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PU.1-mediated upregulation of *CSF1R* is crucial for leukemia stem cell potential induced by MOZ-TIF2

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Leukemias and other cancers possess self-renewing stem cells that help to maintain the cancer[1,2]. Cancer stem cell eradication is thought to be crucial for successful anticancer therapy. Using an acute myeloid leukemia (AML) model induced by the leukemia-associated monocytic leukemia zinc finger (MOZ)-TIF2 fusion protein, we show here that AML can be cured by the ablation of leukemia stem cells. The MOZ fusion proteins MOZ-TIF2 and MOZ-CBP interacted with the transcription factor PU.1 to stimulate the expression of macrophage colony–stimulating factor receptor (CSF1R, also known as M-CSFR, c-FMS or CD115). Studies using PU.1-deficient mice showed that PU.1 is essential for the ability of MOZ-TIF2 to establish and maintain AML stem cells. Cells expressing high amounts of CSF1R (CSF1R^{high} cells), but not those expressing low amounts of CSF1R (CSF1R^{low} cells), showed potent leukemia-initiating activity. Using transgenic mice expressing a drug-inducible suicide gene controlled by the *CSF1R* promoter, we cured AML by ablation of CSF1R^{high} cells. Moreover, induction of AML was suppressed in CSF1R-deficient mice and CSF1R inhibitors slowed the progression of MOZ-TIF2–induced leukemia. Thus, in this subtype of AML, leukemia stem cells are contained within the CSF1R^{high} cell population, and we suggest that targeting of PU.1-mediated upregulation of CSF1R expression might be a useful therapeutic approach.

Chromosomal translocations that involve the *MOZ* gene[3] (official gene symbol *Myst3*) are typically associated with acute myelomonocytic leukemia and predict a poor prognosis[4]. Whereas MOZ is essential for the self-renewal of hematopoietic stem cells[5,6], MOZ fusion proteins enable the transformation of non–self-renewing myeloid progenitors into leukemia stem cells[7]. We previously generated a mouse model for AML by introducing c-Kit$^+$ mouse myeloid stem/progenitor cells infected with a retrovirus encoding MOZ-TIF2 and EGFP into lethally irradiated mice[8].

To identify leukemia-initiating cells (LICs), we investigated the bone marrow cells of these mice for various cell surface markers by FACS analysis. CSF1R^{high} and CSF1R^{low} cells were present in the bone marrow (**Fig. 1a**) and expressed equivalent amounts of MOZ-TIF2 protein (**Fig. 1b**). To determine the LIC activity of these cell populations, we isolated CSF1R^{high} and CSF1R^{low} cells by cell sorting and transplanted limited numbers (10 to 1×10^4 cells) into irradiated mice. One hundred CSF1R^{high} cells were sufficient to induce AML in all transplanted mice (**Fig. 1c**). Conversely, no mice developed AML after 1×10^3 CSF1R^{low} cells were transplanted per mouse, and only half of the mice developed AML with delayed onset when 1×10^4 CSF1R^{low} cells were transplanted (**Fig. 1d**). Thus, the CSF1R^{high} cells showed a >100-fold stronger LIC activity than CSF1R^{low} cells.

FACS analysis indicated that the CSF1R^{high} cell population had the phenotype of both granulocyte-macrophage progenitors (GMPs, Kit$^+$Sca-1$^-$CD16/CD32$^+$) and differentiated monocytes (Mac-1^{low}Gr-1$^+$) (**Supplementary Fig. 1a**). Comparison of the CSF1R^{high} and CSF1R^{low} cell populations indicated that Mac-1 expression was lower in CSF1R^{high} than in CSF1R^{low} cells (**Fig. 1e**). However, we did not observe significant differences between the CSF1R^{high} and CSF1R^{low} cell populations with respect to their cell morphology (**Fig. 1f**), colony-forming ability in methylcellulose medium (**Fig. 1g**), cell cycle distribution (**Supplementary Fig. 1b**) or homeobox A9 (HoxA9) expression (**Supplementary Fig. 1c**). To investigate whether downstream pathways of CSF1R signaling were activated, we measured phosphorylation levels of signal transducer and activator of traranscription-5 (STAT5) and extracellular signal–regulated kinase (ERK) in CSF1R^{high} and CSF1R^{low} cells. STAT5 was highly phosphorylated in the CSF1R^{high} cell population but not in the CSF1R^{low} population, whereas ERK was equivalently phosphorylated in the two cell populations (**Fig. 1h**).

Side population cells, which are present in some types of normal and malignant stem cell populations, were present in the bone marrow of MOZ-TIF2–induced AML mice (**Supplementary Fig. 2a**). Whereas most side population cells were CSF1R^{high}, the non–side population fraction contained both CSFR1^{high} and CSF1R^{low} cells (**Supplementary Fig. 2b**). LICs were approximately tenfold more enriched in the side population fraction than in the non–side population fraction (**Supplementary Fig. 2c,d**). Because the side population fraction was very small (~0.12% of total bone marrow cells), the fraction of LICs in the side population fraction was also small (~1% of all LICs), and most LICs were present in the non–side population fraction (~99%).

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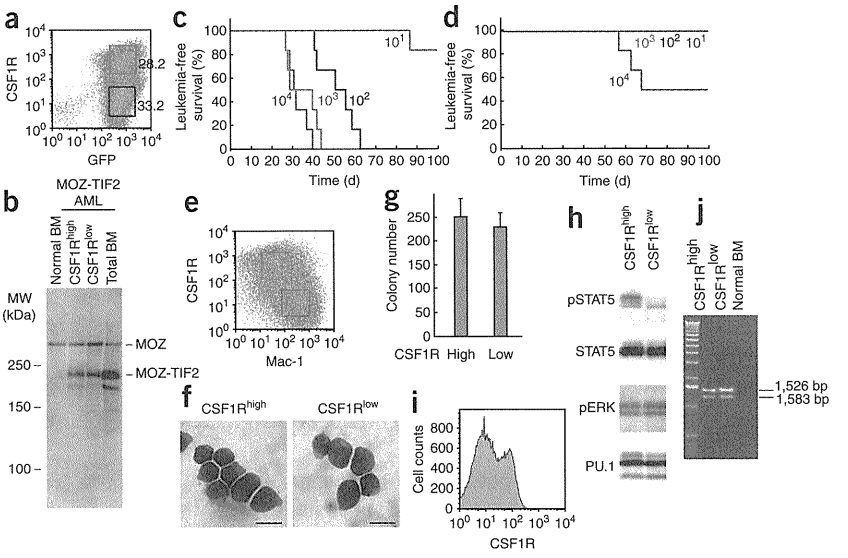

Figure 1 CSF1R^{high} cells show potent leukemia-initiating activity. (**a**) FACS analysis of bone marrow cells from mice with MOZ-TIF2–induced AML for expression of GFP and CSF1R. The red and black boxes signify CSF1R^{high} and CSF1R^{low} cell fractions, respectively. (**b**) Immunoblot analysis of MOZ-TIF2 expression in CSF1R^{high} and CSF1R^{low} cell populations (sorted by flow cytometry) with a MOZ-specific antibody. MW, molecular weight; BM, bone marrow. (**c**,**d**) Leukemia-free survival after the indicated numbers of flow-sorted CSF1R^{high} (**c**) and CSF1R^{low} (**d**) cells were transplanted into sublethally irradiated mice. $n = 6$, $P = 0.0001$ (1×10^4, 1×10^3 and 1×10^2) and 0.3173 (1×10^1) (CSF1R^{high} versus CSF1R^{low} cells). (**e**) FACS analysis of Mac-1 and CSF1R expression in bone marrow cells from mice with MOZ-TIF2–induced AML. The red and blue boxes signify CSF1R^{high} and CSF1R^{low} cell fractions, respectively. (**f**–**h**) CSF1R^{high} and CSF1R^{low} cells were sorted and analyzed for morphology by staining with May-Giemsa (**f**), colony-forming activity in methylcellulose medium (**g**) and levels of total and phosphorylated STAT5, phosphorylated ERK and PU.1 (**h**). Scale bars represent 10 μm in **f**. The error bars represent s.d. in **g**. (**i**) FACS analysis of CSF1R expression in bone marrow cells from an individual with AML with a t(8;16) translocation; the cells were cultured for 3 d in 10 ng ml^{-1} human M-CSF. (**j**) RT-PCR analysis of MOZ-CBP transcripts in CSF1R^{high} and CSF1R^{low} cells of the individual with t(8;16) AML. The results are representative of 25 (**a**,**e**), four (**b**), three (**c**,**d**,**f**–**h**) and two (**i**,**j**) independent experiments.

To determine whether a high level of CSF1R expression also occurs in human AML cells with *MOZ* translocations, we investigated CSF1R expression in bone marrow cells from a subject with AML harboring a t(8;16) translocation, yielding a MOZ-CREB–binding protein (CBP, encoded by the *Crebbp* gene) fusion[9]. FACS analysis indicated that both CSF1R^{high} and CSF1R^{low} cells were present among the bone marrow cells with this translocation (**Fig. 1i**). We detected *MOZ-CBP* fusion transcripts in both the CSF1R^{high} and CSF1R^{low} cell populations (**Fig. 1j**).

These results suggest that leukemia stem cells in this subtype of AML express a high amount of CSF1R, indicating that leukemia might be cured by inducing apoptosis of CSF1R^{high} cells. To test this idea, we used transgenic mice expressing a drug-inducible FK506-binding protein (FKBP)-Fas suicide gene and EGFP under the control of the *CSF1R* promoter[10] (**Fig. 2a**). The suicide gene products are inactive monomers under normal conditions but can be activated by injection of the AP20187 dimerizer, inducing apoptosis of cells expressing high amounts of CSF1R[10]. We infected c-Kit$^+$ bone marrow cells of transgenic mice with the MOZ-TIF2 retrovirus and transplanted them into lethally irradiated wild-type mice. These mice developed AML ~2 months after transplantation. In the bone marrow of these mice, we observed morphologically indistinguishable CSF1R^{high} and CSF1R^{low} cells. As expected, endogenous CSF1R expression was proportional to EGFP and FKBP-Fas expression (**Fig. 2b** and **Supplementary Fig. 3a**).

Next, we transplanted the bone marrow cells of these AML mice (1×10^5 cells per mouse) into secondary sublethally irradiated recipient mice. Seven days after transplantation, we injected the mice with

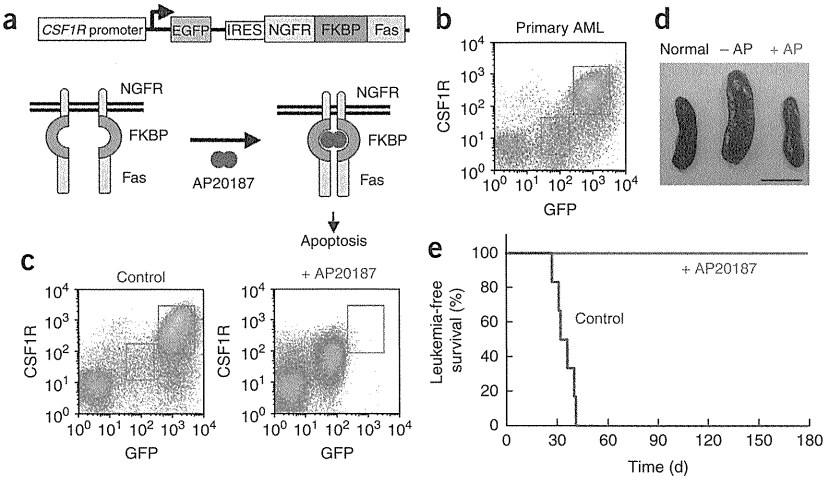

Figure 2 Cure of AML by ablation of CSF1R^{high} cells. (**a**) Top, structure of the *CSF1R* promoter-EGFP-NGFR-FKBP-Fas suicide construct. Bottom, schematic showing the activation of the NGFR-FKBP-Fas fusion protein: in transgenic mice carrying this suicide construct, ablation of cells expressing high levels of CSF1R can be induced by exposure to the AP20187 dimerizer. (**b**) FACS analysis of GFP and CSF1R expression in bone marrow cells of mice with AML 2 months after the transplantation of MSCV-MOZ-TIF2-IRES-GFP–transfected bone marrow cells derived from transgenic mice into lethally irradiated C57BL/6 mice. The red boxes signify CSF1R^{high} and CSF1R^{low} cell fractions. (**c**–**e**) Bone marrow cells (1×10^5) of primary transplanted mice with AML, generated as in **b**, were transplanted into sublethally irradiated C57BL/6 mice. Administration of AP20187 or solvent (control) to the secondary transplanted mice was started by intravenous injection 3 weeks after transplantation. Expression of GFP and CSF1R in bone marrow cells (**c**) and spleen sizes (**d**) were analyzed 4 weeks after transplantation. Scale bars, 1 cm. (**e**) Leukemia-free survival of the untreated ($n = 6$) and AP20187-treated ($n = 6$) secondary transplanted mice. $P < 0.0001$. The results are representative of five (**b**), four (**c**) and three (**d**,**e**) independent experiments.

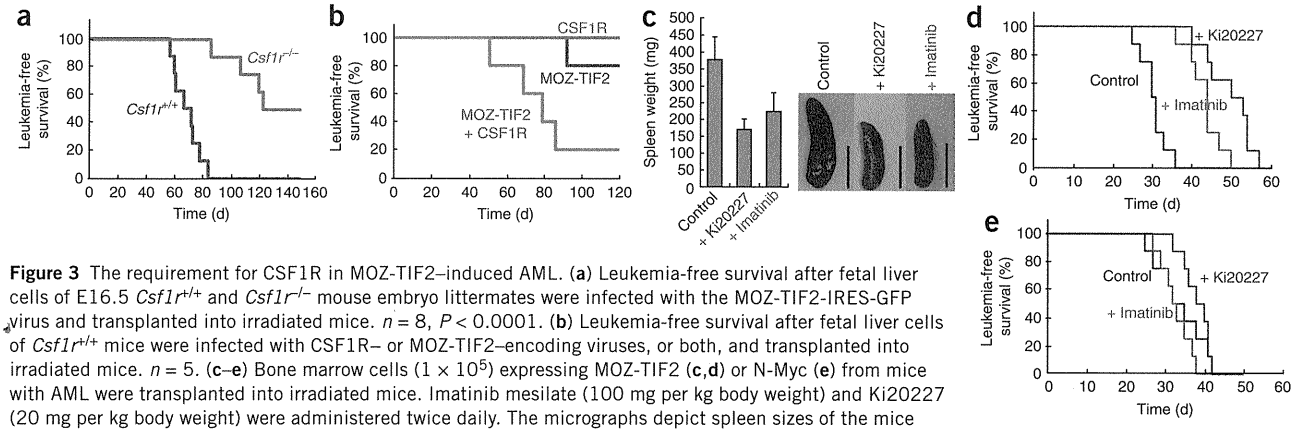

Figure 3 The requirement for CSF1R in MOZ-TIF2–induced AML. (**a**) Leukemia-free survival after fetal liver cells of E16.5 *Csf1r*$^{+/+}$ and *Csf1r*$^{-/-}$ mouse embryo littermates were infected with the MOZ-TIF2-IRES-GFP virus and transplanted into irradiated mice. $n = 8$, $P < 0.0001$. (**b**) Leukemia-free survival after fetal liver cells of *Csf1r*$^{+/+}$ mice were infected with CSF1R– or MOZ-TIF2–encoding viruses, or both, and transplanted into irradiated mice. $n = 5$. (**c–e**) Bone marrow cells (1×10^5) expressing MOZ-TIF2 (**c**,**d**) or N-Myc (**e**) from mice with AML were transplanted into irradiated mice. Imatinib mesilate (100 mg per kg body weight) and Ki20227 (20 mg per kg body weight) were administered twice daily. The micrographs depict spleen sizes of the mice transplanted with MOZ-TIF2–expressing cells, analyzed three weeks after transplantation (**c**). Scale bars, 1 cm. (**d**,**e**) Leukemia-free survival of the control and drug-treated mice was analyzed. In **d**, $n = 8$, $P < 0.0001$ (control versus + Ki20227 and control versus + imatinib). In **e**, $n = 8$, $P = 0.3825$ (control v.s. + Ki20227) and 0.4051 (control versus + imatinib).

AP20187 or a control solvent, as previously described[10]. We observed an increase in the number of CSF1R^{high} cells (**Fig. 2c**) and splenomegaly (**Fig. 2d**) in the control-treated mice 3 weeks after transplantation. However, we detected neither CSF1R^{high} cells nor splenomegaly in the AP20187-treated mice after a 1-week course of treatment (**Fig. 2c,d**). Although we observed CSF1R^{low} cells in the bone marrow and peripheral blood after the 1-week treatment course, we did not detect these cells after three months of treatment (**Fig. 2c** and **Supplementary Fig. 3b**). All control-treated mice developed AML 4–6 weeks after transplantation, but none of the AP20187-treated mice died of AML within 6 months of transplantation (**Fig. 2e**). These results indicate that ablation of the CSF1R^{high} cells was sufficient to cure MOZ-TIF2–induced AML, and that a high level of CSF1R expression is a key contributor to leukemia stem cell potential.

As it has been reported that N-Myc overexpression rapidly causes AML in mice[11], we next tested the specificity of the requirement for CSF1R^{high} cells in AML progression. We transfected the bone marrow cells of suicide gene–expressing transgenic mice with a retrovirus encoding N-Myc and EGFP, and transplanted the cells into lethally irradiated recipient mice, which developed AML. In these mice, GFP$^+$ leukemia cells were Mac1$^+$Gr1$^+$CSF1R$^-$ blast cells (**Supplementary Fig. 4a,b**), and treatment with AP20187 did not affect AML induction (**Supplementary Fig. 4c**). These results indicate a specific role of CSFR expression in MOZ-TIF2–induced AML.

To investigate the role of CSF1R in the development of MOZ-TIF2–induced AML, we infected wild-type and *Csf1r*$^{-/-}$ (ref. 12) mouse fetal liver cells of embryonic day 16.5 (E16.5) littermate embryos with the MOZ-TIF2 virus and transplanted them into lethally irradiated mice. All mice transplanted with wild-type cells developed AML within 3 months (**Fig. 3a**). In contrast, AML induction was initially suppressed in mice transplanted with *Csf1r*$^{-/-}$ cells, but half of the mice developed AML after a longer latency period (**Fig. 3a**). The suppression of AML was rescued by co-infection with the retrovirus encoding CSF1R (**Fig. 3b**). STAT5, which was highly phosphorylated in CSF1R^{high} cells but not in CSF1R^{low} cells (**Fig. 1h**), was phosphorylated in the bone marrow of recipient mice transplanted with *Csf1r*$^{+/+}$ cells but not with *Csf1r*$^{-/-}$ cells (**Supplementary Fig. 5**). To test the specificity of the requirement of CSF1R for AML induction by MOZ-TIF2, we transfected *Csf1r*$^{+/+}$ and *Csf1r*$^{-/-}$ fetal liver cells with the retrovirus encoding N-Myc and transplanted them into irradiated recipient mice. All of the mice transplanted with either *Csf1r*$^{+/+}$ or *Csf1r*$^{-/-}$ cells expressing N-Myc developed AML (**Supplementary Fig. 4d**). These results indicate that CSF1R has a key role in AML induction by MOZ-TIF2, but not by N-Myc.

The above results suggest that signaling through CSF1R might be a therapeutic target for kinase inhibitors in leukemogenesis induced by MOZ fusions. To test this, we used the CSF1R-specific inhibitor Ki20227 (ref. 13) and the tyrosine kinase inhibitor imatinib mesylate (STI571), which inhibits CSF1R1[14–16]. Oral administration of Ki20227 or imatinib inhibited MOZ-TIF2–induced splenomegaly (**Fig. 3c**) and slowed MOZ-TIF2–induced AML onset (**Fig. 3d**). However, the drugs did not affect the progress of N-Myc–induced AML (**Fig. 3e**).

Next, we investigated the molecular mechanism of CSF1R expression in the leukemia cells. Monocyte-specific expression of CSF1R is reportedly regulated by transcription factors such as AML1, PU.1 and CCAAT/enhancer-binding proteins (C/EBPs)[17]. We previously found that MOZ interacts with AML1 and PU.1, but not with C/EBPα or C/EBPε, to stimulate transcription of their target genes[5,18]. Deletion analysis indicated that PU.1 interacted with the N-terminal and central regions of MOZ (**Fig. 4a** and **Supplementary Fig. 6**), and that the acidic amino acid–rich region (DE region) of PU.1 was required for its high-affinity interaction with MOZ (**Fig. 4a** and **Supplementary Fig. 7a–d**). Although binding of PU.1 to N-terminal MOZ (amino acids 1–513) was inhibited by several deletions in the PU.1 protein (**Supplementary Fig. 7c**), binding to full-length MOZ was not completely inhibited by these deletions (**Supplementary Fig. 7b**), suggesting that there may be other PU.1-binding sites in MOZ, its associated proteins or both. A pull-down assay with *Escherichia coli*–produced GST-PU.1 or GST-AML1 and *in vitro*–produced N-terminal MOZ indicated a direct interaction between both PU.1 and MOZ and between AML1 and MOZ (**Supplementary Fig. 8**). However, we cannot rule out a possibility that other factors may facilitate interactions between PU.1 or AML1 and MOZ *in vivo*.

To investigate transcriptional regulation of CSF1R, we performed reporter analysis with a *CSF1R* promoter–luciferase construct and found that MOZ, MOZ-TIF2 and MOZ-CBP could all activate the *CSF1R* promoter in the presence of PU.1 but not in the presence of AML1 (**Fig. 4b**). Moreover, MOZ, MOZ-TIF2 and MOZ-CBP did not activate a *CSF1R* promoter mutant lacking PU.1-binding sites (**Fig. 4c**). These results suggest that MOZ and MOZ fusion

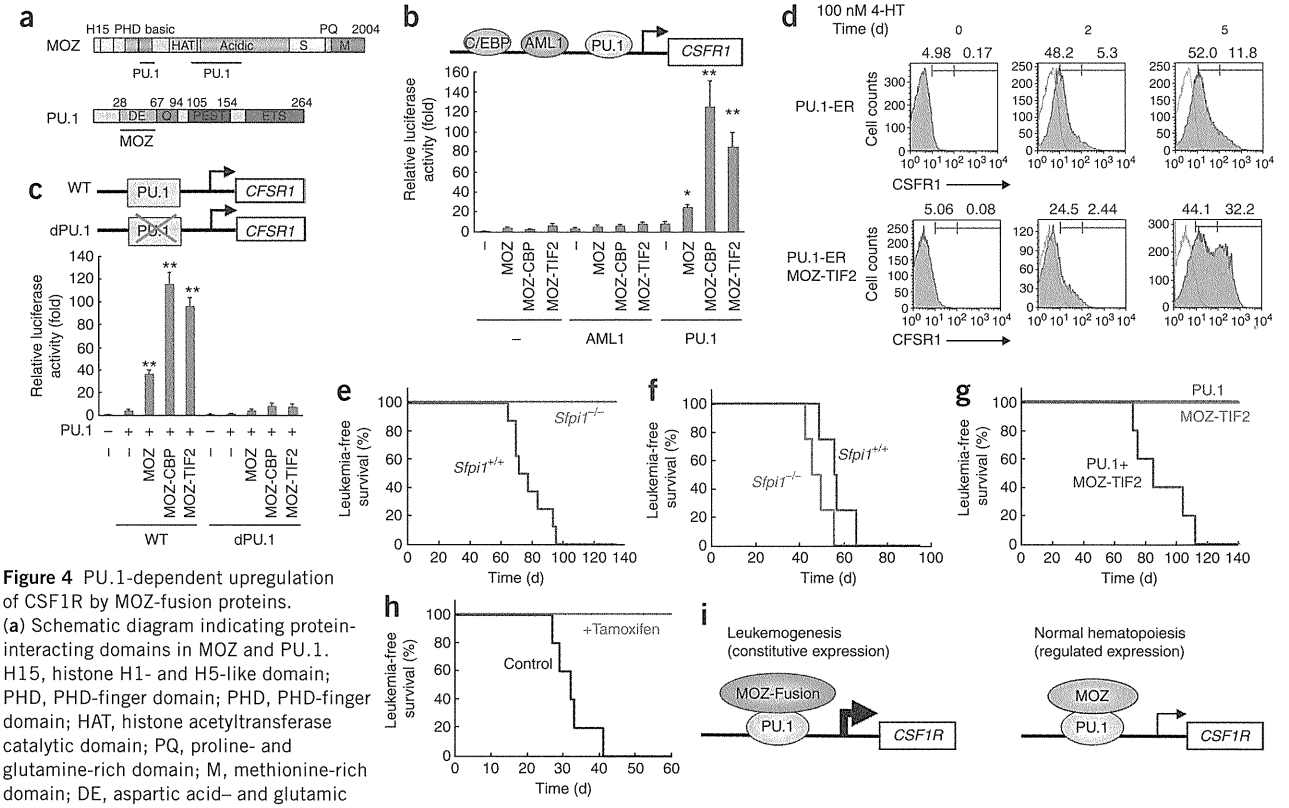

Figure 4 PU.1-dependent upregulation of CSF1R by MOZ-fusion proteins. (**a**) Schematic diagram indicating protein-interacting domains in MOZ and PU.1. H15, histone H1- and H5-like domain; PHD, PHD-finger domain; PHD, PHD-finger domain; HAT, histone acetyltransferase catalytic domain; PQ, proline- and glutamine-rich domain; M, methionine-rich domain; DE, aspartic acid– and glutamic acid–rich domain; Q, glutamine-rich domain; PEST, proline-, glutamic acid–, serine- and threonine-rich domain; ETS, Ets DNA-binding domain. (**b**) Effects of MOZ, MOZ-CBP and MOZ-TIF2 on AML1- and PU.1-mediated transcription of the *CSF1R* promoter. Osteocarcinoma SaOS2 cells were transfected with the CSF1R-luciferase construct and the indicated effector constructs encoding AML1 or PU.1 together with MOZ, MOZ-CBP or MOZ-TIF2. Luciferase activity was analyzed 24 h after transfection. Error bars represent s.d. $*P < 0.01$ and $**P < 0.005$ (comparison to PU.1 only). The results are representative of six independent experiments in which three samples were tested for each group in each experiment. (**c**) PU.1-dependent activation of CSF1R promoter. SaOS2 cells were transfected with the wild-type (WT) *CSF1R*-luciferase construct or its mutant lacking the PU.1-binding site (dPU.1), together with the indicated effectors. Error bars represent s.d. $*P < 0.01$ and $**P < 0.005$ (comparison to PU.1 only). The results are representative of three independent experiments in which three samples were tested for each group in each experiment. (**d**) FACS analysis of CSF1R expression in PUER cells infected with MSCV-GFP (top) or MSCV-MOZ-TIF2-IRES-GFP (bottom) retroviruses and exposed to 100 nM 4-HT for 0, 2 or 5 d. Population (%) of $CSF1R^{high}$ and $CSF1R^{low}$ cells were indicated. The results are representative of three independent experiments. The horizontal lines and the numbers above the graphs indicate $CSF1R^{high}$ (right) and $CSF1R^{low}$ (left) cell fractions and their populations (%), respectively. (**e**,**f**) Leukemia-free survival after fetal liver cells of E12.5 $Sfpi1^{+/+}$ and $Sfpi1^{-/-}$ mouse embryo littermates were infected with either MOZ-TIF2– (**e**) or N-Myc– (**f**) encoding viruses and transplanted into irradiated mice. (**g**) Leukemia-free survival after fetal liver cells of $Sfpi1^{-/-}$ mice were infected with PU.1– or MOZ-TIF2–encoding viruses, or both, and transplanted into irradiated mice. In **e**, $n = 8$, $P < 0.0001$; in **f**, $n = 4$, $P = 0.0943$; in **g**, $n = 5$, $P = 0.0001$ (PU.1 + MOZ-TIF2 versus either PU.1 or MOZ-TIF2). (**h**) Fetal liver cells of E14.5 $Sfpi1^{flox/flox}$ ER-Cre mice were infected with the MOZ-TIF2–encoding virus and transplanted into irradiated mice, which developed AML. The bone marrow cells of these mice were then transplanted into sublethally irradiated wild-type mice. Tamoxifen or solvent (control) was administered to the secondary transplanted mice every 2 d by intravenous injection starting 17 d after transplantation, when GFP^+ cells were detected in peripheral blood. Leukemia-free survival of the secondary transplanted mice is shown. $n = 5$, $P = 0.0018$. (**i**) Model for transcriptional regulation by normal and fusion MOZ proteins. MOZ fusion proteins stimulate constitutive CSF1R expression to induce leukemia (left). Normal MOZ protein controls CSF1R expression by binding to PU.1 to regulate normal hematopoiesis (right).

proteins activate *CSF1R* transcription in a PU.1-dependent manner. It was recently reported that although chromatin reorganization of *Csf1r* requires prior PU.1 expression together with AML1 binding, stable transcription factor complexes and active chromatin can be maintained at the *Csf1r* locus without AML1 once the full hematopoietic program has been established[19]. This might explain why we found that AML1 was not required for MOZ-TIF2–mediated activation of *Csf1r*. Deletion analysis indicated that the DE-rich, Q-rich and ETS DNA-binding domains of PU.1, as well as the histone H1 and H5-like (H15) and the central PU.1-binding domains of MOZ and MOZ fusion proteins, are required for the activation of *CSF1R* transcription (**Supplementary Figs. 7e** and **9**). A truncated version of MOZ (1–1518) lacking the C-terminal region failed to activate transcription, indicating that the transcriptional activity of MOZ-TIF2 and MOZ-CBP, which do not contain that C-terminal region, requires the TIF2 or CBP portion of the fusion protein.

To test the requirement of PU.1 for the expression of endogenous *CSF1R*, we used PU.1-deficient ($Sfpi1^{-/-}$) myeloid progenitors expressing the PU.1–estrogen receptor fusion protein (PUER). Upon restoration of PU.1 activity by exposure to 4-hydroxytamoxifen (4-HT), PUER cells can differentiate into macrophages[20]. We infected PUER cells with the MOZ-TIF2 retrovirus or control retrovirus, sorted them for GFP expression and cultured the GFP^+ cells in the presence of 4-HT. The results of FACS (**Fig. 4d**) and quantitative RT-PCR (**Supplementary Fig. 10**) analyses indicated that CSF1R expression was induced after exposure to 4-HT, and that MOZ-TIF2 enhanced

the PU.1-induced upregulation of CSF1R. Notably, 5 d after exposure to 4-HT, we detected CSF1R^{high} and CSF1R^{low} cells in the population of PUER cells expressing MOZ-TIF2, but only CSF1R^{low} cells were in the control PUER cell population (**Fig. 4d**). We did not detect CSF1R expression before addition of 4-HT, even in PUER cells expressing MOZ-TIF2 (**Fig. 4d**), indicating that functional PU.1 is required for MOZ-TIF2–induced CSF1R expression. Chromatin immunoprecipitation (ChIP) analysis indicated that PU.1, MOZ-TIF2 and possibly endogenous MOZ were recruited to the *Csf1r* promoter in the bone marrow cells of mice with MOZ-TIF2–induced AML (**Supplementary Fig. 11a**). In PUER cells expressing MOZ-TIF2, recruitment of MOZ-TIF2 and MOZ to the *Csf1r* promoter was detected after 4-HT treatment, but not before the treatment (**Supplementary Fig. 11b**), suggesting that the recruitment of MOZ-TIF2 and MOZ is dependent upon functional PU.1.

To determine whether PU.1 is essential for the development of MOZ-TIF2–induced AML, we infected wild-type and *Sfpi1*$^{-/-}$ fetal liver cells of E12.5 littermates with retroviruses encoding MOZ-TIF2 or N-Myc and transplanted them into irradiated mice. Although mice transplanted with *Sfpi1*$^{+/+}$ cells expressing MOZ-TIF2 developed AML 8–14 weeks after transplantation, mice transplanted with *Sfpi1*$^{-/-}$ cells were healthy for at least 6 months (**Fig. 4e**). In contrast, all mice transplanted with either wild-type or *Sfpi1*$^{-/-}$ cells expressing N-Myc developed AML 6–10 weeks after transplantation (**Fig. 4f**). When both PU.1 and MOZ-TIF2 were introduced into PU.1-deficient fetal liver cells, the transplanted mice developed leukemia (**Fig. 4g**). However, introduction of either PU.1 or MOZ-TIF2 alone was not sufficient for AML induction. Thus, we conclude that PU.1 is required for the initiation of MOZ-TIF2–induced AML.

To determine whether PU.1 is also required for the maintenance of MOZ-TIF2–induced AML, we infected fetal liver cells of PU.1 conditional knockout mice (*Sfpi1*$^{flox/flox}$ and expressing estrogen receptor (ER)-Cre) with MOZ-TIF2 and transplanted them into irradiated recipient mice, which developed AML. We next transplanted bone marrow cells of these mice into irradiated secondary recipients and then treated half of the mice with tamoxifen to induce PU.1 deletion. All of the control mice died of AML within 6 weeks, but none of the tamoxifen-treated mice developed AML for at least for 6 months (**Fig. 4h**). These results indicate that PU.1 is also required for the maintenance of MOZ-TIF2–induced AML stem cells.

Taken together, our results indicate that MOZ and its leukemia-associated fusion proteins activate PU.1-mediated transcription of the monocyte-specific gene *Csf1r*. MOZ fusion proteins might constitutively stimulate high *Csf1r* expression to induce AML (**Fig. 4i**). In contrast, we previously found that MOZ fusion proteins inhibit AML1-mediated activation of granulocyte-specific *Mpo* gene transcription[18]. Because MOZ fusion proteins are associated with monocytic leukemia, commitment to the monocytic lineage may be determined by differential regulation of target genes by MOZ fusion proteins (that is, upregulation of monocyte-specific genes such as *Csf1r* and downregulation of granulocyte-specific genes such as that encoding myeloperoxidase). It is also likely that the normal MOZ protein modulates *Csf1r* expression to an appropriate level to regulate normal hematopoiesis (**Fig. 4i**), as *Csf1r* expression was impaired in *MOZ*$^{-/-}$ fetal liver cells (**Supplementary Fig. 12**).

Although AML induction was suppressed in mice transplanted with *Csf1r*$^{-/-}$ cells, half of these mice developed AML, albeit at a longer latency. Thus, MOZ-TIF2 can provoke either a rapid induction of AML in a CSF1R-dependent manner or a slower induction in a CSF1R-independent manner. There are several possibilities to explain this CSF1R independence. First, we observed increased HoxA9 expression in both CSF1R^{high} and CSF1R^{low} cells. HoxA9 overexpression is reportedly not sufficient to induce AML and additional mutations or oncogene activation is required for AML induction in this context[21,22]. Thus, MOZ-TIF2–transfected *Csf1r*$^{-/-}$ cells might require additional mutations to induce leukemia. Second, because we used a retrovirus vector to introduce MOZ-TIF2, it is possible that oncogene activation by retroviral integration might mediate AML pathogenesis.

In conclusion, our results indicate that PU.1-mediated upregulation of *Csf1r* is crucial for leukemia stem cell potential induced by MOZ-TIF2. Our findings add to previous work associating CSF1R with AML. CSF1R upregulation has been reported in human[23–25] and mouse[26] AML. CSF1R is also known as the oncoprotein c-Fms, and transplantation of bone marrow cells expressing the v-fms oncoprotein induces multilineage hematopoietic disorders[27]. A chromosomal translocation resulting in expression of a fusion protein in which RNA-binding motif protein-6 (RBM6) is fused to CSF1R has recently been reported to be associated with AML[28]. CSF1R may thus be crucial for not only leukemia induced by MOZ fusions but also a wider subset of AML.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

Y.A., I.K., T.K. and M.S. conducted experiments in AML mice. Y.A., H. Shima and I.K. performed western blotting, immunoprecipitation, GST pull down, ChIP and reporter assays. P.Z. and D.G.T. conducted experiments in PU.1-deficient mice. E.R.S. designed and performed experiments in CSF1R-deficient mice. K.T. and E.I. analyzed expression of CSF1R in human AML cells. H. Singh designed and performed experiments in PUER cells. H.O. prepared Ki20227. I.K. and Y.A. analyzed data and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Human subjects, mice and cells. The study involving human samples was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine, and all clinical samples were obtained with informed consent. C57BL/6 mice were purchased from CREA Japan. NGF-FKBP-Fas transgenic mice[10] (Jackson Laboratories), *Csf1r*-deficient mice[12] (provided by E.R.S.), PU.1-null (*Sfpi1*$^{-/-}$) and PU.1 conditionally deficient (*Sfpi1* floxed) mice[29] (provided by D.G.T.), CreERT2 knock-in mice (TaconicArtemis GmbH)[30] and MOZ-deficient mice[5] were backcrossed to C57BL/6 mice at least five times. Mouse experiments were performed in a specific pathogen–free environment at the Japan National Cancer Center animal facility according to institutional guidelines and with approval of the Japan National Cancer Center Animal Ethics Committee. PUER cells[20] were provided by H. Singh.

Generation of acute myeloid leukemia mouse models. MSCV-MOZ-TIF2-IRES-EGFP, MSCV-N-Myc-IRES-EGFP, MSCV-CSF1R-pgk-pac and MSCV-PU.1-pgk-pac constructs were generated by inserting cDNAs encoding MOZ-TIF2, N-Myc, CSF1R or PU.1 into the appropriate vector. The constructs were transfected into Plat-E cells[31] cells using the FuGENE 6 reagent (Roche Diagnostics) and supernatants containing retrovirus were collected 48 h after transfection. c-Kit$^+$ cells (1×10^5 cells) were selected from bone marrow or fetal liver cells using CD117-specific MicroBeads (Miltenyi Biotec); the cells were then incubated with retroviruses using RetroNectin (Takara Bio) for 24 h in StemPro-34 serum-free medium (Invitrogen) containing cytokines (20 ng ml^{-1} stem cell factor (PeproTech), 10 ng ml^{-1} interleukin-6 (PeproTech), 10 ng ml^{-1} interleukin-3 (a gift from Kirin Pharmaceuticals)). The infected cells were then transplanted together with bone marrow cells (2×10^5) into lethally irradiated (9 Gy) 6- to 8-week-old C57BL/6 mice by intravenous injection. Secondary transplants were performed by intravenous injection of bone marrow cells from primary AML mice into sublethally irradiated (6 Gy) C57BL/6 mice.

Administration of AP20187, imatinib or Ki20227. AP20187 (a gift from Ariad Pharmaceuticals; 10 mg per kg body weight) was administered daily by intravenous injection for 5 d, and then 1 mg per kg body weight AP20187 was administered every 3 d thereafter as described previously[10]. Mice were orally administered imatinib mesylate (Novartis Pharmaceuticals; 100 mg per kg body weight), Ki20227 (ref. 13) (a gift from Kirin Pharmaceuticals; 20 mg per kg body weight) or solvent twice daily from 7 d after transplantation.

Immunofluorescent staining, detection of side population cells, flow cytometric analysis and cell sorting. Bone marrow cells from mice with AML were preincubated with rat IgG and then incubated on ice with the following staining reagents: antibody to CD115 (AFS98) conjugated to phycoerythrin (PE) (eBioscience), antibody to Mac-1 (M1/70) conjugated to PE-Cy7 (eBioscience), antibody to Gr-1(RB6-8C5) conjugated to allophycocyanin (APC) (BD Pharmingen) and antibody to c-Kit (2B8) conjugated to APC (BD Pharmingen). For the detection of side population cells, bone marrow cells were stained with 5 μg ml^{-1} Hoechst 33342 in the presence or absence of 50 μM verapamil at 37 °C for 60 min. Flow cytometric analysis and cell sorting were performed using the JSAN cell sorter (Baybioscience) and the results were analyzed with FlowJo software (Tree Star).

Reporter analysis. *CSF1R*-luciferase constructs were generated by insertion of *CSF1R* promoter constructs, either wild type or lacking the PU.1-binding site[32], into pGL4.10 (luc2) (Promega). SaOS2 cells (a gift from T. Taya) were transfected with *CSF1R*-luciferase constructs and pGL4.75 (hRL-CMV) (Promega) together with various expression constructs (pLNCX-AML1 (ref. 18), pLNCX-PU.1 (ref. 33), pLNCX-MOZ[18], pLNCX-MOZ-TIF2 (ref. 18) and pLNCX-MOZ-CBP[18]) in 24-well plates, and luciferase activity was assayed 24 h after transfection using the microplate luminometer GLOMAX (Promega). The results shown for the reporter assays represent average values for relative luciferase activity generated from at least three independent experiments; relative values were obtained by normalizing to the luciferase activity of phRL-CMV, which served as an internal control.

Immunoprecipitation and immunoblotting. For Flag tag immunoprecipitation experiments, cells were lysed in a lysis buffer containing 250 mM NaCl, 20 mM sodium phosphate (pH 7.0), 30 mM sodium pyrophosphate, 10 mM NaF, 0.1% NP-40, 5 mM dithiothreitol, 1 mM phenylmethanesulfonylfluoride and Complete protease inhibitor (Roche). Cell lysates were incubated with Flag-specific antibody–conjugated agarose beads (Sigma) and rotated at 10 r.p.m. (TAITEC RT-50) at 4 °C overnight. The adsorbed beads were washed three times with lysis buffer. Precipitated proteins were eluted from the beads by Flag peptide and dissolved with the same volume of 2× SDS sample buffer. When immunoprecipitation was not performed, total protein lysates were prepared in 2× SDS sample buffer. Antibodies were detected by chemiluminescence with ECL plus Detection Reagents (Amersham Biosciences). The primary antibodies used in this study were Flag-specific antibody (M2) (Sigma), hemagglutinin-specific antibody (3F10) (Roche) and MOZ-specific antibody[18], which was generated by immunizing rabbit with peptides corresponding residue 441–460 of human MOZ.

GST pull-down assay. The HindIII-ClaI fragment corresponding to the N-terminal region (1–664) of MOZ was cloned into the pSP64polyA vector. [^{35}S]-MOZ (1–664) was produced by incubating pSP64polyA-MOZ with [^{35}S]-methionine using the TNT Coupled Rabbit Reticulocyte Lysate System (Promega). pGEX-6P-PU.1 and pGEX-6P-AML1 were generated by subcloning full-length human PU.1 and AML1 cDNAs into pGEX-6P (GE Healthcare). GST, GST-PU.1 and GST-AML1 were produced in *Escherichia coli* BL21 containing pGEX-6P, pGEX-6P-PU.1 and pGEX-6P-AML1, respectively. The [^{35}S]-MOZ (1-664) protein was incubated with GST-, GST-PU.1– or GST-AML1–conjugated glutathione-agarose at 4 °C for 60 min in lysis buffer, washed three times with lysis buffer, analyzed by SDS-PAGE and detected by autoradiography.

Statistical analyses. We performed unpaired two-tailed Student's *t* tests for comparisons and a log-rank test for survival data with JMP8 software (SAS Institute).

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Mutations of an E3 ubiquitin ligase *c-Cbl* but not *TET2* mutations are pathogenic in juvenile myelomonocytic leukemia

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Juvenile myelomonocytic leukemia (JMML) is a rare pediatric myeloid neoplasm characterized by excessive proliferation of myelomonocytic cells. When we investigated the presence of recurrent molecular lesions in a cohort of 49 children with JMML, neurofibromatosis phenotype (and thereby *NF1* mutation) was present in 2 patients (4%), whereas previously described *PTPN11*, *NRAS*, and *KRAS* mutations were found in 53%, 4%, and 2% of cases, respectively. Consequently, a significant proportion of JMML patients without identifiable pathogenesis prompted our search for other molecular defects. When we applied single nucleotide polymorphism arrays to JMML patients, somatic uniparental disomy 11q was detected in 4 of 49 patients; all of these cases harbored RING finger domain *c-Cbl* mutations. In total, *c-Cbl* mutations were detected in 5 (10%) of 49 patients. No mutations were identified in *Cbl-b* and *TET2*. *c-Cbl* and *RAS* pathway mutations were mutually exclusive. Comparison of clinical phenotypes showed earlier presentation and lower hemoglobin F levels in patients with *c-Cbl* mutations. Our results indicate that mutations in *c-Cbl* may represent key molecular lesions in JMML patients without *RAS*/*PTPN11* lesions, suggesting analogous pathogenesis to those observed in chronic myelomonocytic leukemia (CMML) patients. (Blood. 2010;115:1969-1975)

Introduction

Juvenile myelomonocytic leukemia (JMML) is a special subtype of myelodysplastic syndrome/myeloproliferative disorder (MDS/MPD) that, analogous to chronic myelomonocytic leukemia (CMML), is characterized by excessive proliferation of myelomonocytic cells, but unlike CMML it occurs in young children and shows characteristic hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF).[1-3] Mutations of genes involved in GM-CSF signal transduction, including *RAS* and *PTPN11*, can be identified in a majority of children with JMML.[3-5] Constitutional mutations of *NF1* can be found in another 10% of patients with JMML.[1,6,7] Recent studies show that a common mechanism of *NF1* inactivation is uniparental disomy (UPD) resulting in duplication of the mutant *NF1* allele.[7,8] *NF1* is a GTPase activating protein for RAS and thereby acts as a tumor suppressor.[9] Oncogenic *RAS* mutations at codons 12, 13, and 61 have been identified in approximately 20% to 25% of patients with JMML.[4,10] These mutations lead to elevated levels of RAS-GTP, the active form of RAS.[11] Somatic mutations in *PTPN11*, coding for tyrosine phosphatase Src homology 2 domain-containing protein, have been reported in 35% of patients with JMML,[5,12,13] and induce hematopoietic progenitor hypersensitivity to GM-CSF due to hyperactivation of the RAS signaling axis.[14,15]

Based on the proposed paradigm that recurrent areas of somatic copy-neutral loss of heterozygosity can point toward the presence of homozygous mutations contained within the corresponding region,[16] we have identified various recurrent areas of acquired segmental UPD, in particular in patients with MDS/MPD, including CMML. Such analyses have shown that, in addition to the recently identified *Jak2V617F* mutation associated with UPD9p, other known mutations can be duplicated by homologous recombination, including, for example, *c-Mpl* (UPD1p), *FLT-3* ITD (UPD13q), *TET2* (UPD4q), and others.[17-22] Based on the observation of recurrent somatic UPD11q23.3, we have discovered homozygous *c-Cbl* mutations in the RING finger domain (RFD) occurring frequently in MDS/MPD and especially CMML or secondary acute myeloid leukemia (AML) evolved from CMML.[23] When we analyzed other members of *Cbl* gene family, mutations were also found in *Cbl-b* and *Cbl-c* and were associated with an indistinguishable clinical phenotype.[24] The *Cbl* gene family codes for E3 ubiquitin ligases (ULs) with the ubiquitination activity mediated via the RFD. They are involved in degradation of activated phosphotyrosine receptors and other phosphotyrosine kinases such as ζ-chain–associated protein kinase 70 involved in signal transduction.[25] Thus, mutations in the RFD can lead to decreased receptor degradation and, analogous to *PTPN11* mutations, result in augmentation of proliferative signals mediated by various growth factor receptors. In a *c-Cbl*$^{-/-}$ mouse model a mild myeloproliferative phenotype with expansion of stem cells and hyperresponsiveness to growth factors is found,[26] whereas a RFD mutant knock-in model shows a severe myeloproliferative phenotype (W. Langdon, University of Western Australia, oral communication, January 2009). These observations, together with the transforming effects of the

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v-Cbl oncogene lacking the RFD, suggest that E3 UL activity is essential for the tumor suppressor function of *c-Cbl,* whereas the N-terminal portion of the protein may be oncogenic.

Based on the morphologic similarities of JMML and typical CMML, presence of growth factor hypersensitivity, and observation of UPD11q in children affected by JMML, we hypothesized that *Cbl* family mutations may also be present in a subset of patients with JMML. Here, we investigated 49 JMML patients with the goals of (1) identifying pathogenic molecular lesions, including mutations in *Cbl* gene family members, and (2) correlating clinical outcomes to presence and location of other pathogenic molecular lesions, including *PTPN11*, *NRAS*, *KRAS*, and *TET2*. Of note is that during review of our paper, *c-Cbl* mutations were reported in JMML.[27]

Methods

Patients

We studied 49 children (32 boys and 16 girls; 1 patient's sex was unknown) with JMML diagnosed between 1988 and 2008 in 28 institutions throughout Japan. Written informed consent for sample collection was obtained at appropriate institutions from patients' parents according to the institutional protocols and the Declaration of Helsinki. The sample repository was located at Nagoya University Graduate School of Medicine. Molecular analysis of the mutational status was approved by the Ethics Committee of Nagoya University Graduate School of Medicine. The diagnosis of JMML was based on the internationally accepted criteria previously published.[26] We excluded patients with Noonan syndrome. The clinical and hematologic characteristics of the patients are summarized in Table 1. The median age at diagnosis was 28 months (range, 1-75 months). Karyotypic abnormalities were detected in 11 patients, including 7 patients with monosomy 7. Two children had clinical evidence of *NF1*. Of 49 patients, 32 underwent hematopoietic stem cell transplantation.

Table 1. Characteristics of JMML patient cohort

Variable	Total cohort, N = 49
Median age at diagnosis, mo (range)	32 (1-75)
Sex, male/female/unknown	32/16/1
NF1 by clinical diagnosis, yes/no	2/47
Median Hb, g/L (range)	0.96 (0.49-1.20)
Median HbF, % (range)	23.6 (1.0-62.0)
Median WBC, $\times 10^9$/L (range)	28.0 (10.9-126.2)
Median monocyte in PB count, $\times 10^9$/L (range)	4.5 (1.0-31.6)
Median plt, $\times 10^9$/L (range)	49 (1.4-320)
Metaphase cytogenetics, no. of patients (%)	
Normal karyotype	35 (71.4)
Monosomy 7	8 (16.3)
Trisomy 8	1 (2.0)
Other abnormalities	3 (6.1)
Unknown	2 (4.1)
Hematopoietic stem cell transplantation	
Yes	32
No	13
Unknown	4
Status at last follow-up	
Alive	24
Dead	21
Unknown	4
Median observation period, mo (range)	14 (1-216)

NF1 indicates neurofibromatosis type1; Hb, hemoglobin; WBC, white blood cell; PB, peripheral blood; and plt, platelet.

SNP-A karyotyping analysis

Mononuclear cells were isolated using Ficoll-Hypaque density gradient centrifugation and cryopreserved until use. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN). High-density Affymetrix single nucleotide polymorphism array (SNP-A; 250 K) was applied as a karyotyping platform to identify loss of heterozygosity (LOH), microamplification, and microdeletion as previously described.[28]

Bioinformatic analysis

Signal intensity was analyzed and SNP calls were determined using Gene Chip Genotyping Analysis Software Version 4.0 (GTYPE). Copy number and areas of UPD were investigated using a Hidden Markov Model and CN Analyzer for Affymetrix GeneChip Mapping 250-K arrays (CNAG Version 3.0) as previously described.[28]

We excluded germline-encoded copy number variation and nonclonal areas of gene copy number–neutral LOH from further analysis using a bioanalytic algorithm based on lesions identified by SNP-A in an internal control series (N = 713) and reported in the Database of Genomic Variants (http://projects.tcag.ca/variation).[29] Through calculation of their average sizes, we defined a maximal size of germline LOH in controls and consequently excluded all defects of this type in patients' samples; according to 95% confidence interval, stretches of UPD larger than 25.8 Mb were considered unlikely of germline origin. In addition, all nonclonal areas of UPD seen in controls were interstitial.

PTPN11, *NRAS*, *KRAS*, *TET2*, and *E3* ubiquitin ligase mutational screening

To screen for *PTPN11* mutations, we polymerase chain reaction amplified genomic DNA corresponding to exons 2, 3, 4, 7, 8, 12, and 13 as previously reported.[12,30,31] *NRAS* and *KRAS* mutations in codons 12, 13, and 61 were identified as previously described[32,33] and were confirmed by sequencing. To screen patients for mutations in E3 ubiquitin ligase genes and *TET2,* direct genomic sequencing of exons constituting the RFD of *Cbl* family members (exons 8 and 9 of *c-Cbl,* exons 9 and 10 of *Cbl-b,* exons 7 and 8 of *Cbl-c,* and exons 3-11 of *TET2*) was performed. For sequencing, 250 ng of polymerase chain reaction product, 3μM original forward or reverse primer, 2 μL of Big Dye Version 3.1 (Applied Biosystems), and 14.5 μL of deionized H_2O were amplified under the following conditions: 95°C (2 minutes) followed by 25 cycles of 95°C (10 seconds), 50°C (5 seconds), and 60°C (4 minutes). Sequencing was performed as previously described.[22]

GM-CSF hypersensitivity assay

GM-CSF hypersensitivity assays were established as described previously.[2] Briefly, we used cytokine-free methocult H4230 (StemCell Technologies), and added 1×10^3 $CD34^+$ bone marrow cells that were prepared by positive selection with magnetic-activated cell sorting beads (Miltenyi Biotec). Recombinant human GM-CSF (R&D Systems) was added at the time the cultures were initiated. Cultures were performed in duplicate, and colonies of 40 or more cells were scored after 14 days of incubation. The data are expressed as percentage of maximal numbers of granulocyte-macrophage colony-forming units (CFU-GMs). This approach more accurately reflects changes in sensitivity and does not bias the results compared with graphing actual counts because most JMML samples had considerably higher total numbers of CFU-GMs than controls, although there was considerable patient-to-patient variability.

Statistical analysis

When appropriate, Kaplan-Meier statistics were applied to assess survival. For comparison of the frequency of mutation or other clinical features between disease groups, categoric variables were analyzed using the Fisher exact test and continuous variables were tested using the Mann-Whitney *U* test.

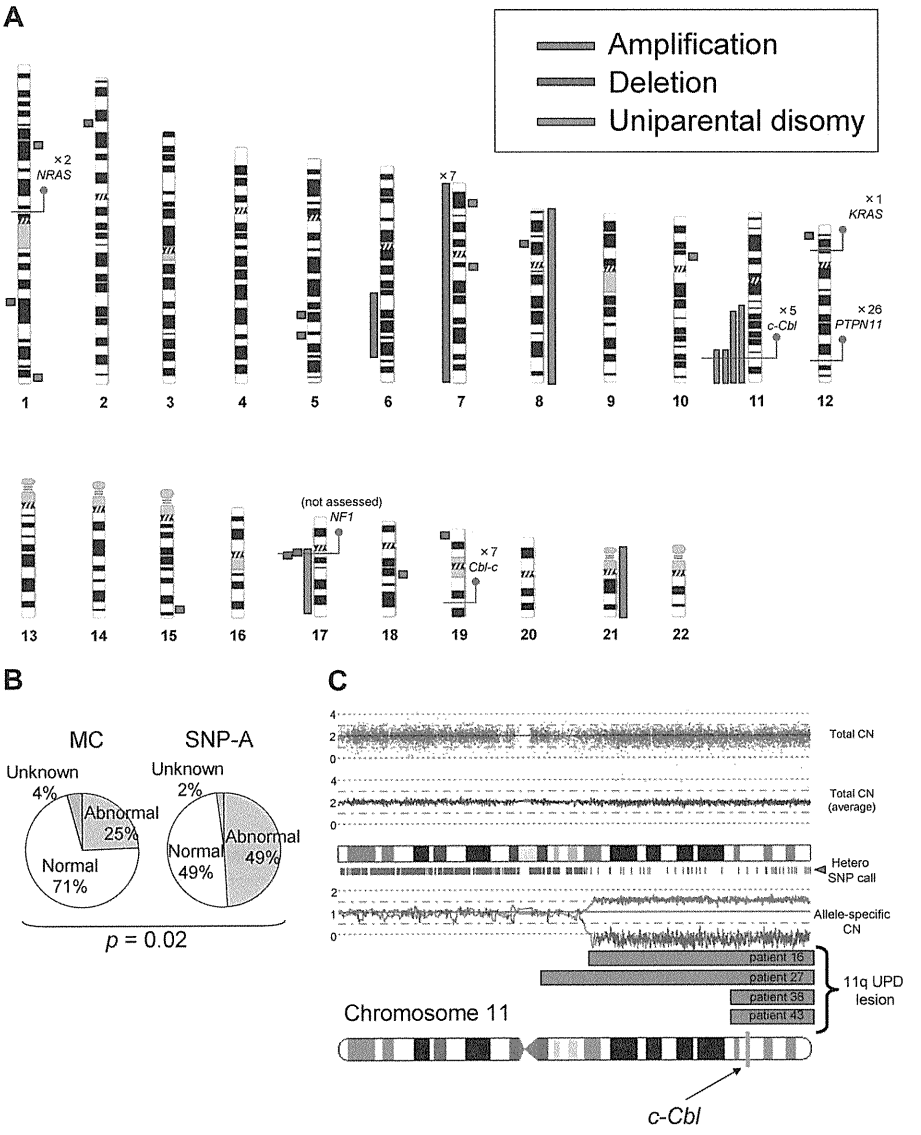


Figure 1. Single nucleotide polymorphism array–based karyotyping of JMML. (A) Genomic distribution and type of lesion identified in patients with JMML by SNP-A analysis. Green bar represent amplification, red shows deletion, and blue corresponds to UPD. Red lines pinpoint the locus of genes discussed in the text, as well as the number of patients mutated at that locus. *NF1* mutational status was not assessed in this cohort. (B) Increased sensitivity of SNP-A for detecting chromosomal lesions. The results of MC (25%) and by SNP-A (49%) from the JMML cohort studied are shown. (C) Representative 250-K SNP-A analysis of UPD11q by CNAG Version 3.0 (patient 16). Both the raw and averaged total copy number (CN) tracks (red dots, blue line) show a normal copy number, whereas heterozygous SNP calls and allele-specific copy number tracks (green dashes, red/green lines) show a reduction in copy number, indicating UPD. The specific localization of 11qUPD in 4 patients (patients 16, 27, 38, and 43) is indicated by the blue bars. The *c-Cbl* locus is indicated on the chromosome 11 idiogram with a yellow line.

Results

Cytogenetic and clinical characterization of JMML patients

First, we performed SNP-A– and metaphase cytogenetics–based analyses. Using conventional metaphase cytogenetics, chromosomal aberrations were found only in a minority of patients (25%). SNP-A–based karyotyping confirmed the results of metaphase cytogenetics, including the presence of monosomy 7 in 7 patients and trisomy 8 in 1 patient. However, due to increased precision and ability to detect copy-neutral loss of heterozygosity of SNP-A, additional lesions were identified by SNP-A in 24 (49%) of 48 patients, including trisomy 21 not detected by metaphase cytogenetics (MC) in 1 patient and microdeletions in 9 patients (Figure 1A), including 1q25.3 (patient 44), 2p22.1 (patient 46), 5q23.1 (patient 20), 5q31.3 (patient 40), 6q21q25.3 (patient 15), 8p21.2 (patient 36), 12p13.2 (patient 3), 17q11.2 (patients 15, 49), and 19p13.3 (patient 2). We also detected microamplifications in 7 patients (Figure 1A), located at 1p31.1 (patient 14), 1q44 (patient 39), 7p21.1 (patient 16), 7q11.22 (patient 17), 10p11.23 (patient 29), 15q26.3 (patient 47), and 18q12.3 (patient 22). The shared copy number–altering lesions included monosomy 7 and loss of 17q11.2, which contained the *NF1* locus. Although we were unable to confirm the somatic nature of the submicroscopic defects due to lack of germline DNA, these lesions did not overlap with copy number variations present in internal control cohort and publicly available databases. Most significantly, we identified UPD in 5 patients (Figure 1A). UPD11q was found in 4 patients, all regions overlapping from 11q23.3 to the telomere. This commonly affected region contained the *c-Cbl* locus (Figure 1A,C). The region of UPD at 17q contained the *NF1* locus and corresponded with clinical neurofibromatosis features. Overall, compared with the results of MC, SNP-A identified significantly more genetic abnormalities (25% vs 49%; $P = .02$; Figure 1B).

Table 2. Patients' mutational status

Patient number	*PTPN11*	*NRAS*	*KRAS*	*c-Cbl*	*Cbl-b*	*TET2*
1	■					
2	■					
3						
4						
5						
6						
7	■					
8	■					
9	■					
10	■					
11						
12						
13	■					
14	■					
15						
16				■		
17	■					
18	■					
19						
20						
21	■					
22	■					
23				■		
24	■					
25			■			
26		■				
27				■		
28	■					
29	■					
30		■				
31	■					
32	■					
33						
34						
35						
36	■					
37	■					
38				■		
39	■					
40	■					
41	■					
42	■					
43				■		
44						
45	■					
46						
47	■					
48						
49	■					
Total	**26**	**2**	**1**	**5**	**0**	**0**

Gray cells represent mutation; and white cells, wild type.

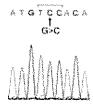
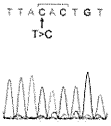
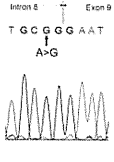
Mutational analysis of patients with JMML

After defining chromosomal defects associated with JMML, we performed mutational analysis of the genes known to be affected by mutations in JMML. *PTPN11* mutations were found in 26 (53%) of 49, whereas *NRAS* and *KRAS* mutations were found in 2 (4%) of 49 and 1 (2%) of 49, respectively (Table 2). None of the patients screened show the presence of *TET2* mutations, previously shown to be present in a significant proportion of patients with MDS/MPD, including CMML.[21] Excluding patients with a neurofibromatosis phenotype, 18 (37%) of 49 of patients did not show any of the known pathogenic defects occurring in JMML.

Identification of *Cbl* gene family mutations in JMML

Previously, homozygous *c-Cbl* mutations in the RFD were identified in patients with MDS/MPD, especially CMML or secondary AML that evolved from CMML.[22] We focused our attention on this gene as UPD11q was found in 4 of 49 JMML patients. Mutational analysis of *Cbl* family genes revealed mutations of *c-Cbl* in 5 (10%) of 49 patients, and no *Cbl-b* mutations (Tables 2-3). *c-Cbl* mutations were heterozygous in 1 patient (patient 23) and homozygous in 4 patients (patients 16, 27, 38, 43; supplemental Table 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). All mutations were located in the RFD (exon 8 and intron 8); 2 patients had an identical homozygous mutation (1111T>C, Tyr371His; patients 27, 38). All 4 patients with a homozygous *c-Cbl* mutation simultaneously harbored UPD11q (supplemental Table 1, Figures 1C-2). In addition, no patient with a *c-Cbl* mutation had mutations in genes known to play a role in JMML (*PTPN11*, *NRAS*, and *KRAS*; Table 2) or had clinical diagnosis of NF1. Excluding patients with a neurofibromatosis phenotype, 13 (26.5%) of 49 of patients did not have the mutation of *PTPN11*, *RAS*, and *c-Cbl* genes.

Table 3. Summary of *c-Cbl* mutations in patients with JMML

Patient number	Pherograms of sequence	*c-Cbl* mutation			
		Homo /Hetero	Nucleotide change	Amino acid change	Location
16		Homo	1202 G>C	Cys401Ser	Exon 8
23		Hetero	1106 del 66bp	Deletion	Exon 8
27		Homo	1111 T>C	Tyr371His	Exon 8
38		Homo	1111 T>C	Tyr371His	Exon 8
43		Homo	1228-2 A>G	Splice acceptor site	Intron 8

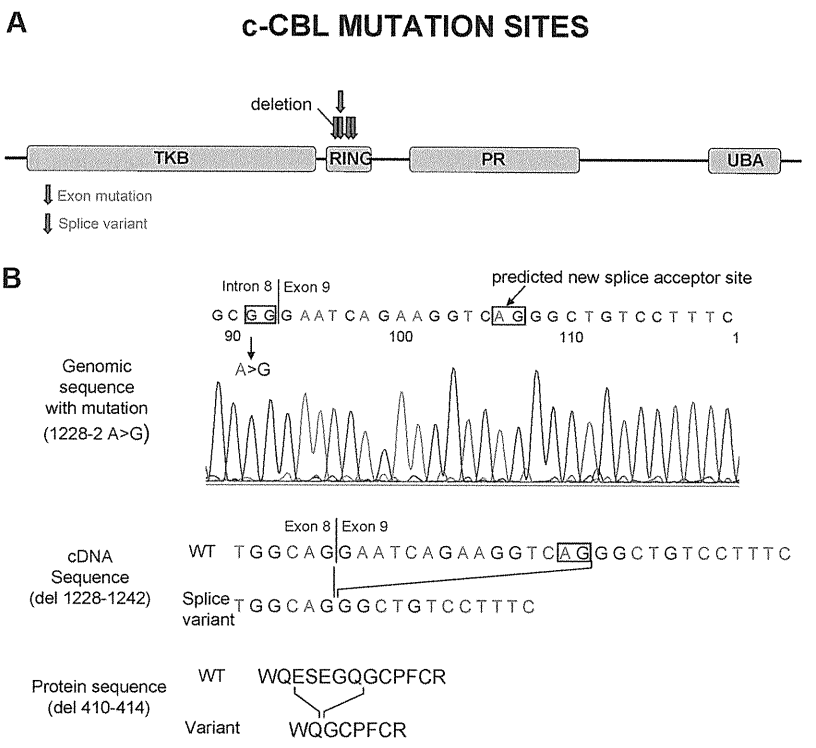


Figure 2. Site of the *c-Cbl* mutations and predicted product of splice variant in the intron 8 splice acceptor site. (A) Localization of the c-Cbl mutations within the predicted protein product. Red arrows show the site of mutations in exon, and blue arrows show the site of splice variant. (B) In patient 48, a homozygous mutation was seen in the intron 8 splice acceptor site of *c-Cbl*. According to http://genome.cbs.dtu.dk/services/NetGene2,[34] this mutation may result in a splice variant, leading to a shorter transcript in RF domain.

When we investigated our cohort for the presence of *Cbl-c* mutations, we found heterozygous frameshift nucleotide variation (1256 insertion C; patients 7, 12, 14, 29, 33, 38, and 46; data not shown). However, *Cbl-c* mutational status of germline though sequencing of nonclonal $CD3^+$ lymphocytes in those patients showed the same frameshift mutation. Consequently, these *Cbl-c* nucleotide exchanges represent rare polymorphisms.

GM-CSF hypersensitivity assay

We also investigated whether JMML-specific GM-CSF is related exclusively to individual types of mutations identified, including c-Cbl mutations. $CD34^+$ bone marrow cells' colony counts are expressed as percentage of maximal (supraoptimal) number of CFU-GMs (colony counts at any given concentration of GM-CSF/colony counts at 10 ng/mL GM-CSF). The colony growth of JMML cells with or without *c-Cbl* mutation did not differ from normal controls in low concentration of GM-CSF. For example at 0.01 ng/mL GM-CSF, colony counts were 55% (± 8%) with *c-Cbl* mutation (n = 4) versus 65% (± 10%) without *c-Cbl* mutation (n = 14) versus 15% (± 5%) in controls (n = 2; $P = .042$). At 0.1 ng/mL GM-CSF, colony counts were 87% (± 6%) versus 83% (± 11%) versus 15% (± 5%; $P = .011$) and at 1.0 ng/mL GM-CSF, 94% (± 11%) versus 93% (± 7%) versus 43% (± 3%; $P = .063$), respectively. Consequently, our results indicate that GM-CSF hypersensitivity of $CD34^+$ cells from JMML patients may be a result of various molecular lesions including *c-Cbl* mutations (Figure 3).

Clinical features associated with *Cbl* gene family mutations

Although different molecular lesions can result in similar clinical phenotypes, specific mutations can modify clinical behavior and morphologic features. Consequently, we analyzed clinical characteristics of patients with specific mutations (Table 4).

We did not find any distinctive morphologic features of patients with *Cbl* gene family mutations and no differences were present in the blood counts at initial presentation. Other variables studied (sex, the presence of cytogenetic abnormalities) also did not differ between patients grouped according to mutational status. However, patients with mutant *c-Cbl* compared with those with wild-type constellation showed earlier presentation (median age at diagnosis, 12 months vs 29 months, $P = .037$) and lower median hemoglobin F (HbF) percentage (3.5% vs 24.9%, $P = .02$), previously shown to correlate with less favorable prognosis.[1,33,35-40] Low HbF values in *c-Cbl* mutant cases were not attributable to monosomy 7, absent in this patient cohort. The probability of 2-year overall survival of *c-Cbl* mutant patients (50.0%; 95% confidence interval [CI], 25.0%-75.0%; n = 4) was similar to that of patients without *c-Cbl* mutations (50.4% [95% CI, 42%-59%]; n = 41). Similarly, when

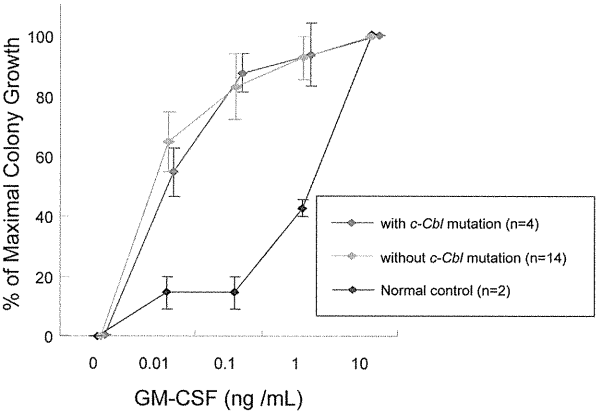


Figure 3. GM-CSF hypersensitivity assay. Colony counts are expressed as percentage of maximal numbers of CFU-GM (colony counts cultured with each concentration of GM-CSF/colony counts cultured with 10 ng/mL GM-CSF). The similar GM-CSF hypersensitivity was seen in JMML patients with or without *c-Cbl* mutation. Error bars represent SE.

Table 4. Comparison of clinical characteristics for JMML patients with and without *c-Cbl* mutation

Variable	With *c-Cbl* mutation, n = 5	Without *c-Cbl* mutation, n = 44	*P*
Median age at diagnosis, mo (range)	12 (8-15)	29 (1-75)	.037
Median HbF, % (range)	3.5 (2.0-7.6)	24.9 (1.0-62.0)	.02

Other variables studied (sex, hemoglobin level, white blood cell count, platelet count, monocyte percentage in peripheral blood, and metaphase cytogenetic abnormalities) do not show statistical significance.

HbF indicates hemoglobin F.

patients with all *Cbl* gene family mutations were analyzed, no distinct clinical features including differences in outcomes were found.

Discussion

The molecular pathogenesis of the often heterogeneous myeloid malignancies is not discernable through traditional morphologic analyses. Conversely, various molecular mechanisms can lead to similar clinical phenotypes and distinct mutational steps can result in various types of functional defects, each requiring distinct therapeutic approaches. Although JMML is associated with mutations in *PTPN11* and *RAS* in a large proportion of cases[3-5,12,13] and mutations of *NF-1* in a smaller fraction,[1,6,7] no specific mutations can be identified in a number of children affected by this disease.

Previously, we identified UPD11q and associated homozygous *c-Cbl* mutations in patients with CMML and secondary AML with monocytoid features.[23] We have also noted that heterozygous mutations of other closely related E3 ULs such as *Cbl-b* and *Cbl-c* may be found in some patients with otherwise indistinguishable morphologic features; these mutations presented in heterozygous constellation as they were not associated with corresponding areas of somatic UPD.[24] We have also found a significant proportion of CMML cases with UPD4q and microdeletions corresponding to the location of *TET2* gene. We have shown that UPD4q is associated with *TET2* mutations but, unlike for *c-Cbl,* heterozygous *TET2* mutations were common.[22]

Based on our progress in CMML, in this article we undertook the molecular analysis of cytogenetic abnormalities and mutational events in the clinically similar syndrome of JMML occurring in children. Using SNP-A analysis we show that patients with JMML, in addition to known typical chromosomal defects, harbor invariant somatic copy-neutral loss of heterozygosity, in particular UPD11q23.3. Based on this finding and the previously shown association of UPD11q with *c-Cbl* mutation, we demonstrated that *c-Cbl* mutations located in the RFD of this gene are found in 5 (10%) of 49 of JMML patients. Since submission of this paper, similar results were reported by Loh et al.[27] Unlike in adult CMML, *TET2* mutations were not identified in JMML, a finding consistent with the absence of UPD4q or del4 in JMML.

Our findings suggest that selective pressure in JMML leads to use of functionally related pathways but may involve distinct genes. In fact, both *c-Cbl* (ubiquitination) and *PTPN11* (dephosphorylation) mutations can lead to the augmentation of growth factor receptor–mediated signals and may explain why GM-CSF hypersensitivity is present in patients with JMML irrespective of whether *c-Cbl, PTPN11,* or *RAS* is mutated.

For *Cbl* mutations, in addition to the impaired degradation of activated growth factor receptors, altered ζ-chain–associated protein kinase 70 activation by c-Cbl may mediate proliferative signals analogous to RAS. Moreover, by binding to Grb2, c-Cbl competes with the guanine-nucleotide-exchange factor son-of-sevenless, thereby blocking signaling through the RAS–mitogen-activated protein kinase pathway and inhibiting proliferation.[25] In agreement with this theory, RFD mutant knock-in mouse experiments suggest that *c-Cbl* deprived of its E3 ligase activity may act as an oncogene, and functional analysis of mutated *c-Cbl* showed that mutated *c-Cbl* has an oncogenic effect.[35] These findings conclusively prove the pathogenic role of *c-Cbl* mutation in hematologic malignancies.

Our earlier studies showed that *c-Cbl* mutations stem from a somatic event and are not present in germline[23]; however, germline DNA was not available from our patients to conduct confirmatory studies. Nevertheless, *c-Cbl* mutations in JMML were similar or identical to those previously shown in CMML, for which the somatic nature has been confirmed through analysis of germline DNA and serial studies. Similarly, *c-Cbl* mutations were present exclusively in the context of UPD11q23.3, shown to occur only as a clonal somatic event. In agreement with a previous report,[7] we have also found UPD17q in association with neurofibromatosis-associated JMML.

Patients with *c-Cbl* mutations show comparable survival as those without *c-Cbl* mutations, but a large fraction of these patients underwent transplantation. However, *c-Cbl* mutations were associated with a younger age of presentation and smaller percentage of HbF. Given that in previous reports an older age at diagnosis and elevated HbF level have been repeatedly described as risk factors for survival in JMML,[1,36-42] lack of these poor prognostic markers in *c-Cbl* patients who demonstrate a similar outcome argues for an unfavorable impact of *c-Cbl* mutation, analogous to adult patients with *c-Cbl.*

In summary, our study describes a novel molecular lesion in children affected by JMML, suggesting similarity in the pathogenesis of a portion of patients with JMML to those with CMML.

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Authorship

Contribution: H. Muramatsu and H. Makishima designed research, performed research, analyzed data, and wrote the paper; A.M.J. and H.C. performed research; C.O. designed research, analyzed data, and wrote the paper; N.Y., Y.X., N.N., A.H., H.Y., Y.T., K.K., and A.M. designed research; S.K. designed research and wrote the paper; and J.P.M. designed research, performed research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Brief report

Autoimmune lymphoproliferative syndrome–like disease with somatic *KRAS* mutation

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Autoimmune lymphoproliferative syndrome (ALPS) is classically defined as a disease with defective FAS-mediated apoptosis (type I-III). Germline *NRAS* mutation was recently identified in type IV ALPS. We report 2 cases with ALPS-like disease with somatic *KRAS* mutation. Both cases were characterized by prominent autoimmune cytopenia and lymphoadenopathy/splenomegaly. These patients did not satisfy the diagnostic criteria for ALPS or juvenile myelomonocytic leukemia and are probably defined as a new disease entity of RAS-associated ALPS-like disease (RALD). (*Blood.* 2011;117(10):2887-2890)

Introduction

Autoimmune lymphoproliferative syndrome (ALPS) is a disease characterized by dysfunction of the FAS-mediated apoptotic pathway,[1,2] currently categorized as: type Ia, germline *TNFRSF6/FAS* mutation; type Ib, germline *FAS ligand* mutation; type Is, somatic *TNFRSF6/FAS* mutation; and type II, germline *Caspase 10* mutation. Patients exhibit lymphadenopathy, hepatosplenomegaly, and autoimmune diseases, such as immune cytopenia and hyper-γ-globulinemia. An additional subclassification has been proposed that includes types III and IV, whereby type III has been defined as that with no known mutation but with a defect in FAS-mediated apoptosis and type IV as one showing germline *NRAS* mutation.[3] Type IV is considered exceptional because the FAS-dependent apoptosis pathway is not involved in the pathogenesis, and this subclass is characterized by a resistance to interleukin-2 (IL-2) depletion-dependent apoptosis. Recent updated criteria and classification of ALPS suggested type IV ALPS as a RAS-associated leukoproliferative disease.[4]

Juvenile myelomonocytic leukemia (JMML) is a chronic leukemia in children. Patients show lymphadenopathy, hepatosplenomegaly, leukocytosis associated with monocytosis, anemia, thrombocytopenia, and occasional autoimmune phenotypes. Approximately 80% of patients with JMML have been shown to have a genetic abnormality in their leukemia cells, including mutations of *NF1*, *RAS* family,[5] *CBL*, or *PTPN11*. The hallmarks of the laboratory findings of JMML include spontaneous colony formation in bone marrow (BM) or peripheral blood mononuclear cells (MNCs) and hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) of $CD34^+$ BM-MNCs.[6]

Germline RAS pathway mutations cause Costello (*HRAS*), Noonan (*PTPN11*, *KRAS*, and *SOS1*), and cardio-facio-cutaneous syndromes (*KRAS*, *BRAF*, *MEK1*, and *MEK2*). Patients with Costello and Noonan syndromes have an increased propensity to develop solid and hematopoietic tumors, respectively[7]; among these tumors, the incidence of JMML in patients with germline mutation of *NF1* or *PTPN11* is well known.

We present 2 cases with autoimmune cytopenia and remarkable lymphadenopathy and hepatosplenomegaly, both of which were identified as having a somatic KRAS G13D mutation without any clinical features of germline *RAS* mutation, such as cardio-facio-cutaneous or Noonan syndrome.

Methods

All studies were approved by the ethical board of Tokyo Medical and Dental University.

Case 1

A 9-month-old boy had enormous bilateral cervical lymphadenopathy and hepatosplenomegaly (supplemental Figure 1A-B, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Blood test revealed the presence of hemolytic anemia and autoimmune thrombocytopenia. Hyper-γ-globulinemia with various autoantibodies was also noted. ALPS and JMML were nominated as the diseases to be differentially diagnosed. Detailed clinical history and laboratory data are provided as Supplemental data. The patient did not satisfy the criteria for the diagnosis of ALPS or JMML as discussed in "Results and discussion."

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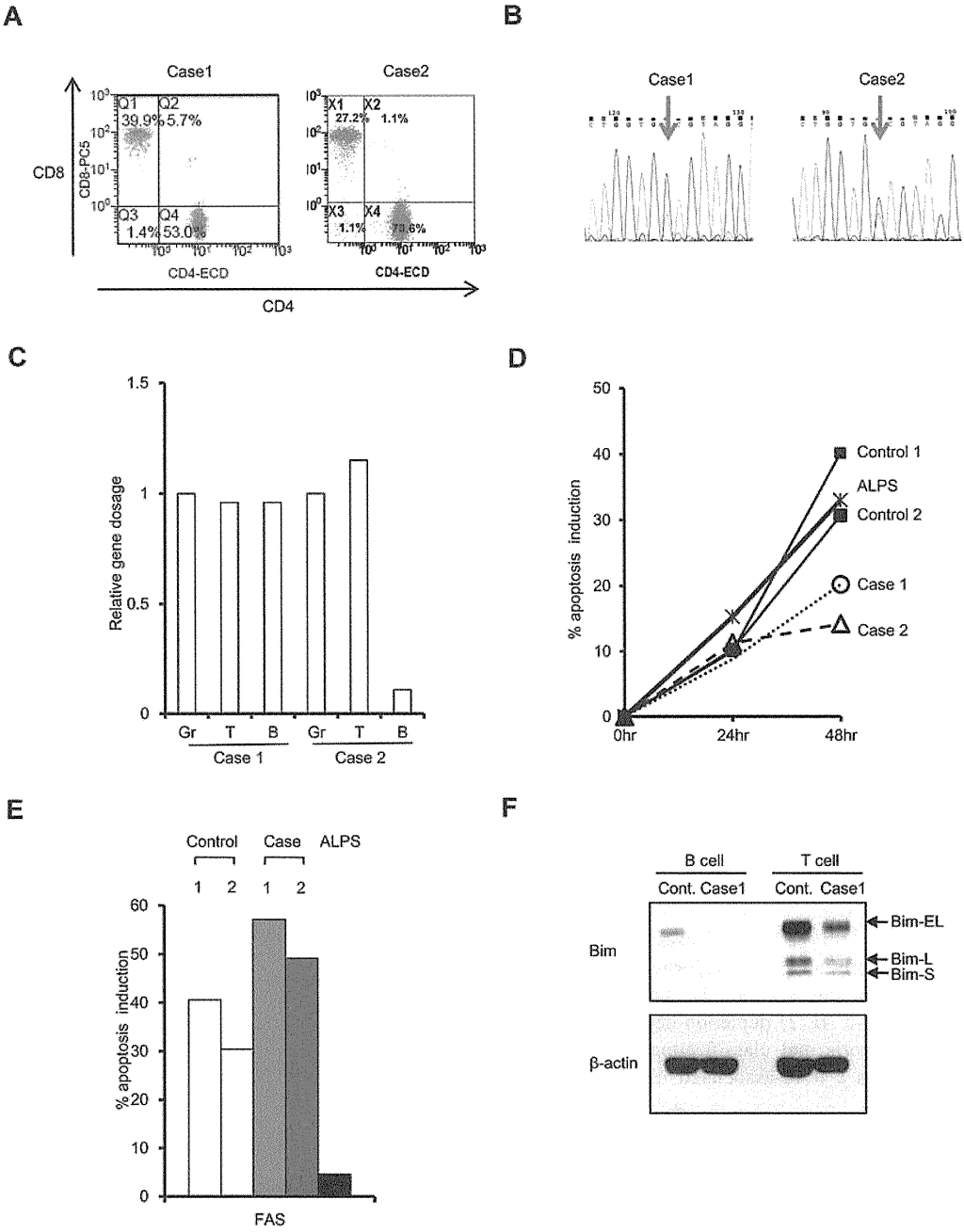

Figure 1. Molecular cell biologic assay of RALD. (A) Flow cytometric analysis of double-negative T cells. CD8 and CD4 double staining was performed in T-cell receptor-αβ-expressing cells. (B) Electropherogram showing KRAS G13D mutation in BM-MNCs in case 1 (left panel) and case 2 (right panel). (C) Gene dosage of mutated allele in granulocytes (Gr), T cells (T), and B cells (B). Relative gene dosage was estimated by a mutant allele-specific polymerase chain reaction method in cases 1 and 2 using albumin gene as internal control. (D) Apoptosis assay using activated T cells. Apoptosis percentage was measured by flow cytometry with annexin V staining 24 and 48 hours after IL-2 depletion. (E) Apoptosis percentage was measured 24 hours after addition of anti-FAS CH11 antibody (final 100 ng/mL). (F) Western blotting analysis of Bim expression.

Case 2

A 5-month-old girl had a fever and massive hepatosplenomegaly (supplemental Figure 1D). She was initially diagnosed with Evans syndrome based on the presence of hemolytic anemia and autoimmune thrombocytopenia with hyper-γ-globulinemia and autoantibodies. Spontaneous colony formation assay and GM-CSF hypersensitivity of BM-MNCs showed positivity. Then, tentative diagnosis of JMML was given, even though she showed no massive monocytosis or increased fetal hemoglobin. Detailed clinical history and laboratory data are provided in supplemental data.

Detailed methods for experiments are described in supplemental data.

Results and discussion

Case 1 showed a high likelihood of being a case of ALPS according to the symptoms and clinical data presented (supplemental Table 1), except for number of double-negative T cells, which was only 1.4% of T-cell receptor-αβ cells (Figure 1A). JMML was also nominated as a disease to be differentiated because remarkable hepatosplenomegaly with thrombocythemia and moderate monocytosis was

noted. However, no hypersensitivity to GM-CSF as determined by colony formation assay for BM-MNCs (data not shown) or phosphor-STAT5 staining (data not shown) was observed. DNA sequence for JMML-associated genes, such as *NRAS, KRAS, HRAS, PTPN11*, and *CBL*, was determined, and KRAS G13D mutation was identified (Figure 1B). The mutation was seen exclusively in the hematopoietic cell lineage, and no mutation was seen in the oral mucosa or nail-derived DNA. Granulocytes, monocytes, T cells, and B cells were all positive for KRAS G13D mutation (data not shown). The proportion of mutated cells in each hematopoietic lineage was quantitated by mutation allele-specific quantitative polymerase chain reaction methods, which revealed that mutated allele was almost equally present in granulocytes, T cells, and B cells (Figure 1C). $CD34^+$ hematopoietic stem cells (HSCs) were also positive for KRAS G13D mutation, and 60% of colony-forming units-granulocyte macrophage (CFU-GM) developed from isolated CD34 cells carried the KRAS G13D mutation (data not shown). These observations suggest that the mutation occurred at the HSCs level, and HSC consists of wild-type and mutant HSCs.

NRAS-mutated type IV ALPS was previously characterized by apoptosis resistance of T cells in IL-2 depletion.[3] Then, activated T cells were subjected to an apoptosis assay by FAS stimulation or IL-2 depletion. Remarkable resistance to IL-2 depletion, but not to FAS-dependent apoptosis (Figure 1D-E), was seen. This was in contrast to T cells from FAS-mutated ALPS type 1a, which showed remarkable resistance to FAS-dependent apoptosis and normal apoptosis induction by IL-2 withdrawal (Figure 1D-E). Western blotting analysis of activated T cells or Epstein-Barr virus-transformed B cells showed reduced expression of Bim (Figure 1F).

In case 2, autoimmune phenotype and hepatosplenomegaly were remarkable, as shown in Supplemental data. The patient was initially diagnosed as Evans syndrome based on the presence of hemolytic anemia and autoimmune thrombocytopenia. Double-negative T cells were 1.1% of T-cell receptor-$\alpha\beta$ cells in the peripheral blood, which did not meet with the criteria of ALPS. Although spontaneous colony formation was shown in peripheral blood- and BM-MNCs, and GM-CSF hypersensitivity was demonstrated in BM-MNCs derived $CD34^+$ cell (supplemental Table 2), she showed no massive monocytosis or increased fetal hemoglobin. Thus, the diagnosis was less likely to be ALPS or JMML. DNA sequencing of JMML-related genes, such as *NRAS, KRAS, HRAS, PTPN11*, and *CBL*, identified somatic, but not germline, KRAS G13D mutation (Figure 1B). KRAS G13D mutation was detected in granulocytes and T cells. Mutation was not identified in B cells by conventional DNA sequencing (data not shown). Mutant allele-specific quantitative polymerase chain reaction revealed that mutated allele was almost equally present in granulocytes and T cells, but barely in B cells (Figure 1C). Activated T cells showed resistance to IL-2 depletion but not to FAS-dependent apoptosis (Figure 1D-E).

Both of our cases were characterized by strong autoimmunity, immune cytopenia, and lymphadenopathy or hepatosplenomegaly with partial similarity with ALPS or JMML. However, they did not meet with the well-defined diagnostic criteria of ALPS[2] or JMML.[6] It is interesting that case 2 presented GM-CSF hypersensitivity, which is one of the hallmarks of JMML. Given the strict clinical and laboratory criteria of JMML and ALPS, our 2 cases should be defined as a new disease entity, such as RAS-associated ALPS-like disease (RALD). Recently defined NRAS-mutated ALPS type IV may also be included in a similar disease entity.

There are several cases of JMML reported simultaneously having clinical and laboratory findings compatible with autoimmune disease.[8,9] Autoimmune syndromes are occasionally seen in patients with myelodysplastic syndromes, including chronic myelomonocytic leukemia.[10] These previous findings may suggest a close relationship of autoimmune disease and JMML. Because KRAS G13D has been identified in JMML,[11-13] it is tempting to speculate that KRAS G13D mutation is involved in JMML as well as RALD. In JMML, erythroid cells reportedly carry mutant RAS, whereas B- and T-cell involvement was variable.[13] In both of our cases, myeloid cells and T cells carried mutant RAS, whereas B cells were affected variably. These findings would support a hypothesis that the clinical and hematologic features are related to the differentiation stages of HSCs where RAS mutation is acquired. JMML-like myelomonocytic proliferation may predict an involvement of RAS mutation in myeloid stem/precursor cell level, whereas ALPS-like phenotype may predict that of stem/precursor cells of lymphoid lineage, especially of T cells. Under the light of subtle differences between the 2 cases presented, their hematologic and clinical features may reflect the characteristics of the stem cell level where *KRAS* mutation is acquired. Involvement of the precursors with higher propensity toward lymphoid lineage may lead to autoimmune phenotypes, whereas involvement of those with propensity toward the myeloid lineage may lead to GM-CSF hypersensitivity while still sharing some overlapping autoimmune characteristics.

One may argue from the other viewpoints with regard to the clinicopathologic features of these disorders. First, transformation in fetal HSCs might be obligatory for the development of JMML[14] and, in HSCs later in life, may not have the same consequences. Second, certain KRAS mutations may be more potent than others. Codon 13 mutations are generally less deleterious biochemically than codon 12 substitutions, and patients with JMML with codon 13 mutations have been reported to show spontaneous hematologic improvement.[12,15] Thus, further studies are needed to reveal in-depth clinicopathologic characteristics in this type of lymphomyeloproliferative disorder.

KRAS mutation may initiate the oncogenic pathway as one of the first genetic hits but is insufficient to cause frank malignancy by itself.[16,17] Considering recent findings that additional mutations of the genes involved in DNA repair, cell cycle arrest, and apoptosis are required for full malignant transformation, one can argue that RALD patients will also develop malignancies during the course of the disease. Occasional association of myeloid blast crisis in JMML and that of lymphoid malignancies in ALPS will support this notion. Thus, the 2 patients are now being followed up carefully. It was recently revealed that half of the patients diagnosed with Evans syndrome, an autoimmune disease presenting with hemolytic anemia and thrombocytopenia, met the criteria for ALPS diagnosis.[18,19] In this study, FAS-mediated apoptosis analysis was used for the screening. Considering the cases we presented, it will be intriguing to reevaluate Evans syndrome by IL-2 depletion-dependent apoptosis assay focusing on the overlapping autoimmunity with RALD.

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Authorship

Contribution: Masatoshi Takagi and S.M. designed entire experiments and wrote the manuscript; K.S., N.M., and Mari Takagi treated patients and designed clinical laboratory test; J.P. performed experiments described in Figure 1B-F; K.M., H.M., and S.D. performed colony and mutational analysis; and M.N., T.M., K.K., S.K., Y.K., and A.T. supervised clinical and immunologic experiments or coordinated clinical information.

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