**Table 3** Best responses to nilotinib (ITT population)

| | CML-CP ($N = 16$) | CML-AP ($N = 7$) | CML-BC ($N = 4$) | Ph+ ALL ($N = 7$) |
|---|---|---|---|---|
| Hematologic response (HR) | 6 (100)[a] | 5 (71) | 2 (50) | 3 (43) |
| Complete hematologic response | 6 (100) | 1 (14) | 1 (25) | – |
| Complete response | – | – | – | 3 (43) |
| Marrow response with no evidence of leukemia | – | 3 (43) | 0 (0) | – |
| Return to chronic phase | – | 1 (14) | 1 (25) | – |
| Stable disease | 0 (0) | 1 (14) | 2 (50) | 1 (14) |
| Progressive disease | 0 (0) | 0 (0) | 0 (0) | 3 (43) |
| Not evaluable/not assessable | 10 (63) | 1 (14) | 0 (0) | 0 (0) |
| Cytogenetic response (CyR) | | | | |
| Major | 15 (94) | 1 (14) | 2 (50) | – |
| Complete | 13 (81) | 1 (14) | 2 (50) | – |
| Partial | 2 (13) | 0 (0) | 0 (0) | – |
| Minor | 0 (0) | 0 (0) | 1 (25) | – |
| Minimal | 1 (6) | 3 (43) | 0 (0) | – |
| None | 0 (0) | 1 (14) | 0 (0) | – |
| Not assessable | 0 (0) | 2 (29) | 1 (25) | – |
| Molecular response (MR) | | | | |
| Major[b] | 13 (81) | 1 (14) | 2 (50) | 1 (17)[c] |
| None | 3 (19) | 6 (86) | 2 (50) | 5 (83)[c] |
| Not evaluable | 0 (0) | 0 (0) | 0 (0) | 1 (14) |

Values are *n* (%)

[a] Of which 6 were evaluable

[b] Major molecular response was defined as a BCR-ABL/BCR ratio ≤0.1%

[c] Of which 6 were evaluable

*ITT* intention-to-treat

most frequent reason for discontinuation was disease progression in 12 patients. Disease progression was seen in 1 patient with CML-CP, 3 patients with CML-AP, 2 patients with CML-BC and 6 patients with Ph+ ALL.

The median duration (range) of nilotinib exposure was 445.5 days (13–1173 days) and that of administration was 428.0 days (13–1173 days). The median daily dose (range) of nilotinib was 750.7 mg/day (284.9–798.6 mg/day) in all patients, consistent with the planned dose of administration (400 mg twice daily = 800 mg/day) in the study protocol. Dose reductions occurred in 27 patients (79.4%) because of adverse events in 19 patients (55.9%), in accordance with the study protocol in 14 patients (41.2%), incorrect administration in 10 patients (29.4%) or incorrect scheduling in 1 patient (2.9%) (multiple dose reductions were possible). Treatment interruption occurred in 17 patients (50.0%) because of adverse events in all 17 patients. Thirteen of these patients showed improvement of adverse events and were able to restart nilotinib administration at a lower dose.

## Efficacy

### *CML-CP*

The best responses (HR, CyR and MR) in the ITT population are shown in Table 3. All 6 CML-CP patients without CHR at baseline achieved CHR. The median time

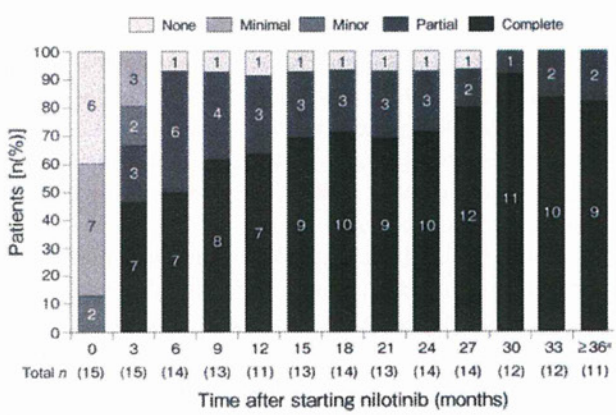


**Fig. 1** Cytogenetic responses in CML-CP patients. [a]Including up to and beyond 36 months

(range) to CHR was 28 days (28–56 days). Of these, 5 patients showed sustained response up to the last evaluation, while the remaining patient discontinued treatment on Day 787 because of disease progression. The duration of CHR in that patient was 478 days. MCyR was achieved in 15 patients (93.8%) and the response was sustained at the last evaluation in 13 patients. CCyR was achieved in 13 patients (81.3%) and the response was sustained at the last evaluation in 11 patients. The median time (range) to MCyR or CCyR was 84 days (28–178 days) and 97 days (57–847 days), respectively. The rate of CyR in evaluable patients at each time point is shown in Fig. 1. Thirteen

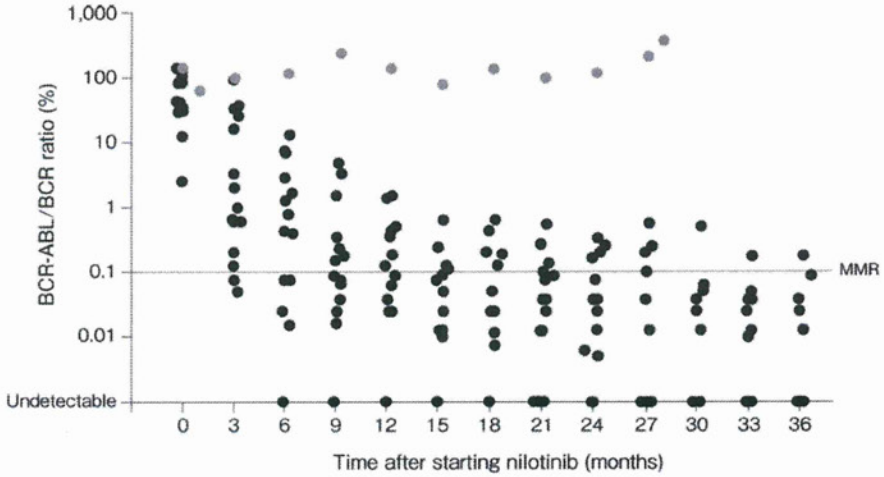


**Fig. 2** Molecular responses during the 36-month study in patients with CML-CP. *MMR* major molecular response

patients continued treatment at 36 months or later. Among them, 11 patients were evaluated as showing cytogenetic response, all of whom achieved MCyR, including 9 with CCyR. The figure shows that the proportion of CCyRs increased with nilotinib treatment period.

The BCR-ABL/BCR ratio in CML-CP patients over time is shown in Fig. 2. The BCR-ABL/BCR ratio gradually decreased from baseline with long-term nilotinib treatment in all patients except one with baseline or newly detected mutations. An approximately 1-log reduction in BCR-ABL/BCR ratio from baseline at 6 months and an approximately 2-log reduction at 12 months were observed. MMR was achieved in 13 patients (81.3%) and was sustained at the last evaluation in 11 patients. The median time (range) to MMR was 248 days (84–852 days) in these CML-CP patients.

Among CML-CP patients, 3 patients discontinued nilotinib treatment. One patient discontinued treatment on Day 176 to undergo allogeneic hematopoietic stem cell transplantation (allo-HSCT). Another patient once achieved CCyR but discontinued treatment on Day 787 because of disease progression, as mentioned above. This patient had a newly detected mutation (F359V). Another patient withdrew consent on Day 931.

*CML-AP*

Among 7 CML-AP patients, 5 patients (71.4%) achieved HR, including CHR in 1 patient, marrow response with no evidence of leukemia in 3 patients, and return to chronic phase in 1 patient. Of the remaining 2 patients, 1 had stable disease and 1 was not evaluable. Of the 5 patients with HR, 1 patient with CHR and another 2 patients with HR experienced sustained response at the last evaluation or at discontinuation of treatment. In the remaining 2 patients, the duration of HR was 29 and 57 days, respectively. Minimal CyR was observed in 3 patients (42.9%). One patient with CHR achieved CCyR (14.3%). This patient also achieved MMR, which was sustained at the last evaluation.

*CML-BC*

Among 4 CML-BC patients, 2 patients (50.0%) achieved HR, including CHR in 1 patient and return to chronic phase in 1 patient. They also achieved CCyR and MMR. In both patients, MCyR was sustained until discontinuation of treatment to undergo allo-HSCT (on Day 247) in the first patient, or because of increasing blast numbers in bone marrow (on Day 168) in the second patient. The remaining 2 patients (50.0%) experienced stable disease and one of them achieved minor CyR.

*Ph+ ALL*

Among 7 patients with relapsed/refractory Ph + ALL, 1 of 5 patients (20.0%) without MRD experienced HR (complete response [CR]), which was sustained for 108 days. Three patients experienced disease progression and 1 experienced stable disease. Both patients with MRD achieved HR (CR). In one of these patients, CR was sustained for 58 days, but treatment was discontinued on Day 109 because of encephalitis. In the other patient, CR was sustained for 470 days, but treatment was discontinued on Day 644 because of disease progression. MMR was achieved in 1 patient with MRD, while the other patient with MRD achieved MMR at baseline and was thus considered not evaluable.

BCR-ABL mutations

*Detection of new mutations*

The development of new BCR-ABL mutations during the administration of nilotinib in this study is shown in

**Table 4** Detection of new BCR-ABL mutations

| Stage | Mutation | Day of detection | Baseline mutation | Achieved MMR | Outcome |
|---|---|---|---|---|---|
| CML-CP | F359V | 174 | M244V | No | Disease progression |
| CML-CP | E255K | 340 | None | Yes | Continued |
| CML-BC | T315I/Y253H | 168 | F317L | Yes | Disease progression |
| Ph+ ALL | T315I | 16 | E255K/E255V/G250E | No | Disease progression |
| Ph+ ALL | E255V | 57 | E459K | No | Disease progression |
| Ph+ ALL | T315I | 43 | None | No | Disease progression |
| Ph+ ALL | E255K/E255V | 135 | NA | No | Disease progression |

*MMR* major molecular response, *NA* Not assessable

Table 4. New mutations were detected in 7 patients during nilotinib treatment. Among them, the T315I mutation occurred in 3 patients and nilotinib was discontinued in these patients because of disease progression. Three of the 4 patients with mutations other than T315I also discontinued treatment because of disease progression. The remaining patient continued treatment.

*CML-CP*

Among 16 CML-CP patients, MMR was observed in 4 of 5 patients (80.0%) with BCR-ABL mutations at baseline or emerging during the treatment period. As shown in Table 4, new mutations were detected in 2 patients.

One patient had a baseline M244V mutation and achieved minimal CyR on Day 87; however, an F359V mutation was also detected on Day 174. From Day 426, only the F359V mutation was detected and the M244V mutation was not; this patient was withdrawn from the study because of disease progression on Day 787 (see "CML-CP" under the heading Efficacy). In another patient without baseline mutation, E255K was detected only once on Day 340. This patient achieved MMR on Day 511, which was sustained at the last evaluation, and the mutation was not detected again after achievement of MMR. In 1 patient with an imatinib resistance-associated mutation (F359I) at baseline, the mutation could not be detected after commencing nilotinib treatment, which led to MMR that had been sustained for 666 days at the last evaluation.

*CML-AP/-BC and Ph+ ALL*

Among 7 CML-AP patients, no new mutations were detected. As shown in Table 4, among 4 CML-BC patients, new mutations were detected in 1 patient with the F317L mutation at baseline. This patient achieved CCyR and MMR on Day 56; however, Y253H and T315I mutations were detected on Day 168 followed by disease progression on Day 171. Among 7 Ph+ ALL patients, new mutations were detected in 4 patients, all of whom experienced disease progression.

Safety analysis

All adverse events regardless of drug relationship occurring at a frequency ≥20% and those of grade 3/4 are summarized in Table 5 (adverse events and adverse drug reactions occurring in ≥10% of subjects are shown in Supplemental Tables 1 and 2, respectively, while all adverse events of grade 3 or worse are shown in Supplemental Table 3). Adverse events occurred in all of the patients. The most common non-hematologic events were rash (64.7%), nasopharyngitis (58.8%), nausea and headache (47.1% each), and vomiting (41.2%). Hematologic events included leukopenia (47.1%), neutropenia (47.1%), thrombocytopenia (47.1%) and anemia (38.2%).

Adverse events of grade 3/4 occurred in 29/34 patients (85.3%). The most frequent grade 3/4 non-hematologic events were abnormal hepatic function, hyponatremia and pneumonia (11.8% each). Grade 3/4 hematologic events included neutropenia (47.1%), leukopenia (41.2%), thrombocytopenia (32.4%), anemia (29.4%) and lymphopenia (11.8%). The most common biochemical grade 3/4 events were decreased blood phosphorus levels (14.7%), hyperglycemia and increased lipase levels (11.8% each).

*Serious adverse events*

Thirty-four serious adverse events occurred in 19 patients. Among these, 21 events in 12 patients were considered possibly related to nilotinib. Two of these patients discontinued nilotinib treatment because of serious adverse events considered to be related to the drug. One, with CML-BC, developed back pain (non-serious) and discontinued treatment. Two days later, this patient developed cardiac tamponade and pericardial effusion, and died because of heart failure. The other, with Ph+ ALL, developed encephalitis and also discontinued treatment. Furthermore, one CML-CP patient developed acute pancreatitis reported as a serious adverse event that resolved

**Table 5** Non-hematologic, hematologic and biochemical adverse events with a frequency ≥20% for all grades

| Total $N = 34$ | All grades | | | | | Grade 3/4 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CML-CP *n* (%) | CML-AP *n* (%) | CML-BC *n* (%) | Ph+ ALL *n* (%) | Total *n* (%) | CML-CP *n* (%) | CML-AP *n* (%) | CML-BC *n* (%) | Ph+ ALL *n* (%) | Total *n* (%) |
| Non-hematologic events | | | | | | | | | | |
| Rash | 9 (56.3) | 5 (71.4) | 3 (75.0) | 5 (71.4) | 22 (64.7) | 1 (6.3) | 0 (0.0) | 1 (25.0) | 0 (0.0) | 2 (5.9) |
| Nasopharyngitis | 15 (93.8) | 3 (42.9) | 2 (50.0) | 0 (0.0) | 20 (58.8) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Headache | 7 (43.8) | 2 (28.6) | 3 (75.0) | 4 (57.1) | 16 (47.1) | 0 (0.0) | 0 (0.0) | 1 (25.0) | 1 (14.3) | 2 (5.9) |
| Nausea | 6 (37.5) | 3 (42.9) | 4 (100.0) | 3 (42.9) | 16 (47.1) | 0 (0.0) | 0 (0.0) | 1 (25.0) | 0 (0.0) | 1 (2.9) |
| Vomiting | 6 (37.5) | 3 (42.9) | 2 (50.0) | 3 (42.9) | 14 (41.2) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Pyrexia | 4 (25.0) | 1 (14.3) | 4 (100.0) | 4 (57.1) | 13 (38.2) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Constipation | 8 (50.0) | 2 (28.6) | 1 (25.0) | 1 (14.3) | 12 (35.3) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Hyperbilirubinemia | 5 (31.3) | 3 (42.9) | 1 (25.0) | 1 (14.3) | 10 (29.4) | 2 (12.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (5.9) |
| Hyperglycemia | 8 (50.0) | 1 (14.3) | 1 (25.0) | 0 (0.0) | 10 (29.4) | 2 (12.5) | 1 (14.3) | 1 (25.0) | 0 (0.0) | 4 (11.8) |
| Malaise | 8 (50.0) | 0 (0.0) | 0 (0.0) | 2 (28.6) | 10 (29.4) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Back pain | 6 (37.5) | 0 (0.0) | 2 (50.0) | 1 (14.3) | 9 (26.5) | 0 (0.0) | 0 (0.0) | 1 (25.0) | 0 (0.0) | 1 (2.9) |
| Pruritus | 3 (18.8) | 2 (28.6) | 1 (25.0) | 3 (42.9) | 9 (26.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Abnormal hepatic function | 5 (31.3) | 0 (0.0) | 1 (25.0) | 2 (28.6) | 8 (23.5) | 1 (6.3) | 0 (0.0) | 1 (25.0) | 2 (28.6) | 4 (11.8) |
| Conjunctivitis | 7 (43.8) | 1 (14.3) | 0 (0.0) | 0 (0.0) | 8 (23.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Diarrhea | 3 (18.8) | 2 (28.6) | 1 (25.0) | 2 (28.6) | 8 (23.5) | 0 (0.0) | 0 (0.0) | 1 (25.0) | 1 (14.3) | 2 (5.9) |
| Anorexia | 5 (31.3) | 1 (14.3) | 0 (0.0) | 1 (14.3) | 7 (20.6) | 1 (6.3) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (2.9) |
| Arthralgia | 5 (31.3) | 2 (28.6) | 0 (0.0) | 0 (0.0) | 7 (20.6) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Eczema | 6 (37.5) | 0 (0.0) | 1 (25.0) | 0 (0.0) | 7 (20.6) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Hypokalemia | 1 (6.3) | 2 (28.6) | 2 (50.0) | 2 (28.6) | 7 (20.6) | 0 (0.0) | 1 (14.3) | 0 (0.0) | 1 (14.3) | 2 (5.9) |
| Insomnia | 2 (12.5) | 2 (28.6) | 1 (25.0) | 2 (28.6) | 7 (20.6) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Pharyngitis | 4 (25.0) | 0 (0.0) | 0 (0.0) | 3 (42.9) | 7 (20.6) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Hematologic events | | | | | | | | | | |
| Leukopenia | 7 (43.8) | 3 (42.9) | 2 (50.0) | 4 (57.1) | 16 (47.1) | 5 (31.3) | 3 (42.9) | 2 (50.0) | 4 (57.1) | 14 (41.2) |
| Neutropenia | 7 (43.8) | 3 (42.9) | 2 (50.0) | 4 (57.1) | 16 (47.1) | 7 (43.8) | 3 (42.9) | 2 (50.0) | 4 (57.1) | 16 (47.1) |
| Thrombocytopenia | 7 (43.8) | 3 (42.9) | 2 (50.0) | 4 (57.1) | 16 (47.1) | 3 (18.8) | 3 (42.9) | 2 (50.0) | 3 (42.9) | 11 (32.4) |
| Anemia | 5 (31.3) | 2 (28.6) | 3 (75.0) | 3 (42.9) | 13 (38.2) | 3 (18.8) | 2 (28.6) | 2 (50.0) | 3 (42.9) | 10 (29.4) |
| Biochemical events | | | | | | | | | | |
| Increased bilirubin | 6 (37.5) | 1 (14.3) | 1 (25.0) | 2 (28.6) | 10 (29.4) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Increased alanine aminotransferase | 3 (18.8) | 0 (0.0) | 2 (50.0) | 3 (42.9) | 8 (23.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (14.3) | 1 (2.9) |
| Increased lipase | 5 (31.3) | 1 (14.3) | 1 (25.0) | 1 (14.3) | 8 (23.5) | 3 (18.8) | 1 (14.3) | 0 (0.0) | 0 (0.0) | 4 (11.8) |

The table includes drug-related and non-related adverse events combined

following nilotinib dose interruption. This patient restarted nilotinib at 400 mg once daily, which was then increased to 400 mg twice daily, and the subject completed study treatment. QT interval prolongation occurred in 1 CML-CP patient and nilotinib treatment was interrupted. This patient restarted nilotinib at 400 mg once daily and continued treatment without QT interval prolongation.

*Adverse events by time-points*

Among the CML-CP patients, the incidences of blood/lymphatic system disorders, gastrointestinal disorders, laboratory abnormalities, and skin/subcutaneous tissue disorders in Cycles 1–12 in the first year of treatment were 68.8, 87.5, 62.5 and 75.0%, respectively. The incidences of these events were much lower during Cycles 13–24 (20.0, 40.0, 40.0 and 53.3%, respectively) and Cycles 25 or later (20.0, 73.3, 26.7, 46.7%) in the second year of treatment. Gastrointestinal disorders showed higher incidence in Cycles 25 or later (3 years or more of treatment) and, in particular, the incidence of constipation was as high as 26.7%. Fewer patients with CML-AP, CML-BC, and Ph+ ALL continued treatment beyond Cycle 24, so no significant difference in the

incidence of these adverse events between time-points was observed.

## Discussion

Here, we report the long-term efficacy and tolerability profiles of nilotinib in 34 patients with imatinib-resistant or -intolerant Ph+ CML or relapsed/refractory Ph+ ALL. In comparison with the findings obtained at 12 months [22], there were few occurrences of new adverse events during the 36-month study.

In the phase I/II clinical trial of nilotinib [22], the drug was found to be generally safe and well-tolerated in patients with imatinib-resistant or -intolerant CML, and those with relapsed/refractory Ph+ ALL. The tolerability of nilotinib up to doses of 400 mg twice daily was confirmed in Japanese patients. The dose intensity of nilotinib increased with increasing dose within the investigated dose range, and the 400 mg twice-daily dose regimen gave the highest exposure.

In the present extension study, in CML-CP patients, CCyR was achieved in 13/16 patients (81.3%) and CCyR was achieved rapidly, within a median of approximately 3 months. Furthermore, MMR (defined as a BCR-ABL/BCR ratio $\leq$0.1%) was also achieved in 13/16 patients (81.3%). These results compare favorably with those reported after 24 months of nilotinib treatment in another study of imatinib-resistant or -intolerant CML-CP [20]. In that study, 44% (141/321) of patients achieved CCyR and 28% (82/294) of patients achieved MMR. Comparable rates of HR, CyR and MMR during nilotinib therapy in CML-CP were reported in other studies. In this analysis, 13/16 patients achieved MMR and, in some patients, the BCR-ABL transcript level was undetectable by quantitative RT-PCR.

One CML-AP patient who responded well to nilotinib and achieved CCyR was treated with nilotinib for 3 years. This suggests that nilotinib has long-term benefits for the treatment of some patients with CML-AP. The findings in Ph+ ALL and CML-BC patients in this study are similar to those reported in other studies [26]. Although the sample size is small, the results obtained in 4 CML-BC patients and 7 Ph+ ALL patients suggest that, in some patients, nilotinib may be an effective drug for the treatment of imatinib-resistant or -intolerant CML-BC and Ph+ ALL. Further studies are needed in patients with advanced CML to verify these results. All 5 Ph+ ALL patients without MRD in this study were previously treated with imatinib, and only 1 patient (20.0%) achieved HR. The other 4 patients ultimately discontinued treatment because of disease progression. In contrast, both Ph+ ALL patients with MRD achieved HR. The small sample size in this study meant that patients with imatinib-resistant or -intolerant disease were considered together, not separately.

As reported previously [28], imatinib resistance or intolerance, or the presence of baseline BCR-ABL mutations associated with imatinib resistance, did not affect the response to nilotinib. We detected 5 new mutations in 7 patients after starting nilotinib treatment. T315I, which is the mutation associated with the most resistance to currently available TKIs, was detected in 3 patients (8.8%) with CML-BC or Ph+ ALL; these patients discontinued treatment because of disease progression. Three of the 4 patients who developed other mutations also discontinued treatment, and the remaining patient, who had an E255K mutation, achieved MMR. These findings are consistent with previous studies suggesting that patients with the T315I mutation have a poor response to nilotinib [12, 19].

Two types of amino acid substitution at F359, F359V and F359I, were detected in this study. A CML-CP patient with baseline M244V mutation later harbored an F359V mutation (detected on Day 174) and showed poor response to nilotinib treatment; this patient experienced disease progression, as seen in other patients with the F359V mutation described in previous reports [29]. On the other hand, another patient who had F359I mutation at baseline achieved MMR. A previous study [30] showed that the F359I mutation is moderately sensitive to nilotinib ($IC_{90}$ value = 433 nM). Nevertheless, in the present study, nilotinib treatment was effective, and sustainable MMR was observed in the patient with F359I mutation at baseline.

A recent study also described that CML patients with baseline mutations on imatinib treatment were more likely to relapse because of the development of other mutations after receiving dasatinib or nilotinib as second-line treatment [31]. Although the sample size of our study was small, only one CML-CP patient with a BCR-ABL mutation showed disease progression while the others completed study treatment. The effects of BCR-ABL mutation on the efficacy of treatment may differ depending on not only the type of mutation, but also the disease type and stage.

Adverse events of any grade occurred in all of the patients, regardless of drug relationship, and adverse events of grade 3/4 occurred in 29/34 patients (85.3%). The most common hematologic or non-hematologic adverse events included rash, nasopharyngitis, nausea, headache, vomiting, leukopenia, neutropenia and thrombocytopenia. Hematologic adverse events were commonly of grade 3/4 severity, similar to previously reported findings [19–21, 25, 26, 28]. Abnormal biochemical findings included hyperbilirubinemia, hyperglycemia and increased lipase. The rates of abnormal hematologic/blood biochemical findings were similar to those reported in a 12-month study [22] and in a global phase II study [19–21]. Most of these events

were not serious. The majority of adverse events did not require treatment discontinuation, interruption or dose reduction. Taken together, these findings are comparable with those reported in global phase I and II clinical studies [19–21, 25, 26] and a retrospective multicenter analysis [28]. During the 36-month observation period, only one patient with CML-BC died. Death resulted from heart failure due to cardiac tamponade and pericardial effusion occurring after discontinuation of nilotinib treatment.

Hematological and cytogenetic effects of nilotinib have been already observed in studies of up to 12 months [22] or 24 months in duration [20]. We have extended these findings in Japanese patients with imatinib-resistant or -intolerant Ph+ CML (CP, AP, or BC) or relapsed/refractory Ph+ ALL treated with nilotinib 400 mg twice daily for up to 36 months in this study. Importantly, nilotinib was shown to be effective as a second-line treatment for patients who failed to respond to previous imatinib treatment and who were considered to have a poor prognosis, with many patients achieving HR and CyR, which were maintained until last observation. No safety concerns arose over 36 months of treatment that were not apparent during the first 12 months of treatment. Most adverse events resolved following nilotinib dose interruption, dose reduction or supportive care.

The median daily dose of nilotinib (750.7 mg; range 284.9–798.6 mg) was below the prescribed dose (800 mg), mainly as a result of dose reductions in response to adverse events. In a previous study of nilotinib in Japanese newly diagnosed CML patients [24], the median dose was 730 mg (range, 644–794 mg) in the group administered nilotinib 400 mg twice daily; this dose was not considered particularly low, providing dose intensities similar to those in the overall population. The dose reduction in that study [24] was similar to that in ours.

Nilotinib was approved in Japan for the treatment of patients with CML-CP or CML-AP, but not patients with CML-BC or Ph+ ALL. The results of this study update provide further evidence supporting the use of nilotinib in Japanese patients with CML-CP or CML-AP. Our results also suggest that nilotinib may be useful for the treatment of patients with CML-BC or Ph+ ALL. Indeed, efficacy was observed in some CML-BC and Ph+ ALL patients; however, it remains to elucidate for which patient populations this drug would be most suitable in CML-BC and Ph+ ALL.

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**Conflict of interest** Taro Amagasaki and Aira Wanajo are employees of Novartis Pharmaceuticals. The other authors have no conflicts of interest to disclose.

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# Imatinib mesylate directly impairs class switch recombination through down-regulation of AID: its potential efficacy as an AID suppressor

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Brief report

# Imatinib mesylate directly impairs class switch recombination through down-regulation of AID: its potential efficacy as an AID suppressor

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**Activation-induced cytidine deaminase (AID) is essential for class switch recombination and somatic hypermutation. Its deregulated expression acts as a genomic mutator that can contribute to the development of various malignancies. During treatment with imatinib mesylate (IM), patients with chronic myeloid leukemia often develop hypogammaglobulinemia, the mechanism of which has not yet been clarified. Here, we provide evidence that class switch recombination on B-cell activation is apparently inhibited by IM through down-regulation of AID. Furthermore, expression of E2A, a key transcription factor for AID induction, was markedly suppressed by IM. These results elucidate not only the underlying mechanism of IM-induced hypogammaglobulinemia but also its potential efficacy as an AID suppressor. (*Blood*. 2012;119(13): 3123-3127)**

## Introduction

Activation-induced cytidine deaminase (AID) is essential for class switch recombination (CSR) and somatic hypermutation.[1] Deregulated expression of AID acts as a genomic mutator and can contribute to tumorigenesis through genomic recombination and aberrant somatic hypermutation.[2-4] E2A, which harbors 2 binding sites in the AID promoter, is the crucial transcription factor for induction of AID.[5] Imatinib mesylate (IM) has diverse immunomodulatory effects,[6,7] including reduction of T-cell proliferation and inhibition of T-cell effector functions.[8,9] Previously, we reported that serum titers of IgG and IgA, but not IgM, were significantly lower in chronic myeloid leukemia patients treated with IM versus those treated with IFN-α,[10] suggesting that IM impairs CSR. In the present study, we investigated the effects of IM on CSR both in vitro and in vivo. Here, we present evidence that IM inhibits CSR through down-regulation of AID expression in splenic B cells.

## Methods

### Mouse immunization

Eight-week-old mice were immunized as previously reported,[1] with or without 50 mg/kg imatinib mesylate. The experiments were approved by the Committee of Animal Care at the Institute of Medical Science, University of Tokyo.

### Immunohistochemistry

Immunostaining for AID was performed on frozen sections following the manufacturer's instructions using an AID antibody (H-80; Santa Cruz Biotechnology).

Primer sequences, reagents, and more detailed methods are shown in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

## Results and discussion

CSR is induced in splenic B cells by stimulation with IL-4 and lipopolysaccharide (LPS).[11] After stimulation with IL-4 and LPS for 72 hours, IM decreased the proportion of IgG1-positive B cells dose-dependently. The proportion of B cells expressing surface IgG1 was approximately 16% without IM but was significantly reduced to approximately 3% with 10μM IM (Figure 1A). In the present culture system, only B cells can survive and proliferate,[1] suggesting that IM may act directly on B cells and inhibit their CSR.

Next, we examined expression of the germline transcript directed by the I promoter of IgG1 and AID, both of which are essential for CSR after B-cell stimulation.[12] Expression of AID was suppressed by IM dose-dependently (Figure 1B), whereas the IgG1 germline transcripts were not decreased by IM (Figure 1C). Likewise, IgA CSR in CH12F3-2A cells was impaired by IM in a dose-dependent manner (Figure 1D). These results

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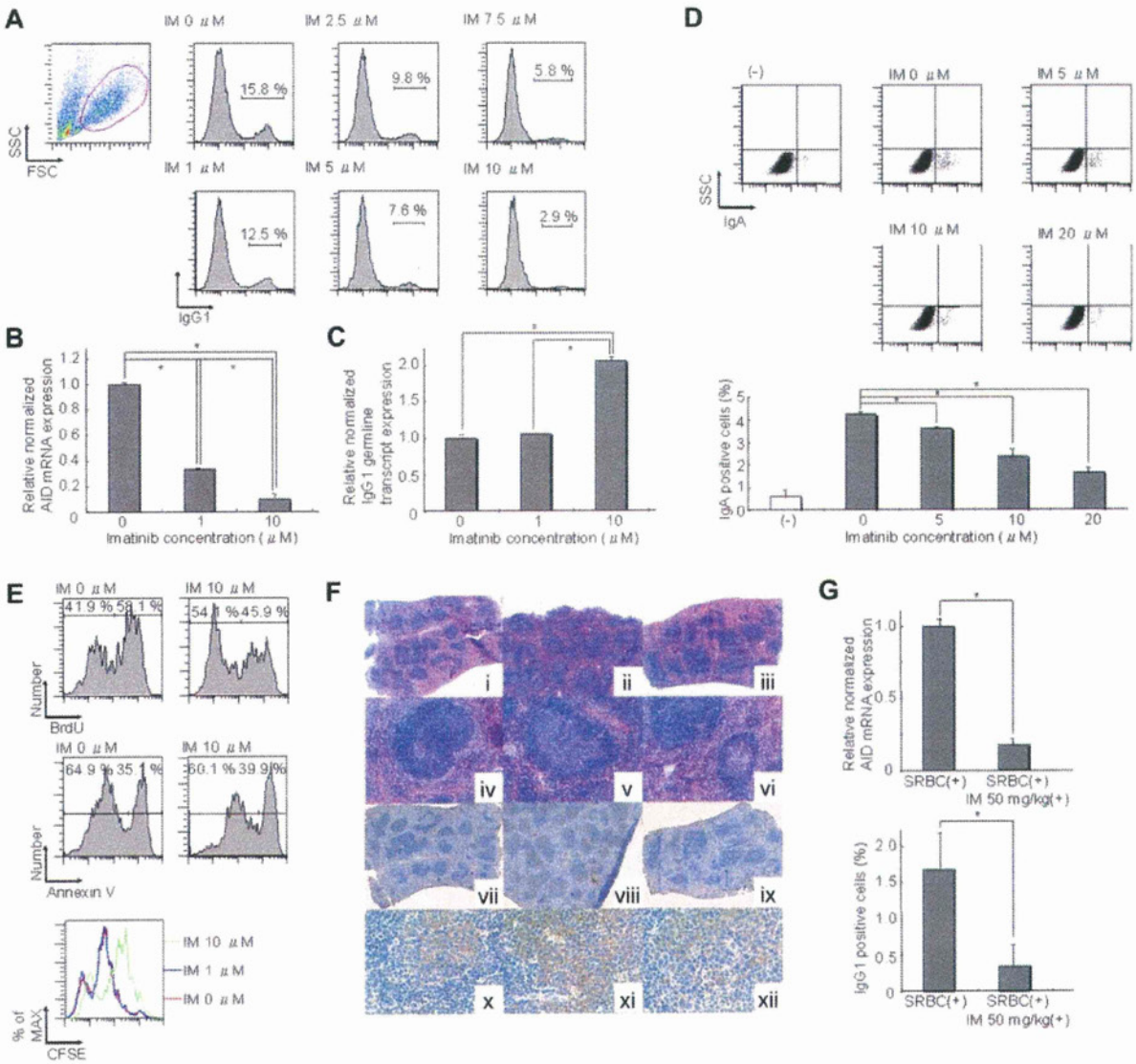


**Figure 1. IM directly inhibits CSR in activated B cells through down-regulation of AID.** (A) IgG1 expression levels in spleen cells cultured in conditioning medium containing 12.5 μg/mL LPS and 7.5 ng/mL IL-4 with 0, 1, 2.5, 5, 7.5, and 10μM IM for 72 hours were 15.8%, 12.5%, 9.8%, 7.6%, 5.8%, and 2.9% of untreated controls, respectively. Reduction of IgG1 expression was induced by IM dose-dependently. (B) Real-time RT-PCR in spleen cells cultured in conditioning medium containing IL-4 and LPS for 72 hours indicated that expression of AID was decreased by IM dose-dependently. Significant differences were found between 0 and 1μM or 10μM IM. $^*P < .05$. The y-axis represents AID mRNA levels relative to the no-IM control. The levels of AID mRNA at each IM concentration were calculated relative to the internal control (GAPDH); n = 6. (C) The level of the germline transcript of IgG1 in spleen cells cultured in conditioning medium containing IL-4 and LPS for 72 hours was not decreased in contrast to AID mRNA expression levels, which were decreased by IM in a dose-dependent manner. Significant differences were found between 0, 1, and 10μM IM. $^*P < .05$. The y-axis represents expression levels of the IgG1 germline transcript relative to the no-IM control in the same manner as that in panel B; n = 4. (D) IgA expression levels in CH12F3-2A cells cultured in conditioning medium containing 7.5 μg/mL IL-4, 0.3 ng/mL TGF-β1, and 40% CD40 ligand with 0, 5, 10, and 20μM IM for 72 hours were reduced in an IM dose-dependent manner. (E) Cell proliferation, division, and apoptosis in spleen cells cultured in conditioning medium containing IL-4 and LPS for 72 hours were investigated using BrdU, annexin V, and CFSE assays. The BrdU incorporation rate of 10μM IM was 45.9%, whereas that of 0μM IM was 58.1%. Cell fluorescence of 10μM IM using the CFSE assay was shifted, but that of 1μM IM was not shifted to the right compared with that of 0μM IM. Annexin V analysis of 10μM IM was 39.9%, whereas that of 0μM IM was 35.1%. These results indicate that IM affects cell proliferation but not apoptosis. (F) Immunohistochemical analysis of spleens from mice that were administered SRBC with or without IM. Serial sections of spleens were prepared from nonimmunized (i,iv,vii,x), SRBC-immunized (ii,v,viii,xi), or SRBC-immunized + IM (50 mg/kg; iii,vi,ix,xii) animals. (i-vi) H&E staining. (vii-xii) Immunohistochemical analysis of AID. Low-power fields are shown in panels i to iii and vii to ix. High-power fields are shown in subpanels iv to vi and x to xii. Individual germinal centers from SRBC-immunized IM (+) mice were significantly smaller than those from SRBC-immunized IM (−) mice and were comparable with those from nonimmunized mice. AID expression, which was induced in germinal center-activated B cells, was barely detectable in spleens of IM-treated mice but was strongly positive in those of nontreated mice. Moreover, IM significantly suppressed AID expression, even in the residual germinal centers. (G) Real-time RT-PCR analysis of AID and FACS analysis of IgG1 expression of spleen cells harvested from SRBC-immunized mice with or without 50 mg/kg IM. The top panel shows relative normalized AID mRNA expression, and the bottom panel shows surface IgG1 expression of total splenocytes. A significant difference was found between SRBC (+) and SRBC (+) IM 50 mg/kg (+) for both AID and IgG1. $^*P < .05$. The y-axis represents the relative ratio of the relative expression level of AID mRNA (top panel) and the percentage of surface IgG1 expression of total splenocytes (bottom panel). Normalized values obtained for SRBC (+) IM 50 mg/kg (+) were derived from SRBC (+); n = 2.

showed that AID, but not the germline transcript, was responsible for inhibition of CSR by IM. BrdU, CFSE, and annexin V analysis revealed that IM affected proliferation but not apoptosis (Figure 1E). Importantly, 1μM of IM did not decrease proliferation but down-regulates AID (Figure 1B,E). In addition, 5-fluorouracil decreased proliferation but did not down-regulate AID (supplemental Figure 1), suggesting that proliferation is not necessarily coupled with expression of AID. Therefore, it is

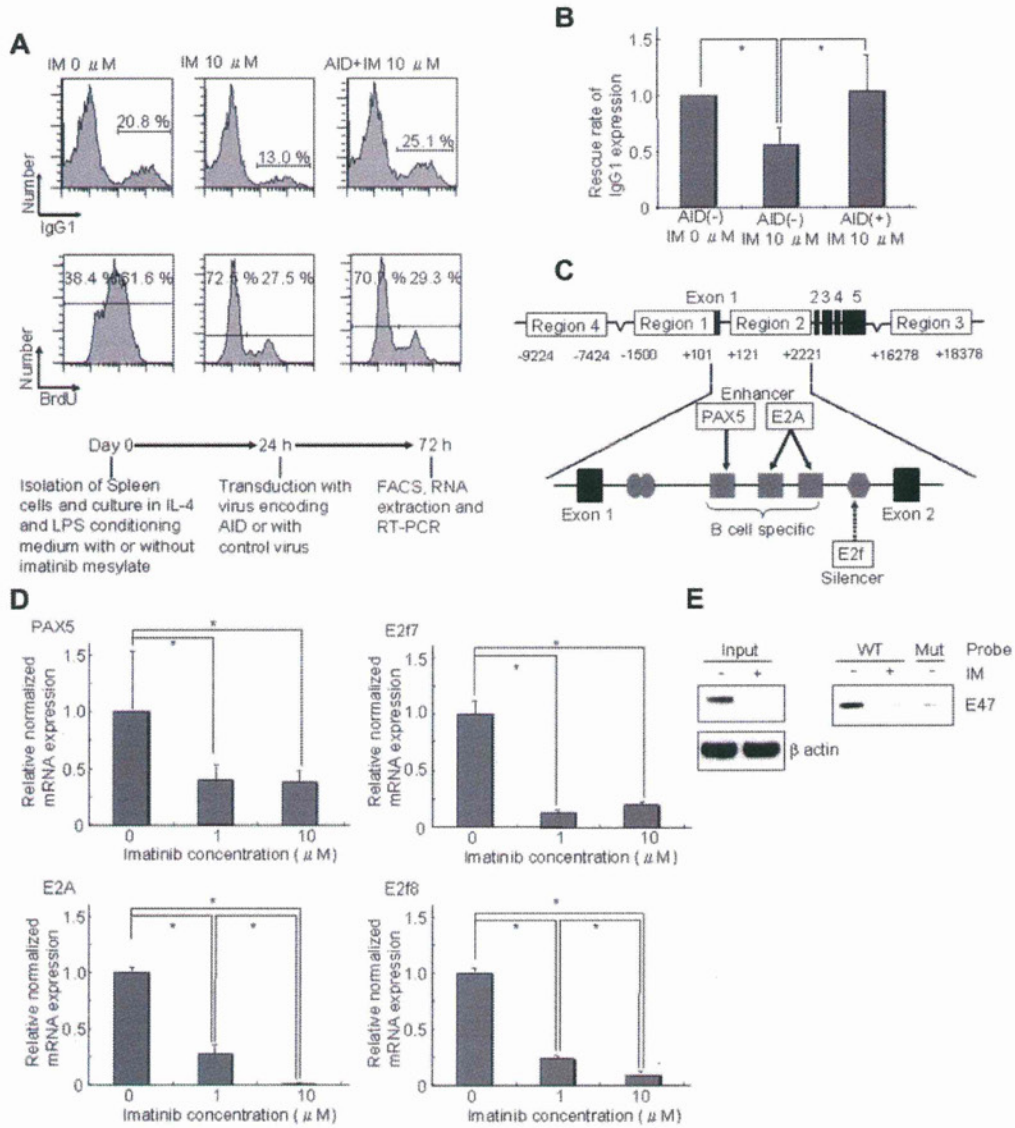


**Figure 2. Down-regulation of AID mediated by E2A, Pax5, E2f7, and E2f8 is responsible for CSR impairment by IM.** (A) Ectopic expression of AID completely rescued reduction of IgG1 expression caused by IM. Spleen cells were cultured in IL-4 and LPS conditioning medium with or without IM. After 24 hours of prestimulation culture, cells were transduced with retrovirus encoding AID-eGFP or retrovirus encoding eGFP only (control). After a further 48 hours, IgG1 expression was analyzed (bottom panel). A mononuclear cell fraction based on forward scatter/side scatter profiles was gated and sequentially subdivided into an eGFP-positive fraction. This eGFP-positive fraction was analyzed. Ectopic AID expression with 10μM IM increased IgG1 expression from 13.0% to 25.1% versus 20.8% without IM. The BrdU assay revealed that DNA synthesis decreased in the 10μM IM culturing condition. Although the BrdU assay was similar with or without ectopic expression of AID, IgG1 expression was completely rescued by ectopic expression of AID. (B) Average of the IgG1 expression rescue rate among 4 rescue experiments. IgG1 expression of AID(+) IM at 10μM was completely rescued by ectopic expression of AID. (C) Schema illustrating transcriptional binding sites in the *Aicda* gene promoter region, focusing particularly on region 2 in the first intron. PAX5 and E2A activate the *Aicda* promoter, whereas E2f7 and E2f8 have silencing effects. (D) The expression levels of 4 transcriptional factors (PAX5, E2A, E2f7, and E2f8) in spleen cells cultured in conditioning medium containing IL-4 and LPS for 72 hours were determined by real-time RT-PCR. All were reduced by IM. E2A expression was most markedly reduced.*$P < .05$. The y-axis represents mRNA levels of the PAX5, E2A, E2f7, and E2f8 relative to the no-IM control. Levels of each transcriptional factor mRNA were calculated relative to the internal control (GAPDH); n = 2. (E) Protein expression and DNA-binding activity of E2A in spleen cells cultured in conditioning medium containing IL-4 and LPS for 72 hours. The E2A gene encodes 2 transcription factors: E12 and E47. Western blot analysis revealed that expression of E47 in splenocytes cultured in conditioning medium containing LPS and IL-4 was down-regulated by IM to a barely detectable level. DNA affinity precipitation analysis of the same cell extracts using biotinylated E-box probe and its mutant revealed that E-box binding activity of E47 in the extracts was similarly reduced by IM.

possible to differentiate the effect of IM on proliferation and AID expression.

To further confirm that CSR is impaired by IM through down-regulation of AID in vivo, immunohistochemical analysis was performed on splenic tissues from nonimmunized and sheep red blood cells–immunized C57BL/6 mice with or without IM treatment (Figure 1F). The individual germinal centers from SRBC-immunized IM (+) mice were significantly smaller than those from SRBC-immunized IM (−) mice and comparable with those from nonimmunized mice (Figure 1F). As expected from these findings, AID expression, which is induced in germinal center-activated B cells, was barely detectable in the spleens of IM-treated mice but was strongly positive in those of nontreated mice. In addition, IM significantly suppressed AID expression,

even in the residual germinal centers. Expression of AID was confirmed by real-time RT-PCR analysis. The results of IgG1 expression did not conflict with these results (Figure 1G). Compatible with the results obtained by in vitro stimulation of spleen cells, IM down-regulated expression of IgG1 as well as AID. Although enlargement of germinal center formation has been reported in AID knockout mice,[1] it is assumed that the immunomodulatory effects of IM on B cells, T cells, and dendritic cells[6,7] resulted in impairment of germinal center formation in our system.

Furthermore, we investigated whether ectopic expression of AID could rescue inhibition of CSR by IM. IgG1 expression in spleen cells decreased with IM treatment, whereas ectopic expression of AID completely rescued impairment of CSR under the condition that cell proliferation was suppressed by IM (Figure 2A-B). The results indicated that impairment of CSR by IM was at least in part the result of down-regulation of AID.

Finally, we examined the mechanism of down-regulation of AID by IM. Recently, Tran et al reported that *Aicda* regulation involved derepression by several layers of positive regulatory elements in addition to the 5′-promoter region.[5,13] Promoter region 2 in the first intron contains the functional binding elements for the ubiquitous silencers c-Myb and E2f and for the B cell–specific activators Pax5 and E2A (Figure 2C).[5,13] Surprisingly, all of these transcription factors were down-regulated by IM. Among them, expression of E2A was most markedly reduced (to 1 of 500) by IM (Figure 2D). We further found that levels of E2A protein as well as E-box binding activity were markedly reduced by IM (Figure 2E), suggesting that down-regulation of E2A by IM causes significant suppression of AID.

For the first time, our findings elucidate a mechanism of hypogammaglobulinemia caused by IM, which has been observed frequently in IM-treated chronic myeloid leukemia patients.[8,9] Its adverse effects as well as the immunomodulatory functions of each drug and their underlying mechanisms must be examined in more extensive studies.

AID was previously reported to be induced by BCR-ABL1 in $Ph1^+$ pre B-ALL cell lines and inhibited by IM through ID2 up-regulation. Interestingly, neither PAX5 nor E2A showed changes in expression.[14] In the present study using normal mature B cells, PAX5 and E2A levels were significantly decreased by IM, whereas ID2 was not increased (data not shown). PDGFR[15] and c-kit,[16] kinases that are also inhibited by IM, were not expressed in mature B cells. Together, these results could be induced by the off-target multikinase inhibitory effects of IM. The results of microarray analysis (supplemental Table 1; supplemental Figures 2-3) are consistent with this hypothesis. All microarray data are available for viewing on the Gene Expression Omnibus under accession number GSE35559.

Inappropriate expression of AID affects many diseases, such as malignancy and autoimmune diseases.[17,18] It probably also affects allergic disorders because AID is also essential to CSR from IgM to IgE, deregulation of which is an important causative factor of allergic disorders. The results of the present study suggest that IM, which has been used safely for several decades in clinical settings, can be used for various diseases involving AID. Indeed, dramatic resolution by IM has been reported in several cases of rheumatoid arthritis or asthma complicated with chronic myeloid leukemia.[19,20]

In conclusion, suppression of AID by IM is responsible for CSR impairment, leading to the frequent adverse effects of IM. IM may also be clinically useful as an AID suppressor.

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## Authorship

Contribution: T.K., J.L., T.S., A.K., and M.T. designed, performed, and analyzed the experiments and wrote the manuscript; T.T., H.N., Y.A., K.Y., N.O., and N.N. contributed vital reagents; K.A. collected the clinical samples; and A.T. supervised the research.

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# Leukemogenic Fusion Gene (p190 BCR-ABL) Transduction into Hematopoietic Stem/Progenitor Cells in the Common Marmoset

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## ABSTRACT

Patients with Philadelphia chromosome (p190 BCR-ABL fusion gene)-positive acute lymphoblastic leukemia have a poor prognosis despite intensive therapeutic intervention. In this study, we attempted to develop a leukemia nonhuman primate model that mimics various human systems. Hematopoietic stem/progenitor cells in the common marmoset were transduced with a lentiviral vector containing the p190 BCR-ABL fusion gene by *ex vivo* transduction or *in vivo* direct bone marrow injection. In the latter model, BCR-ABL gene expression was maintained for more than one and a half years. One marmoset unexpectedly developed myelofibrosis-like disease. However, none of the marmosets have developed leukemia to date. In conclusion, we successfully achieved sustained p190 BCR-ABL gene expression *in vivo*. However, a genetic mutation in addition to p190 BCR-ABL may be required for the malignant transformation of hematopoietic stem/progenitor cells in the common marmoset during the short observation period. This novel *in vivo* approach will help develop a marmoset leukemia model in the future.

**Keywords:** Leukemia; Lentiviral Vector; Myelofibrosis; Common Marmoset

## 1. Introduction

Many preclinical *in vivo* studies have been conducted in mice because they are easy to breed and their biology and genetics are well-characterized. However, humans and mice differ genetically, pathophysiologically and pharmacokinetically, which makes it difficult to extrapolate the results from mouse models for direct clinical applications in humans. Large animals, especially nonhuman primates, are more closely related to humans. Moreover, because of their long life span, nonhuman primates can be treated and monitored over a long period, which presents opportunities for time-varying sampling of their blood and bone marrow. Thus, the development of non-human primate models that mimic human pathophysiology and pharmacokinetics will significantly further our understanding of human diseases. Particularly, genetically modified primates will be a powerful human disease model that can be used to preclinically assess the safety and efficacy of developing drugs.

Currently, Old World primates, such as the rhesus monkey (*Macaca mulatta*) and cynomolgus monkey (*Macaca fascicularis*), are commonly used for research [1-3]. However, these primates have several disadvantages, such as a slow sexual maturation period of approximately three years, fewer offspring over the female lifespan, and difficulty in handling.

The common marmoset (*Callithrix jacchus*) is a small New World primate that has attracted considerable attention as a potential animal for biomedical research [4,5]. The common marmoset is small, weighing approximately 350 - 400 g, relatively easy to breed, has a short gestation period of approximately 144 days, reaches sexual maturity at 12 - 18 months, and produces 40 - 80 offspring during the female lifespan. Thus, although marmosets are

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not as closely related to humans as apes or Old World primates, they are valuable as a potential primate model of human disease.

In this study, we attempted to establish a marmoset leukemia model by introducing a fusion gene that causes leukemia in humans. The Philadelphia chromosome (Ph) contains one of several forms of BCR and c-ABL gene fusions, and these fusions substantially contribute to the pathogenesis of chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL). The p190 BCR-ABL fusion gene, in which BCR exon 1 is joined to ABL exon 2 (e1a2) and produces the p190 protein, is detected in 20% - 35% of ALL patients, and the prognosis of these patients is particularly poor [6,7]. Several treatments, such as allogeneic hematopoietic stem cell transplantation and novel small molecules that directly target the p190 BCR-ABL fusion gene have been developed to treat this refractory disease [8]. Transduction of the p190 BCR-ABL fusion gene is reportedly sufficient to cause leukemia in mice [9-11]. However, to date, there are no reports of a primate leukemia model. Establishing a marmoset model of this disease will be useful to test the efficacy of current and future treatments. Therefore, we transduced the p190 BCR-ABL fusion gene into marmoset $CD34^+$ hematopoietic stem/progenitor cells using a lentiviral vector and examined the occurrence of leukemogenic events.

## 2. Materials and Methods

### 2.1. Cell Lines

Ba/F3 cells, a mouse interleukin-3 (mIL-3)-dependent hematopoietic cell line, were maintained in RPMI (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and mIL-3 (10 ng/mL) at 37°C in 5% $CO_2$ and passaged twice every week.

### 2.2. Animals and Preparation of Bone Marrow and Peripheral Blood Mononuclear Cells

Common marmosets were purchased from the Division of Animal Experimentation, Central Institute for Experimental Animals (Kawasaki, Japan), and bred at the animal center at our institute. In this study five animals were used including one control marmoset. No. 591 (male, 2 years and 6 months old) and No. 2338 (female, 4 years and 9 months old) were used as *ex vivo* BCR-ABL transduction models. No. 2129 (female, 5 years and 7 months old) and No. 2223 (female, 5 years and 3 months old) were used as BCR-ABL direct *in vivo* injection models. The study protocol was approved by the animal ethical committee of the University of Tokyo.

Bone marrow samples were collected by flushing the femurs of euthanized animals or aspirating the femoral bone marrow with an aspiration needle (Task, Tochigi, Japan). Peripheral blood samples were collected with heparin. Mononuclear cells (MNCs) in each sample were isolated by density-gradient centrifugation with Lymphoprep (Axis-Shield, Oslo, Norway). The cells were frozen in liquid nitrogen until further use.

### 2.3. Construction, Production and Transduction of Lentiviral Vector

Third generation, VSV.G pseudotyped lentiviral vectors were produced by transiently cotransfecting four plasmids into 293T cells as previously described [12]. Briefly, the p190 BCR-ABL fusion gene driven by a CMV or PGK promoter (HIV-CMV/PGK-BCR-ABL) was inserted into the transfer vector [13]. This plasmid was co-transfected into 293T cells using the calcium-phosphate method. The viral supernatant was harvested 48 and 72 hrs post transfection. The viral pellet was collected by ultra-centrifuging the supernatant and then stored at −80°C. The DNA titer, which is known to reflect the amount of transducible vector genome, was determined by real-time quantitative PCR as previously described [14]. For transduction, a cell pellet ($2 \times 10^5$) was mixed and incubated with concentrated viral supernatant (20 μL) for 2 hrs in a 37°C incubator. The cells were generally infected *in vitro* with an MOI (multiplicity of infection) of 2.

### 2.4. Detection of p190 BCR-ABL Transgene Expression

RNA extraction and reverse transcription were performed as previously described using an RNA/DNA extraction kit (Qiagen, Hilden, Germany) and SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Nested PCR amplification of p190 BCR-ABL was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) with primers that specifically amplify the e1a2 transcripts. A 50 μL reaction mixture containing 2 mM of each dNTP, 25 mM $MgCl_2$, 10× PCR buffer, 0.5 μM primers, 1.25 U AmpliTaq Gold (Applied Biosystems) and 10 ng cDNA was subjected to 40 cycles of denaturation (95°C, 30 sec), annealing (61°C, 30 sec), and extension (72°C, 30 sec) and another 40 cycles with the inner primer set of denaturation (95°C, 30 sec), annealing (57°C, 30 sec), and extension (72°C, 30 sec). The final products were analyzed on a 1% agarose gel stained with ethidium bromide. The outer BCR-ABL primer set was forward primer (5'-CGC TCT CCC TCG CAG AAC TC-3') and reverse primer (5'-GGA GTG TTT CTC CAG ACT GTT GAC TG-3'), while the inner primer set was forward primer (5'-AAC AGT CCT TCG ACA GCA GCA-3') and reverse primer (5'-GCG TGA TGT AGT TGC TTG GGA-

 

3'). Sequencing analysis showed that the amplified products were compatible with the p190 BCR-ABL fusion gene (data not shown). Expression of p190 BCR-ABL protein was confirmed by western blot analysis in our previous report [15]. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was simultaneously amplified as an internal control. In some experiments, real-time quantitative PCR analysis of p190 BCR-ABL was performed as previously described [16].

## 2.5. Enrichment of $CD34^+$ Bone Marrow Cells

Marmoset bone marrow $CD34^+$ cells were isolated with the immunobeads system using streptavidin microbeads (MACS, Miltenyi Biotec, Sunnyvale, CA, USA). Marmoset bone marrow MNCs were prepared from fresh bone marrow samples and stained with a biotin-labeled anti-marmoset CD34 monoclonal antibody (clone MA24) for 30 min at 4°C [17]. The cells were washed and incubated with streptavidin-conjugated microbeads for 15 min at 4°C. The $CD34^+$ cells were washed and separated using immunomagnetic columns (MACS) according to the manufacturer's instructions.

## 2.6. *Ex Vivo* p190 BCR-ABL Transduction and Autologous Peripheral Blood Stem Cell Transplantation (PBSCT)

The time course and treatment protocol are described in **Table 1**. Briefly, 10 μg/kg/day of recombinant human granulocyte colony-stimulating factor (G-CSF, Roche, Basel, Switzerland) was subcutaneously administered to individual marmosets for five days. Peripheral blood (1 mL) was collected daily from the femoral vein of each marmoset for seven consecutive days. MNCs were isolated by Ficoll-Hypaque centrifugation. Red cell lysis was performed when necessary. The cells were frozen using a programmed freezer and stored in liquid nitrogen until use. Ninety milligrams of busulfan (Sigma Aldrich, St Louis, MO, USA) was dissolved in 15 mL of DMA/PEG (1:2) solution, and a 6 mg/mL busulfan solution was made. All marmosets received the busulfan solution (10 mg/kg) from day −3 to day −2 before PBSCT as previously described [18,19].

Three days before transplantation, the MNCs were thawed and incubated overnight in IMDM supplemented with 10% FBS and human cytokines (10 ng/mL hSCF, hIL-3 and hTPO) as previously described with some modifications [12]. After pre-stimulation, the cells were collected and centrifuged. The cell pellet was incubated with the concentrated viral supernatant at 37°C in 5% $CO_2$ for 2 hrs. The cells of two marmoset (No. 591 and 2338) were infected with the CMV-p190 BCR-ABL lentiviral vector. Then, the cells were cultured in IMDM supplemented with 10% FBS and human cytokines (hSCF, hIL-3, hTPO) in 24-well plates for 48 hrs. The transduced cells were collected, filtered with 40 μm nylon mesh, washed with serum-free IMDM and suspended in 1.5 mL normal saline. The cells ($2 \times 10^6$) were transplanted via the femoral vein using a syringe and 27 G needle.

## 2.7. Direct Injection of Lentiviral Vectors into the Bone Marrow

The time course and treatment protocol are described in **Table 2**. Briefly, marmosets received 25 mg/body of 5-fluorouracil (Kyowa Hakko Kirin, Tokyo, Japan) on day −5 and prednisolone from day −3 to day 1. After anesthesia, *in vivo* transduction of bone marrow cells was performed by directly injecting the viral supernatant into the bone marrow cavity using an aspiration needle on day 0. One marmoset (No. 2129) received the CMV-p190 BCR-ABL lentiviral vector, whereas the other (No. 2223) received the PGK-p190 BCR-ABL lentiviral vector. All marmosets received both oral fluconazol (2 mg/body/day) and intramuscular injections of ampicillin (10 mg/body/day) from day −5 to day 7 and pentamidine (1.5 mg/body/day) once on day −5.

## 2.8. Colony Formation Assay

A colony formation assay (progenitor cell assay) was performed as previously described with minor modifications [17]. Bone marrow cells were washed twice, plated in methylcellulose containing human hematopoietic

**Table 1. Time coure and treatment of *ex vivo* BCR-ABL transduction method.**

| Treatment | −14 | −13 | −12 | −11 | −10 | −9 | −8 | −7 | −6 | −5 | −4 | −3 | −2 | −1 | 0 | (days) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G-CSF (10 μg/body) | • | • | • | • | • | | | | | | | | | | | |
| PBMC collection | | | | | • | • | • | • | • | • | • | | | | | |
| Busulfan (10 mg/body) | | | | | | | | | | | | • | • | | | |
| *ex vivo* gene transduction (p190 BCR-ABL) | | | | | | | | | | | | | • | • | | |
| PBSCT | | | | | | | | | | | | | | | ▼ | |

G-CSF: granulocyte colony stimulationg factor; PBMC: peripheral blood mononuclear cell; PBSCT: peripheral blood stem cell transplantation.

 

**Table 2. Time coure and treatment of BCR-ABL direct *in vivo* injection method.**

| Treatment | –5 | –4 | –3 | –2 | –1 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | (days) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5FU (25 mg/body) | • | | | | | | | | | | | | | |
| PSL (8 mg/body) | | | • | • | • | | | | | | | | | |
| PSL (4 mg/body) | | | | | | • | | | | | | | | |
| PSL (2 mg/body) | | | | | | | • | | | | | | | |
| BM injection (p190 BCR-ABL) | | | | | | ▼ | | | | | | | | |
| Pentamidine (1.5 mg/body) | • | | | | | | | | | | | | | |
| ABPC (10 mg/body) | • | • | • | • | • | • | • | • | • | • | • | • | • | |
| FCZ (2 mg/body) | • | • | • | • | • | • | • | • | • | • | • | • | • | |

5FU: 5-fluorouracil, PSL: predonisolone, BM: bone marrow, ABPC: ampicillin, FCZ: fluconazole

cytokines (Methocult GF+, StemCell Technologies, Vancouver, BC, Canada) and incubated at 37°C in 5% $CO_2$. Then, $1.5 \times 10^3$ MACS-sorted $CD34^+$ cells or $3 \times 10^4$ non-sorted cells were plated in one dish. On day 14, individual colonies were picked to identify BCR-ABL gene transduction by RT-PCR and examined by May-Giemsa staining.

## 3. Results

### 3.1. Lentiviral Vector Expressing p190 BCR-ABL Functionally and Efficiently Transduced Hematopoietic Stem/Progenitor Cells

First, we produced third generation VSV.G pseudotyped lentiviral vectors expressing the p190 BCR-ABL fusion gene (HIV-CMV/PGK-BCR-ABL). Plasmids including the lentiviral transfer vector were transduced into 293T cells by the calcium phosphate method. After 48 and 72 hrs, the viral supernatants were collected and ultra-centrifuged. The DNA titers of HIV-CMV-BCR-ABL and HIV-PGK-BCR-ABL were $5.59 \times 10^7$/ml and $2.53 \times 10^8$/ml, respectively. To determine that the vector functions properly, Ba/F3 cells, a mIL-3-dependent murine hematopoietic cell line, were transduced with this vector and cultured without mIL-3. These cells rapidly expanded after 12 days, indicating that p190 BCR-ABL gene expression allowed the Ba/F3 cells to grow autonomously regardless of the promoter used (**Figure 1(a)**). RT-PCR confirmed that p190 BCR-ABL was expressed in the transduced Ba/F3 cells (**Figure 1(b)**). We then performed quantitative RT-PCR (**Figure 1(c)**). Although the MOI of the lentiviral vector was not high, p190 BCR-ABL expression was detected in the Ba/F3 cell line. Next, to check whether marmoset hematopoietic stem/progenitor cells were efficiently transduced with the lentiviral vector, MACS-sorted bone marrow $CD34^+$ cells were transduced with the lentiviral vector (HIV-CMV/PGK-BCR-ABL) and subjected to the colony formation assay (**Figure 1(d)**). In more than 80% of colonies examined, p190 BCR-ABL transduction was detected regardless of the promoter. Taken together, the above findings indicate that this oncogene was efficiently transduced into marmoset stem/progenitor cells.

### 3.2. *Ex Vivo* BCR-ABL Transduction Method

To establish a marmoset leukemia model, we first tried the *ex vivo* BCR-ABL transduction method (**Table 1**). After mobilizing the hematopoietic stem/progenitor cells by administering G-CSF, peripheral blood MNCs were collected according to the schedule in **Table 1**. The collected cells were pre-cultured with cytokines (10 ng/ml hSCF, hIL-3 and hTPO) to improve the transduction efficiency. After the cells were transduced with the lentiviral vector containing the p190 BCR-ABL fusion gene *ex vivo*, they were transplanted into common marmosets that were previously treated with pre-conditioning busulfan (**Table 1**). On days 28 and 56 post transplantation, BCR-ABL expression was detected by RT-PCR in the peripheral blood MNCs of two marmosets (**Figure 2(a)**, upper). The time course for BCR-ABL expression after transplantation is shown in **Figure 2(a)** (lower). Although we expected stable BCR-ABL expression, BCR-ABL expression disappeared after days 56 and 100 in two marmosets. Because this result was thought to partially result from low-level transgene expression due to ineffective gene transduction in hematopoietic stem/progenitor cells, we changed the gene transduction method.

### 3.3. *In Vivo* BCR-ABL Direct Transduction Method

Previous reports showed that directly injecting lentiviral vectors *in vivo* resulted in stable gene expression [20].

 

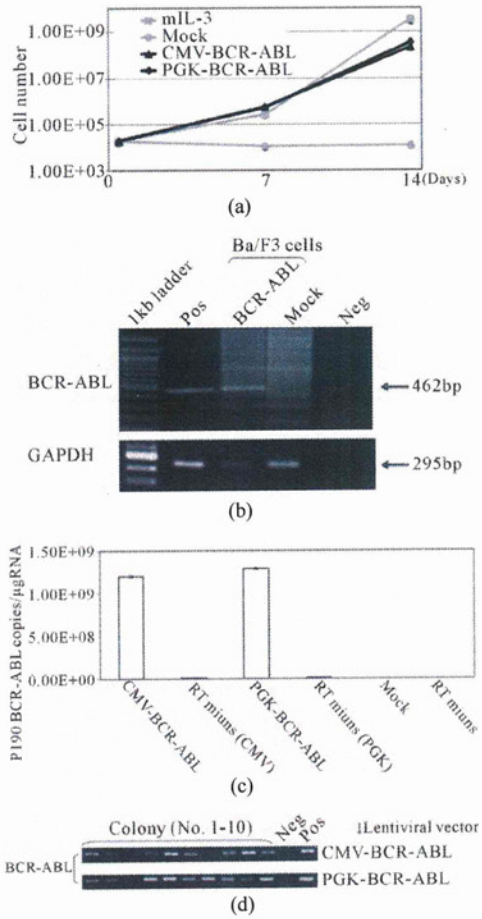


**Figure 1. (a) Ba/F3, a murine hematopoietic cell line that is dependent on mouse interleukin-3 (mIL-3), was transduced with the p190 BCR-ABL lentiviral vector (HIV-CMV/ PGK-BCR-ABL) and cultured without mIL-3. As positive and negative controls, mock transfected cells were cultured with or without mIL-3. The number of live cells was determined by the trypan blue exclusion method. All experiments were done in triplicate and also repeated three independent times, and data were plotted as mean ± SD; (b) Detection of the BCR-ABL fusion gene in transduced Ba/F3 cells. Three days after lentiviral transduction, RT-PCR for the BCR-ABL gene was performed in BCR-ABL- or mock-transduced cell lines. Pos: positive control (KOPN30 cell line harbouring p190 BCR-ABL), Neg: negative control (ddw: deionized distilled water); (c) Quantitative analysis of transduced p190 BCR-ABL fusion gene expression. Three days after lentiviral transduction, quantitative RT-PCR for p190 BCR-ABL was performed. RT minus indicates the control PCR reaction without reverse transcriptase; (d) Lentiviral transduction of the BCR-ABL gene into colony-forming hematopoietic stem/progenitor cells. MACS-sorted marmoset CD34$^+$ cells were transduced with the lentiviral vector expressing p190 BCR-ABL under a CMV or PGK promoter, and then 1.5 × 10$^3$ cells were plated in methylcellulose containing human hematopoietic growth factors. After 14 days of culture, colonies were randomly picked and examined for BCR-ABL gene expression by RT-PCR.**

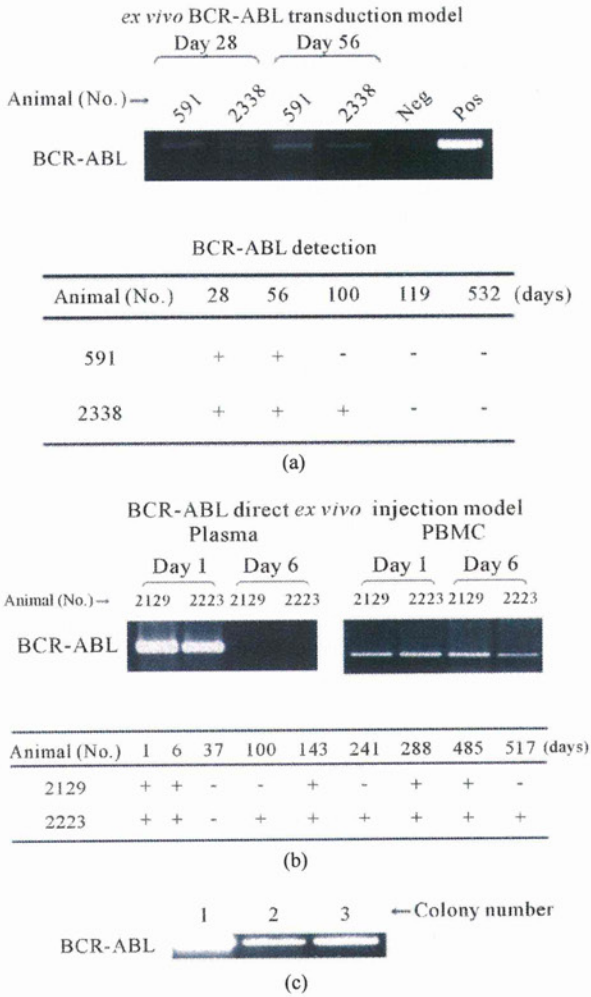


**Figure 2. (a) Detection of the BCR-ABL fusion gene with the *ex vivo* BCR-ABL transduction method. Upper: RT-PCR of the BCR-ABL fusion gene in samples on day 28 and 56 after the BCR-ABL-transduced CD34$^+$ cells were transplanted. Lower: detection of BCR-ABL in samples from day 28 to 532 in two marmosets. BCR-ABL expression disappeared in two marmosets after day 56 and 100. GAPDH expression was confirmed for all samples (data not shown). No. 591 and No. 2338: CMV-p190 BCR-ABL; (b) Detection of BCR-ABL fusion gene with the *in vivo* BCR-ABL direct transduction method. Upper: RT-PCR of the BCR-ABL fusion gene in plasma and PBMC samples on days 1 and 6. Lower: detection of BCR-ABL in samples from day 1 to day 517 in two marmosets. Compared to the *ex vivo* BCR-ABL transduction method, two marmosets maintained long-term BCR-ABL expression. No. 2129: CMV-p190 BCR-ABL. No. 2223: PGK-p190 BCR-ABL; (c) Detection of the BCR-ABL fusion gene in colony-forming hematopoietic stem/progenitor cells. Bone marrow MNCs were isolated from marmoset No. 2223 on day 517, and 3 × 10$^4$ cells were plated in methylcellulose containing several human hematopoietic growth factors. After 14 days of culture, colonies were randomly picked and examined for the BCR-ABL gene. Three out of nine colonies were positive for BCR-ABL.**

 

Therefore, we administered an immunosuppressive pretreatment therapy consisting of 5-fluouracil and prednisolone and then injected the lentiviral vector into the bone marrow cavity (**Table 2**). BCR-ABL was detected in the plasma on day 1 but not day 6. In contrast, BCR-ABL was detected in peripheral blood MNCs on day 1 and 6 in two marmosets. **Figure 2(b)** (lower) shows the time course of BCR-ABL expression after injection. With this *in vivo* BCR-ABL direct transduction method, BCR-ABL expression was sustained in two marmosets (No. 2129: positive until day 485, No. 2223: positive until day 517). To determine if the p190 BCR-ABL fusion gene was successfully transduced into hematopoietic stem/progenitor cells, we performed a colony formation assay. Thirty thousand bone marrow MNCs were isolated from marmoset No. 2223 on day 517 and plated in methylcellulose. After 14 days of culture, random colonies were examined for BCR-ABL gene expression. Three of nine colonies were positive for BCR-ABL expression (**Figure 2(c)**). In the same experiment, all colonies for the control marmoset were negative for BCR-ABL expression (data not shown).

### 3.4. Myelofibrosis in a Marmoset That Was Directly Transduced with BCR-ABL *in Vivo*

Marmoset No. 2223, which received a direct *in vivo* injection and maintained BCR-ABL expression, became lethargic and lost weight. A blood cell count indicated anemia and thrombocytopenia (**Figure 3(a)**). This marmoset was sacrificed on day 686 post injection and examined pathologically. BCR-ABL expression was detected in the spleen, liver, kidney, heart and peripheral blood MNCs by RT-PCR. However, the bone marrow sample was negative for BCR-ABL. Hematoxylin-Eosin staining of the bone marrow showed that the bone marrow cavity was replaced by a marked noncellular component (**Figure 3(b)**). Bone marrow fibrosis was confirmed by Masson staining. An examination of the liver revealed extramedullary hematopoiesis. Naphthol AS-D chloroacetate (ASD) and Myeloperoxidase (MPO) staining confirmed that the cells were of myeloid lineage (**Figure 3(c)**).

## 4. Discussion

Previously, we reperted the usefulness of the common marmoset as a hematopoietic stem/progenitor cell transplantation model, analytic results of major histocompatibility antigens, and the production of anti-marmoset CD34 monoclonal antibodies [4,17,21,22]. These studies helped to establish and analyze a primate disease model. In this study, we lentivirally transduced common marmoset hematopoietic cells with the p190 BCR-ABL fu-

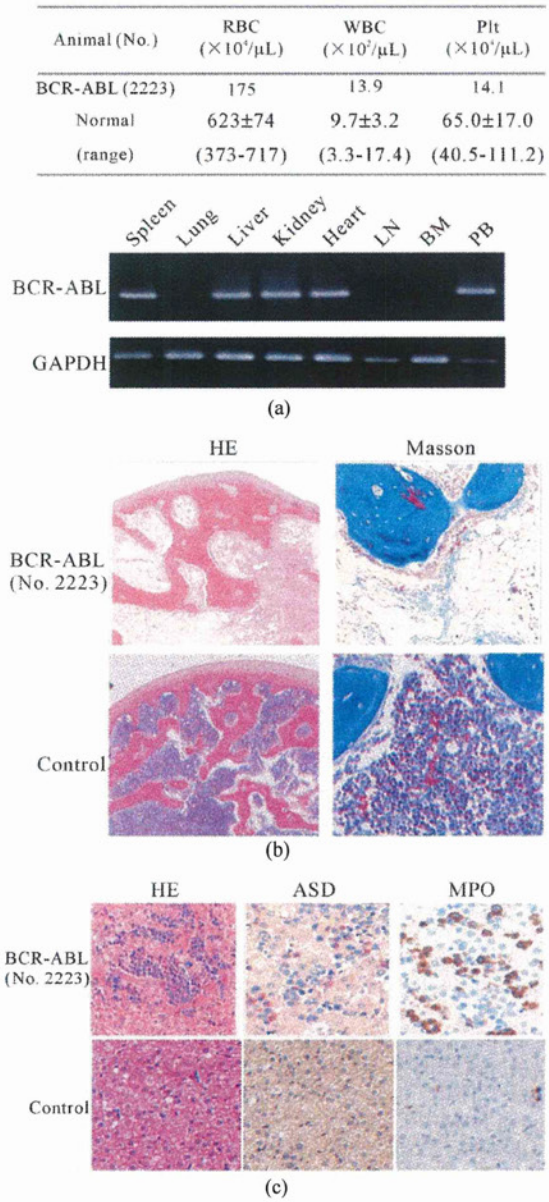


**Figure 3. Pathological examination of marmoset No. 2223 (transduced using the *in vivo* BCR-ABL direct transduction method) with myelofibrosis-like disease. (a) Upper: Peripheral blood count of No. 2223 on day −7 before sacrifice. Data on the blood cell count of healthy marmosets were obtained from a textbook. Lower: The marmoset was sacrificed and pathologically examined on day 686 after p190 BCR-ABL was directly injected into the bone marrow. BCR-ABL gene expression was detected by a RT-PCR method in several organs. No. 2223: PGK-p190 BCR-ABL; (b) Left: Hematoxylin-Eosin (HE) staining of the bone marrow. Right: Masson staining. Bone marrow fibrosis is stained in blue. Upper: Experimental marmoset (No. 2223). Lower: Control marmoset; (c) Extramedullary hematopoiesis in the liver in the experimental marmoset (upper). ASD: Naphthol AS-D chloroacetate stain, MPO: Myeloperoxidase stain.**

 

sion gene using either an *ex vivo* BCR-ABL transduction method or *in vivo* BCR-ABL direct transduction method. Then, we examined the expression of the fusion oncogene in transduced progenitor cells *in vivo* and monitored the marmosets for leukemogenic events.

With the *ex vivo* BCR-ABL transduction method, BCR-ABL expression was not detected by RT-PCR after day 100 (**Figure 2(a)**). This lack of sustained expression may result from a low gene transduction efficiency of hematopoietic stem/progenitor cells even though the high transduction efficiency at progenitor levels. Additionally, the transduced cells may have been immunologically rejected. Anti-human G-CSF neutralizing antibodies appeared in the transplanted marmosets even with an immunosuppressive pre-conditioning regimen (data not shown). The presence of these antibodies supports the latter possibility. Majority of the marmosets used in this study were four to six years old, which is an immunologically competent and equivalent to an adult human. Treating the marmosets with immune suppressants might solve this problem.

Previously, our group successfully transduced G-CSF-mobilized hematopoietic stem/progenitor cells in a marmoset model with the MDR1 gene using a retroviral vector [21]. In this study, a consistent increase in progenitor cells in the peripheral blood was observed using the same G-CSF mobilization protocol. Retroviral vectors mainly transduce dividing cells. In contrast, lentiviral vectors transduce both dividing and non-dividing cells [12,20]. And recent reports showed that the retroviral vector, but not lentiviral vector, is integrated near transcriptional genes and induce leukemia [23]. Moreover, in a previous study, we successfully transduced cord blood hematopoietic stem/progenitor cells using a lentiviral vector [12]. For these reasons, we chose a lentiviral vector to transduce p190 BCR-ABL in order to obtain a high transduction efficiency. However, the p190 BCR-ABL gene is approximately 6 kilobase pairs in length, and its large size hinders the production of a high-titer lentiviral vector. This limitation may be one reason why BCR-ABL was expressed for a limited time with the *ex vivo* BCR-ABL transduction method (**Figure 2(a)**).

This was our first attempt to directly inject a lentiviral vector *in vivo* into the bone marrow of common marmosets, although a previous study reported efficient gene transduction by direct injection into the central nervous system of rats [24]. BCR-ABL was detected in the plasmaon day 1 but not day 6. It is likely that vectors injected into the bone marrow, which is rich in blood vessels, transiently leaked into the circulation. Of note, we observed long-term BCR-ABL expression in hematopoietic cells (**Figure 2(b)**). Further optimization of the experimental procedures, such as altering the pre-conditioning regimen, using a higher titer vector, and treating with immunosuppressive drugs after injecting the lentiviral vector, might be effective to obtain stable gene expression in hematopoietic stem/progenitor cells *in vivo*.

To date, mouse models have been predominantly used to study human cancers [25]. However, primates are more genetically related to humans than rodents. For this reason, much effort has focused on establishing a primate model that can be used to more precisely evaluate new cancer therapies in pre-clinical studies. However, thus far, there have been no successful reports of a primate model that mimics human cancers, and this study also faced challenges. The primary reason for these difficulties may be because primate and rodent hematopoietic stem/progenitor cells have different susceptibilities to oncogene transduction [26,27]. In gene therapy clinical trials in France for X chromosome-linked severe combined immune deficiency, leukemia developed two to three years after the common $\gamma$ chain receptor gene was transduced into hematopoietic stem/progenitor cells [26-28]. In contrast, in a mouse model of oncogene transduction, the duration of developing leukemia is generally less than one year [9-11]. Therefore, these findings support the hypothesis mentioned above.

Moreover, the susceptibility to malignant transformation by each oncogene reportedly differs based on age in humans. For example, for the chromosomal translocations in ALL, MLL-AF4 is dominant in infants, TEL-AML1 in children, and BCR-ABL in adults [29]. Furthermore, cord blood cells were used in all previous reports of BCR-ABL transduction into human hematopoietic stem/progenitor cells in a SCID mouse model [30]. There have been no studies that used adult hematopoietic progenitor cells. In this study, all of the marmosets were adults. Considering these findings, it will be important to consider using younger marmosets in future studies.

Furthermore, it is well known that multiple genetic mutations are required for leukemogenesis [31]. The number of required gene mutations may not be the same between primates and rodents. In the marmoset model, subsequent gene mutations in addition to p190 BCR-ABL may be required for malignant transformation [32]. Thus, it may be necessary to test the cotransduction of p190 BCR-ABL and another oncogene, mutated tumor suppressor gene, or anti-apoptosis gene in order to achieve leukemogenesis in the marmoset model.

Unexpectedly, one marmoset that was transduced using the *in vivo* BCR-ABL direct transduction method developed myelofibrosis-like disease (**Figures 3(a)-(c)**). BCR-ABL gene expression was detected in various organs. However, BCR-ABL was not expressed in the lung, lymph nodes and bone marrow. BCR-ABL expression could be detected where extramedullar hematopoiesis

 