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RESEARCH ARTICLE

THE KINASE-ACTIVATING PATHWAYS AND SENSITIVITIES TO TKIS VARY BETWEEN FUSION SITES OF RCSD1–ABL1 IN PH-LIKE ACUTE LYMPHOBLASTIC LEUKEMIA

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ABSTRACT

RCSD1–ABL1-positive acute lymphoblastic leukemia (ALL) is included in Philadelphia chromosome-like (Ph-like) ALL. We have experienced a case of RCSD1–ABL1-positive ALL which was resistant to dasatinib which is a tyrosine kinase inhibitor (TKI) although previous study showed RCSD1–ABL1-positive ALL is sensitive to dasatinib. The aim of this study is to examine the varieties of sensitivity to TKIs in RCSD1–ABL1-positive ALL.
Ba/F3 cells were transduced with retroviral vectors expressing fusion of RCSD1 exon 3/ABL1 exon 4 (R3A4; 3042 bp). Leukemogenicity was examined *in vitro* and *in vivo.* Phosphorylation antibody array and Western blotting assay were performed in R3A4-Ba/F3 to examine the kinase-activating pathways.
Sensitivities to TKIs of R3A4-Ba/F3 were also examined.
R3A4-Ba/F3 showed leukemogenicity both *in vitro* and *in vivo*.Phosphorylation antibody array showed Tyk2 activation in R3A4-Ba/F3. Western blotting showed that the leukemogenicity of R3A4 involves the Tyk2-STAT2 pathway. R3A4-Ba/F3 was resistant to imatinib and dasatinib but sensitive to pan-JAK family, including Tyk2 inhibitor. Previous opened R3A4 fusion whose fusion point is different from ours was reported to activate STAT5, which was inhibited by dasatinib.
Taken together, these findings suggest that the kinase-activating pathways and sensitivities to TKIs vary between fusion sites of RCSD1–ABL1 in Ph-like ALL.

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INTRODUCTION

ABL1 is a protooncogene, located at 9q34, which encodes a protein with tyrosine kinase (TK) activity. The major fusion partner of ABL1 is BCR, which is involved in the t(9;22)(q34;q11.2) translocation, also known as a Philadelphia (Ph) chromosome. A BCR–ABL1 fusion gene in t(9;22) (q34;q11.2) is present in more than 90% of patients with chronic myelogenous leukemia (CML) and in 20% of B-cell acute lymphoblastic leukemia (B-ALL) patients (de Klein A ,*et.al*,1982; de Klein A, *et.al,*1986). The BCR–ABL1 gene results in formation of a chimeric BCR–ABL1 fusion transcript, which encodes a constitutively active ABL1 tyrosine kinase.

BCR–ABL1-like, or Philadelphia chromosome-like (Ph-like), ALL is one subtype of precursor B-cell ALL. Patients with Ph-like ALL do not have the BCR–ABL1 fusion protein expressed from the t(9;22)(q34;q11.2) Ph chromosome, but have a gene expression profile similar to that of patients with BCR–ABL1 ALL (Mulligan CG, *et.al,* 2009; Den Boer ML*, et.al,* 2009).

Ph-like ALL was reported to be associated with poor prognosis (Mullighan CG, *et.al,* 2009; Den Boer ML*, et.al,* 2009; van der Veer A, *et.al,* 2013; Loh ML, *et.al,* 2013; Kiyokawa N, *et.al,* 2013; Te Kronnie G, *et.al,* 2013). A detailed genomic analysis

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of 154 patients with Ph-like ALL was recently begun (Roberts KG , *et.al,* 2014) and kinase-activating alternations were identified in 91% of patients with Ph-like ALL, with rearrangements involving ABL1, ABL2, CSF1R, JAK2, and PDGFRB, PTK2B, TSLP, or TYK2 (Roberts KG , *et.al,* 2014). Expression of ABL1, ABL2, CSF1R, JAK2, and PDGFRB fusions resulted in cytokine-independent proliferation through phosphorylation of STAT5 (Roberts KG, *et.al,* 2014).

Among Ph-like ALL, we focused on the RCSD1–ABL1 fusion gene. RCSD1 involved in a t(1;9)(q24;q34) translocation is one of several novel ABL1 fusion partners (Mustjoki S, *et.al,* 2009; Inokuchi K, *et.al,* 2011; De Braekeleer E, *et.al,* 2013).

In a previous study, an RCSD1–ABL1-expressing cell line showed sensitivity to dasatinib, which is a tyrosine kinase inhibitor (TKI) (Roberts KG, *et.al,* 2014). The dasatinib sensitivity of RCSD1–ABL1-positive ALL was also described in a previous case report (Mustjoki S, *et.al,* 2009). In our case, however, RCSD1–ABL1-positive ALL showed little sensitivity to imatinib or dasatinib, and showed rapid clonal evolution (Inokuchi K, *et.al,* 2011). The present study was performed to examine the reasons of the difference of sensitivities to TKIs and clinical courses of RCSD1–ABL1-positive ALL between previous reported cases and our cases.

MATERIALS AND METHODS

Cell culture

The murine bone marrow-derived pro-B-cell line, Ba/F3, was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The cell lines were used within 6 months after receipt or resuscitation. Ba/F3 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1.0 ng/mL of recombinant IL3 (Kirin Inc., Tokyo, Japan) in a 5% CO_2 incubator at 37°C.

Generation of retroviral vectors expressing RCSD1–ABL1

We found a fusion gene in a case of RCSD1–ABL1-positive ALL (Figure 1a). The fusion was consisted of exon 3 of RCSD1/exon 4 of ABL1 (R3A4; 3042 bp).

Although both fusion of RCSD1-ABL1of previous report and our case consists of exon 3 of RCSD1/exon 4 of ABL1, the fusion points are different from each other (Inokuchi K, *et.al,* 2011; Roberts KG, *et.al,* 2013) (Table.1). To analyze the functions of R3A4 of our case, we generated ecotropic retroviral vectors expressing Mock (control) or R3A4 of our case. Briefly, retroviral vector plasmids expressing Mock or R3A4 were constructed by inserting PCR-amplified fragments into the cloning site of the pDON-5 Neo plasmid (pDON-5 Neo/Mock or R3A4). Primers for PCR amplification of RCSD1 and ABL1 were designed in accordance with the previously reported sequence (Inokuchi K, *et.al,* 2011). Retroviral vectors were prepared by transiently transfecting G3T-hi cells (2×10^6 cells/6-cm collagen-coated laboratory dish) with 2 µg of pGP Vector (gag-pol gene expression vector), 1 µg of pE-Eco Vector (ecotropic envelope gene expression vector), and 2 µg of pDON-5 Neo/Mock or pDON-5 Neo/R3A4 using TransIT-293 transfection reagent (Mirus Bio LLC, Madison, WI, USA). Twenty-four hours after transfection, the medium was replaced with fresh medium, and culture was continued for 24 hours. The supernatant was collected from each dish 48 hours after transfection, and filtered through a 0.8/0.2 µm filter. This retrovirus solution was divided into aliquots of 1 mL/vial and stored at –80°C.

Establishment of RCSD/ABL-expressing Ba/F3 cells

Ba/F3 cells were infected with the retroviral vectors using polybrene reagent, and lines with stable expression of the desired gene were selected on G418 (Geneticin)-supplemented medium. Briefly, aliquots of 1×10^5 cells were suspended in 1.0 mL of retrovirus solution diluted 1:4 in medium and seeded into each well of untreated 6-well plates. Polybrene was added to a final concentration of 8 µg/mL. Approximately 6 hours after viral infection, 1 mL of medium was added to each well. Cells were collected by pipetting 24 hours after virus infection, and after sedimentation of the cells by centrifugation, the culture supernatant was removed and seeding was repeated by changing to medium supplemented with 400 µg/mL G418. For the subsequent 2 – 3 days, while performing subculture, transgenic cells were selected. Cells that had proliferated and become drug-resistant were recovered 9 days after the start of drug selection.

In vitro *analysis of Mock-Ba/F3 and R3A4-Ba/F3 cell growth*

The two generated cell lines, Mock-Ba/F3 and R3A4-Ba/F3 were incubated *in vitro* without IL3 for 48 hours to determine their IL3 independence. After 48-hour incubation, trypan blue exclusion assay was performed to count living cells.

Receptor tyrosine kinase phosphorylation profiling by antibody arrays

We examined receptor tyrosine kinase activity of each Ba/F3 by phosphorylation antibody array (RayBiotech, Norcross, GA, USA) in accordance with the manufacturer's protocol.

Western blotting analysis

Western blotting (WB) analysis was performed as described previously (Tamai H, *et.al,* 2012). Briefly, R3A4-Ba/F3 or Mock (control)-Ba/F3 cells were seeded into 6-well plates (3×10^5 cells/well), incubated for 24 hours in standard normal culture medium, and collected for WB analysis. Equal aliquots of lysates from cell lines were subjected to 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and immunoblotted with the following primary antibodies: phosphor-Tyk2 antibody (Abcam, Cambridge, MA, USA), Tyk2 antibody, phosphor stat antibody sampler (STAT 1/2/3/4/5 antibody and phospho- STAT 1/2/3/4/5 antibody), and anti-β-actin (Merck Millipore, Billerica, MA, USA).

Tyrosine kinase inhibitors assays

To assess the sensitivity of R3A4-Ba/F3 to TKIs, TKI assays were performed as described previously (Roberts KG , *et.al,* 2014). Briefly, R3A4-Ba/F3 cells in culture were harvested at 1×10^6 cells per tube and treated with the TKIs, imatinib (Santa Cruz Biotechnology, Dallas, TX, USA), dasatinib (Santa Cruz Biotechnology), or JAK-Inhibitor I (Calbiochem, Darmstadt, Germany), which is pan-JAK family, including Tyk2 inhibitor, for 1 hour, and the cell viabilities were determined.

Murine transplantation experiments with Mock-Ba/F3 and R3A4-Ba/F3

For *in vivo* analysis, 2×10^8/body of Mock-Ba/F3 or R3A4-Ba/F3 cells were injected intraperitoneally (ip) into groups of five severe combined immune deficiency (SCID) mice. Overall survival (OS) was determined by recording the day of death or sacrifice (day 100) where applicable. All animal experiments were performed in accordance with the regulations established by the Ethics Committee of Nippon Medical School and were approved by the Animal Care and Use Committee of Nippon Medical School.

Statistical analysis

The results of cell growth and gene expression assays were analyzed by Student's *t* test, assuming unequal variances and two-tailed distributions. Data are shown as the means ± standard deviation of at least three samples. For survival analyses, event time distributions were estimated using the Kaplan–Meier method, and differences in survival rates were compared using the log-rank test. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

RESULTS

***Only R3A4-Ba/F3 acquired significant independence from IL3* in vitro**

Figure 1b shows the growth curves of Mock-Ba/F3 and R3A4-Ba/F3 without IL3. Only R3A4-Ba/F3 acquired significant independence from IL3 (Mock *vs.* R3A4, $P = 0.001$). Based on these results, we concluded that only R3A4-Ba/F3 has leukemogenicity.

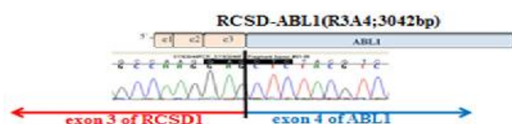

Fig. 1a RCSD1–ABL1 fusion of our RCSD1–ABL1-positive ALL
RCSD1–ABL1 fusion of our case consists of exon 3 of RCSD1 and exon 4 of ABL1 (R3A4; 3 042 bp).
Fig. 1b *Growth curves of our R3A4-Ba/F3 and Mock-Ba/F3 without IL3 (each* n *= 3)*

Phosphorylation Antibody Array

Next, to analyze phosphorylation as a cause of the leukemogenicity of R3A4-Ba/F3, we compared Mock-Ba/F3 and R3A4-Ba/F3 by phosphorylation antibody array. The data obtained using the phosphorylation antibody array are shown in Figure 2a. We focused on the markedly increased phosphorylation of Tyk2 in R3A4-Ba/F3 cells in comparison with that in Mock-Ba/F3.

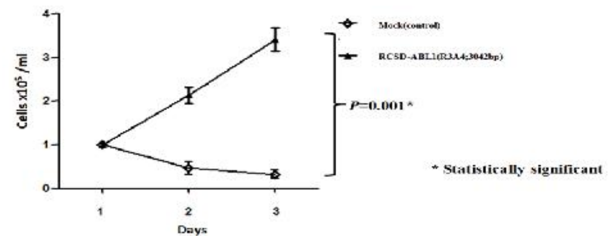

Fig. 2
a. Phosphorylation antibody array results
Phosphorylation of Tyk2 was increased markedly in our R3A4-Ba/F3 cells in comparison with that in Mock-Ba/F3.
b. Western blotting analysis results
WB analysis indicated that our R3A4 induced phosphorylation of Tyk2 followed by phosphorylation of STAT2.

Western blotting analysis

Next, we performed WB analysis to confirm the phosphorylation of Tyk2 in R3A4-Ba/F3. WB analysis indicated that R3A4 induced upregulation of Tyk2 followed by upregulation of STAT2 (Figure 2b). Other STATs (STAT1/3/4/5) were not upregulated in R3A4-Ba/F3 in comparison with those in Mock-Ba/F3 (data not shown).

Tyrosine kinase inhibitor assays

Next, we performed tyrosine kinase inhibitor assays to examine the sensitivity of R3A4-Ba/F3 cells to TKIs. Figure 3 shows the sensitivity of R3A4-Ba/F3 cells to the TKIs imatinib, dasatinib, and JAK-Inhibitor I, which is a pan-JAK family, including Tyk2 inhibitor. R3A4-Ba/F3 showed sensitivity only to JAK-Inhibitor I.

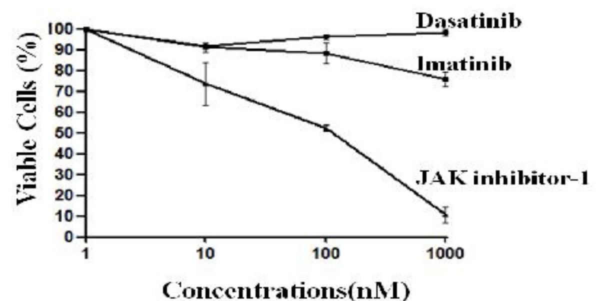

Fig. 3 Tyrosine kinase inhibitors assays results

The sensitivity of our R3A4-Ba/F3 cells showed sensitivity not to dasatinib or imatinib but only to JAK-Inhibitor I.

Overall survival curves of Ba/F3-injected mice

Finally, to examine the leukemogenicity of Mock-Ba/F3 and R3A4-Ba/F3 *in vivo*, we transplanted these cells into SCID mice. *In vivo*, R3A4-Ba/F3-injected mice showed significantly shorter survival times than Mock-Ba/F3-injected mice (42 *vs.* > 100 days, respectively, $P = 0.0009$). (Figure 4a).

Histopathological analysis of Ba/F3-injected mice (macro)

Mice injected with R3A4- Ba/F3 cells showed hepatosplenomegaly in comparison with Mock- Ba/F3-injected mice (Figure 4b).

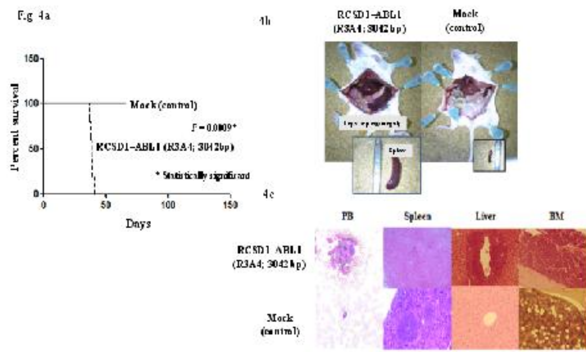

Fig. 4 Comparison among mice injected with R3A4-Ba/F3 and Mock-Ba/F3.

a. Overall survival: R3A4-Ba/F3-injected mice showed significantly shorter survival times than Mock-Ba/F3-injected mice (42 days *vs.* > 100 days, $P = 0.0009$) (each $n = 5$).

b. Histopathology (macro): Only R3A4-Ba/F3-injected mice showed hepatosplenomegaly.

c. Histopathology (micro): Only R3A4-Ba/F3-injected mice showed leukemic changes.

Only R3A4-Ba/F3-injected mice showed leukemia cells in peripheral blood, destruction of spleen, and leukemic infiltration of liver and bone marrow.

Fig2

a

	Mock	R3A4
POS	14,700.88	14,700.88
NEG	471.75	328.04
ABL1	0.00	325.46
ACK1	0.00	823.00
ALK	0.00	0.00
Axl	0.00	155.96
Blk	24.25	19.33
BMX	0.00	0.00
Btk	0.00	0.00
Csk	0.00	318.37
Ddr	0.00	0.00
EGFR	0.00	371.22
EphA1	0.00	0.00
EphA2	0.00	0.00
EphA3	0.00	0.00
EphA4	0.00	0.00
EphA5	0.00	0.00
EphA6	0.00	0.00
EphA7	0.00	48.98
EphA8	0.00	0.00
EphB1	0.00	0.00
EphB2	0.00	0.00
EphB3	0.00	0.00
EphB4	4,967.75	4,171.71
EphB6	0.00	0.00
ErbB2	0.00	0.00
ErbB3	0.00	0.00
ErbB4	0.00	0.00
FAK	0.00	0.00
FER	0.00	0.00
FGFR1	31.25	0.00
FGFR2	0.00	0.00
FGFR2 (α isoform)	0.00	0.00
Fgr	506.25	0.00

	Mock	R3A4
FRK	0.00	0.00
Fyn	286.25	0.00
Hck	0.00	0.00
HGFR	0.00	0.00
IGF-1R	0.00	0.00
Insulin R	0.00	0.00
Itk	261.75	0.00
JAK1	23.75	0.00
JAK2	2,910.75	2,068.13
JAK3	0.00	0.00
LCK	0.00	0.00
LTK	0.00	0.00
Lyn	3,315.75	1,442.99
MATK	259.75	0.00
M-CSFR	287.75	0.00
MUSK	0.00	0.00
NGFR	26.25	0.00
PDGFR-α	0.00	0.00
PDGFR-β	0.00	0.00
PYK2	0.00	0.00
RET	21.75	0.00
ROR1	0.00	0.00
ROR2	119.25	0.00
ROS	0.00	154.03
RYK	0.00	0.00
SCFR	0.00	0.00
SRMS	44.25	213.32
SYK	0.00	0.00
Tec	631.25	0.00
TNK1	1,974.75	855.87
TRKB	0.00	391.64
TXK	2,017.25	2,557.29
Tyk2	0.00	1,328.48
TYRO10	0.00	0.00
VEGFR2	0.00	0.00
VEGFR3	0.00	528.47
ZAP70	0.00	385.40

b

Mock R3A4

P-Tyk2

Tyk2

P-STAT2

STAT2

β-actin

Histopathological analysis of Ba/F3-injected mice (micro)

Leukemic changes, destruction of the spleen, and tumor invasion into the liver were observed only in mice injected with R3A4- Ba/F3 cells, but not in those injected with Mock- Ba/F3 cells (Figure 4c).

DISCUSSION

This is the first report which showed the kinase-activating pathways and sensitivities to TKIs vary between fusion sites of RCSD1–ABL1 in Ph-like ALL.

Precious study by Roberts KG, *et al*. about 154 patients with Ph-like leukemia showed expression of ABL1, ABL2, CSF1R, JAK2, and PDGFRB fusions resulted in cytokine-independent proliferation and activation of phosphorylated STAT5. Cell lines and human leukemic cells expressing ABL1, ABL2, CSF1R, and PDGFRB fusions were sensitive *in vitro* to dasatinib, EPOR and JAK2 rearrangements were sensitive to ruxolitinib, and the ETV6–NTRK3 fusion was sensitive to crizotinib (Roberts KG , *et.al,* 2014). This study is epoch-making because it opened targetable use of TKIs in patients with Ph-like leukemia whose prognosis are very poor. However, as mentioned above, RCSD1-ABL1 fusion of our case was resistant to dasatinib both *in vitro* and in clinical course although RCSD1-ABL1 fusion is sensitive to dasatinib in the study by Roberts KG, *et al.*

The reason of this difference of sensitivity to dasatinib seems to come from the difference of fusion points of R3A4 of RCSD1-ABL1 (Table 1).

	sequence of RCSD1-ABL1 fusion
R3A4 of Roberts KG, et al (ref. 14)	GGTGAGGAG-CTCTACGTC
Our R3A4	GCCAAGGAG-CTCTACGTC

Table 1 The difference of fusion site of R3A4

Roberts *et al*. also reported R3A4, the fusion point of which is different from that in our case (Roberts KG, *et.al,* 2012). This R3A4-transduced Ba/F3 by Roberts *et al.* was reported that STAT5 is phosphorylated and sensitive to dasatinib (Roberts KG, *et.al,* 2014). However, our R3A4-transduced Ba/F3 showed phosphorylation not of STAT5 but of STAT2 following Tyk2 phosphorylation. Our R3A4-transduced Ba/F3 showed sensitivity not to dasatinib but to pan-JAK family, including Tyk2 inhibitor.

Our findings, together with this previous report, suggest that the kinase-activating pathways and sensitivities to TKIs vary between fusion sites of RCSD1–ABL1 in Ph-like ALL. Ruxolitinib which is one of JAK family inhibitors and used for myelofibrosis may be effective to RCSD1–ABL1-positive acute lymphoblastic leukemia.

These observations raise questions regarding why RCSD1–ABL1 fusion has such heterogeneity in comparison with other ABL1 fusion genes, including BCR–ABL1. Seven genes including BCR-ABL1, ETV6-ABL1, ZMIZ1-ABL1, EML1-ABL1, NUP214-ABL1, SFPQ- ABL1 *and* RCSD1-ABL1 are known to fuse to the ABL1 gene[15]. The breakpoint occurs in intron 1 or 2 of ABL1 in five different types of fusion gene. Breakpoint in intron 3 of the ABL1 gene was found in the sole case of ALL associated with a SFPQ–ABL1 fusion and in all cases with RCSD1–ABL1 thus far studied. All of the fusion genes result from the joining of the 5' sequence of the partner gene with the 3' sequence of the ABL1 gene. As a consequence, the fusion protein contains part of the SH2 domain of ABL1, the SH1 domain including tyrosine kinase function, the three nuclear localization signal domains, the three DNA-binding regions, and the F-actin-binding domain (De Braekeleer E, *et.al,* 2013). A previous study indicated that the SH2 domain of BCR–ABL1 was required for efficient induction of CML-like disease in mice (Roumiantsev S, *et.al,* 2001). However, it was not required for transformation of primary bone marrow B-lymphoid progenitors *in vitro* or for induction of B-lymphoid leukemia in mice (Roumiantsev S, *et.al,* 2001). This could explain why all patients reported to date carrying the RCSD1–ABL1 gene were diagnosed with B-cell ALL.

Furthermore, the retained N-terminal part of the partner proteins contain a coiled-coil domain or a helix-loop-helix domain that is necessary for oligomerization of the fusion ABL1 protein and required for its tyrosine kinase activation (De Braekeleer E, *et.al,* 2013). However, although its structure is not clear, the RCSD1 protein does not have a coiled-coil domain or a helix-loop-helix domain (Eyers CE, *et.al,* 2005). Therefore, it is likely that the RCSD1–ABL1 protein exerts its transforming effects through different mechanisms. Further studies including structural arrangement of RCSD1–ABL1 are needed to determine the function of RCSD1–ABL1 and to lead to the development of novel therapies for ALL with RCSD1–ABL1.

Authorship

Contribution: H.T. designed research, performed experiments, analyzed data, and wrote the paper; M.T.and T.K. performed experiments and analyzed data; and H.Y., K.M., K.D., and K.I. analyzed data and wrote the paper.

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Conflict-of-interest disclosure

The authors have no conflict-of-interest to disclose.

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