, Germany), and subjected to chromatography on miniMACS magnetic cell separation columns (Miltenyi Biotec). The purity of the resultant $CD34^+$ cell fractions was confirmed by staining with Wright–Giemsa solution and analysis of CD34 expression by flow cytometry (data not shown).

BOSC23 [11] and 3T3 cell lines were obtained from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM)–F12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and 2 mM L-glutamine.

Construction of a retroviral cDNA expression library

A cDNA library was constructed essentially as described previously [12–14]. In brief, total RNA was extracted from the $CD34^+$ cells of a patient with BAL with the use of an RNeasy Mini column and RNase-free DNase (Qiagen, Valencia, CA) and was subjected to first-strand cDNA synthesis with PowerScript reverse transcriptase, the SMART IIA oligonucleotide, and CDS primer IIA (all from Clontech, Palo Alto, CA). The resulting cDNAs were amplified for 20 cycles with 5′ PCR primer IIA and a SMART PCR cDNA synthesis kit (Clontech), with the exception that LA *Taq* polymerase (Takara Bio, Shiga, Japan) was substituted for the Advantage 2 DNA polymerase provided with the kit. The amplified cDNAs were treated with proteinase K, rendered blunt-ended with T4 DNA polymerase, and ligated to the BstXI-adaptor (Invitrogen). Unbound adaptors were removed with the use of a cDNA size-fractionation column (Invitrogen), and the remaining cDNAs were ligated into the BstXI site of the pMXS retroviral plasmid (kindly provided by T. Kitamura, Institute of Medical Science, University of Tokyo). The resulting pMXS-cDNA plasmids were introduced into ElecroMax DH10B cells (Invitrogen) by electroporation.

Focus formation assay

BOSC23 cells (2.0×10^6) were seeded into a 6-cm culture dish and transfected with 2 μg of retroviral plasmids mixed with 0.5 μg of the pGP plasmid, 0.5 μg of the pE-eco plasmid (both from Takara Bio), and 18 μl of Lipofectamine reagent (Invitrogen). After 2 days, polybrene (Sigma, St. Louis, MO) was added to the culture supernatant at a concentration of 4 μg/ml, and the supernatant was then used to infect 3T3 cells for 48 h. The culture medium of the 3T3 cells was then changed to DMEM-F12 supplemented with 5% calf serum and 2 mM L-glutamine, and the cells were cultured for 2 weeks.

Recovery of cDNA from transformed foci

Transformed 3T3 cell clones were harvested with a cloning syringe and cultured independently in 10-cm culture dishes. Genomic DNA was extracted from each clone by standard procedures and then subjected to PCR with 5′ PCR primer IIA and LA *Taq* polymerase for 50 cycles of 98°C for 20 s and 68°C for 6 min. Amplified DNA fragments were purified by gel electrophoresis and ligated into the pT7Blue-2

vector (EMD Biosciences, San Diego, CA) for nucleotide sequencing.

Tumorigenicity assay in nude mice

3T3 cells (2×10^6) infected with a retrovirus encoding P2RY8 were resuspended in 500 μl of phosphate-buffered saline and injected into each shoulder of a nu/nu Balb-c mouse (6 weeks old). Tumor formation was assessed after 3 weeks.

Luciferase reporter assays

PathDetect In Vivo Signal Transduction Pathway *Trans*-Reporting Systems (Stratagene, La Jolla, CA) were used to measure the activity of the transcription factors CREB, Elk-1, and c-Jun. BOSC23 cells (5.0×10^5) were seeded into 6-cm culture dishes and transfected with a mixture of 500 ng of the P2RY8 expression vector, 125 ng of the PathDetect *Trans*-Reporter Activator plasmid (pFA-CREB, pFA-Elk1, or pFA-cJun), 125 ng of the PathDetect *Trans*-Reporter plasmid pFR-Luc, 7 ng of the pGL4 vector (Promega), and 4.5 μl of Lipofectamine reagent. Similarly, the activation of p53, Rb, and E2F proteins was assessed with the use of a Mercury Pathway Profiling System (Clontech); the P2RY8 expression vector (500 ng) and pGL4 (7 ng) were thus introduced into BOSC23 cells together with 125 ng of pp53-TA-Luc, pRb-TA-Luc, or pE2F-TA-Luc vectors.

PathDetect *Cis*-Reporting Systems (Stratagene) were used to evaluate the transcriptional activities of the serum response element (SRE) and an NF-κB binding sequence. BOSC23 cells were transfected with a mixture of 500 ng of the P2RY8 expression vector, 125 ng of the PathDetect *Cis*-Reporter plasmid (pNF-κB-Luc or pSRE-Luc), 7 ng of pGL4, and 4.5 μl of Lipofectamine. The transcriptional activities of enhancer-promoter fragments of the c-Fos [15], c-Myc [16], and Bcl-x_L [17] genes were similarly assessed with luciferase expression vectors controlled by the corresponding enhancer-promoter elements (pFL700, pHXLuc, and pBclxl-Luc, respectively).

After transfection for 2 days, the BOSC23 cells were solubilized with lysis buffer (Promega), and luciferase activities were measured with a Luciferase Assay System (Promega). Firefly luciferase activity was normalized relative to that of the corresponding Renilla luciferase activity.

Quantitative reverse transcription (RT)-PCR analysis

Portions of oligo(dT)-primed cDNAs produced by reverse transcription were subjected to PCR with a QuantiTect SYBR Green PCR kit (Qiagen) and an amplification protocol comprising incubation at 94°C for 15 s, 60°C for 30 s, and 72°C for 60 s. Incorporation of the SYBR Green dye into PCR products was monitored in real time with an ABI PRISM 7900HT sequence detection system (PE Applied Biosystems, Foster City, CA), thereby allowing determination of the threshold cycle (C_T) at which exponential amplification of PCR products begins. The C_T values for cDNAs corresponding to the β-actin gene (*ACTB*) and to *P2RY8* were used to calculate the abundance of the latter mRNA relative to that of the former. The oligonucleotide primers used for PCR were 5′-CCATCATGAAGTGTGA CGTGG-3′ and 5′-GTCCGCCTAGAAGCATTT GCG-3′ for *ACTB* and 5′-TTCCTCTTCACCATC TTCATCCTG-3′ and 5′-CGTGGTAGTAGCTC TTGCCGTAGA-3′ for *P2RY8*.

Results

Screening for transforming genes with the focus formation assay

To screen for transforming genes in BAL, we constructed a cDNA expression library from a $CD34^+$ cell fraction purified from mononuclear cells of bone marrow from a patient with this condition. Full-length cDNAs were preferentially amplified by the SMART protocol (Clontech) and were ligated into the retroviral vector pMXS. We obtained a total of 4.2×10^6 colony-forming units of independent plasmid clones, from which we randomly selected 30 clones and examined the incorporated cDNAs. An insert of ≥500 bp was present in 27 (90%) of the plasmid clones, and the average size of these inserts was 1.94 kbp.

Introduction of the library plasmids into a packaging cell line generated a recombinant ecotropic retroviral library, which was then used to infect mouse NIH 3T3 fibroblasts. After culture of the infected cells for 2 weeks, we identified a total of 67 transformed foci (Figure 1). No foci were observed for 3T3 cells infected with the empty virus. Each transformed focus was isolated, expanded individually, and used to prepare genomic DNA. PCR amplification of the inserts yielded a total of 30 cDNA fragments, each of which was ligated into a cloning vector and subjected to nucleotide sequencing from both ends. Screening of these cDNA sequences against the public nucleotide sequence databases revealed that they showed >95% sequence identity to five independent known genes.

To confirm the transforming ability of the isolated cDNAs, we again ligated them into the pMXS vector and used the corresponding retroviruses to infect

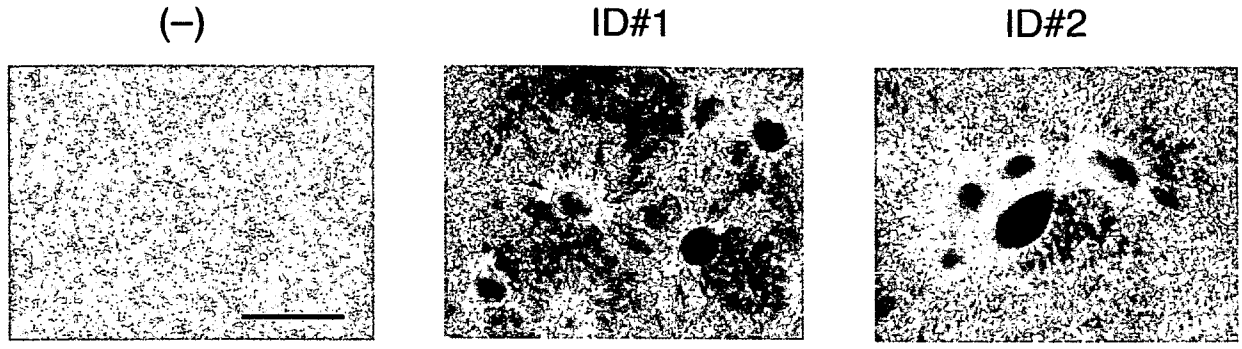

Figure 1. Screening for transforming genes from a patient with BAL. Mouse 3T3 cells were infected with a recombinant retrovirus library or the corresponding empty virus (–). Complementary DNAs for *P2RY8* and *KRAS2* were recovered from 3T3 clones ID No. 1 and ID No. 2, respectively. The photographs show the infected 3T3 cells after culture for 2 weeks. Scale bar, 1 mm.

3T3 cells. Two of the five independent genes, *P2RY8* (GenBank accession no. NM_178129) and *KRAS2* (GenBank accession no. NM_004985), reproducibly induced the formation of transformed foci in 3T3 cells. Further sequencing of the *KRAS2* cDNA revealed that it contained a point mutation that results in a glycine to alanine substitution at codon 12, a change that is known to activate KRAS2 [18]. Given that, as far as we were aware, transforming activity had not previously been described for P2RY8, we focused on P2RY8 in further analyses.

Confirmation of the transforming activity of P2RY8

The complete nucleotide sequence was determined for the *P2RY8* cDNA isolated from 3T3 clone ID No. 1, revealing that the cDNA comprised 4208 bp encoding for a full-length protein of 359 amino acids with a sequence identical to that of P2RY8. Within the protein-coding region, the cDNA sequence differed by only one nucleotide compared with that of the *P2RY8* sequence in the database; the T at nucleotide position 697 in the reported sequence (NM_178129) is replaced with a C in the determined sequence. However, this change does not affect the amino acid sequence of P2RY8 and may therefore reflect a single nucleotide polymorphism. There was no difference in the nucleotide sequence of the 5′ untranslated region between our *P2RY8* cDNA and the deposited one. There were, however, four changes in the 3′ untranslated region of our cDNA compared to that of the reported one; deletion of a A at the position 3599 in the deposited cDNA sequence, a G-to-A transition at the position 3749, deletion of a AA at the positions of 4169–4170, and deletion of a C at the position of 4194. While all of these changes are located at the AT-rich region close to the polyadenylation signal, it is possible that some of these changes may affect the quantity of the protein product.

To confirm that overexpression of wild-type P2RY8 induces transformation of 3T3 cells, we repeated the focus formation assay with a retrovirus generated from the isolated *P2RY8* cDNA. Infection of the cells with the retrovirus resulted in the generation of many foci (data not shown). The transforming potential of P2RY8 was also evaluated in a tumorigenicity assay with athymic nude mice. The animals were thus injected subcutaneously with 3T3 cells infected with the retrovirus encoding P2RY8. Tumor formation was observed at all 10 injection sites. In contrast, no tumors were detected at sites injected with 3T3 cells infected with the empty virus (Figure 2). Injection of nude mice with 3T3 cells infected with a virus encoding v-Ras also induced the formation of subcutaneous tumors at four out of four injection sites (data not shown).

Signal transduction pathways downstream of P2RY8

To identify signal transduction pathways activated by P2RY8 overexpression, we performed a series of luciferase reporter assays. First, the activity of several signaling molecules involved in cell growth or apoptosis was measured by two commercially available systems [Figure 3(A)]. With the use of PathDetect In Vivo Signal Transduction Pathway *Trans*-Reporting Systems (Stratagene), a fusion *trans*-activator protein comprised of the yeast GAL4 DNA-binding domain and the activation domain of either c-Jun, CREB or Elk-1 was expressed in BOSC23 cells, together with the firefly luciferase under the control of the yeast GAL4 binding sequence. Coexpression of P2RY8 resulted in the activation of the *trans*-activator for CREB or Elk-1, which thereby induced the marked production of the luciferase protein [Figure 3(A)].

With the Mercury Pathway Profiling System (Clontech), a plasmid containing the target sequence of p53, Rb, or E2F (each fused to the luciferase cDNA) was introduced into BOSC23 cells with or

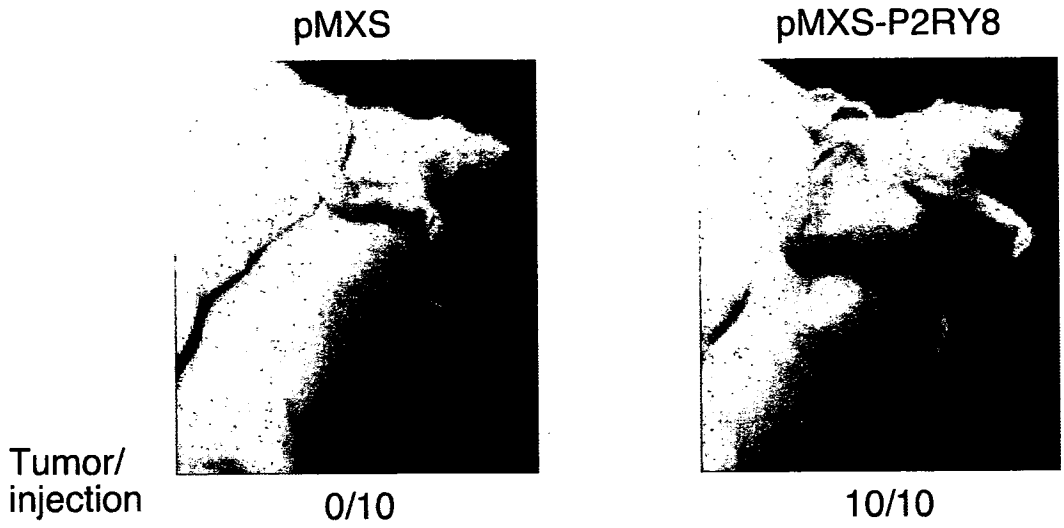

Figure 2. Tumorigenicity assay with nude mice. 3T3 cells infected with a retrovirus encoding P2RY8 (pMXS-P2RY8) or the corresponding empty virus (pMXS) were injected into the shoulder of nu/nu mice. Tumor formation was examined after 3 weeks. The number of tumors formed at the 10 injection sites is indicated.

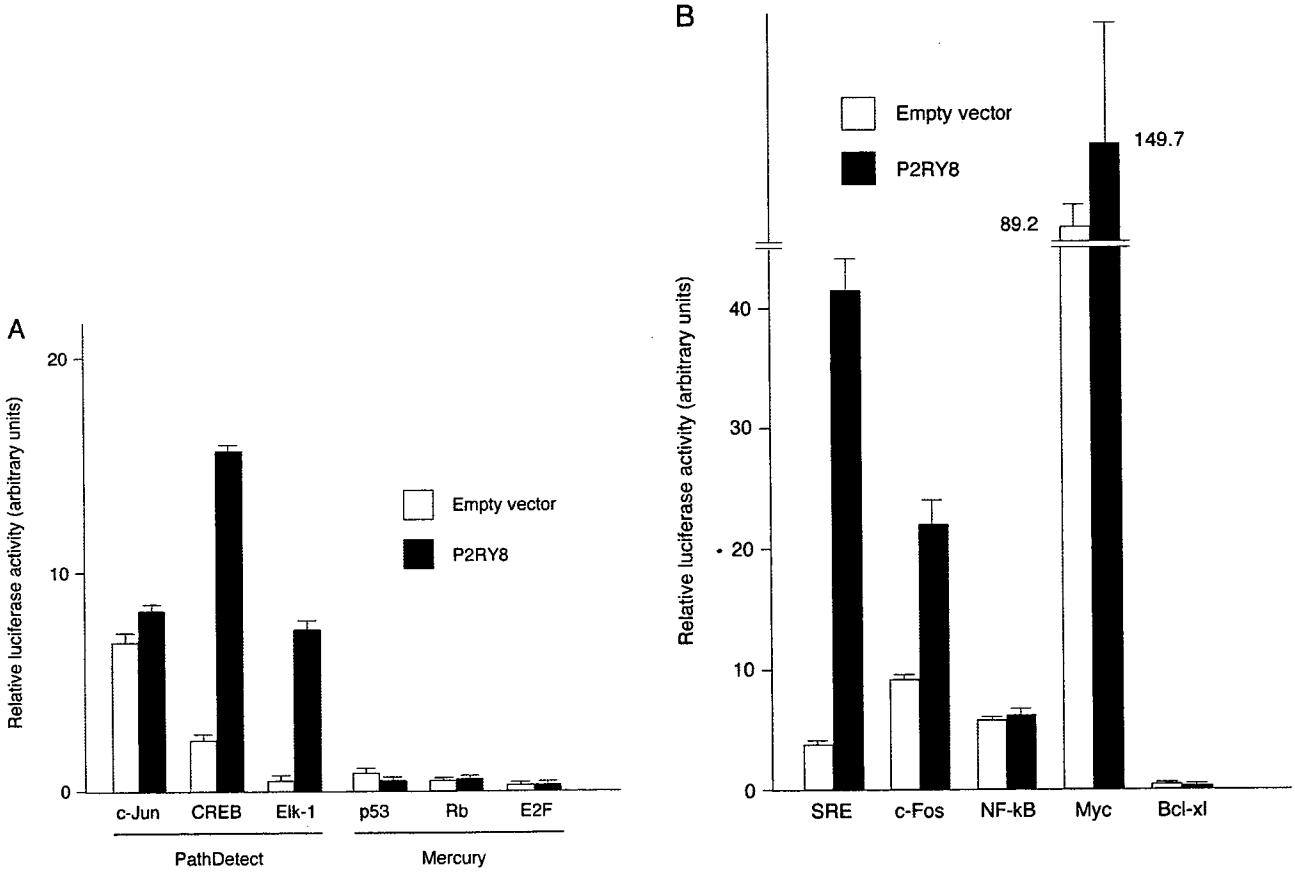

Figure 3. Activation of signal transduction pathways by P2RY8. (A) The trans-activation activities of the transcription factors (c-Jun, CREB, and Elk-1) were assessed with the PathDetect In Vivo Signal Transduction Pathway *Trans*-Reporting Systems (PathDetect) in BOSC23 cells transfected with a P2RY8 expression vector or the corresponding empty vector. Similarly, the activity of p53, Rb, or E2F was measured with the use of Mercury Pathway Profiling System (Mercury). The activity of firefly luciferase in cell lysates was normalized by that of Renilla luciferase. Data are means + SD of values from three experiments. (B) The SRE sequence, the enhancer/promoter fragment of the c-Fos, c-Myc, or Bcl-x_L gene, or the NF-κB binding sequence was fused to the cDNA of firefly luciferase, and was introduced into BOSC23 cells. The activity of firefly luciferase in cell lysates was normalized by that of Renilla luciferase. Data are means + SD of values from three experiments.

without the expression plasmid for P2RY8. As demonstrated in the right panel of Figure 3(A), none of the target sequences were activated by P2RY8.

Next, the activity of enhancer/promoter sequences was measured with *cis* reporting systems. The SRE fragment, the enhancer/promoter sequence for c-Fos, c-Myc, or Bcl-x_L gene, or the NF-κB binding sequence was individually fused to the luciferase cDNA, and was introduced into BOSC23 cells. While coexpression of P2RY8 failed to substantially affect the activity of NF-κB or that of the enhancer/promoter fragments of Bcl-x_L, it resulted in activation of SRE and the enhancer/promoter fragments of the c-Fos and c-Myc genes [Figure 3(B)].

Expression of P2RY8 in leukemia specimens

Given that overexpression of *P2RY8* was found to induce cell transformation, we compared the abundance of *P2RY8* mRNA by quantitative RT-PCR analysis among $CD34^+$ progenitor cells isolated from the bone marrow of healthy volunteers ($n=3$) as well as from individuals with AML developed from myelodysplastic syndrome (MDS) ($n=3$), AML developed from myeloproliferative disorder (MPD) ($n=1$), de novo AML ($n=15$), ALL ($n=4$), or BAL ($n=2$, including the patient used for cDNA library construction).

Overall, the amount of *P2RY8* mRNA in $CD34^+$ progenitor cells was increased in patients with acute leukemia compared with that in healthy volunteers, and this increase was apparent across all the subtypes of leukemia (de novo or secondary AML, ALL, and BAL) examined [Figure 4(A)]. To determine whether an increased expression of *P2RY8* is associated with unfavorable characteristics of leukemia, we compared the amount of *P2RY8* mRNA between untreated patients and those with relapsed or refractory leukemia. The abundance of *P2RY8* mRNA was indeed significantly greater ($P=0.0054$) in $CD34^+$ cells from the latter group of patients than in those from the former group [Figure 4(B)]. To examine further the relation between a high level of *P2RY8* expression and poor clinical outcome, we compared the survival of leukemia patients with a *P2RY8/ACTB* mRNA ratio in blasts of ≥ 0.006 with that of those with a ratio of < 0.006. Kaplan–Meier analysis revealed that the patients with a low level of *P2RY8* expression showed a significantly better survival ($P < 0.05$) than did those with a high level of *P2RY8* expression [Figure 4(C)].

Discussion

We constructed a retroviral cDNA expression library with $CD34^+$ cells isolated from the bone marrow of a patient with BAL. Given that the plasmid library was shown to harbor cDNA inserts in $\geq 90\%$ of the clones and that the overall independent clone number was >4 million, the cDNA expression library likely represented most of the mRNAs in the $CD34^+$ cells. A 3T3 focus formation assay with the recombinant viruses generated from the plasmids identified *KRAS2* with an activating mutation and a full-length *P2RY8* cDNA as having transforming activity. The transforming activity of wild-type P2RY8 was confirmed with both the focus formation assay and a tumorigenicity assay in nude mice. We also examined the expression level of *P2RY8* in 3T3 cells by quantitative RT-PCR as in Figure 4(A). Although 3T3 cells infected with an empty virus had no detectable amount of *P2RY8* mRNA, the cells infected with the virus expressing P2RY8 had an abundant expression of *P2RY8* message (*P2RY8/ACTB* mRNA ratio was 48.7 ± 6.1, mean $\pm$ SD).

P2RY8 is presumed to function as a transmembrane receptor for adenosine and uridine nucleotides and to couple with heterotrimeric G-proteins [9]. Upon binding to nucleotides, P2Y receptors trigger activation of heterotrimeric G-proteins and thereby evoke intracellular signaling through adenylyl cyclase, extracellular signal-regulated kinases (ERKs), and/or phospholipase C [8,19]. Purinergic receptor signaling has been shown to be associated with a variety of cellular responses including cell growth [20,21], differentiation [22], migration [23], and apoptosis [24]. In addition, *XlP2Y* (a homolog of *P2RY8* in *Xenopus laevis*) is expressed in the neural plate of developing embryos [25] and *P2RY8* is disrupted in individuals with X-linked mental retardation [10], observations that suggest an important role for *P2RY8* in neural development. Despite the absence of evidence for a direct linkage between *P2RY8* and oncogenic activity, several reports have suggested the potential relationship between purinergic receptors and leukemia. Extracellular ATP, for instance, suppresses the growth of, and induces the differentiation of, HL-60 leukemia cells [26]. Further, *P2X7* message was shown abundant in some leukemic cells [27]. Our current data indicates the possible contribution of another purinergic receptor, P2RY8, to leukemogenesis. However, it should be noted that, in our hands, a forced expression of *P2RY8* in a mouse cell line, 32Dcl3 [28], failed to abrogate an interleukein-3-dependency for cell growth, and to suppress granulocyte colony-stimulating factor-dependent differentiation toward terminal granulocytes (data not shown). Thus, despite the transforming activity, P2RY8 may require other genetic events to confer full properties as leukemia onto blood cells.

Screening for downstream components of P2RY8 signaling in cells with reporter constructs revealed

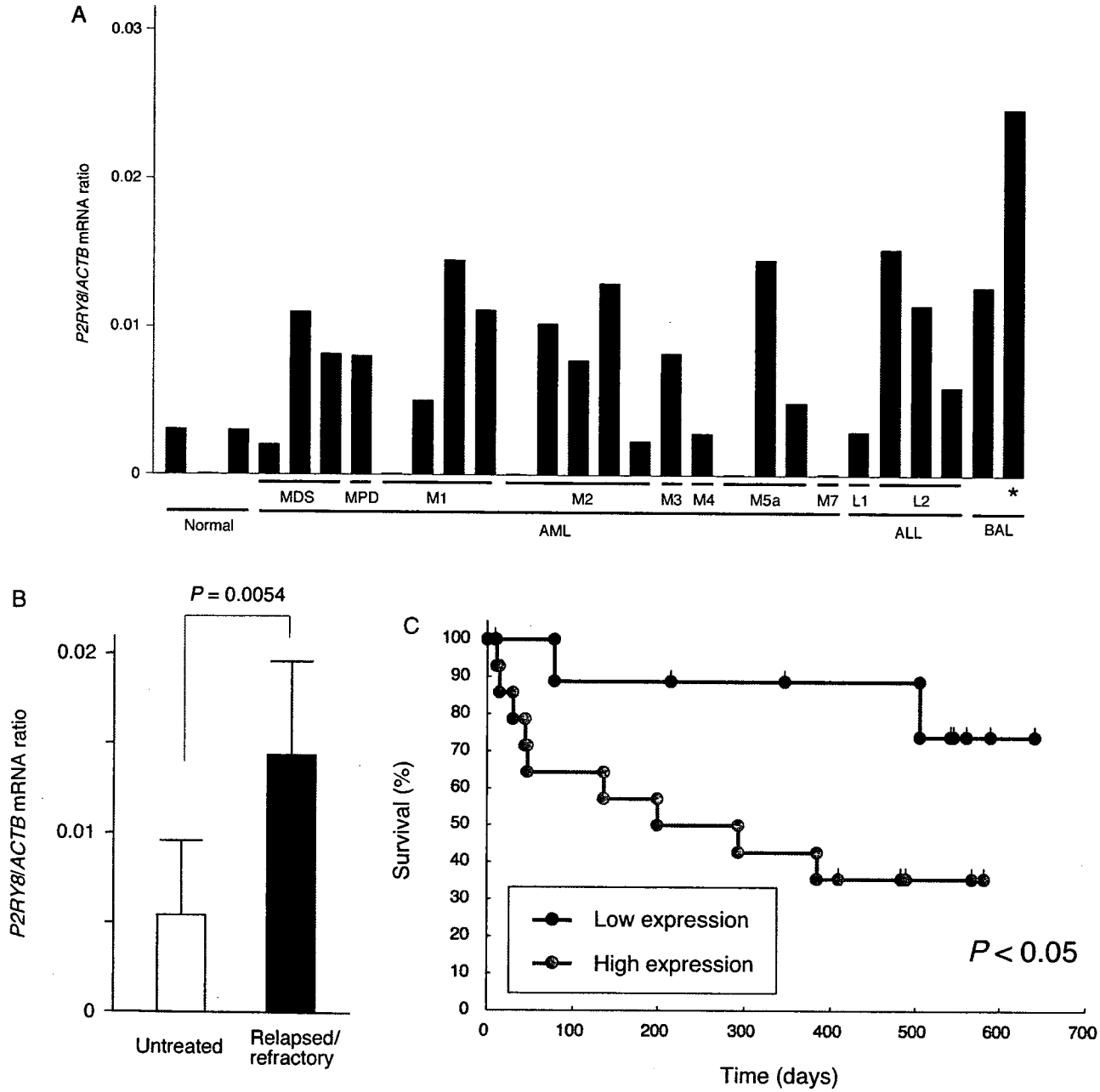

Figure 4. Quantitation of *P2RY8* mRNA in blasts of patients with various types of acute leukemia. (A) The amount of *P2RY8* mRNA in $CD34^+$ cell fractions purified from healthy volunteers (normal) or from patients with MDS-derived AML, MPD-derived AML, de novo AML of various FAB subtypes (M1 to M7), ALL of FAB subtypes L1 or L2, or BAL was determined by real-time RT-PCR analysis. The ratio of the abundance of *P2RY8* mRNA to that of *ACTB* mRNA was calculated as 2^n, where n is the C_T value for *ACTB* cDNA minus the C_T value for *P2RY8* cDNA. The asterisk indicates the patient from whom the cDNA library was constructed. (B) Comparison of the *P2RY8/ACTB* mRNA ratio (mean + SD) between untreated patients ($n = 16$) and those with relapsed or refractory leukemia ($n = 8$). The significance of the difference in the ratio between the two groups was assessed by Student's t-test. (C) Kaplan–Meier plots of the survival of patients with a high level of *P2RY8* expression (*P2RY8/ACTB* mRNA ratio of ≥ 0.006, $n = 15$) compared with that of those with a low level of *P2RY8* expression (*P2RY8/ACTB* mRNA ratio of < 0.006, $n = 10$). The significance of the difference between the two groups was evaluated by the log-rank test.

that the trans-activation activities of CREB and Elk-1 were increased by P2RY8 expression, as were the transcriptional activities of the SRE as well as of enhancer/promoter fragments of the c-Fos and c-Myc genes. Given that both CREB and Elk-1 bind to and activate the promoter of the c-Fos gene and that the SRE is present within this promoter [29,30], these data collectively indicate that c-Fos is a target and effector of P2RY8 signaling. These data are in good agreement with the finding by Muscella et al. that c-Fos can be activated by another purinergic receptor, P2RY6 [19]. Given that the c-Myc gene, an

immediate-early response gene in growth signaling, was also transcriptionally activated by P2RY8, both c-Myc and c-Fos may play an important role in P2RY8-induced malignant transformation.

The second messengers for transformation, acting downstream to P2RY8, are not elucidated yet. The activities of protein kinase C (PKC) were, however, shown essential in the P2RY6-evoked cell proliferation and ERK activation [19]. Since P2RY8 activity may also induce an increase in Ca_i^{2+} [25], it is possible that P2RY8 activates PKC and triggers the machinery for cell proliferation as well.

The finding that overexpression of *P2RY8* results in cell transformation led us to examine whether this gene is overexpressed in leukemic blasts. Quantitative RT-PCR analysis revealed that this was indeed the case, at least for the cells of some patients. A possible contribution of P2RY8 to leukemogenesis was further supported by the observation that patients with a high level of *P2RY8* expression had a poorer prognosis than did those with a lower level. It will be of interest to examine whether the level of *P2RY8* expression is increased in other types of human cancer as well. Especially, given the presumed role of P2RY8 in neural development, it is possible that some brain tumors may have an abundant expression of P2RY8. Our findings implicate the purinergic receptor family in cancer and suggest that P2RY8 is a potential therapeutic target in some types of leukemia.

Acknowledgements

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References

1. Legrand O, Perrot JY, Simonin G, Baudard M, Cadiou M, Blanc C, et al. Adult biphenotypic acute leukaemia: an entity with poor prognosis which is related to unfavourable cytogenetics and P-glycoprotein over-expression. Br J Haematol 1998;100:147–155.
2. Owaidah TM, Al Beihany A, Iqbal MA, Elkum N, Roberts GT. Cytogenetics, molecular and ultrastructural characteristics of biphenotypic acute leukemia identified by the EGIL scoring system. Leukemia 2006;20:620–626.
3. Jaffe ES, Harris NL, Stein H, Vardiman JW, editors. Pathology and genetics of tumours of haematopoietic and lymphoid tissues. Lyon: IARC Press; 2001.
4. Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A, et al. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia 1995;9:1783–1786.
5. Killick S, Matutes E, Powles RL, Hamblin M, Swansbury J, Treleaven JG, et al. Outcome of biphenotypic acute leukemia. Haematologica 1999;84:699–706.
6. Carbonell F, Swansbury J, Min T, Matutes E, Farahat N, Buccheri V, et al. Cytogenetic findings in acute biphenotypic leukaemia. Leukemia 1996;10:1283–1287.
7. Aaronson SA. Growth factors and cancer. Science 1991;254:1146–1153.
8. Burnstock G. Introduction: P2 receptors. Curr Top Med Chem 2004;4:793–803.
9. Burnstock G. Pathophysiology and therapeutic potential of purinergic signaling. Pharmacol Rev 2006;58:58–86.
10. Cantagrel V, Lossi AM, Boulanger S, Depetris D, Mattei MG, Gecz J, et al. Disruption of a new X linked gene highly expressed in brain in a family with two mentally retarded males. J Med Genet 2004;41:736–742.
11. Pear WS, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. Proc Natl Acad Sci USA 1993;90:8392–8396.
12. Kisanuki H, Choi YL, Wada T, Moriuchi R, Fujiwara SI, Kaneda R, et al. Retroviral expression screening of oncogenes in pancreatic ductal carcinoma. Eur J Cancer 2005;41:2170–2175.
13. Fujiwara S, Yamashita Y, Choi YL, Wada T, Kaneda R, Takada S, et al. Transforming activity of the lymphotoxin-beta receptor revealed by expression screening. Biochem Biophys Res Commun 2005;338:1256–1262.
14. Choi YL, Moriuchi R, Osawa M, Iwama A, Makishima H, Wada T, et al. Retroviral expression screening of oncogenes in natural killer cell leukemia. Leuk Res 2005;29:943–949.
15. Hu Q, Milfay D, Williams LT. Binding of NCK to SOS and activation of *ras*-dependent gene expression. Mol Cell Biol 1995;15:1169–1174.
16. Takeshita T, Arita T, Higuchi M, Asao H, Endo K, Kuroda H, et al. STAM, signal transducing adaptor molecule, is associated with Janus kinase and involved in signaling for cell growth and c-myc induction. Immunity 1997;6:449–457.
17. Grillot DAM, Gonzalez-Garcia M, Ekhterae D, Duan L, Inohara N, Ohta S, et al. Genomic organization, promoter region analysis, and chromosome localization of the mouse *bcl-x* gene. J Immunol 1997;158:4750–4757.
18. Shih TY, Weeks MO. Oncogenes and cancer: the p21 ras genes. Cancer Invest 1984;2:109–123.
19. Muscella A, Greco S, Elia MG, Storelli C, Marsigliante S. Differential signalling of purinoceptors in HeLa cells through the extracellular signal-regulated kinase and protein kinase C pathways. J Cell Physiol 2004;200:428–439.
20. Van Kolen K, Slegers H. Atypical PKCzeta is involved in RhoA-dependent mitogenic signaling by the P2Y(12) receptor in C6 cells. FEBS J 2006;273:1843–1854.
21. Wagstaff SC, Bowler WB, Gallagher JA, Hipskind RA. Extracellular ATP activates multiple signalling pathways and potentiates growth factor-induced c-fos gene expression in MCF-7 breast cancer cells. Carcinogenesis 2000;21:2175–2181.
22. Adrian K, Bernhard MK, Breitinger HG, Ogilvie A. Expression of purinergic receptors (ionotropic P2X1-7 and metabotropic P2Y1-11) during myeloid differentiation of HL60 cells. Biochim Biophys Acta 2000;1492:127–138.
23. Bagchi S, Liao Z, Gonzalez FA, Chorna NE, Seye CI, Weisman GA, et al. The P2Y2 nucleotide receptor interacts with alphav integrins to activate Go and induce cell migration. J Biol Chem 2005;280:39050–39057.
24. Sellers LA, Simon J, Lundahl TS, Cousens DJ, Humphrey PP, Barnard EA. Adenosine nucleotides acting at the human P2Y1 receptor stimulate mitogen-activated protein kinases and induce apoptosis. J Biol Chem 2001;276:16379–16390.

25. Bogdanov YD, Dale L, King BF, Whittock N, Burnstock G. Early expression of a novel nucleotide receptor in the neural plate of Xenopus embryos. J Biol Chem 1997;272:12583–12590.
26. Conigrave AD, van der Weyden L, Holt L, Jiang L, Wilson P, Christopherson RI, et al. Extracellular ATP-dependent suppression of proliferation and induction of differentiation of human HL-60 leukemia cells by distinct mechanisms. Biochem Pharmacol 2000;60:1585–1591.
27. Zhang XJ, Zheng GG, Ma XT, Yang YH, Li G, Rao Q, et al. Expression of P2X7 in human hematopoietic cell lines and leukemia patients. Leukemia Res 2004;28:1313–1322.
28. Greenberger JS, Sakakeeny MA, Humphries RK, Eaves CJ, Eckner RJ. Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell lines. Proc Natl Acad Sci USA 1983;80:2931–2935.
29. De Cesare D, Jacquot S, Hanauer A, Sassone-Corsi P. Rsk-2 activity is necessary for epidermal growth factor-induced phosphorylation of CREB protein and transcription of c-fos gene. Proc Natl Acad Sci USA 1998;95:12202–12207.
30. Janknecht R, Nordheim A. MAP kinase-dependent transcriptional coactivation by Elk-1 and its cofactor CBP. Biochem Biophys Res Commun 1996;228:831–837.

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ARTICLES

Identification of the transforming *EML4–ALK* fusion gene in non-small-cell lung cancer

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Improvement in the clinical outcome of lung cancer is likely to be achieved by identification of the molecular events that underlie its pathogenesis. Here we show that a small inversion within chromosome 2p results in the formation of a fusion gene comprising portions of the echinoderm microtubule-associated protein-like 4 (*EML4*) gene and the anaplastic lymphoma kinase (*ALK*) gene in non-small-cell lung cancer (NSCLC) cells. Mouse 3T3 fibroblasts forced to express this human fusion tyrosine kinase generated transformed foci in culture and subcutaneous tumours in nude mice. The *EML4–ALK* fusion transcript was detected in 6.7% (5 out of 75) of NSCLC patients examined; these individuals were distinct from those harbouring mutations in the epidermal growth factor receptor gene. Our data demonstrate that a subset of NSCLC patients may express a transforming fusion kinase that is a promising candidate for a therapeutic target as well as for a diagnostic molecular marker in NSCLC.

Lung cancer remains the leading cause of cancer deaths in western countries[1]. Patients with NSCLC, which accounts for ~80% of lung cancer cases, are often diagnosed at advanced stages of the disease. Given that conventional chemotherapeutic regimens only marginally improve the outcome of such individuals, their median survival time is less than one year after diagnosis (ref. 2). A subset of NSCLCs was recently shown to harbour activating mutations in the epidermal growth factor receptor gene (*EGFR*)[3,4]; such cancers are responsive to gefitinib, a specific inhibitor of the tyrosine kinase activity of EGFR. The efficacy of targeting key 'growth drivers' in cancer treatment is further exemplified by chronic myeloid leukaemia, for which another tyrosine kinase inhibitor, STI571, is highly effective in reducing the number of cancer cells[5]. However, *EGFR* mutations are associated preferentially with NSCLC of non-smokers and Asians[4,6]. Few oncogenes have thus been identified for NSCLC in individuals with a smoking habit, who constitute most cases of the disease.

Retrovirus-mediated complementary DNA expression systems allow expression of the encoded proteins in most of the targeted cells. Through modification of the method used in ref. 7, we have achieved reliable amplification of cDNAs from small quantities of clinical specimens as well as the generation of retroviral libraries for expression of these cDNAs[8–10]. Application of such a cDNA expression library prepared from an NSCLC specimen to a focus formation assay with mouse 3T3 fibroblasts has now led to the identification of a fusion oncogene.

Identification of *EML4–ALK*

To isolate novel transforming genes in NSCLC, we generated a retroviral cDNA expression library from a lung adenocarcinoma specimen surgically resected from a 62-yr-old man with a history of smoking (patient 33). In construction of the library, we used the SMART method (Clontech) for preferential amplification of full-length cDNAs from limited amounts of clinical specimens[7]; this resulted in the production of $>1.4 \times 10^6$ independent plasmid clones. Infection of mouse 3T3 fibroblasts with the recombinant retroviruses that were based on these plasmids led to the formation of many transformed foci, from which insert cDNAs were recovered with the polymerase chain reaction (PCR).

One of the amplified cDNAs comprised 3,926 base pairs (bp) and contained an open reading frame for a protein of 1,059 amino acids (Fig. 1a and Supplementary Fig. 1). The amino-terminal portion (residues 1–496) of the predicted protein is identical to that of human EML4 (GenBank accession number NM_019063), whereas the carboxy-terminal portion (residues 497–1059) is identical to the intracellular domain (residues 1058–1620 of the wild-type protein) of human ALK (GenBank accession number AB209477), suggesting that the cDNA is derived from a fusion product of *EML4* and *ALK* (Fig. 1a, b). EML4 belongs to the family of echinoderm microtubule-associated protein-like proteins[11] and is composed of an N-terminal basic region (isoelectric point, 10.2), a hydrophobic echinoderm microtubule-associated protein-like protein (HELP) domain[12] and WD repeats[13] (Fig. 1b). In the predicted fusion protein, the N-terminal half of EML4 encompassing the basic region, the HELP domain and a portion of the WD-repeat region is fused to the intracellular juxtamembrane region of ALK.

ALK was first identified as a fusion partner of nucleophosmin (NPM) in anaplastic large-cell lymphoma with a t(2;5) chromosome rearrangement[14,15]. Other chromosome translocations involving the *ALK* locus were subsequently identified in the same lymphoma subtype as well as in inflammatory myofibroblastic tumours[16]. The fusion point of ALK is conserved among most of these chimaeric tyrosine kinases, including EML4–ALK (resulting in fusion of the

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a

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KPQGQREKKEESHSHDQSPQIRASPSPQPSSQPLQIHRQTPESKNATPTKSIKRPSPAEK  180
SHNSWENSDDSRNKLSKIPSTPKLIPKVTKTADKHKDVIINQEGEYIKMFMRGRPITMFI  240
PSDVDNYDDIRTELPPEKLKLEWAYGYRGKDCRANVYLLPTGEIVYFIASVVVLFNYEER  300
TQRHYLGHTDCVKCLAIHPDKIRIATGQIAGVDKDGRPLQPHVRVWDSVTLSTLQIIGLG  360
TFERGVGCLDFSKADSGVHLCVIDDSNEHMLTVWDWQKKAKGAEIKTTNEVVLAVEFHPT  420
DANTIITCGKSHIFFWTWSGNSLTRKQGIPGKYEKPKFVQCLAFLGNGDVLTGDSGGVML  480
IWSKTTVEPTPGKGPKVYRRKHQELQAMQMELQSPEYKLSKLRTSTIMTDYNPNYCFAGK  540
TSSISDLKEVPRKNITLIRGLGHGAFGEVYEGQVSGMPNDPSPLQVAVKTLPEVCSEQDE  600
LDFLMEALIISKFNHQNIVRCIGVSLQSLPRFILLELMAGGDLKSFLRETRPRPSQPSSL  660
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YYRKGGCAMLPVKWMPPEAFMEGIFTSKTDTWSFGVLLWEIFSLGYMPYPSKSNQEVLEF  780
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GPLVEEEEKVPVRPKDPEGVPPLLVSQQAKREEERSPAAPPPLPTTSSGKAAKKPTAAEV  900
SVRVPRGPAVEGGHVNMAFSQSNPPSELHRVHGSRNKPTSLWNPTYGSWFTEKPTKKNNP  960
IAKKEPHERGNLGLEGSCTVPPNVATGRLPGASLLLEPSSLTANMKEVPLFRLRHFPCGN L020
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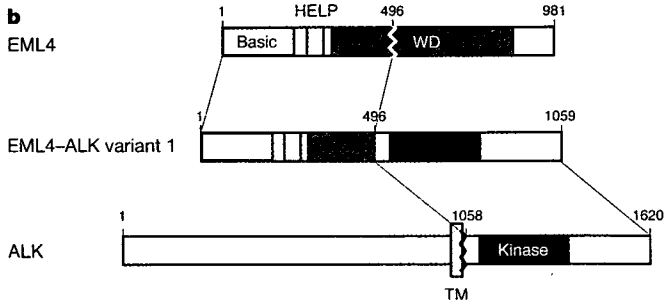
b



c

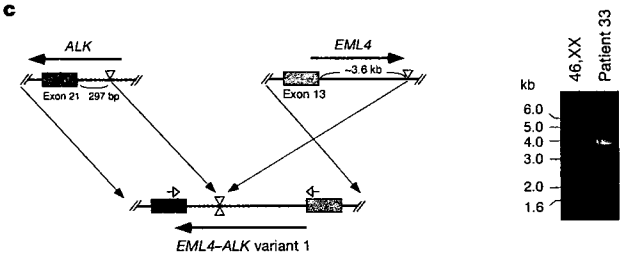


Figure 1 | Gene fusion between *EML4* and *ALK*. **a**, Amino acid sequence of the EML4–ALK protein (variant 1). Residues corresponding to EML4 or to ALK are shown in blue or red, respectively. Lys 589 in the ATP-binding site is boxed. **b**, Fusion of the N-terminal portion of EML4 (comprising the basic region, the HELP domain and part of the WD-repeat region) to the intracellular region of ALK (containing the tyrosine kinase domain). TM, transmembrane domain. **c**, Both the *ALK* gene and the *EML4* gene map to chromosome 2p, but have opposite orientations. In the NSCLC patient 33, *EML4* is disrupted at a position ~3.6 kb downstream of exon 13 and is ligated to a position 297 bp upstream of exon 21 of *ALK*, giving rise to the *EML4–ALK* (variant 1) fusion gene (left panel). Filled and open horizontal arrows indicate the direction of transcription and the positions of the Fusion-genome primers, respectively (Supplementary Fig. 1). PCR with these Fusion-genome primers and genomic DNA of patient 33 generated a single product of ~4 kb (right panel); this product was not detected with control DNA of a healthy female (46,XX).

entire intracellular kinase domain of ALK to the corresponding partner), and the kinase activity of NPM–ALK was shown to be essential for the proliferation of lymphoma cells positive for this construct[17].

Given that *EML4* and *ALK* each map to the short arm of chromosome 2 (2p21 and 2p23, respectively, separated by a distance of ~12 megabases, Mb) but have opposite orientations, either gene might have been inverted to generate the *EML4–ALK* fusion gene (Fig. 1c). To address this issue directly, we amplified the genomic fusion point between *EML4* and *ALK* using genomic DNA of patient 33 as the template. This approach led to the identification of a ~4-kilobase (kb) product (Fig. 1c). Nucleotide sequencing of this genomic fragment revealed that intron 13 of *EML4* is disrupted at a point ~3.6 kb downstream of exon 13 and is inverted to connect to a position 297 bp upstream of exon 21 of *ALK* (Fig. 1c and Supplementary Data), yielding *EML4–ALK* variant 1 (the structure of variant 2 is addressed below). To determine whether the chromosome rearrangement in this specimen is a simple inversion within 2p, we attempted to detect the other connection point between *EML4* and *ALK* in chromosome 2 by PCR amplification of the *ALK–EML4* cDNA or gene. However, neither of the corresponding PCR products was obtained (data not shown). It thus remains undetermined whether the NSCLC cells of patient 33 harbour a simple inv(2)(p21p23) or whether they contain complex chromosome translocations involving 2p.

Transforming activity of EML4–ALK

To confirm the transforming potential of EML4–ALK, we generated expression plasmids for wild-type EML4, wild-type ALK, EML4–ALK, EML4–ALK(K589M) (in which Lys 589 in the ATP-binding site of the kinase domain is replaced with Met), NPM–ALK and v-Ras, and introduced them individually into mouse 3T3 fibroblasts. Transformed foci were readily identified for the cells expressing EML4–ALK, NPM–ALK or v-Ras, but not for those expressing EML4, ALK or EML4–ALK(K589M) (Fig. 2a). Subcutaneous injection of the transfected 3T3 cells into nude mice also revealed that only those expressing EML4–ALK, NPM–ALK or v-Ras formed tumours (Fig. 2a). These data thus showed that EML4–ALK possesses transforming activity that is dependent on its catalytic activity. We also found that fusion to EML4 results in redistribution of the kinase domain of ALK from the cell membrane to the cytoplasm (Supplementary Fig. 2), as revealed by monitoring the fluorescence of the corresponding proteins tagged with enhanced green fluorescent protein.

To identify the domains of EML4 required for the transforming activity of the EML4–ALK fusion protein, we generated expression plasmids for EML4–ALK with internal deletions of the basic domain (ΔBasic, lacking residues 31–140), of the HELP domain (ΔHELP, lacking residues 220–296) or of the WD repeats (ΔWD, lacking residues 305–475) (Fig. 2b). Injection of 3T3 cells expressing the deletion constructs into nude mice revealed that deletion of the WD repeats allowed tumour formation at all injection sites, but that the tumours were smaller than those formed by cells expressing full-length EML4–ALK (Fig. 2b). Tumours were even smaller for cells expressing ΔHELP and were undetectable for those expressing ΔBasic. All domains of EML4 thus seem to contribute to the oncogenic potential of EML4–ALK, with the basic domain being the most important.

Gene fusion often results in activation of tyrosine kinases through oligomerization mediated by the fusion partner. Although little is known regarding the dimerization potential of EML4, our results (Fig. 2b) suggested that the basic domain derived from EML4 may mediate EML4–ALK dimerization. To examine this possibility, we transfected HEK 293 cells with expression vectors for both Myc-epitope-tagged EML4–ALK and Flag-epitope-tagged full-length, ΔBasic, ΔHELP or ΔWD forms of EML4–ALK. Immunoprecipitation of cell lysates with antibodies to Myc and probing of the resulting precipitates with antibodies to Flag revealed that Myc-epitope-tagged EML4–ALK was associated with substantial amounts of each of the Flag-tagged EML4–ALK constructs with the exception of ΔBasic (Fig. 2c). Immunoblot analysis of immunoprecipitates prepared from the same cell lysates with antibodies to Flag confirmed that the various Flag-tagged EML4–ALK constructs were expressed at similar levels. Similar results were obtained in a reciprocal experiment in which anti-Flag immunoprecipitates were probed with anti-Myc (Supplementary Fig. 3). These results thus indicated that the basic domain indeed has an important role in dimerization of EML4–ALK.

To examine directly whether internal deletions affect the enzymatic activity of EML4–ALK, we expressed Flag-tagged full-length or truncated forms of the fusion protein in HEK 293 cells and prepared immunoprecipitates from cell lysates with anti-Flag. The resulting precipitates were then subjected to an *in vitro* kinase assay with the synthetic YFF peptide[18], which is based on the activation loop of the catalytic domain of ALK. Deletion of the basic domain resulted in a marked decrease (~84%) in the catalytic activity of EML4–ALK (Fig. 2d). This low level of kinase activity of the ΔBasic mutant was consistent with its residual ability to dimerize with the full-length

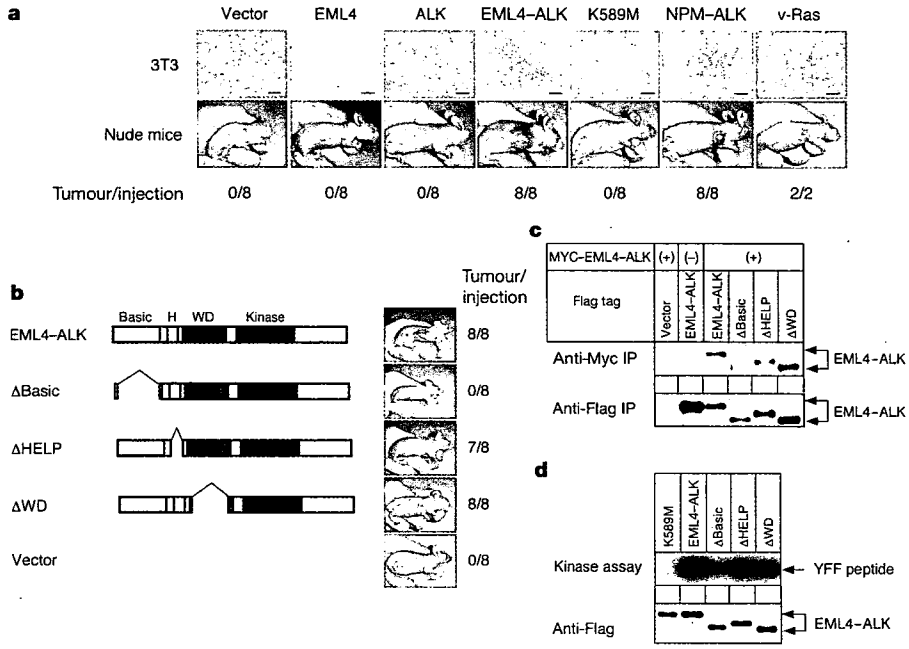


Figure 2 | Transforming activity of EML4–ALK variant 1. **a**, Expression vectors for EML4, ALK, EML4–ALK, EML4–ALK(K589M), NPM–ALK and v-Ras (or the corresponding empty vector) were introduced individually into 3T3 cells, and the cells were photographed after three weeks of culture (upper panels). Scale bars, 100 μm. The same set of transfected cells was also injected subcutaneously into nude mice, and tumour formation was examined after 20 days (lower panels). The number of tumours formed after eight or two injections is indicated. **b**, Schematic representations of EML4–ALK and its deletion mutants are shown on the left. Tumour formation in nude mice was examined as in **a** for 3T3 cells transfected with expression vectors for the indicated forms of EML4–ALK (right). H, HELP domain. **c**, Expression vectors for Flag-tagged EML4–ALK or its deletion mutants were introduced into HEK 293 cells with (+) or without (–) a vector for Myc-epitope-tagged EML4–ALK. Cell lysates were subjected to immunoprecipitation (IP) with antibodies to Myc or to Flag, and the resulting precipitates were subjected to immunoblot analysis with anti-Flag. The positions of EML4–ALK and its mutants are shown on the right. **d**, Expression vectors for Flag-tagged EML4–ALK, EML4–ALK(K589M) or its deletion mutants were introduced into HEK 293 cells. Immunoprecipitates prepared from cell lysates with anti-Flag were subjected to an *in vitro* kinase assay with the synthetic YFF peptide (upper panel) or to immunoblot analysis with anti-Flag (lower panel).

protein (Fig. 2c and Supplementary Fig. 3). Deletion of either the HELP or WD domain reduced the kinase activity of EML4–ALK by ~50% (Fig. 2d), whereas the transforming activity of the ΔHELP mutant was reproducibly lower than that of ΔWD in both the focus formation (data not shown) and tumorigenicity (Fig. 2b) assays. The molecular basis of this discrepancy between kinase and transforming activities remains to be determined.

Detection of *EML4–ALK* in clinical specimens

We next evaluated the frequency of *EML4–ALK* gene fusion in NSCLC. A consecutive panel of NSCLC specimens ($n = 33$) obtained in one hospital was examined for the presence of *EML4–ALK* messenger RNA, wild-type *ALK* mRNA and mutations within the *EGFR* and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue (*KRAS*) genes. The *EML4–ALK* fusion mRNA was readily detected by PCR with reverse transcription (RT–PCR) analysis as a 247-bp product in three patients (9.1%), including the patient who served as the source for the retroviral library (Fig. 3a). Sequencing of the PCR products amplified from each of these three patients confirmed the presence of *EML4–ALK* variant 1 cDNA (Supplementary Data); patients 20 and 39 had squamous cell carcinoma and adenocarcinoma of the lung, respectively.

We also amplified the genomic fragments corresponding to exons 18, 19 and 21 of *EGFR* and determined their nucleotide sequences in all 33 patients. This revealed the presence of *EGFR* mutations—all of which were deletions or nucleotide substitutions within exon 19—in six individuals (18.2%) (Supplementary Table 1). Notably, the patient population harbouring *EGFR* mutations did not overlap with that harbouring the *EML4–ALK* fusion gene, showing that *EML4–ALK*-positive cancer is a novel subclass within NSCLC. A *KRAS* mutation (Val 12 to Cys 12 substitution) was detected in two individuals, neither of whom harboured *EGFR* mutations or the *EML4–ALK* fusion gene. Wild-type *ALK* mRNA was detected in 8 of the 33 specimens (24%) of this cohort. A moderate level of *ALK* mRNA in lung cancer specimens has also been detected by serial analysis of gene expression studies (http://cgap.nci.nih.gov/SAGE/AnatomicViewer), although it is not clear whether such profiling of the 3′ end of mRNAs actually detected mRNAs for wild-type *ALK*, *EML4–ALK* or even other *ALK* fusion genes.

To determine whether fusion of *EML4* to *ALK* is specific to NSCLC, we used RT–PCR to attempt to detect the fusion mRNA in cancer specimens from 39 patients with acute myeloid leukaemia, 69 patients with non-Hodgkin's lymphoma, 93 patients with gastric carcinoma and 60 patients with colorectal carcinoma. However, none of these 261 specimens yielded the *EML4–ALK* cDNA (data not shown), indicating that *EML4–ALK* has a high level of specificity to NSCLC.

Further screening for the *EML4–ALK* fusion cDNA with the same RT–PCR primer set in a different cohort of NSCLC patients ($n = 42$) resulted in the identification of a larger PCR product (~1 kb) in another two individuals with lung adenocarcinoma. Nucleotide sequencing of these PCR products revealed that exon 20 of *EML4* was fused to exon 21 of *ALK* (Supplementary Fig. 4 and Supplementary Data), indicative of diversity in the breakpoint region within *EML4*. PCR analysis of genomic DNA from one of these two patients further revealed the breakpoints in *EML4* and *ALK* as well as the formation of both *EML4–ALK* and *ALK–EML4* fusion genes, thus demonstrating the presence of an inv(2)(p21p23) rearrangement in this individual (Supplementary Fig. 4 and Supplementary Data). We here refer to the initially identified *EML4–ALK* gene, in which intron 13 of *EML4* is fused to intron 20 of *ALK*, as variant 1, and to the *EML4–ALK* gene, in which intron 20 of *EML4* is fused to intron 20 of