leukemia (ALL) gene expression signatures classify an independent cohort of adult ALL patients. *Leukemia*, **18,** 63–71.

Kohlmann, A., Schoch, C., Dugas, M., Schnittger, S., Hiddemann, W., Kern, W. & Haferlach, T. (2005) New insights into MLL gene rearranged acute leukemias using gene expression profiling: shared pathways, lineage commitment, and partner genes. *Leukemia*, **19,** 953–964.

Krishnamachary, B., Zagzag, D., Nagasawa, H., Rainey, K., Okuyama, H., Baek, J.H. & Semenza, G.L. (2006) Hypoxia-inducible factor-1-dependent repression of E-cadherin in von Hippel-Lindau tumor suppressor-null renal cell carcinoma mediated by TCF3, ZFHX1A, and ZFHX1B. *Cancer Research*, **66,** 2725–2731.

Martineau, M., Berger, R., Lillington, D.M., Moorman, A.V. & Secker-Walker, L.M. (1998) The t(6;11)(q27;q23) translocation in acute leukemia: a laboratory and clinical study of 30 cases. *Leukemia*, **12,** 788–791.

Meshinchi, S., Woods, W.G., Stirewalt, W.D., Sweetser, D.A., Buckley, J.D., Tjoa, T.K., Bernstein, I.D. & Radich, J.P. (2001) Prevalence and prognostic significance of FLT3 internal tandem duplication in pediatric acute myeloid leukemia. *Blood*, **97,** 89–94.

Miyoshi, A., Kitajima, Y., Sumi, K., Sato, K., Hagiwara, A., Koga, Y. & Miyazaki, K. (2004) Snail and SIP1 increase cancer invasion by upregulating MMP family in hepatocellular carcinoma cells. *British Journal of Cancer*, **90,** 1265–1273.

Mowat, D.R., Wilson, M.J. & Goossens, M. (2003) Mowat-Wilson syndrome. *Journal of Medical Genetics*, **40,** 305–310.

Mullighan, C.G., Goorha, S., Radtke, I., Miller, C.B., Coustan-Smith, E., Dalton, J.D., Girtman, K., Mathew, S., Ma, J., Pounds, S.B., Su, X., Pui, C.H., Relling, M.V., Evans, W.E., Shurtleff, S.A. & Downing, J.R. (2007) Genome-wide analysis of genetic alterations in acute lymphoblastic leukemia. *Nature*, **446,** 758–764.

Nagao, T. (1995) Footprints of the Children's Leukemia Study Group of Japan. *The Japanese Journal of Pediatric Hematology*, **9,** 332–340.

Pallisgaard, N., Hokland, P., Riishoj, D.C., Pedersen, B. & Jorgensen, P. (1998) Multiplex reverse transcription-polymerase chain reaction for simultaneous screening of 29 translocations and chromosomal aberrations in acute leukemia. *Blood*, **92,** 574–588.

Paschka, P., Marucci, G., Ruppert, A.S., Mrozek, K., Chen, H., Kittles, R.A., Vukosavljevic, T., Perrotti, D., Vardiman, J.W., Carroll, A.J., Kolitz, J.E., Larson, R.A. & Bloomfield, C.D. (2006) Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv(16) and t(8;21): a Cancer and Leukemia Group B Study. *Journal of Clinical Oncology*, **24,** 3904–3911.

Pui, C.H., Kane, J.R. & Crist, W.M. (1995) Biology and treatment of infant leukemias. *Leukemia*, **9,** 762–769.

Pui, C.H., Raimondi, S.C., Srivastava, D.K., Tong, X., Behm, F.G., Razzouk, B., Rubnitz, J.E., Sandlund, J.T., Evans, W.E. & Ribeiro, R. (2000) Prognostic factors in infants with acute myeloid leukemia. *Leukemia*, **14,** 684–687.

Pui, C.H., Gaynon, P.S., Boyett, J.M., Chessells, J.M., Baruchel, A., Kamps, W., Silverman, L.B., Biondi, A., Harms, D.O., Vilmer, E., Schrappe, M. & Camitta, B. (2002) Outcome of treatment in childhood acute lymphoblastic leukaemia with rearrangements of the 11q23 chromosomal region. *Lancet*, **359,** 1909–1915.

Ross, M.E., Mahfouz, R., Onciu, M., Liu, H., Zhou, X., Song, G., Shurtleff, S.A., Pounds, S., Cheng, C., Ma, J., Ribeiro, R.C., Rubnitz, J.E., Girtman, K., Williams, W.K., Raimondi, S.C., Liang, D.C., Shih, L.Y., Pui, C.H. & Downing, J.R. (2004) Gene expression profiling of pediatric acute myelogeneous leukemia. *Blood*, **104,** 3679–3687.

Salto-Tellez, M., Shelat, S.G., Benoit, B., Rennert, H., Carroll, M., Leonard, D.G., Nowell, P. & Bagg, A. (2003) Multiplex RT-PCR for the detection of leukemia-associated translocations. *Journal of Molecular Diagnostics*, **5,** 231–236.

Satake, N., Maseki, N., Nishiyama, M., Kobayashi, H., Sakurai, M., Inaba, H., Katano, N., Horikoshi, Y., Eguchi, H., Miyake, M., Seto, M. & Kaneko, Y. (1999) Chromosome abnormalities and MLL rearrangements in acute myeloid leukemia of infants. *Leukemia*, **13,** 1013–1017.

Schoch, C., Kohlmann, A., Schnittger, S., Brors, B., Dugas, M., Mergenthaler, S., Kern, W., Hiddemann, W., Eils, R. & Haferlach, T. (2002) Acute myeloid leukemias with reciprocal rearrangements can be distinguished by specific gene expression profiles. *Proceedings of the National Academy of Sciences of the United States of America*, **99,** 10008–10013.

Shimada, A., Taki, T., Tabuchi, K., Tawa, A., Horibe, K., Tsuchida, M., Hanada, R., Tsukimoto, I. & Hayashi, Y. (2006) KIT mutations, and not FLT3 internal tandem duplication, are strongly associated with a poor prognosis in pediatric acute myeloid leukemia with t(8;21): a study of the Japanese Childhood AML Cooperative Study Group. *Blood*, **107,** 1806–1809.

Taki, T., Ida, K., Bessho, F., Hanada, R., Kikuchi, A., Yamamoto, K., Sako, M., Tsuchida, M., Seto, M., Ueda, R. & Hayashi, Y. (1996) Frequency and clinical significance of the MLL gene rearrangements in infant acute leukemia. *Leukemia*, **10,** 1303–1307.

Tsukimoto, I., Tawa, A., Hanada, R., Tabuchi, K., Kigasawa, H., Tsuchiya, S., Tsuchida, M., Yabe, H., Nakayama, H., Kudo, K., Kobayashi, R., Hamamoto, K., Imaizumi, M., Morimoto, A. & Horibe, K. (2005) Excellent outcome of risk stratified treatment for childhood acute myeloid leukemia-AML99 trial. For the Japanese Childhood AML Cooperative Study Group. *Blood*, **106.** (Abstract 889).

Tsutsumi, S., Taketani, T., Nishimura, K., Ge, X., Taki, T., Sugita, K., Ishii, E., Hanada, R., Ohki, M., Aburatani, H. & Hayashi, Y. (2003) Two distinct gene expression signatures in pediatric acute lymphoblastic leukemia with MLL rearrangements. *Cancer Research*, **63,** 4882–4887.

Tusher, V.G., Tibshirani, R. & Chu, G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proceedings of the National Academy of Sciences of the United States of America*, **98,** 5116–5121.

Valk, P.J., Verhaak, R.G., Beijen, M.A., Erpelinck, C.A., Doorn-Khosrovani, S.B., Boer, J.M., Beverloo, H.B., Moorhouse, M.J., van der Spek, P.J., Lowenberg, B. & Delwel, R. (2004) Prognostically useful gene-expression profiles in acute myeloid leukemia. *The New England Journal of Medicine*, **350,** 1617–1628.

Wakamatsu, N., Yamada, Y., Yamada, K., Ono, T., Nomura, N., Taniguchi, H., Kitoh, H., Mutoh, N., Yamanaka, T., Mushiake, K., Kato, K., Sonta, S. & Nagaya, M. (2001) Mutations in SIP1, encoding Smad interacting protein-1, cause a form of Hirschsprung disease. *Nature Genetics*, **27,** 369–370.

Weisser, M., Kern, W., Rauhut, S., Schoch, C., Hiddemann, W., Haferlach, T. & Schnittger, S. (2005) Prognostic impact of RT-PCR-based quantification of WT1 gene expression during MRD monitoring of acute myeloid leukemia. *Leukemia*, **19,** 1416–1423.

Welbron, J.L., Jenks, H.M. & Hagemeijer, A. (1993) Unique clinical features and prognostic significance of the translocation (6;11) in acute leukemia. *Cancer Genetics and Cytogenetics*, **65,** 125–129.

Yagi, T., Morimoto, A., Eguchi, M., Hibi, S., Sako, M., Ishii, E., Mizutani, S., Imashuku, S., Ohki, M. & Ichikawa, H. (2003)

Identification of a gene expression signature associated with pediatric AML prognosis. *Blood*, **102**, 1849–1856.

Yeoh, E.J., Ross, M.E., Shurtleff, S.A., Williams, W.K., Patel, D., Mahfouz, R., Behm, F.G., Raimondi, S.C., Relling, M.V., Patel, A., Cheng, C., Campana, D., Wilkins, D., Zhou, X., Li, J., Liu, H., Pui, C.H., Evans, W.E., Naeve, C., Wong, L. & Downing, J.R. (2002) Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. *Cancer Cell*, **1**, 133–143.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Expression profiles of AML-M4/M5.

Figure S2. The significance of *MLL* gene rearrangements partners.

Table SI. Clinical information of paediatric patients enrolled in this study.

Table SII. Clinical information of adult patients enrolled in this study.

Table SIII. Primer sequences used for quantitative RT-PCR.

Table SIV. Top 50 probe sets in overexpressed genes in 10 typical infants.

Table SV. Top 50 probe sets in differentially expressed genes between subgroup A and subgroup C patients.

Table SVI. Differentially expressed genes between *MLL*gr(+) and *MLL*gr(−) patients.

Table SVII. Differentially expressed genes among *MLL*gr(+) patients.

Table SVIII. The numbers of probe sets showing differential expression among *MLL*gr(+) patients.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Leukemia & Lymphoma, December 2008; 49(12): 2359–2364

informa
healthcare

ORIGINAL ARTICLE: RESEARCH

Abnormal cytoplasmic dyslocalisation and/or reduction of nucleophosmin protein level rarely occurs in myelodysplastic syndromes

YUICHI ISHIKAWA[1]*, JINGLAN XU[1]*, GYOSUKE SAKASHITA[2], TAKESHI URANO[2], TATSUYA SUZUKI[1], AKIHIRO TOMITA[1], HITOSHI KIYOI[3], SHIGEO NAKAMURA[4], & TOMOKI NAOE[1]

[1]*Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya, Japan,* [2]*Department of Biochemistry, Shimane University School of Medicine, Izumo, Japan,* [3]*Department of Infectious Diseases, Nagoya University Hospital, Showa-ku, Nagoya, Japan, and* [4]*Department of Pathophysiology, Nagoya University Hospital, Showa-ku, Nagoya, Japan*

(Received 6 September 2008; accepted 8 October 2008)

Abstract

The Nucleophosmin1 (*NPM1*) gene located in chromosome 5q35 is affected by chromosomal translocation, mutation and deletion in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). *NPM1* haploinsufficiency reportedly causes MDS-like disorders in knockout mice. Here, we studied mRNA and protein expression in bone marrow (BM) samples from 36 patients with MDS. The *NPM1* expression levels of mRNA and protein were not related to chromosome 5 abnormalities and were almost the same as those in normal BM and AML cells. However, the protein levels in AML cells with *NPM1* mutations were slightly lower than in those without mutation. Immunochemical studies showed no difference in the staining intensity and subcellular localisation between MDS and normal BM cells. It was concluded that abnormal cytoplasmic localisation and/or significant reduction of NPM1 protein level rarely occurs in MDS. The increase in the number of nuclear NPM1-positive cells may be related to the progression of MDS.

Keywords: *Nucleophosmin, myelodysplastic syndrome, immunohistochemical staining*

Introduction

Nucleophosmin1 (NPM1) is a nucleolar phosphoprotein with multiple biological roles [1–5]. In hematological malignancies, the *NPM1* is affected by chromosomal translocation, mutation and deletion [6–8]. The *NPM1* on 5q35 is translocated with the *ALK* in anaplastic large cell lymphoma with t(2;5) [9]. The *MLF1* and *RARA* genes are fused with *NPM1* in myelodysplastic syndrome (MDS)/acute myeloid leukemia (AML) with t(3;5) [10] and acute promyelocytic leukemia with t(5;17) [11], respectively. Most importantly, mutations in exon 12 have been found in a significant proportion of *de novo* AML cases, especially in those with a normal karyotype [12–16]. Mutant NPM is aberrantly localised in the cytoplasm, but the molecular mechanisms associated with leukemia remain unclear. The knockout mice model of the *NPM1* gene suggests that $NPM1^{+/-}$ haploinsufficiency causes hematological disorders like MDS [17]. Because the *NPM1* gene locus on 5q35 is often deleted in MDS and AML, loss-of-heterozygosity and/or mutation have been studied in human MDS samples [18]. According to previous reports, however, mutation of the *NPM1* exon 12 is very rare in MDS either in combination with 5q abnormality or not. Furthermore, the promoter region of *NPM1* is rarely methylated [19], suggesting that *NPM1* may not simply have a role as a tumor suppressor gene in MDS. To further clarify the involvement of *NPM1* in MDS, we generated a new antibody to NPM and

Correspondence: Yuichi Ishikawa, Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8560, Japan. Tel: +81-52-744-2955. Fax: +81-52-744-2801. E-mail: yishikaw@med.nagoya-u.ac.jp
*These authors equally contributed to this work.

ISSN 1042-8194 print/ISSN 1029-2403 online
DOI: 10.1080/10428190802541815

analysed the expression levels and subcellular localisation of NPM in MDS.

Materials and methods

Patient samples

NPM1 expression and mutation were analysed in bone marrow (BM) cells and specimens from 36 patients with newly diagnosed MDS at the Nagoya University Hospital. Diagnosis was made according to the FAB classification. MDS patients consisted of 24 men and 12 women with a median age of 58 years (range, 28–89 years). Four secondary AML patients with MDS were also studied. BM mononuclear cells were harvested by standard Ficoll/Paque density gradient centrifugation (Amersham Pharmacia Biosciences, Roozendaal, The Netherlands) and were suspended in RPMI1640 medium supplemented with 10% fetal bovine serum, 100 IU/mL of penicillin G and 100 μg/mL of streptomycin. Informed consent was obtained from all patients to use their samples for banking and molecular analysis, and approval was obtained from the ethical committee of Nagoya University School of Medicine for these studies.

Antibodies

Anti-NPM mouse monoclonal antibody (NPM9.2.6) specific for bacterially expressed NPM1 was generated, then, purified from BL21 transformed with pGEX 4T-2 carrying full-length human NPM1. To determine the epitope of this antibody, HeLa cells were transfected for 48 h with human NPM1, NPM1.2 and their truncated mutants were tagged with EGFP. Cells were then lysed with SDS-sample buffer followed by sonication. Western blot analysis was performed with anti-NPM 9.2.6 and anti-EGFP rabbit polyclonal antibody (MBL). To characterise the cross-reactivity between species, lysates were prepared from HeLa and NIH3T3 cells. Western blot analysis was performed with anti-NPM 9.2.6, anti-B23 mouse monoclonal antibody (B0556, Sigma) and anti-αtubulin mouse monoclonal antibody (T6199, Sigma).

Characterisation of anti NPM 9.2.6 monoclonal antibody

Anti-NPM mouse monoclonal antibody 9.2.6 can recognise both human NPM1 and NPM1.2, the epitope of which is located in the region of 170–189 aa (Supplement Figure 1A). However, this antibody cannot recognise mouse NPM (Supplement Figure 1B, upper panel) despite the expression level being comparable between the cell lines (Supplement Figure 1B, middle panel).

Real time quantitative PCR

Total RNA was extracted from the samples by using a QIAamp RNA Blood Mini Kit (Qiagen, Chatsworth, CA). cDNA was synthesised from each RNA using a random primer and Moloney murine leukemia virus reverse transcriptase (SuperScript II; Invitrogen) according to the manufacturer's recommendations. The expression level of NPM1 transcripts were quantified using a real-time fluorescence detection method on an ABI prism7000 sequence detection system following the manufacturer's recommendations (Applied Biosystem, Foster City, CA). PCR procedures were carried out at 50°C for 2 min, 95°C for 10 min, followed by 40 PCR cycles at 95°C for 15 s and 60°C for 1 min. The housekeeping gene, GAPDH, served as a control for cDNA quality. Each gene expression level was analysed in duplicate and the expression level was calculated as previously described [20].

Screening for mutation of the NPM1 gene

For the screening of *NPM1* mutations, we amplified genomic DNA corresponding to exon 12 of *NPM1* by PCR using the primers NPM1-F, 5′-TTAACTCTCTGGTGGTAGAATGAA-3′ and NPM1-R, 5′-CAAGACTATTTGCCATTCC TAAC-3′, as previously reported [6]. Amplified products were separated through agarose gel, purified using a QIAquick gel extraction kit (Qiagen) and directly sequenced on a DNA sequencer (310; Applied Biosystems) using a BigDye terminator cycle sequencing kit (Applied Biosystems). If mutations were found by direct sequencing, the fragments were cloned into a pGEM-T Easy vector (Promega, Madison, WI), then transfected into *Escherichia coli* strain DH5α. At least four recombinant colonies were selected and plasmid DNA was prepared using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced.

Immunohistochemical staining

Samples were fixed with ice-cold 4% paraformaldehyde for 16–24 h, embedded in paraffin, sectioned transversely (thickness, 3 μm) and processed so that immunohistochemical techniques could be used to determine the localisation of NPM. After removal of paraffin with xylene and dehydration with a series of ethanol solutions, the tissue sections were subjected to microwave irradiation (750 W) for 15 min in 0.01 mol/L citrate buffer (pH 6.0). The sections were then placed in

an automated immunostainer (Ventana Medical Systems, Tucson, AZ) as described [21]. For negative controls, primary antibodies were replaced with mouse IgG. We investigated a single case twice for NPM expression. The entire section was screened to find the region with the highest immunostaining.

Immunoblotting

A total of $1-5\times10^6$ fresh or frozen cells were directly lysed in sample buffer and then subjected to SDS-polyacrylamide gel electrophoresis on a 10% gel and the separated proteins were then transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA). The membrane was incubated at room temperature first for 1 h with 5% non-fat milk and 0.1% Tween-20 in Tris-buffered saline and then kept overnight with the appropriately diluted mouse monoclonal antibodies in the same solution. After washing, the membrane was incubated for 1 h with diluted horseradish peroxidase-conjugated mouse antibody to mouse IgG (MBL) and immune complexes were then detected with ECL reagents (Amersham).

Results

The expression level of the *NPM1* transcripts in MDS cells was studied and compared with those in normal and AML cells (Figure 1). In normal peripheral blood, the median expression level of the *NPM1* transcripts was 8.0 (range, 3.2–9.5) $\times 10^6$, 9.4 (range, 1.6–12) $\times 10^6$, 10.8 (7.2–22) $\times 10^6$ copies/μg RNA in normal peripheral blood cells, cord blood cells and BM cells, respectively. The median expression levels in MDS cells was 2.0 (range, 0.2–83) $\times 10^6$ copies/μg RNA, which was a lower level than that those of normal cells, but the expression levels of *NPM1* transcripts were not statistically different between MDS samples and normal BM samples ($p=0.163$, by Mann–Whitney test). In two patients with secondary AML carrying a 5q abnormality, the mRNA level was not lower. Mutations in exon 12 of the NPM1 gene were analysed in 36 patients with RA, RAEB and RAEB-T. In two (5%) patients with RAEB, 4-bp was inserted into the common site of exon 12, and mRNA levels in both cases were the same as for those without the mutation.

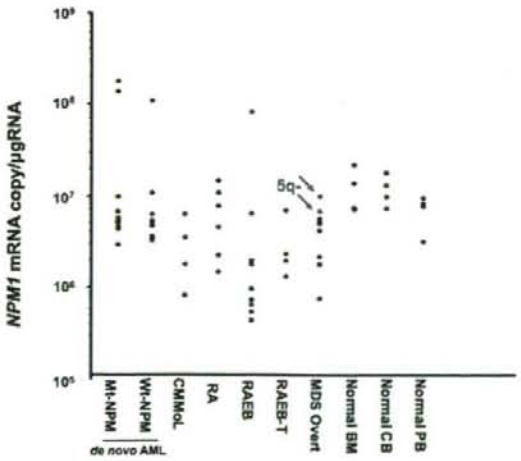


Figure 1. Real-time quantitative PCR of NPM1 transcripts. Distribution of the expression level of the NPM1 mRNA in AML, MDS and normal samples are indicated, the arrowed dots indicate that the patients carrying a 5q- abnormality. There were no significant differences in the sequence of exon12 of *NPM1*, the FAB type and normal samples.

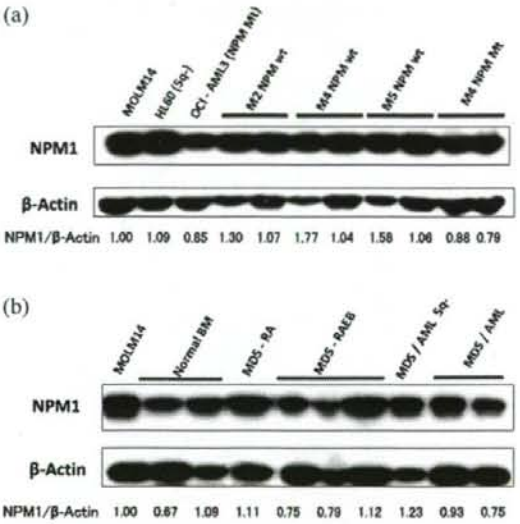


Figure 2. NPM1 protein detected by immunoblotting. The expression level of NPM1 protein in clinical samples and cell lines was analysed by immunoblotting. Protein signal intensities were measured and normalised with the signal intensities of β-actin. The normalised NPM1 β-actin ratio is indicated below, the ratio of MOLM14 cell is used as a control. (A) In AML cell line, the expression level of NPM1 in HL60 cell carrrying 5q deletion was almost equally to that of MOLM14 cell carrying intact chromosome 5, each cell lines possesed wild-type NPM1. Although in OCI–AML3 cell carrying *NPM1* mutation, the slightly lower expression of NPM1 protein level was observed. In AML samples, immunoblotting analysis of M2, M4 with and M5 according to FAB classification samples with wild-type *NPM1* revealed that the expression level of NPM1 protein was almost at the same level as the FAB type. In AML samples with mutated *NPM1*, the expression level of NPM1 protein was slightly lower than that in AML cells without *NPM1* mutation, in spite of the same FAB type. (B) The expression level of NPM1 protein was compared in MDS samples according to FAB type and normal bone marrow mononuclear cells. There was no significance in the difference in expression level of NPM1 protein between MDS samples with or without 5q- and normal BM samples.

To study the levels of NPM1 protein expression, a total of 21 samples from healthy volunteers, AML patients and MDS patients were subjected to immunoblot analysis. Anti-NPM antibody (9.2.6) detected bands at a molecular weight of 37 kDa, corresponding to NPM, in all samples. The NPM level in AML cells with *NPM1* mutation was slightly lower than that in AML cells without *NPM1*

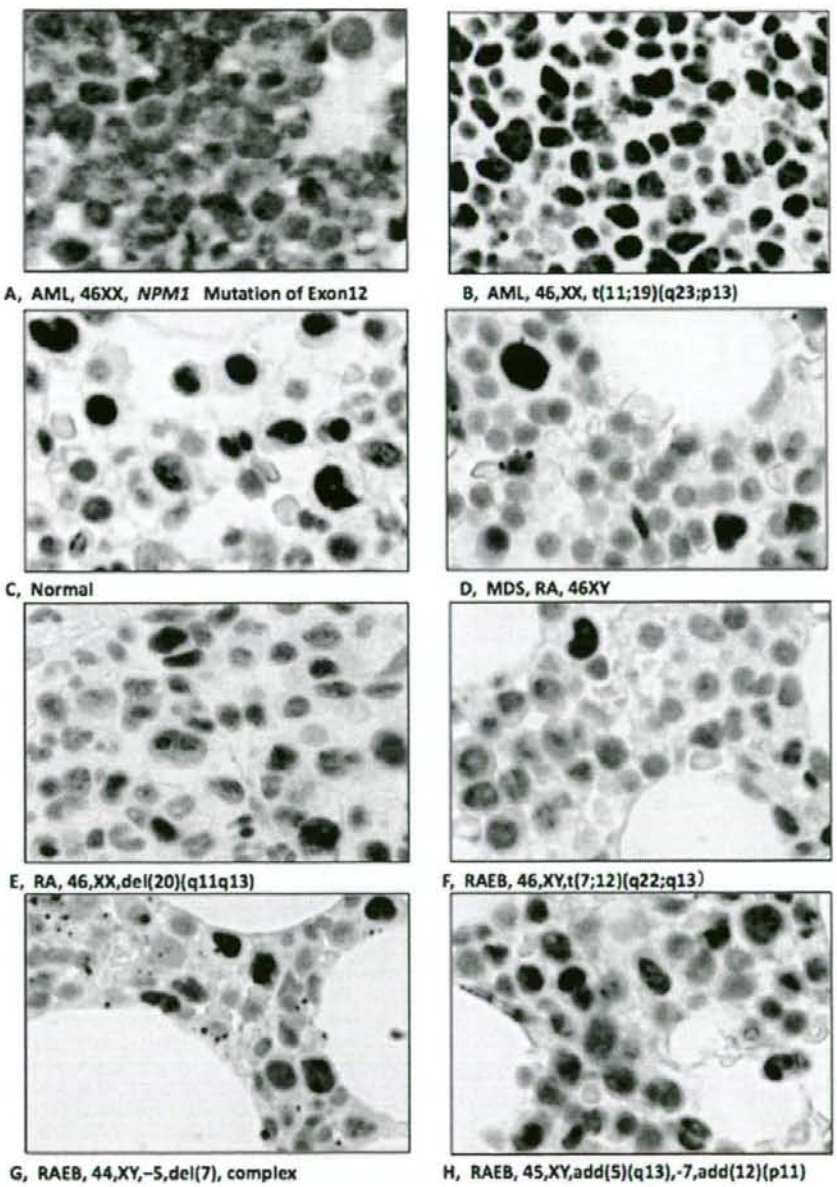


Figure 3. Immunohistochemistry of NPM1. The immunohistochemical studies of the NPM1 in AML, MDS and normal samples are indicated with karyotype. (A) In AML with *NPM1* mutation, NPM was diffusely stained in a microgranular pattern in the cytoplasm as well as the nucleus, as reported previously. (B) In AML without *NPM1* mutation, nuclear and nucleolar staining was clearly detected in leukemia cells. (C) In Normal BM cells, nuclear and nucleolar staining was detected in a part of lymphoid and myeloid cells. (D–H) In MDS, positive nuclear staining was observed in relatively large myeloid cells, which was similar to the staining pattern in normal BM.

mutation (mean relative intensity: 0.85 *vs.* 1.08, $p = 0.01$, Figure 2A). In MDS, the expression levels varied among samples but the expression level was similar to that of AML cells without *NPM1* mutation (0.93 *vs.* 1.08, $p = 0.13$, Figure 2A). No aberrant bands were detected in these samples (Figure 2B).

Using newly generated anti-NPM monoclonal antibody (NPM 9.2.6), both NPM1.1 and NPM1.2 proteins were detected by immunoblot analysis and immunohistochemical techniques. In normal BM cells, NPM1 was clearly detected in mononuclear myeloid cells but not in polymorphonuclear cells and erythroblasts. Lack of NPM1 expression in the polymorphonuclear cells was confirmed using normal peripheral blood by immunoblot analysis and immunohistochemical techniques (Supplement Figure 2). In AML specimens with *NPM1* mutation (Figure 3A) as a control, diffusely stained NPM1 could be observed as a microgranular pattern in the cytoplasm as well as the nucleus, as reported previously. In AML specimens without *NPM1* mutation (Figure 3B) as another control, nuclear and nucleolar staining was clearly detected in leukemia cells.

In MDS specimens (Figure 3D–H), positive nuclear staining was observed in relatively large myeloid cells, which was fundamentally similar to the staining pattern in normal BM (Figure 3C). However, the percentage of NPM-positive cells in the specimen was significantly decreased, which is explained by the percentage of mature cells such as polymorphonuclear cells and lymphocytes, as well as erythroblasts. There are no specimens in which NPM showed subcellular localisation which was different from normal BM cells.

Discussion

MDS is a clonal hematopoietic stem cell disorder characterised by multi-lineage dysplasia and pancytopenia in which the molecular mechanism is still mainly unclear, and even that which is understood is, quite heterogeneous [22]. Recently, it was suggested that *NPM1* might play the role of a tumor-suppressor gene in MDS in light of findings that $NPM1^{+/-}$ heterozygous mice develop a hematologic syndrome with features of human MDS [17]. During a long-time observation, $NPM1^{+/-}$ mice developed lymphoid malignancies and solid tumors, in addition to myeloid malignancies [23]. Malignant cells displayed multiple centrosomes and retained the wild-type allele and NPM1 protein with normal subcellular localisation and expression level. If this haploinsufficiency model is applicable to human MDS, the expression level of NPM1 must be critical for development or suppression of MDS. According to Knudson's two-hit model, two successive events, such as a deletion, mutation or methylation, in both alleles of a tumor-suppressor gene are required to turn a normal cell into a cancer cell [23]. In this case, the tumor-suppressor protein almost loses its function entirely. Because NPM1 has a critical role in ribosome biogenesis and cell proliferation, a haploinsufficiency model rather than Knudson's two-hit model may fit better with the above mice model. So far no study has carefully investigated the expression of NPM1 in human MDS samples. Here, we indicated that NPM1 protein levels were not significantly decreased in MDS cells irrespective of the presence of a 5q abnormality. Although accurate quantification of protein expression is difficult in clinical samples, our study showed that NPM1 levels in AML cells with *NPM1* mutation were lower than that in AML cells without *NPM1* mutation. Mutated NPM protein might have a shorter half-life than wild-type NPM as well as being localised in the cytoplasm. But, it is needed to study more samples to elucidate the differences of NPM protein levels in AML cells with or without *NPM1* mutation.

The immunohistochemical study clearly showed that NPM was expressed scarcely in granulocytes, moderately in lymphocytes and erythroblasts and abundantly in myeloblasts, supporting the role of NPM1 in proliferation and differentiation. Because the amount of *NPM1* transcripts are decreased but still expressed in granulocytes, the NPM protein level may be controlled by a post-transcriptional manner. In MDS specimens, the staining pattern and intensity were almost similar to the normal BM. Accordingly, low expression levels in immunoblots might reflect the increased mature fractions in the BM cells. In conclusion, cytoplasmic localisation and/or significant reduction of NPM1 protein level rarely occur in MDS. The expression level of NPM1 should be further studied in the stem/progenitor cell fraction of MDS.

Acknowledgments

This study was supported by Grant-in-Aids from the National Institute of Biomedical Innovation (to T.N.), for Scientific Research (to T.N., T.U. and G.S.) and the Japan Leukemia Research Fund (to T.U.).

References

1. Korgaonkar C, Hagen J, Tompkins V, Frazier AA, Allamargot C, Quelle FW, et al. Nucleophosmin (B23) targets ARF to nucleoli and inhibits its function. Mol Cell Biol 2005;25:1258–1271.
2. Kurki S, Peltonen K, Latonen L, Kiviharju TM, Ojala PM, Meek D, et al. Nucleolar protein NPM interacts with HDM2 and protects tumor suppressor protein p53 from HDM2-mediated degradation. Cancer Cell 2004;5:465–475.

3. Colombo E, Marine JC, Danovi D, Falini B, Pelicci PG. Nucleophosmin regulates the stability and transcriptional activity of p53. Nat Cell Biol 2002;4:529–533.
4. Borer RA, Lehner CF, Eppenberger HM, Nigg EA. Major nucleolar proteins shuttle between nucleus and cytoplasm. Cell 1989;56:379–390.
5. Chan WY, Liu QR, Borjigin J, Busch H, Rennert OM, Tease LA, et al. Characterization of the cDNA encoding human nucleophosmin and studies of its role in normal and abnormal growth. Biochemistry 1989;28:1033–1039.
6. 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;352:254–266.
7. Grisendi S, Mecucci C, Falini B, Pandolfi PP. Nucleophosmin and cancer. Nat Rev Cancer 2006;6:493–505.
8. Naoe T, Suzuki T, Kiyoi H, Urano T. Nucleophosmin: a versatile molecule associated with hematological malignancies. Cancer Sci 2006;97:963–969.
9. Morris SW, Kirstein MN, Valentine MB, Dittmer K, Shapiro DN, Look AT, et al. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. Science 1995;267:316–317.
10. Yoneda-Kato N, Look AT, Kirstein MN, Valentine MB, Raimondi SC, Cohen KJ, et al. The t(3;5)(q25.1;q34) of myelodysplastic syndrome and acute myeloid leukemia produces a novel fusion gene, NPM-MLF1. Oncogene 1996;12:265–275.
11. Redner RL, Rush EA, Faas S, Rudert WA, Corey SJ. The t(5;17) variant of acute promyelocytic leukemia expresses a nucleophosmin-retinoic acid receptor fusion. Blood 1996;87:882–886.
12. Thiede C, Koch S, Creutzig E, Steudel C, Illmer T, Schaich M, et al. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). Blood 2006;107:4011–4020.
13. Verhaak RG, Goudswaard CS, van Putten W, Bijl MA, Sanders MA, Hugens W, et al. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. Blood 2005;106:3747–3754.
14. 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;106:3733–3739.
15. Dohner K, Schlenk RF, Habdank M, Scholl C, Rucker FG, Corbacioglu A, et al. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. Blood 2005;106:3740–3746.
16. Suzuki T, Kiyoi H, Ozeki K, Tomita A, Yamaji S, Suzuki R, et al. Clinical characteristics and prognostic implications of NPM1 mutations in acute myeloid leukemia. Blood 2005;106:2854–2861.
17. Grisendi S, Bernardi R, Rossi M, Cheng K, Khandker L, Manova K, et al. Role of nucleophosmin in embryonic development and tumorigenesis. Nature 2005; 437:147–153.
18. Berger R, Busson M, Baranger L, Helias C, Lessard M, Dastugue N, et al. Loss of the NPM1 gene in myeloid disorders with chromosome 5 rearrangements. Leukemia 2006;20:319–321.
19. Oki Y, Jelinek J, Beran M, Verstovsek S, Kantarjian HM, Issa JP. Mutations and promoter methylation status of NPM1 in myeloproliferative disorders. Haematologica 2006;91:1147–1148.
20. Ozeki K, Kiyoi H, Hirose Y, Iwai M, Ninomiya M, Kodera Y, et al. Biologic and clinical significance of the FLT3 transcript level in acute myeloid leukemia. Blood 2004;103:1901–1908.
21. Xu JL, Lai R, Kinoshita T, Nakashima N, Nagasaka T. Proliferation, apoptosis, and intratumoral vascularity in multiple myeloma: correlation with the clinical stage and cytological grade. J Clin Pathol 2002;55:530–534.
22. Corey SJ, Minden MD, Barber DL, Kantarjian H, Wang JC, Schimmer AD. Myelodysplastic syndromes: the complexity of stem-cell diseases. Nat Rev Cancer 2007;7:118–129.
23. Sportoletti P, Grisendi S, Majid SM, Cheng K, Clohessy JG, Viale A, et al. Npm1 is a haploinsufficient suppressor of myeloid and lymphoid malignancies in the mouse. Blood 2008;111:3859–3862.

Int J Hematol (2008) 88:471–475
DOI 10.1007/s12185-008-0221-1

RAPID COMMUNICATION

Retention but significant reduction of BCR-ABL transcript in hematopoietic stem cells in chronic myelogenous leukemia after imatinib therapy

Akihiro Abe · Yosuke Minami · Fumihiko Hayakawa · Kunio Kitamura · Yuka Nomura · Makoto Murata · Akira Katsumi · Hitoshi Kiyoi · Catriona H. M. Jamieson · Jean Y. J. Wang · Tomoki Naoe

Received: 5 May 2008 / Revised: 24 October 2008 / Accepted: 31 October 2008 / Published online: 29 November 2008

Abstract Chronic myelogenous leukemia (CML) is effectively treated with imatinib mesylate (IM), a small molecule inhibitor of the BCR-ABL tyrosine kinase that is expressed in the entire hematopoietic compartment including stem cells (HSC) and progenitors in CML patients. While IM induces disease remission, it does not appear to eradicate BCR-ABL-positive stem cells. We investigated the residual CML cells in HSC and myeloid progenitors isolated using fluorescence-activated cell sorting after IM-therapy. Quantitative real-time polymerase chain reaction detecting *BCR-ABL* transcripts showed that CML progenitors were eradicated within 12 months while the BCR-ABL-positive HSC remained. However, IM-therapy continuation could significantly decrease the ratio of *BCR-ABL* to *BCR* also in the HSC population. Our results implicate that the sorted and purified stem cells are useful for more sensitive quantification of BCR-ABL-positive minimal residual disease.

Keywords CML · Imatinib · Leukemic stem cells · MRD

1 Introduction

The clinical success of the ABL kinase inhibitor imatinib mesylate (IM) in chronic myelogenous leukemia (CML) serves as a model for molecular targeted therapy of cancer [1–5]. However, despite unprecedented rates of complete cytogenetic response, residual disease remains detectable in the majority of patients [6–8], with disease recurrence upon discontinuation of IM-therapy [9–11]. It has been reported that primitive quiescent, malignant hematopoietic progenitor cells from patients with CML are insensitive to IM [12]. Recently, granulocyte–macrophage progenitors (GMP) with an aberrant potential for self-renewal were detected in CML blast crisis (BC) [7, 13], indicating GMP might also function as leukemia stem cells. Mathematical models of clinical response to IM-therapy have also suggested that CML stem cells may be resistant to this drug, thus accounting for the persistence of minimal residual disease and the development of drug resistance [14]. In this study, we investigated the residual disease in hematopoietic stem cells (HSC) and myeloid progenitors from patients with CML chronic phase (CP) after IM-therapy, and show retention but significant reduction of *BCR-ABL* transcript in HSC.

Electronic supplementary material The online version of this article (doi:10.1007/s12185-008-0221-1) contains supplementary material, which is available to authorized users.

A. Abe (✉) · Y. Minami · F. Hayakawa · Y. Nomura · M. Murata · A. Katsumi · T. Naoe
Department of Hematology and Oncology,
Nagoya University Graduate School of Medicine,
65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan
e-mail: aakihiro@med.nagoya-u.ac.jp

Y. Minami · C. H. M. Jamieson · J. Y. J. Wang
Division of Hematology–Oncology,
Department of Medicine and Moores Cancer Center,
University of California at San Diego School of Medicine,
La Jolla, CA, USA

K. Kitamura
Department of Hematology,
Ichinomiya Municipal Hospital,
Ichinomiya, Japan

H. Kiyoi
Department of Infectious Disease,
Nagoya University School of Medicine,
Nagoya, Japan

2 Study design

2.1 Patients and evaluation

Patients with a confirmed diagnosis of CML were investigated at indicated points before and after the start of IM-therapy. Bone marrow samples were harvested after written informed consent. Hematologic, cytogenetic and molecular responses were determined according to the European LeukemiaNet recommendations [15]. Briefly, complete hematological response (CHR) was defined as disappearance of signs and symptoms of disease, no splenomegaly, and complete blood counts within institutional normal limits. Complete cytogenetic response (CCR) was defined as 0% Ph metaphases among at least 20 metaphases in the bone marrow. Major molecular response (MMR) was defined either by *BCR-ABL* transcript levels below 100 copy per microgram of RNA quantified with reverse-transcriptase-polymerase-chain-reaction (RT-PCR) or transcription-mediated amplification (TMA) [16], or by 3 log reduction from initial levels at diagnosis [17, 18]. Quantification of the *BCR-ABL* transcripts by TMA method was performed using Amp-CML kit (Fujirebio, Tokyo, Japan).

2.2 Separation of HSC and progenitors

For the detection of MRD of HSC or progenitors from CML CP after IM-treatment, the mononuclear cells were freshly prepared within 24 hr after bone marrow harvest. For the detection of *BCR-ABL* and *BCR* transcripts of HSC or progenitors from CML CP before IM-treatment, if the fresh bone marrow samples were not available, frozen cells were thawed and subjected to FACS analysis. Mononuclear cells were stained with lineage-associated PE-Cy5.5-conjugated antibodies including CD2, CD3, CD4, CD8, CD14, CD19, CD20 and CD56 from Caltag (South San Francisco, CA). Flow-cytometric analysis and cell sorting were performed as previously published [12, 19]. The cells with the lineage cocktail antibodies were further incubated either with HSC-associated antibodies consisting of APC-conjugated anti-CD34 (HPCA-2; BD Pharmingen, San Diego, CA), biotinylated anti-CD38 (Caltag), FITC-labeled CD47 and phycoerythrin-conjugated anti-CD90 (Thy-1) followed by staining with streptavidin-Cy7PE (Invitrogen, Carlsbad, CA) to visualize CD38-biotin-stained cells or with progenitor-associated antibodies consisting of APC-conjugated anti-CD34, biotinylated anti-CD38, streptavidin-Cy7PE, phycoerythrin-conjugated anti-IL-3 receptor (9F5; BD Pharmingen) and FITC-conjugated anti-CD45RA (MEM56; Caltag).

Unstained samples and isotype controls were included to assess background fluorescence. After staining, cells were analyzed and sorted by using FACSAria (BD Immunocytometry Systems, San Jose, CA). HSC identified as $CD34^+CD38^-Lin^-$, were separated to Thy-1^+ (HSC/Thy-1^+) and Thy-1^- (HSC/Thy-1^-) cells. Common myeloid progenitors (CMP) were identified based on $CD34^+CD38^+$ IL-$3R\alpha^+CD45RA^-Lin^-$ staining, and their progeny including GMP were $CD34^+CD38^+$IL-$3R\alpha^+CD45RA^+Lin^-$, whereas megakaryocyte/erythroid progenitors (MEP) were identified based on $CD34^+CD38^+$IL-$3R\alpha^-$ $CD45RA^-Lin^-$ staining [20].

2.3 Quantification of *BCR-ABL* transcripts

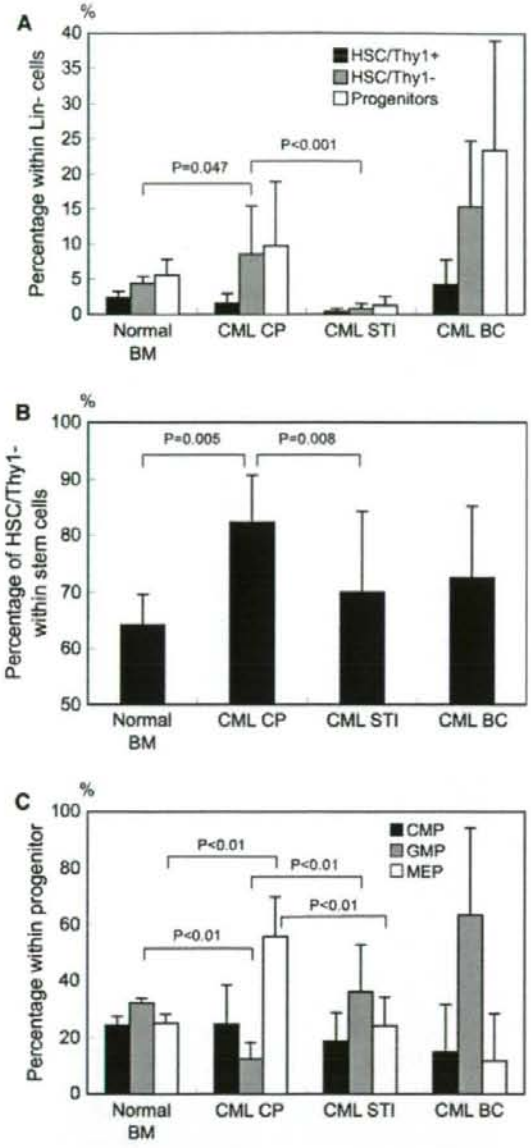
RNA was isolated from HSC/Thy-1^+, HSC/Thy-1^-, CMP, GMP, or MEP using the RNA STAT-60™ (TEL-TEST, INC. Friendswood, TX), and reversely transcribed into cDNA using TaqMan Gold RT-PCR Kit ™ with random hexamers (Applied Biosystems, Foster City, CA). Primers and probes used in this study were described previously as *BCR-ABL* [21], and *BCR* [12]. Quantitative RT-PCR analysis of the expression of *BCR-ABL* and *BCR* was performed with 50 cycles of two-step PCR (15 s at 95°C and 60 s at 60°C) after initial denaturation (95°C for 10 min) using an ABI Prism 7700 Sequence Detector System (Applied Biosystems). *BCR* was used as the control gene and the *BCR-ABL* levels for each sample were expressed as a ratio of *BCR-ABL* to *BCR*.

Quantification standards were prepared by cloning PCR products of *BCR-ABL* and *BCR* from CML samples. Each PCR product was cloned into pBluescript sk(-) vector by the TA cloning method, sequenced and ligated into the same vector. The resulting plasmids were digested with the appropriate restriction enzymes and used for stable standards to keep the same copy number of *BCR-ABL* and *BCR*.

3 Results and discussion

3.1 Analysis of HSC and progenitor profiles

FACS analysis revealed higher levels of the HSC/Thy-1^- cells and progenitors ($CD34^+CD38^+Lin^-$ cells) in bone marrow from patients with CML CP than in normal bone marrow although the level of long term HSC (HSC/Thy-1^+) in CML CP is similar with normal bone marrow (Fig. 1a). The proportion of HSC/Thy-1^- within stem cells ($CD34^+CD38^-Lin^-$) was expanded in CML CP (Fig. 1b). After the IM-therapy the proportion of progenitor pools ($CD34^+Lin^-$ cells) within Lin^- were remarkably reduced, especially that of HSC/Thy-1^- cells and progenitor cells. They are even significantly lower compared with their counterparts in normal bone marrow. This would seem to



◀ **Fig. 1** Analysis of the proportion of HSC and progenitors. (**a**) Mean proportion of HSC/Thy-1$^-$ ($CD34^+CD38^-$Thy-1$^-$Lin$^-$), HSC/Thy-1$^+$ ($CD34^+CD38^-$Thy-1$^+$Lin$^-$), and progenitors ($CD34^+CD38^+$Lin$^-$). Percentage of individual population is expressed as the percentage within Lin$^-$ cells in bone marrow from 3 control subjects, 14 patients with CML CP, 6 patients with CML BC and 14 samples from 10 patients after treatment with imatinib (CML STI) for 6 months and over. (**b**) Mean proportion of HSC/Thy-1$^-$ within $CD34^+CD38^-$Lin$^-$ cells. (**c**) Mean proportion of myeloid progenitor populations, including common myeloid progenitors (CMP), granulocyte–macrophage progenitors (GMP), and megakaryocyte–erythroid progenitors (MEP). Percentage of individual progenitors within $CD34^+CD38^+$Lin$^-$ population is depicted. *Error bars* represent standard deviation. Statistical analyses were performed with the use of Excel software and Student's two-tailed unpaired *t* test

indicate that imatinib suppresses progenitor pools more than matured pools of hematopoietic cells. With based on the nucleated cell number of bone marrow samples, it was estimated that the absolute number of HSC/Thy-1$^+$ was increased in CML CP, but decreased during IM-therapy (data not shown).

The proportion of MEP was increased and that of GMP was decreased in bone marrow from patients with CML CP as compared with their normal counterparts, which were consistent with previously described results (Fig. 1c) [12, 22]. The reason for the reduced proportion of GMP and the increased proportion of MEP in CML CP is not clear. One hypothesis is a differentiation block of late erythropoietic progenitors and a promoted differentiation of GMP with expansion failure as suggested previously [23, 24]. Another unique characteristic of CML is an increased proliferation of the granulocytic cell lineage with marked basophilia and eosinophilia. The detection of their progenitors has been reported [25, 26] and progenitors of basophil and eosinophil have been identified within GMP, whereas GMP are failed to expand in CML. Analysis of individual progenitors may clear the abnormal proliferation and differentiation destined for basophil and eosinophil in future study.

Expanded HSC/Thy-1$^-$ and progenitors were inhibited and the ratio of MEP and GMP was normalized after IM-therapy. Increased population of HSC/Thy-1$^-$ within HSC in CML CP was also inhibited after IM-therapy. These data suggested that the progenitor pools of CML are expanded in HSC/Thy-1$^-$ population.

3.2 Analysis of *BCR-ABL* transcripts in each population after IM-therapy

We compared the *ABL*, *BCR* or *GAPDH* genes as internal control for the quantification and evaluation of *BCR-ABL* mRNA expression in HSC from CML CP before IM-therapy. When we got less number of sorted cells from frozen samples, there were some cases, in which *BCR-ABL* transcript was positive but *ABL* transcript was negative. The transcript level of *GAPDH* was about 20–200 times higher than that of *BCR-ABL*, so that the *BCR-ABL/GAPDH* ratio was significantly affected by the number of sorted cells especially in cases that the sorted cell counts were small. On the other hand, the transcript level of *BCR-ABL* was within 0.2–2 times of *BCR* transcript and the ratio of *BCR-ABL* to *BCR* was not affected by the sorted cell counts. For these reasons, we believed that the *BCR* transcript as an internal control is the best for our study. An

Case	Age at diagnosis	Sex	Karyotype at diagnosis	Periods of IM-therapy (months)	Clinical response	Frozen or fresh cells	viability (%)	BCR-ABL/BCR					
								Total BM	HSC/ Thy-1+	HSC/ Thy-1-	CMP	GMP	MEP
1	51	M	46,XY,t(9;22)	0		frozen	24.1	0.23	0.44	0.24	0.60	0.47	0.51
				6	MMR	fresh	85.7	0	0.10	NA	0	0	0
				8	MMR	fresh	85.0	0	0.016	0	0	0	0.0018
				12	MMR	fresh	81.9	0	0.012	0	0	0	0
2	57	F	46,XX,t(9;22)	0		fresh	43.2	0.49	0.27	0.73	0.23	0.52	0.25
				1	CHR	fresh	76.3	0.016	0.19	0.42	0.30	0.032	1.00
				6	MMR	fresh	82.4	0.00040	0.0016	0.0017	0	0	0.0012
3	38	M	46,XY,t(9;22)	0		frozen	64.3	0.58	0.39	0.19	0.83	1.62	0.30
				10	MMR	fresh	79.3	0	0	0.00018	0	0	0
4	55	M	46,XY,t(9;22)	0		frozen	19.9	0.15	0.55	0.66	0.83	0.44	0.72
				54	MMR	fresh	78.3	0.00023	0	0.0026	0	0	0
5	63	F	46,XX,t(9;22)	3	CCR	fresh	56.7	0.013	0.30	0.068	0.15	0	0.17
				6	MMR	fresh	67.3	0	0.0069	0.0012	0	0	0
6	32	M	46,XY,t(9;22)	0		frozen	50.6	0.15	0.23	0.37	0.41	0.32	0.44
				50	MMR	fresh	72.9	0.00063	0.0049	0.0010	0.0012	0.0038	0.00032
7	63	F	46,XX,t(9;22)	0		frozen	24.2	0.68	1.83	1.31	0.70	1.09	0.71
				6	MMR	fresh	60.5	0	0.0028	0.0044	0.0052	0.0081	0.0014
				12	MMR	fresh	49.6	0	0	0.00052	0	0	0
8	43	M	46,XY,t(9;22)	7	MMR	fresh	79.7	0.00004	0.0049	0.017	0.015	0.0036	0
				12	MMR	fresh	59.9	0	0.00016	0	0	0	0
9	25	M	46,XY,t(9;22)	0		frozen	50.6	0.24	0.29	1.31	0.27	0.30	0.36
				18	MMR	fresh	76.6	0	0	0	0	0	0

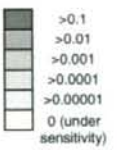


Fig. 2 Characteristics of the patients and results of quantitative PCR. Patient profiles, the time points of examination, the conditions of samples and the cell viability are shown. Residual CML cells are evaluated by the ratio of *BCR-ABL* to *BCR*. The percentage of cell viability was determined by dividing the number of PI negative cells by the total cell counts. Sorted cell numbers are shown in Supplementary Table 1. *CHR* complete hematological response, *CCR* complete cytogenetic response, *MMR* major molecular response, *NA* not available

another advantage to use the *BCR* is that the level of minimal residual CML cells are able to be estimated as two times of the *BCR-ABL/BCR* ratio, if we assume that the promoter activities of *BCR* and *BCR-ABL* are the same and the mRNA stability of the transcripts are the same.

Quantitative real-time polymerase chain reaction detecting *BCR-ABL* transcripts showed that BCR-ABL-positive progenitors in bone marrow were eradicated within 12-month in 5 patients. BCR-ABL-positive cells, however, remained in the stem cell population (Fig. 2). They were positive even after achieving undetectable levels of *BCR-ABL* transcript in total RNA isolated from the bone marrow. Differential retention of CML cells in HSC/Thy-1^+ or HSC/Thy-1^- were observed in case 1, 8 or 3, 4, 7, respectively. MRD in HSC/Thy-1^+ was under the detectable level in case 3, 4, 7 although the MRD in HSC/Thy-1^- was positive. In those cases, HSC/Thy-1^+ population was much smaller than HSC/Thy-1^- population as the mean proportion of sorted cell number of HSC/Thy-1^+ to that of HSC/Thy-1^- was 0.24 (SD = 0.13) (Supplementary Table 1). When MRD in stem cell was small, the detected MRD in stem cell population in patient with MMR was sometimes on border line of our technical sensitivity, so that MRD in HSC/Thy-1^+ might become under the detectable level due to the paucity of the sorted cells in these cases.

3.3 Ratio of *BCR-ABL/BCR* transcripts in each population after IM-therapy (Fig. 2)

The ratio of *BCR-ABL* and *BCR* was significantly decreased with the continuation of imatinib, however the retention of BCR-ABL-positive cells was observed in HSC/Thy-1^- or HSC/Thy-1^+ populations except case 9. In case 6, residual CML was still detected in all populations after 50 months of IM-therapy although the *BCR-ABL/BCR* ratio was significantly lower. The *BCR-ABL/BCR* values are known to correlate with the percentage of the Philadelphia chromosome in matched bone marrow samples [9–11]. We also examined *ABL* transcript as an internal control. However, in the case that we got less number of sorted cells after IM-therapy, we could not detect *ABL* transcript even if we could detect *BCR-ABL* transcript (data not shown).

It has been reported that primitive quiescent leukemic progenitor cells of CML patients are insensitive to IM [27], and Ph positive cells are persistent in the bone marrow of CML patients even in complete cytogenetic remission following IM-therapy [7, 13]. Mathematical models of clinical response to IM-therapy have also suggested that CML stem cells may be resistant to this drug [28]. However, there have been no reports investigating the direct quantification of Ph positive stem cells in bone marrow

during IM-therapy. We purified individual HSC and progenitors populations using fluorescence-activated cell sorting and quantified the ratio of *BCR-ABL/BCR*, which correlates closely with the CML cell ratio in the bone marrow. The BCR-ABL-positive cells were always detected in progenitor pools even after the CCR was achieved. When the MMR was achieved, the retention of CML remained in the HSC. The retention in HSC was observed both in Thy-1^+ and Thy-1^-, or each individual population. Recently, this Thy-1^- population in human cord blood was detected as candidate human multipotent progenitors (MPP). However, we could not determine the role of Thy-1 expression in residual CML cells. Importantly, IM-therapy continuation could significantly decrease the ratio of *BCR-ABL* to *BCR* also in the HSC population. In this report, we demonstrated the sorted and purified stem cells are useful for more sensitive quantification of BCR-ABL-positive minimal residual disease.

Acknowledgments We thank Ryohei Tanizaki at Nagoya University, and DJ Young at UCSD for their technical assistance. This work was supported by Grants-in-Aid from the National Institute of Biomedical Innovation

References

1. Sawyers C. Targeted cancer therapy. Nature. 2004;432:294–7.
2. Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. N Engl J Med. 2006;355:2408–17.
3. Kantarjian HM, Cortes J. New strategies in chronic myeloid leukemia. Int J Hematol. 2006;83:289–93.
4. Deininger MW. Optimizing therapy of chronic myeloid leukemia. Exp Hematol. 2007;35:144–54.
5. Savona M, Talpaz M. Getting to the stem of chronic myeloid leukaemia. Nat Rev Cancer. 2008;8:341–50.
6. Cortes J, O'Brien S, Kantarjian H. Discontinuation of imatinib therapy after achieving a molecular response. Blood. 2004;104:2204–5.
7. Michor F, Hughes TP, Iwasa Y, Branford S, Shah NP, Sawyers CL, et al. Dynamics of chronic myeloid leukaemia. Nature. 2005;435:1267–70.
8. Merante S, Orlandi E, Bernasconi P, Calatroni S, Boni M, Lazzarino M. Outcome of four patients with chronic myeloid leukemia after imatinib mesylate discontinuation. Haematologica. 2005;90:979–81.
9. Graham SM, Jorgensen HG, Allan E, Pearson C, Alcorn MJ, Richmond L, et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood. 2002;99:319–25.
10. Jiang X, Smith C, Eaves A, Eaves C. The challenges of targeting chronic myeloid leukemia stem cells. Clin Lymphoma Myeloma. 2007;7:71–80.
11. Pellicano F, Holyoake TL. Stem cells in chronic myeloid leukaemia. Cancer Biomark. 2007;3:183–91.
12. Jamieson CH, Ailles LE, Dylla SJ, Muijtjens M, Jones C, Zehnder JL, et al. Granulocyte–macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. N Engl J Med. 2004;351:657–67.
13. Roeder I, Horn M, Glauche I, Hochhaus A, Mueller MC, Loeffler M. Dynamic modeling of imatinib-treated chronic myeloid leukemia: functional insights and clinical implications. Nat Med. 2006;12:1181–4.
14. Baccarani M, Saglio G, Goldman J, Hochhaus A, Simonsson B, Appelbaum F, et al. Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. Blood. 2006;108:1809–20.
15. Langabeer SE, Gale RE, Harvey RC, Cook RW, Mackinnon S, Linch DC. Transcription-mediated amplification and hybridisation protection assay to determine BCR-ABL transcript levels in patients with chronic myeloid leukaemia. Leukemia. 2002;16:393–9.
16. Hughes T, Deininger M, Hochhaus A, Branford S, Radich J, Kaeda J, et al. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. Blood. 2006;108:28–37.
17. Goldman JM. How I treat chronic myeloid leukemia in the imatinib era. Blood. 2007;110:2828–37.
18. Jamieson CH, Gotlib J, Durocher JA, Chao MP, Mariappan MR, Lay M, et al. The JAK2 V617F mutation occurs in hematopoietic stem cells in polycythemia vera and predisposes toward erythroid differentiation. Proc Natl Acad Sci USA. 2006;103:6224–9.
19. Manz MG, Miyamoto T, Akashi K, Weissman IL. Prospective isolation of human clonogenic common myeloid progenitors. Proc Natl Acad Sci USA. 2002;99:11872–7.
20. Ishikawa I, Kato C, Harigae H, Sugawara T, Tomiya Y, Yamada M, et al. Dose modification of imatinib by monitoring the level of BCR-ABL transcript in chronic myelogenous leukemia. Tohoku J Exp Med. 2006;210:355–63.
21. Branford S, Hughes TP, Rudzki Z. Monitoring chronic myeloid leukaemia therapy by real-time quantitative PCR in blood is a reliable alternative to bone marrow cytogenetics. Br J Haematol. 1999;107:587–99.
22. Diaz-Blanco E, Bruns I, Neumann F, Fischer JC, Graef T, Rosskopf M, et al. Molecular signature of CD34(+) hematopoietic stem and progenitor cells of patients with CML in chronic phase. Leukemia. 2007;21:494–504.
23. Eaves AC, Eaves CJ. Abnormalities in the erythroid progenitor compartments in patients with chronic myelogenous leukemia (CML). Exp Hematol. 1979;7(Suppl 5):65–75.
24. Eaves AC, Barnett MJ, Ponchio L, Cashman JD, Petzer AL, Eaves CJ. Differences between normal and CML stem cells potential targets for clinical exploitation. Stem Cells. 1998;16 (Suppl 1):77–83.
25. Leary AG, Ogawa M. Identification of pure and mixed basophil colonies in culture of human peripheral blood and marrow cells. Blood. 1984;64:78–83.
26. Denburg JA, Telizyn S, Messner H, Lim B, Jamal N, Ackerman SJ, et al. Heterogeneity of human peripheral blood eosinophil-type colonies: evidence for a common basophil-eosinophil progenitor. Blood. 1985;66:312–8.
27. Bhatia R, Holtz M, Niu N, Gray R, Snyder DS, Sawyers CL, et al. Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment. Blood. 2003;101:4701–7.
28. Majeti R, Park CY, Weissman IL. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell Stem Cell. 2007;1:635–45.

Int J Hematol (2008) 88:460–462
DOI 10.1007/s12185-008-0185-1

LETTER TO THE EDITOR

Mutational analysis of *SOS1* gene in acute myeloid leukemia

Ryohei Tanizaki · Akira Katsumi · Hitoshi Kiyoi · Shinji Kunishima · Toshihiro Iwasaki · Yuichi Ishikawa · Miki Kobayashi · Akihiro Abe · Tadashi Matsushita · Takashi Watanabe · Tetsuhito Kojima · Kozo Kaibuchi · Seiji Kojima · Tomoki Naoe

Received: 20 July 2008 / Revised: 3 September 2008 / Accepted: 9 September 2008 / Published online: 30 October 2008

Ras proteins are small GTPases that transduce signals regulating cell proliferation, survival, and differentiation. Mutations in Ras are the most common oncogenetic alterations found in human cancers [1]. Activating mutations of *NRAS* occur in approximately 10–14% of acute myeloid leukemia (AML) [2, 3] and 6% of myelodysplastic syndrome [3]. In addition to the somatic mutations in *RAS* genes, other components of the Ras pathway, such as *PTPN11* (encoding protein tyrosine phosphatase SHP2) [4], *NF1* [5] and *BRAF* [6] were found in human malignancies.

The activation state of Ras is controlled by the cycle of hydrolysis of GTP catalyzed by GTPase activating proteins (GAPs), and by the replacement of bound GDP with GTP, which is catalyzed by guanine nucleotide exchanging factors (GEFs). The best studied mechanism of Ras activation is Son of sevenless 1 (SOS1), and mutations of *SOS1* gene were reported in approximately 10% of Noonan syndrome (NS) cases [7–9]. NS is associated with the increased risk of juvenile myelomonocytic leukemia (JMML), leading us to examine whether *SOS1* mutation plays certain roles in leukemogenesis. Recent reports claimed no mutations of *SOS1* gene in either 49 JMML [10], 29 chronic myelomonocytic leukemia [11], and 63 AML samples [11]. To determine the incidence of *SOS1* mutations in leukemia, we screened a larger number of patients; 210 bone marrow samples including 155 newly diagnosed AML, 20 AML transformed from MDS, and 35 JMML patients.

The diagnosis of AML is based on the French–American–British classification. The study population included 155 patients with newly diagnosed de novo AML (M0;9, M1;27, M2;49, M3;15, M4;22, M5;11, M6;2, M7;2 and 18

R. Tanizaki · A. Katsumi (✉) · T. Iwasaki · Y. Ishikawa · M. Kobayashi · A. Abe · T. Matsushita · T. Naoe
Department of Hematology and Oncology,
Nagoya University Graduate School of Medicine,
65 Tsurumai-Cho, Showa-ku, Nagoya 466–8550, Japan
e-mail: katsumi@med.nagoya-u.ac.jp

H. Kiyoi
Department of Infectious Diseases,
Nagoya University Graduate School of Medicine,
Nagoya, Japan

S. Kojima
Department of Pediatrics,
Nagoya University Graduate School of Medicine,
Nagoya, Japan

T. Watanabe · K. Kaibuchi
Department of Molecular Pharmacology,
Nagoya University Graduate School of Medicine,
Nagoya, Japan

T. Watanabe
Department of Institute for Advanced Research,
Nagoya University Graduate School of Medicine,
Nagoya, Japan

T. Kojima
Department of Pathophysiological Laboratory Sciences,
Nagoya University Graduate School of Medicine,
Nagoya, Japan

S. Kunishima
Department of Hemostasis and Thrombosis,
Clinical Research Center, National Hospital Organization,
Nagoya Medical Center, Nagoya, Aichi, Japan

Table 1 Primer pairs used to amplify the *SOS1* coding sequence (primer sequence 5′→3′)

Name	Exon	Forward	Reverse
hSOS1-7	7	AATACAGCCTCACTGAATTAATGT	CCTTGGCGGTATCTGTCTTTCTAT
hSOS1-11	11	GCTCTAGGTTTTCTGTCATC	CCATGCAGGAAAGAAAATCA
hSOS1-14	14	TGTGATAAACATTTATGTTTGATT	CGTGTTGGTACTTTTATTACAT
hSOS1-15	15	GTCATGCGTGTGACTTTAAACC	CCTATAAGGCAGAAATCAGTATTTCA
hSOS1-15	17	TTATTGGAAAAACTCTAAAACCT	TCTAATAGGGAATGAAAGTTCA
hSOS1-11-*Psi*I	11	GGATGCTTGATGTAACAATGCTACAGG	GCGTACATATGGTACGTAAGCCTCTTT

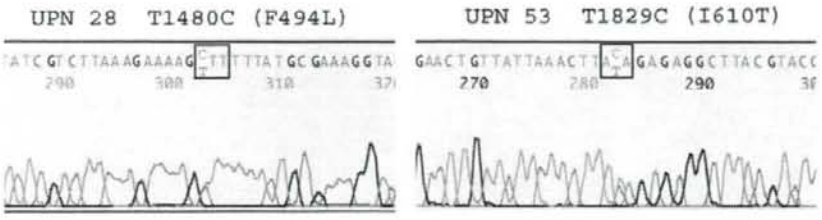


Fig. 1 *SOS1* variants found in AML patients. DNA sequence analysis of patients' *SOS1* gene. A T to C transition at nucleotide 1480 causes F494L (*left*), and a T to C transition at 1829 in exon 11 of *SOS1* gene causes I610T (*right*), each found heterozygously in a single AML patient

with unknown or unclassified subtype), 20 patients of AML transformed from MDS and 35 patients with JMML. Bone marrow (BM) samples from patients were subjected to Ficoll–Hypaque (Pharmacia LKB, Upsala, Sweden) density gradient centrifugation. All samples taken at diagnosis were confirmed to contain more than 90% of the leukemia cells after enrichment by centrifugation. Informed consent was obtained from all patients to use their samples for banking and molecular analysis, and approval for these studies was obtained from the Ethics committee of Nagoya University.

Exon 7,11,14,15 and 17, mutational hot spots of *SOS1* gene in NS [8, 9] was amplified by PCR using the primer described in Table 1. Exon 11 amplicons were directly sequenced by a DNA sequencer (310; Applied Biosystems, Foster City, CA, USA). The amplicons of exon 7, 14, 15 and 17 were screened by denaturing HPLC (DHPLC) analysis using the WAVE Maker System (Transgenomic Inc., San Jose, CA, USA) as described previously [12].

We have discovered two previously unreported missense variants 1480T → C (F494L) and 1829T → C (I610T) in exon 11, each found heterozygously in a single AML patient, respectively (Fig. 1; Table 2). F494L was also found in the post-remission bone marrow samples of the same patient (data not shown). Exon 11 of the *SOS1* gene was amplified from 200 anonymous control genomic DNA samples with either primer pairs hSOS1-11 or hSOS1-11-*Psi*I (Table 1), and digested with *Hind*III or *Psi*I to further investigate occurrence of the 1480T → C (F494L) and 1829T → C (I610T) missense changes,

Table 2 Clinical characteristics of AML with *SOS1* variants

UPN	*SOS1* variant	Age, years	FAB	Karyotype	Other gene mutations
28	F494L	65	M4	Normal	*FLT3*/ITD, *NPM*
53	I610T	19	M1	Normal	*FLT3*/ITD, *MLL*/PTD

respectively. The heterozygous substitution 1829T → C was found in one out of 200 normal individuals, whereas 1480T → C was not found in the same population (data not shown). These variants were not reported in JSNP, a database of Japanese single nucleotide polymorphisms (http://snp.ims.u-tokyo.ac.jp/index.html), and a recent report demonstrated that DNA sequences from 810 primary malignancies could not detect these *SOS1* variants [11]. These findings suggest that F494L and I610T are rare polymorphic variants rather than somatic mutations. *NRAS* mutation was not found in these two patients (data not shown).

Ras GEF activity of SOS1 is controlled by two sites: a catalytic sites that forms a stable interaction with nucleotide-free Ras and an allosteric site that potentiates exchange activity through the binding of nucleotide-bound Ras [13]. The former is located in Cdc25 domain, and the allosteric site is located between REM domain and Cdc25 domain. The amino acid residues F494 and I610 are located in the PH and REM domain, respectively, and these two residues are conserved in many species including

mouse, rat and chicken. Structural data indicated that catalytic output of SOS1 is autoinhibited through DH-PH-mediated blockade of the allosteric site [14]. These reports suggest that SOS1 variants may abrogate its autoinhibition, increase Ras activation and downstream signaling [8, 9]. As these patients demonstrate neither NS nor hereditary gingival fibromatosis phenotype, the significance of these polymorphic variants remains uncertain. Further functional assays including Ras activation are required to elucidate the roles of SOS1 in AML.

Acknowledgments We are indebted to the individuals who participated in the study and the physicians who referred the subjects. We thank Ms. Satomi Yamaji, Kyoko Hirano and Chika Wakamatsu for their excellent technical help. This work was supported by Grants-in-Aid from the National Institute of Biomedical Innovation, the Scientific Research of the Ministry of Education, Culture, Sports, Science and Technology, Japan and the Sankyo Memorial Foundation.

References

1. Bos JL. Ras oncogenes in human cancer: a review. Cancer Res. 1989;49:4682–9.
2. Kiyoi H, Naoe T, Nakano Y, et al. Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. Blood. 1999;93:3074–80.
3. Bacher U, Haferlach T, Kern W, Haferlach C, Schnittger S. A comparative study of molecular mutations in 381 patients with myelodysplastic syndrome and in 4130 patients with acute myeloid leukemia. Haematologica. 2007;92:744–52. doi:10.3324/haematol.10869.
4. Tartaglia M, Mehler EL, Goldberg R, et al. Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. Nat Genet. 2001;29:465–8. doi:10.1038/ng772.
5. Cichowski K, Santiago S, Jardim M, Johnson BW, Jacks T. Dynamic regulation of the Ras pathway via proteolysis of the NF1 tumor suppressor. Genes Dev. 2003;17:449–54. doi:10.1101/gad.1054703.
6. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature. 2002;417:949–54. doi:10.1038/nature00766.
7. Shannon K, Bollag G. Sending out an SOS. Nat Genet. 2007;39:8–9. doi:10.1038/ng0107-8.
8. Roberts AE, Araki T, Swanson KD, et al. Germline gain-of-function mutations in SOS1 cause Noonan syndrome. Nat Genet. 2007;39:70–4. doi:10.1038/ng1926.
9. Tartaglia M, Pennacchio LA, Zhao C, et al. Gain-of-function SOS1 mutations cause a distinctive form of Noonan syndrome. Nat Genet. 2007;39:75–9. doi:10.1038/ng1939.
10. Kratz CP, Niemeyer CM, Thomas C, et al. Mutation analysis of Son of sevenless in juvenile myelomonocytic leukemia. Leukemia. 2007;21:1108–9.
11. Swanson KD, Winter JM, Reis M, et al. SOS1 mutations are rare in human malignancies: implications for Noonan Syndrome patients. Genes Chromosomes Cancer. 2008;47:253–9. doi:10.1002/gcc.20527.
12. Kiyoi H, Yamaji S, Kojima S, Naoe T. JAK3 mutations occur in acute megakaryoblastic leukemia both in Down syndrome children and non-Down syndrome adults. Leukemia. 2007;21:574–6. doi:10.1038/sj.leu.2404527.
13. Margarit SM, Sondermann H, Hall BE, et al. Structural evidence for feedback activation by Ras.GTP of the Ras-specific nucleotide exchange factor SOS. Cell. 2003;112:685–95. doi:10.1016/S0092-8674(03)00149-1.
14. Sondermann H, Soisson SM, Boykevisch S, Yang SS, Bar-Sagi D, Kuriyan J. Structural analysis of autoinhibition in the Ras activator Son of sevenless. Cell. 2004;119:393–405. doi:10.1016/j.cell.2004.10.005.

Bioorganic & Medicinal Chemistry Letters 18 (2008) 5472–5477

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Novel and orally active 5-(1,3,4-oxadiazol-2-yl)pyrimidine derivatives as selective FLT3 inhibitors

Hiroshi Ishida [a,*], Shoichi Isami [a], Tsutomu Matsumura [a], Hiroshi Umehara [a], Yoshinori Yamashita [a], Jiro Kajita [a], Eiichi Fuse [a], Hitoshi Kiyoi [b], Tomoki Naoe [c], Shiro Akinaga [a], Yukimasa Shiotsu [a], Hitoshi Arai [a]

[a] Pharmaceutical Research Center, Kyowa Hakko Kogyo Co., Ltd, 1188 Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8731, Japan
[b] Department of Infectious Diseases, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8560, Japan
[c] Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8560, Japan

ARTICLE INFO

Article history:
Received 10 April 2008
Revised 26 August 2008
Accepted 6 September 2008
Available online 11 September 2008

Keywords:
FLT3 inhibitor
5-(1,3,4-Oxadiazol-2-yl)pyrimidine

ABSTRACT

5-(1,3,4-Oxadiazol-2-yl)pyrimidine derivative **1** was identified as a new class of FLT3 inhibitor from our compound library. With the aim of enhancement of antitumor activity of **2** prepared by minor modification of**1**, structure optimization of side chains at the 2-, 4-, and 5-positions of the pyrimidine ring of **2** was performed to improve the metabolic stability. Introduction of polar substituents on the 1,3,4-oxadiazolyl group contributed to a significant increase in the metabolic stability. As a result, a series of compounds showed increased efficacy against MOLM-13 xenograft model in mice by oral administration.

FLT3 (FMS-like receptor tyrosine kinase 3) also referred to as fetal liver kinase-2 (FLK-2) or stem cell kinase 1 (STK-1) is a class III receptor tyrosine kinase together with KIT, FMS, and platelet-derived growth factor receptor.[1,2] In 1996, FLT3/ITD (Internal Tandem Duplication) mutation was found in AML (Acute Myeloid Leukemia) cells. This mutation is formed by the duplication of juxtamembrane domain-coding sequence in a direct head-to-tail orientation.[3] The ITD mutation occurs in 15–35% of AML patients and promotes ligand-independent dimerization, auto-phosphorylation, and constitutive activation of the FLT3 receptor.[4] Patients carrying the ITD mutations are found to have an increased incidence of leukocytosis and a decreased overall survival when compared with patients without ITD mutations.[5] Therefore, inhibition of activated and mutated FLT3 kinase is effective strategy for the treatment of AML. To date, several FLT3 inhibitors have been subjected to clinical trials in several hematopoietic malignancies including AML.[6–9]

In the course of our discovery study for FLT3 inhibitors, the 5-(1,3,4-oxadiazol-2-yl)pyrimidine derivative **1** was identified as a primary hit compound via high-throughput screening of our compound library. In our preliminary SAR investigations, we found the compound **2** which showed about 70-fold more potent FLT3 inhibitory activity than **1** (Fig. 1).

Compound **2** showed not only FLT3 inhibitory activity but antiproliferative activity against MOLM-13 cells, a human AML cell line expressing ITD activating mutation.[10] This compound also showed

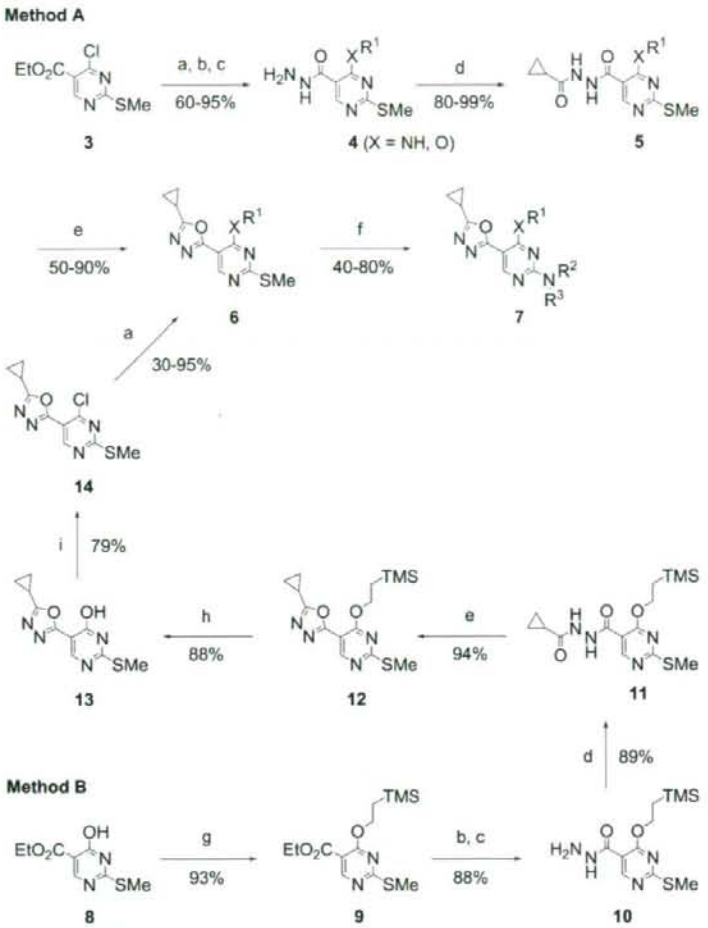
Figure 1. FLT3 inhibitors identified via high-throughput screening (**1**) and subsequent analog screening (**2**).

modest antitumor activity against MOLM-13 xenograft model in SCID mice. However, **2** was found metabolically unstable in both human and mice liver microsomes, and oxidative reaction of the *n*-propylamino group at the 4-position and the 4-(2-aminoethyl)pyridyl group at the 2-position were shown to be major metabolic pathways from the analysis of metabolite profiles. Thus, further structure optimization to acquire metabolic stability was required to enhance in vivo activity, and we started the structure optimization study of **2**. We herein report the synthesis and evaluation of novel 5-(1,3,4-oxadiazol-2-yl)pyrimidine derivatives as FLT3 inhibitors.

The synthesis of derivatives with modified side chains at the 2- and 4-positions is outlined in Scheme 1. Functionalization of the 4-position of a commercially available ethyl 4-chloro-2-methylthiopyrimidine-5-carboxylate **3** followed by hydrolysis of the ester group and condensation with hydrazine monohydrate gave the pyrimidinecarboxylic acid hydrazide **4**. Cyclization reaction[11] of **5**

* Corresponding author. Tel.: +81 55 989 2025; fax: +81 55 986 7430.
E-mail address: hiroshi.ishida@kyowa.co.jp (H. Ishida).

0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.09.031



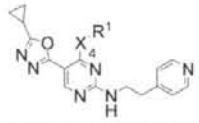
Scheme 1. Reagents and conditions: (a) R^1ONa (for X = O) or R^1NH_2 (for X = NH), Et_3N, THF; (b) 2.0 mol/L NaOH aq, EtOH; (c) i—CDI, THF; ii—$NH_2NH_2 \cdot H_2O$, THF; (d) cyclopropanecarbonyl chloride, $CHCl_3$, satd $NaHCO_3$ aq, 0 °C; (e) PPh_3, CCl_4, Et_3N, CH_3CN; (f) i—mCPBA, CH_2Cl_2; ii—R^2R^3NH, Et_3N, THF; (g) PPh_3, DEAD, 2-(trimethylsilyl)ethanol, toluene; (h) 1.0 mol/L TBAF in THF; (i) $POCl_3$, 60 °C.

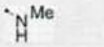
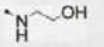
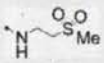
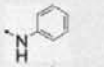
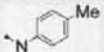
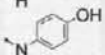
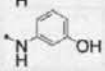
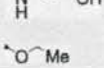
obtained by acylation of the hydrazide moiety in **4** provided the 5-(1,3,4-oxadiazol-2-yl)pyrimidine derivatives **6**. Subsequent substitution reaction at the 2-position of **6** afforded the desired compounds **7** (Method A). Alternatively, to aim for more efficient optimization at the 4-position by introducing the side chain in the latter stage of the reaction route, another method was examined (Method B). Introduction of a (2-trimethylsilyl)ethoxy group[12] to the 4-position of **8** prepared by the reported procedure[13] was performed using Mitsunobu reaction, followed by conversion to **12** according to Method A. Then, removal[14] of the (2-trimethylsilyl)ethyl moiety and treatment with $POCl_3$ yielded **14**, which was converted to **7** via formation of **6**.

Our SAR study was started on the 4-position. These results are summarized in Table 1. Shortening the carbon chain (**7a**) or introduction of a polar group (**7b**) resulted in a significant increase in the metabolic stability. However, their FLT3 kinase inhibitory activity and anti-proliferative activity against MOLM-13 were less potent compared to that of **2**. A similar tendency was also observed in the ethoxy analog **7i**. Anti-proliferative activity of the anilino analogs (**7d**–**7h**) was maintained. Among them, a 4-hydroxyanilino analog **7g** showed the most potent FLT3 inhibitory activity as well as anti-proliferative activity against MOLM-13 and good stability in human microsomes. However, compound **7g** was not expected to show in vivo activity, since **7g** was not detected in plasma after oral administration in mice (data not shown). From these results, we chose *n*-propylamino, anilino, and methylamino groups as desirable substituents for further optimization study because **2**, **7a**, and **7d** show either potent anti-proliferative activity against MOLM-13 (**2**, **7d**) or good metabolic stability (**7a**).

With the results in mind, SAR investigations on the 2-position were also examined for structural tolerability of the pyridyl moiety with the anilino group at the 4-position, which was identified as potent anti-proliferative activity in Table 1. These results are summarized in Table 2. The compounds were evaluated for anti-proliferative activity against MOLM-13 cells preferentially to identify more active compound against tumor cells. In contrast to the 4-position, structure tolerance for anti-proliferative activity in the 2-position was quite limited. Replacement with pyridyl *N*-oxide (**7j**), pyrimidyl (**7k**), and 4-aminophenyl (**7q**) analogs improved metabolic stability, but significantly lowered anti-proliferative activity. As mentioned above, oxidative reaction of the 4-(2-aminoethyl)pyridyl moiety at the 2-position of **2** was observed in the metabolite analysis. Introduction of a methyl group to aim for blocking the oxidative reaction of the nitrogen atom, however,

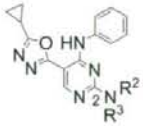
Table 1
SAR for the C-4 modified analogs

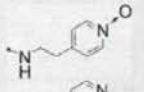
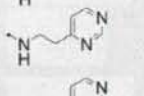
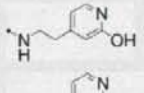
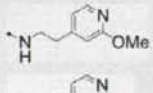
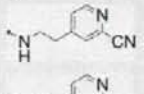
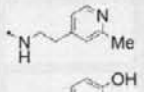
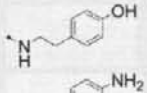
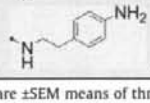


Compound	XR^1	FLT3 IC_{50}[a] (nmol/L)	MOLM-13 GI_{50}[b] (nmol/L)	HLM CL[c] (h/L/kg)
2		4.9 ± 1.0	36 ± 3.4	13
7a		35 ± 10	139 ± 14	1.2
7b		63 ± 10	123 ± 7.7	1.2
7c		515 ± 146	588 ± 235	n.t.[d]
7d		47 ± 7.4	40 ± 6.3	19
7e		134 ± 31	40 ± 8 7	13
7f		106 ± 30	23 ± 1.8	11
7g		4.8 ± 1.2	3.2 ± 0.34.8	
7h		34 ± 17	10 ± 1.5	16
7i		80 ± 14	263 ± 139	1.4

IC_{50} and GI_{50} values are ±SEM means of three experiments.
[a] See Ref. 15 for assay details.
[b] See Ref. 16 for assay details.
[c] See Ref. 17 for assay details.
[d] n.t., not tested.

Table 2
SAR for the C-2 and C-4 modified analogs



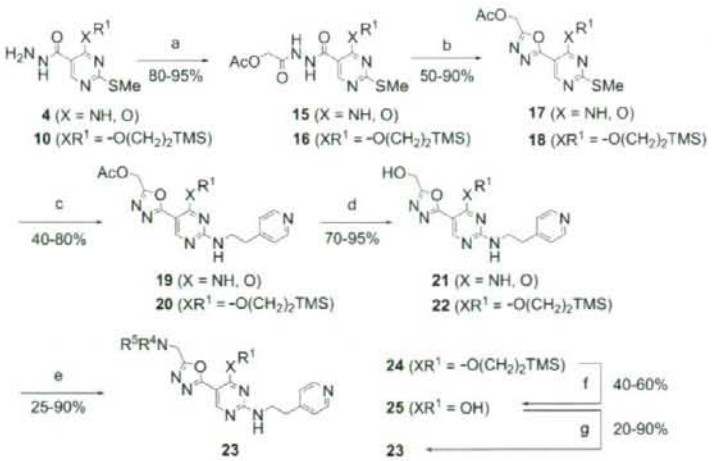
Compound	NR^2R^3	MOLM-13 GI_{50}[a] (nmol/L)	HLM CL[b] (h/L/kg)
7j		3610 ± 657	0.2
7k		775 ± 205	4.4
7l		113 ± 6.6	2.5
7m		999 ± 254	n.t.[c]
7n		>10,000	n.t.
7o		137 ± 10	22
7p		44 ± 6.7	5.1
7q		287 ± 33	4.1

GI_{50} values are ±SEM means of three experiments.
[a] See Ref. 16 for assay details.
[b] See Ref. 17 for assay details.
[c] n.t., not tested.

did not improve metabolic stability (**7o**). 2-Methoxypyridyl (**7m**) and 2-cyanopyridyl (**7n**) analogs also showed diminished activity. Only 2-hydroxypyridyl (**7l**) and 4-hydroxyphenyl (**7p**) analogs satisfied both criteria of anti-proliferative activity and microsomal stability. However, compounds **7l** and **7p** were not detected in plasma after oral administration in mice likewise **7g** (data not shown). From the results, we estimated the 4-(2-aminoethyl)pyridyl group was quite important to show in vivo activity.

Through these unsatisfied results, we decided to examine modification of the side chain on the 1,3,4-oxadiazolyl group at the 5-position, since SAR investigations on the position were carried out to a lesser extent. Thus, we planned to reduce their lipophilicity through introduction of polar substituents at the end of the 5-position to improve metabolic stability. To incorporate the above design into the structure, we developed new synthetic routes as shown in Scheme 2. Treatment of compounds **4** used as starting materials with acetoxyacetyl chloride afforded the corresponding **15** followed by the cyclization reaction to give **17**. Compounds **19** were obtained by introduction of the side chain at the 2-position of **17**. Hydrolysis of the acetyl group of **19** gave **21** followed by conversion to mesylates and subsequent substitution reaction with various amines provided **23**. Synthesis of **23** with XR^1 = OH could be alternatively achieved. Compounds **24** were prepared from **10** according to the above method, which underwent further deprotection of the hydroxy group to give **25**. Conversion of a 4-hydroxy group of the pyrimidine ring to mesylates and substitution reaction[18] with amines provided the desired compounds **23** successfully.

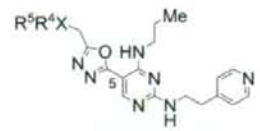
These compounds were evaluated for anti-proliferative activity against MOLM-13 cells. Table 3 summarizes the SAR studies for the substituted 1,3,4-oxadiazolyl group. We identified the 5-position with extensive structural tolerability. Polar substituents on the side chain of the 1,3,4-oxadiazolyl group were well tolerated for anti-proliferative activity against MOLM-13 cells. It is also noteworthy that the microsomal intrinsic clearance values of these compounds varied depending on the side chains. The distinct improvement of metabolic stability by the introduction of dimethylamino (**23a**), morpholino (**23b**), and 4-acetylpiperazinyl (**23d**) groups was not observed. Meanwhile, the introduction of hydroxy (**21a**), 4-methylpiperazinyl (**23c**), piperazinyl (**23f**), and 2-(dimethylamino)ethylamino (**23i**) groups increased metabolic stability. Above all, compound**23f** gave a best result and substituted or unsubstituted piperazinyl groups were shown to be suitable for the improvement of metabolic stability. We therefore tried to explore a combination of the side chains at the 4- and 5-positions which showed both potent activity and good metabolic stability. Table 4 summarizes the SAR studies for the 4-position in combination with the favorable piperazinyl groups on the side chain of the 5-position, which were identified in Table 3. Reduction of lipophil-



Scheme 2. Reagents and conditions: (a) $AcOCH_2COCl$, $CHCl_3$, satd $NaHCO_3$ aq, 0 °C; (b) PPh_3, CCl_4, Et_3N, CH_3CN; (c) i–mCPBA, CH_2Cl_2; ii–4-(2-aminoethyl)pyridine, THF; (d) 2.0 mol/L NaOH aq, MeOH; (e) i–Ms_2O, Et_3N, CH_2Cl_2; ii–R^4R^5NH, THF; (f) 1.0 mol/L TBAF in THF; (g) i–Ms_2O, Et_3N, CH_2Cl_2; ii–R^1NH_2 (for X = NH), Et_3N, CH_2Cl_2.

icity at the 4-position (e.g., **23c** vs **23j** or **23k**, **23h** vs **23r**) was found also effective for metabolic stabilization, which was already shown in Table 1. Incorporation of F atom to aim for blocking

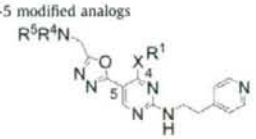
Table 3
SAR for the C-5 modified analogs



Compound	XR^4R^5	MOLM-13 GI_{50}[a] (nmol/L)	HLM CL[b] (h/L/kg)	clogp[c]
21a		58 ± 8.6	2.1	0.74
23a		73 ± 8.1	11	1.61
23b		43 ±4.2	10	1.52
23c		19 ± 3.6	4.9	1.97
23d		66 ± 4.6	8.2	1.11
23e		57 ± 4.3	3.0	1.43
23f		29 ± 1.9	1.1	1.51
23g		74 ± 5.8	3.0	0.82
23h		43 ± 8.1	6.9	2.55
23i		78 ± 4.1	3.0	1.53

GI_{50} values are ±SEM means of three experiments.
[a] See Ref. 16 for assay details.
[b] See Ref. 17 for assay details.
[c] Calculated by Daylight.[19]

Table 4
SAR for the C-4 and C-5 modified analogs



Compound	XR^1	NR^4R^5	MOLM-13 GI_{50}[a] (nmol/L)	HLM CL[b] (h/L/kg)	clogp[c]
23j			262 ± 51	<0.1	0.91
23k			102 ± 5.1	2.7	1.44
23l			52 ± 4.4	5.2	2.0
23m			308 ± 55	0.7	1.51
23n			49 ± 4.0	1.5	1.29
23o			50 ± 7.7	6.3	1.37
23p			74 ± 5.8	2.3	2.43
23q			99 ± 4.9	<0.1	0.93
23r			94 ± 10	0.8	2.02

GI_{50} values are ±SEM means of three experiments.
[a] See Ref. 16 for assay details.
[b] See Ref. 17 for assay details.
[c] Calculated by Daylight.[19]