Myeloid progenitors with *PTPN11* and non-RAS pathway gene mutations are refractory to treatment with 6-mercaptopurine in juvenile myelomonocytic leukemia

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

Juvenile myelomonocytic leukemia (JMML) is a fatal, mixed myeloproliferative and myelodysplastic disorder occurring in the infancy and early childhood. Children with JMML have mutually exclusive genetic abnormalities in granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling pathways: inactivation of the *NF1* or mutations in *PTPN11*, *NRAS*, *KRAS*, and *CBL*.[1,2] A whole-exome sequencing study, performed by Sakaguchi et al.[3] has recently demonstrated that in addition to the high frequency of RAS pathway mutations, mutations in *SETBP1* and *JAK3* are common recurrent secondary events, and that these events may be involved in tumor progression, and are associated with poor clinical outcomes. The *SETBP1* and *JAK3* mutations have been also reported in the other hematological malignancies.[4-9]

We have previously reported 7 cases of patients (5 with *PTPN11* mutation; 1 with *NRAS* mutation) with significant chromosomal changes after chemotherapy or allogeneic hematopoietic stem cell transplantation (HSCT).[10] In addition, we observed a loss of wild-type *NRAS* locus and monosomy 7 after blastic crisis in a patient with JMML and a heterozygous *NRAS* mutation.[11]

The present study aimed to evaluate whether JMML clones with the RAS pathway-associated gene mutation coexist at the onset with those harboring both the RAS pathway-associated and non-RAS pathway gene mutations, and examine 6-mercaptopurine (6-MP)-susceptibility of these two clone types.

First, we examined the presence of *JAK3* and *SETBP1* mutations in 29 patients with JMML (20 patients with *PTPN11* mutations; and 9 with *NRAS* or *KRAS* mutations), including 7 patients who acquired chromosomal abnormalities during the clinical course.[10] The study was approved by the Institutional Review Board of Shinshu University. Informed consent was obtained from the guardians of the patients in

accordance with institutional guidelines. DNA was extracted from peripheral blood mononuclear cells (PBMNCs) obtained at diagnosis and/or after chemotherapy. Exons 2-6 of *SETBP1* and exons 2-24 of *JAK3* were amplified by PCR, using primer pairs described previously.[3, 6] The amplicons were subjected to direct sequencing from both directions using an automatic DNA sequencer. Among 29 patients, 4 patients with *PTPN11* mutations had heterogeneous *JAK3* mutation and/or *SETBP1* mutation (Table 1). These genetic data were obtained from PBMNCs collected at diagnosis in cases no. 1 and 2, and from those after chemotherapy in case nos. 3 and 4. The *PTPN11* mutations in the four cases were considered to be acquired according to the data reported previously.[12, 13] Case nos. 1 and 3 harbored *JAK3* R657Q, and case no. 2 harbored *SETBP1* D868N mutations. Case no. 4 harbored both *JAK3* R657Q and *SETBP1* G870R. Two patients were older than 24 months at the onset of the disease. Only one patient had platelet counts of $<33 \times 10^9$/L, whereas fetal hemoglobin (HbF) levels of 3 patients were >15%. Chromosomal changes were observed in two patients (case nos. 3 and 4) after chemotherapy. Three patients who received allogeneic HSCT are alive and disease-free. The lack of residual disease was confirmed by allele specific quantitative PCR[14] for *PTPN11* mutation in case nos. 1 and 2, and by fluorescence in situ hybridization for sex chromosomes using more than 500 cells in case no. 3.

We then investigated whether JMML clones harboring both *PTPN11* mutation and the non-RAS pathway gene mutations coexisted with those harboring only *PTPN11* mutation at onset. PBMNCs (1×10^4) maintained in liquid nitrogen were plated in dishes containing methylcellulose medium supplemented with 10 ng/ml of GM-CSF. GM colonies were individually lifted after 12 days, and single cell suspensions were

prepared. Sequence analyses were then performed on individual GM colony-constituent cells, as described previously.[10] As presented in Figure 1, 16 of 34 GM colonies derived from PBMNCs obtained at diagnosis of case no. 1 had both JAK3 mutation and *PTPN11* mutations. The identical number of GM colonies was positive for *PTPN11* mutation but negative for *JAK3* mutation. In case no. 2, both *PTPN11* and *SETBP1* mutations were found in 16 of 27 GM colonies derived from PBMNCs obtained at onset, whereas the remaining 11 GM colonies had only *PTPN11* mutation. There were no GM colonies harboring the mutated non-RAS pathway gene and wild-type *PTPN11* gene in these patients. Interestingly, the frequency of GM colonies with both *PTPN11* mutation and the non-RAS pathway mutation significantly increased (>80%) between 1.5−4 months after treatment with only 6-MP in both the cases (p = 0.0032 in case no. 1 and p = 0.0093 in case no. 2). The chi-square test was used to determine the significance of differences. PBMNCs from case no. 3 were obtained 22 months after treatment with 6-MP, which also yielded two types of GM colonies (Figure 1C). In case no. 4, we found heterogeneous mutations in all 3 gene types (*PTPN11*, *JAK*, and *SETBP1*) in 38 of 40 GM colonies grown from PBMNCs obtained 16 months after repeated chemotherapy including 6-MP (Figure 1D). The remaining 2 colonies had mutated *PTPN11* and *JAK3*, where the *SETBP1* was wild-type.

Using liquid cultures, we finally examined whether GM progenitor cells with both non-RAS pathway mutation and *PTPN11* mutation exhibited a susceptibility to 6-MP different from those with only *PTPN11* mutation. Appropriate aliquots of 6-MP (Sigma Chemical, St. Louis, MO) were dissolved in 1N sodium hydroxide, and then diluted with alpha-medium.[10] To examine susceptibility to 6-MP, PBMNCs (1×10^4) were cultured in a dish containing 10 ng/ml of GM-CSF with or without 6-MP (30 μM).

Number of GM colonies from PBMNCs obtained at onset in case no. 1 was decreased to one-third by the addition of 6-MP (30 μM). Nevertheless, exposure to 6-MP significantly increased the proportion of GM colonies with both *PTPN11* and *JAK3* mutations ($p = 0.0130$, Figure 1E).

From the data that *SETBP1* and *JAK3* mutations have lower allele frequencies (difference not statistically significant for *SETBP1*) than the RAS pathway mutations (*PTPN11*, *NF1*, and *NRAS*/*KRAS*), Sakaguchi et al. inferred that the *SETBP1* and *JAK3* mutations represent secondary genetic hits that contribute to clonal evolution after the main tumor population is established.[3] In this study, genetic analyses of individual GM colonies clearly revealed that GM progenitor cells harboring both *PTPN11* and the non-RAS pathway gene mutations (*JAK3* or *SETBP1*) and cells harboring only *PTPN11* mutation coexisted at onset (cases no. 1 and 2). Nevertheless, there were no GM colonies harboring the mutated non-RAS pathway gene and wild-type *PTPN11*. Thus, *SETBP1* and *JAK3* mutations appear to be the second genetic aberration in some JMML children with *PTPN11* mutation. Nevertheless, it is necessary to exclude a possibility of prenatal origin of JMML clone with both *PTPN11* and non-RAS pathway gene mutations; which can be confirmed using Guthrie cards (dried blood spots), as we previously described.[14]

In case nos. 1 and 2, the percentage of GM colonies with both *PTPN11* and non-RAS pathway mutations increased substantially several months after treatment with only 6-MP in comparison with the percentage at diagnosis. Furthermore, the addition of 6-MP to a liquid culture containing PBMNCs obtained at onset of case no. 1 and supplemented with GM-CSF significantly increased the proportion of GM colonies with *PTPN11* and *JAK3* mutations. Since treatment with 6-MP was continued up to the

beginning of preparative conditioning for allogeneic HSCT in case nos. 1, 2 and 3, we could not examine whether the growth advantage of the subclone harboring both the mutated non-RAS pathway gene and *PTPN11* mutation decreased in the absence of therapeutic pressure. Accordingly, JMML clones with *SETBP1* mutation and/or *JAK3* mutation in addition to *PTPN11* mutation appear to be refractory to 6-MP. Allogeneic HSCT may be capable to eliminate such 6-MP-resitant JMML clones because 3 of the children are alive and disease-free after HSCT. Further large-scale studies are needed to establish accurately the relationship between acquisition of the non-RAS pathway mutations and post-transplant outcomes in patients with *PTPN11* mutations.

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AUTHOR CONTRIBUTIONS

KK and KM designed and performed research, collected samples, analyzed data, and wrote the paper. YN designed research. CI performed research. TK, KH, SS, MT, KY, RY, KS, and SS collected samples and analyzed data. All the authors read and approved the manuscript.

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Figure Legend

Figure 1 Genetic analyses of individual GM colonies generated from PBMNCs of JMML children with *PTPN11* mutations

(A-D) Proportion of GM colonies harboring both *PTPN11*and non-RAS pathway gene mutations to those harboring only *PTPN11* mutation in 4 children with JMML. GM colonies were generated from PBMNCs obtained at diagnosis and after treatment with 6-MP in case nos. 1 and 2. GM colonies were analyzed only after chemotherapy in cases no. 3 and 4.

(E) Comparison of susceptibility to 6-MP between GM progenitor cells harboring *PTPN11* and *JAK3* mutations and those with only PTPN11 mutation in case no. 1. PBMNCs (1×10^4) were cultured in a dish containing 10 ng/ml of GM-CSF with or without 6-MP (30 μM). mt, mutant type; wt, wild type. The values in parentheses are the numbers of GM colonies examined.

Table 1 Clinical and genetic characteristics of 4 JMML children with both *PTPN11* mutation and non-RAS pathway gene (*JAK3* and *SETBP1*) mutations

Case (no.)	Sex	Age (mo)	WBC (10^9/l)	Mono (%)	Hb (g/dl)	Plt (10^9/l)	HbF (%)	*PTPN11*	*JAK3*	*SETBP1*	Karyotype at Dx	Treatment	Chromosomal changes after treatment	Outcome (interval after Dx)
1	M	28	69.5	6	9.4	36	20.4	1508G > C	R657Q	wt	46XY	6MP HSCT	(-)	alive (+46 mo)
2	F	27	11.6	36	11.2	39	25.6	182A > T	wt	D868N	46XX	6-MP HSCT	(-)	alive (+166 mo)
3	F	3	53.3	12	9.3	60	8.9	226G > C	R657Q	wt	46XX	6-MP HSCT	(+)[§]	alive (+252 mo)
4	F	19	37.3	15	9.6	21	24.0	1508G > C	R657Q	G870R	46XX	6-MP VP-16, IC	(+)[¶]	dead (29 mo)

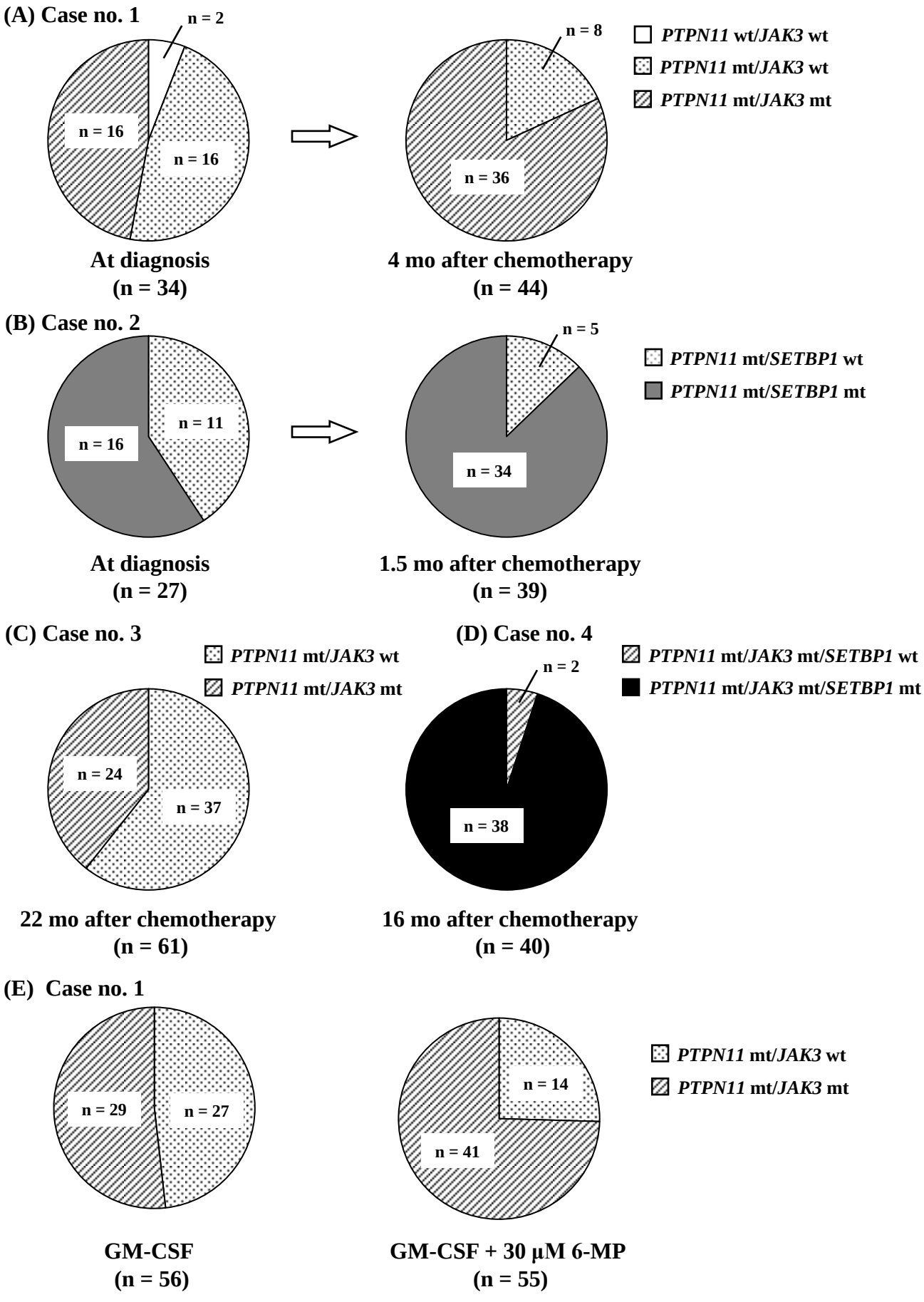
Case nos. 2, 3, and 4 have been reported previously.[10]

Clinical and cytogenetic findings at diagnosis are presented, except that mutation analyses of *JAK3* and *SETBP1*were performed using PBMNCs obtained 22 months after chemotherapy in case no. 3, and 16 months after chemotherapy in case no. 4. In addition, chromosomal changes were examined after chemotherapy and compared with that at diagnosis.

[§] 46,XX,add(7)(q22) appeared 18 months after treatment with 6-MP; [¶] 45,XX,t(4;15)(q2?;q2?) ,-7 appeared 16 months after chemotherapy.

Dx, diagnosis; Hb, hemoglobin; HSCT, hematopoietic stem cell transplantation; IC, intensive chemotherapy; 6-MP, 6-mercaptopurine; mo, month; Mono, monocytes; Plt, platelets; VP-16, etoposide; WBC, white blood cell count; wt, wild-type.

Figure 1


(A) Case no. 1
n = 2
n = 16
n = 16
n = 8
n = 36
PTPN11 wt/JAK3 wt
PTPN11 mt/JAK3 wt
PTPN11 mt/JAK3 mt
At diagnosis
(n = 34)
4 mo after chemotherapy
(n = 44)
(B) Case no. 2
n = 11
n = 16
n = 5
n = 34
PTPN11 mt/SETBP1 wt
PTPN11 mt/SETBP1 mt
At diagnosis
(n = 27)
1.5 mo after chemotherapy
(n = 39)
(C) Case no. 3
PTPN11 mt/JAK3 wt
PTPN11 mt/JAK3 mt
n = 24
n = 37
22 mo after chemotherapy
(n = 61)
(D) Case no. 4
PTPN11 mt/JAK3 mt/SETBP1 wt
PTPN11 mt/JAK3 mt/SETBP1 mt
n = 2
n = 38
16 mo after chemotherapy
(n = 40)
(E) Case no. 1
n = 29
n = 27
n = 14
n = 41
PTPN11 mt/JAK3 wt
PTPN11 mt/JAK3 mt
GM-CSF
(n = 56)
GM-CSF + 30 µM 6-MP
(n = 55)