Table 1. Loss of non-inherited maternal HLA allele expression in infants 'maternal' cancer cells

	HLA-A	HLA-B	DRB1
Father	A2/A33	B61/B58	DR8/DR13
Mother	A24/**A11**	B60/**B67**	DR9/**DR15**
Patient	A24/A33 2402/3303	B60/B58 4001/5801	DR9/DR13 0901/1302
Patient's jaw mass	A24/-2402/-	B60/-4001/-	DR9/-0901/-

Alleles were serotyped or genotyped (2402/3303, 4001/5801, 0901/1302) (see *SI Text*). Insufficient material was available to genotype parental samples. Alleles in bold are maternal HLA alleles lost from maternal cancer cells transmitted to infant.

(>95% maternal cells). STR analysis of the PE sample showed a mixture of alleles for most STR markers analyzed. These mixtures were identified either because of the presence of three different alleles in some markers, or because those markers with only two alleles presented an imbalance in the ratio of the peak areas (Fig. 2). These findings were consistent with the presence of two genetically different cell populations in the PE specimen–one maternal, one infant offspring (see Fig. 2 legend).

HLA Analysis. Survival of maternal cells in the offspring presumably requires some form of immunological acceptance or tolerance of cells expressing foreign, non-inherited maternal MHC antigens. We serotyped samples from both parents for HLA-A, HLA-B, and DRB1, and both serotyped and genotyped the infant's normal blood and lymphoma cells. The result was that the *BCR-ABL1*-positive jaw tumor had selectively deleted or lost the HLA alleles that were not inherited by the daughter (Table 1). The nature of this genetic lesion in the infant tumor cancer cells was further explored by high resolution SNP arrays. In the absence of normal germline maternal and infant DNA and only small quantities of degraded DNA from the maternal leukemia biopsy, we elected to analyze the infant lymphoma (jaw) sample for genome-wide lack of heterozygosity (LOH) in comparison with pooled normal control DNA. Table 2 summarizes the LOH analysis of the infant lymphoma. Recurrent deletions of *IKZF1* and *CDKN2A/B* have previously been described in *BCR-ABL1* ALL (7), as have deletions of *EBF1* and *RAG1/2* in B lineage childhood ALL (8). In addition to these anticipated oncogenic or 'driver' deletions, we observed a large region of homozygosity on the short (p) arm of chromosome 6 including the whole HLA locus. This loss was accompanied by duplication of the other parental 6p region resulting in uniparental disomy. A large genomic deletion, including the HLA loci, therefore accounts for the loss of maternal HLA alleles.

Discussion

These data unambiguously mark the infant cancer as of maternal origin. Some 17 cases of probable metastasis to the fetus have now been recorded (1, 9, and current report), the first being in 1866, most being either melanoma (#6), a cancer with a notoriously metastatic proclivity, or leukemia/lymphoma (#8). Given the phenotypic features described in these cases, it is likely that they were all, as presumed, of maternal origin rather than coincidental cancers. Genetic markers can unambiguously resolve cellular origins in this context. In three of the reported leukemia/lymphoma cases, the male infant bone marrow contained cells with an XX karyotype (1). Whilst these most probably do reflect maternal leukemia/lymphoma cells, it cannot be excluded that they were non-malignant, normal maternal cells or infant male cells in which the Y chromosome was lost and X was duplicated. These are, individually, not rare events in leukemia (10, 11), although they seldom occur together in one clone. Other prior evidence for a maternal origin was the identification in a case of NK cell lymphoma of a specific chromosome translocation t(X;1)(q22;q12) in the maternal lymphoma and in three metaphases of the infant tumor (12).

The rarity of materno-fetal transmission of cancer is a testimony to the efficacy of the placental barrier and perhaps to immunosurveillance. In the present case, an additional feature was the selective loss in the infant maternally-derived tumor cells of maternal HLA alleles that were not inherited by the infant (Tables 1 and 2). Loss of HLA would be expected to render the transmitted cancer cells immunologically inert (13). HLA loci encoded cell surface proteins provide the major antigenic targets for allograft recognition and rejection, so it is likely that HLA deletion in this case contributed to successful transmission of the maternal leukemic cells. However, given the large size of the 6p deletion, it is possible that other gene losses could have contributed to the apparent lack of immuno-surveillance. Other unusual situations where cancer cell transmission occurs all appear to involve immunological invisibility (14): inter-monozygotic twin transmission in utero (4), immunosuppressed recipients of cancer-infiltrated donor organs (15), down-regulated MHC antigen expression in venereal sarcoma in dogs (2), and lack of MHC diversity in the Tasmanian devil (*Sarcophilus harrisii*) with transmissible facial tumors (16). In a recent report (17), loss of allorecognition of leukemic cell HLA by T cells, in a transplant context, also occurred by acquired uniparental disomy of chromosome 6p in the leukemic cells, as in the present study. It is possible that materno-feto transfer of cancer cells is more common than is reflected in the frequency of clinically diagnosed cases and that immuno-surveillance is the principal constraint.

Table 2. Loss of heterozygosity analysis of infant tumor lymphoma

Region	LOH	Gene(s)
5p33.3	Loss	*EBF1*
6p25.3–21.1	UPD	*HLA*, and many other genes
7p14.1	Loss	*TCRG*
7p12.2	Loss	*IKZF1*
9p21.3–12	Loss	*MTAP, CDKN2A/B, PAX5*
11p12	Loss	*RAG1/2*
14q11.2	Loss	*TCRA*
14q32.33	Loss	*IGH*
15q22.33	Loss	*SMAD3*
22q11.22	Loss	*IGL*

Loss of heterozygosity analysis of the infant tumor lymphoma in comparison with unpaired control DNA. UPD, uniparental disomy (see *Materials and Methods* for details).

Materials and Methods

Detection and Amplification of *BCR-ABL1* Genomic Breakpoints. For detection and amplification of DNA breakpoints, ranging from 300 bp to 12 kbp the Expand Long Template PCR kit (Roche) with System 2 was used, with an annealing temperature of 64 °C. To cover the *BCR* and *ABL1* regions, within which breakpoints can occur, 21 *BCR* forward primers and 20 *ABL1* reverse primers were used in multiplex, combining each *BCR* forward primer with 4 mixes of 5 *ABL1* reverse primers.

The child's genomic breakpoint was initially amplified using *BCR* 3C F (GGGCTCATTTTCACTGGATGGAC) and the *ABL1* D reverse primer mix, and

upon split out PCR, a band was amplified with *BCR* 3C F and *ABL1* 1D R (AGC CAT AAC CAT TCT CCC AAG CA). The breakpoint was confirmed by re-amplification and sequencing of the breakpoint in both the original and WGA amplified patient sample with *BCR* 3C F (GGGCTCATTTTCACTGGATGGAC), and a breakpoint specific *ABL1* reverse primer (TTC AGG GGC CTT GGA TCA GAC TA) determined from sequencing the original cloned product. Forward and reverse primers for blood spot PCR were respectively (GATCCTTTTAAAT-AGGCAAG) and (GTAATGCCAAAAATAACACT).

Fifteen polymorphic STR markers were amplified in the paternal blood DNA and patient's PE and PB DNA samples using the Powerplex-16 system (Promega).

Genome Mapping Analysis. Mapping analysis was performed using 500 ng of tumor DNA from the infant lymphoma. DNA was prepared according to manufacturer's instructions using the GeneChip mapping 500K assay protocol for hybridization to GeneChip Mapping 250K Nsp and Sty arrays (Affymetrix). Briefly, genomic DNA was digested in parallel with restriction endonucleases *Nsp*I and StyI, ligated to an adaptor, and subjected to PCR amplification with adaptor-specific primers. The PCR products were digested with *DNase*I and labeled with a biotinylated nucleotide analog. The labeled DNA fragments were hybridized to the microarray, stained by streptavidin–phycoerythrin conjugates, and washed using the Affymetrix Fluidics Station 450 then scanned with a GeneChip scanner 3000 7G.

Copy Number and LOH Analysis. SNP genotypes were obtained using Affymetrix GCOS software (version 1.4) to obtain raw feature intensity and Affymetrix GTYPE software (version 4.0) using the Dynamic Model algorithm with a call threshold of 0.33 to derive SNP genotypes. The sample was analyzed using CNAG 3.0 (http://plaza.umin.ac.jp/genome), comparing tumor sample with unpaired control DNA to determine copy number and LOH caused by imbalance (18).

HLA Typing. Serotyping was by microdroplet lymphocyte cytotoxicity (19). Genotyping was carried out using a reversed SSO HLA DNA typing method using fluorescent microspheres and a flow analyzer (20).

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ORIGINAL ARTICLE

Clinical significance of minimal residual disease in patients with t(8;21) acute myeloid leukemia in Japan

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Abstract To examine the prognostic significance of minimal residual disease (MRD) in t(8;21) acute myeloid leukemia (AML), 96 bone marrow samples from 26 Japanese patients in complete remission (CR) were analyzed regarding the *RUNX1/MTG8* transcript using real-time reverse transcriptase polymerase chain reaction assay. All patients were treated with intensive chemotherapy. The median copy number of the *RUNX1/MTG8* transcript, measured after each treatment course decreased over time. However, an increase in the MRD level was documented in three patients after the second consolidation, and all of them subsequently relapsed. The relapse-free survival (RFS) did not differ between the patients whose MRD levels were below or above 1,000 copies/μg after the first consolidation, with respective 2-year rates of 62 and 86% ($P = 0.21$). With respect to the MRD level after induction therapy, our data also failed to show any favorable effect of a lower MRD on RFS. Although these findings need to be confirmed with a larger number of patients, our data indicate that the MRD level at a given time during the early course in CR does not predict the outcome in Japanese patients.

Keywords Acute myeloid leukemia · t(8;21) · *RUNX/MTG8* · Minimal residual disease · Prognosis

1 Introduction

t(8;21)(q22;q22) is one of the most common karyotype abnormalities in acute myeloid leukemia (AML), occurring

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in 7–8% of adult patients [1–3]. This translocation leads to the formation of the chimeric *RUNX1(AML1)/MTG8(ETO)* transcript, which enables detection by polymerase chain reaction (PCR) assay. Since the introduction of real-time reverse transcriptase (RT)-PCR [4], prognostic significance of minimal residual disease (MRD) quantified using this method has been intensively investigated. Several studies from Western countries showed that MRD levels during or after treatment are associated with a risk of relapse on the basis of results from 21–51 patients [5–9].

We previously reported that Japanese patients with t(8;21) AML could have a more favorable outcome than the Western patients [10]. Marcucci et al. [11] also showed the difference in the outcome between the white and non-white patients enrolled in successive Cancer and Leukemia Group B trials. Given that clinical characteristics of t(8;21) AML can differ according to ethnicities, prognostic significance of MRD may also differ between Japanese and Western patients.

Here we examine the relationship between MRD status during intensive chemotherapy and the outcome in Japanese patients with t(8;21) AML.

2 Patients and methods

2.1 Study patients

We retrospectively reviewed the medical records of a total of 46 adults, who were newly diagnosed to have t(8;21) AML, at nine collaborating hospitals between January 2000 and December 2005. Induction therapy was given to 45 patients, and 41 (91%) achieved complete remission (CR). Data on MRD after the first or second consolidation were available for 27 of the 41 CR patients. We excluded one patient who received low-dose cytarabine-containing therapy, leaving 26 patients eligible for this study. We did not exclude any patient who relapsed after the first consolidation therapy. All patients provided their informed consent before the initiation of any medical procedure.

2.2 Diagnosis of t(8;21) AML and MRD evaluation

The diagnosis of t(8;21) AML was established based on chromosomal analysis (G-banding) and/or detection of the *RUNX1/MTG8* fusion gene by real-time RT-PCR. The molecular quantification of the *RUNX1/MTG8* fusion gene was performed as described previously [12]. The results were reported as the number of transcript copies, which were normalized by means of *GAPDH* and then converted into copies/μg RNA. The molecular quantification of the *RUNX1/MTG8* fusion gene was conducted each time after the induction and consolidation therapies. Bone marrow samples were used for all the MRD analyses.

2.3 Statistical analysis

The relapse-free survival (RFS) was calculated as the time from diagnosis to relapse or death, using the Kaplan–Meier product limit method. A log rank test was applied to assess the difference between the groups. The estimated survival was calculated as of 7 May 2008. Differences in distribution of categorical variables were compared with the Fisher's exact test. All analyses were conducted using the STATA version 9.2 software program (StataCorp, College Station, TX).

3 Results

3.1 Patient characteristics

The characteristics of the 26 patients are shown in Table 1. The median age was 50 years (range, 25–64 years), with 19 males and 7 females. Details of treatments are also summarized in Table 1. For induction therapy, 24 received idarubicin and cytarabine, and 2 received daunorubicin and cytarabine. Consolidation therapy included high-dose cytarabine in 12, and standard-dose cytarabine in 14 patients. The median follow-up of the surviving patients was 39.2 months (range, 14.0–92.4 months).

3.2 Clinical outcome

Of the 26 patients, 17 had continued first CR until the time of last observation. Relapse occurred in the remaining nine patients at a median of 9.9 months (range, 7.5–81.6 months). Five patients died due to the primary disease ($n = 3$), sudden cardiac disorder ($n = 1$) and cardiac arrhythmia ($n = 1$). The probability of RFS was 73% at 2 years for the entire population. The rate was 67% for patients who received high-dose cytarabine for consolidation therapy, whereas it was 79% for patients who received standard-dose cytarabine ($P = 0.87$).

3.3 Kinetics of MRD of each patient

The MRD levels were measured in a total of 96 samples from the 26 patients. Samples were available from 18 patients after induction therapy, 20 patients after the first consolidation, 18 patients after the second consolidation, and 13 patients after the third consolidation. The kinetics of MRD of each patient is shown in Fig. 1. The median copy number of the *RUNX1/MTG8* transcript decreased over time, for example 4,750 copies/μg after induction, 480 copies/μg after the first consolidation, 240 copies/μg after the second consolidation, and <100 copies/μg after the third consolidation. All the 15 patients whose MRD data

Table 1 Characteristics of the patients with t(8;21) AML at diagnosis

Variables		Number
Age (years)	Median, range	50 (25–64)
Sex	Male/female	19/7
Karyotypic abnormality[a]		
(A) t(8;21)(q22; q22) without additional karyotypic abnormality		7
(B) t(8;21)(q22; q22) with loss of sex (Y) chromosome		6
(C) t(8;21)(q22; q22) with abnormal chromosome 9		2
(D) t(8;21)(q22; q22) with ≥3 additional abnormalities		7
(E) t(8;21)(q22; q22) with loss of X chromosome		1
(F) Other karyotypic abnormality[b]		1
White blood cell count (/μL)	Median, range	7750 (900–54970)
Lactate dehydrogenase level (IU/L)	Median, range	441 (186–3354)
Extramedullary involvement	Present/absent	5/21
Induction therapy		
Idarubicin 12 mg/m^2 d1–3 + cytarabine 100 mg/m^2 d1–7		24
Daunorubicin 50 mg/m^2 d1–5 + cytarabine 100 mg/m^2 d1–7		2
Consolidation therapy	High-dose cytarabine-based chemotherapy	12
	No. of courses (2/3/4)	1/8/3
	Standard cytarabine-based chemotherapy	14
Hematopoietic stem cell transplantation	In first complete remission (autologous/allogeneic)	2/1
	In other stage (autologous/allogeneic)	0/3[c]

[a] Two patients were diagnosed by the detection of *RUNX1/MTG8* fusion gene using reverse transcriptase-polymerase chain reaction

[b] 46,XX,t(2;19)(q37;p13),t(8;21)(q22;q22)

[c] Patients who underwent allogeneic stem cell transplantations in second complete remission

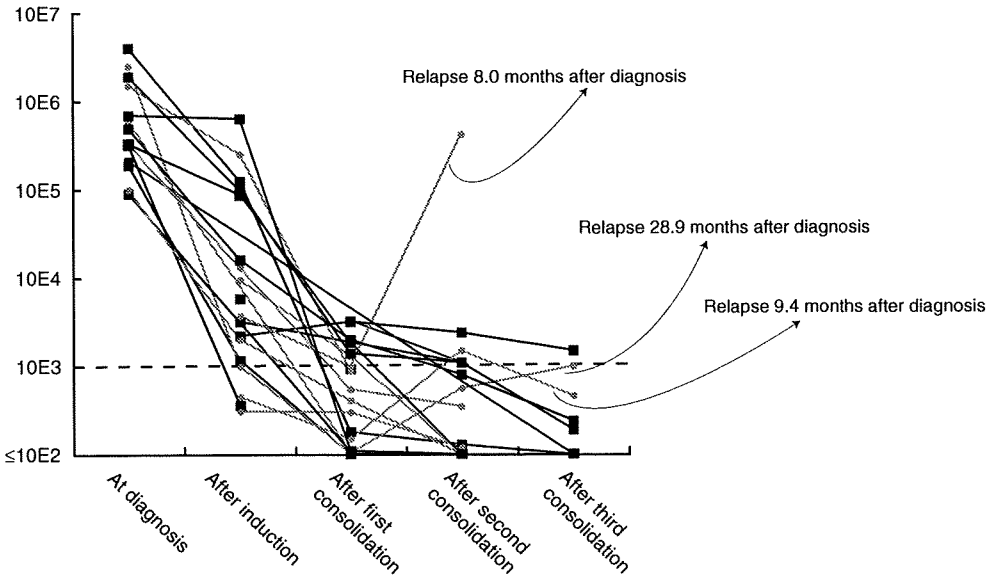

Fig. 1 Kinetics of the *RUNX1/MTG8* level in bone marrow. Kinetics of the *RUNX1/MTG8* level (copies/μg RNA) is shown for the 17 patients who remained in remission (*squares*) and for the 9 patients who had experienced a relapse (*circles*). The increases of the *RUNX1/MTG8* level were documented in three patients and all of them subsequently relapsed

were available both after induction and first consolidation showed reduction in varying degrees. On the other hand, the increments were documented in 3 of the 16 patients who had MRD levels measured both after the first and second consolidation, and all of them subsequently relapsed (Fig. 1).

3.4 Effect of MRD level on relapse-free survival

We next evaluated the prognostic relevance of the MRD level at a specific time point. Given that an increase in the MRD level was observed in none of the patients after the first consolidation, but in three patients after the second

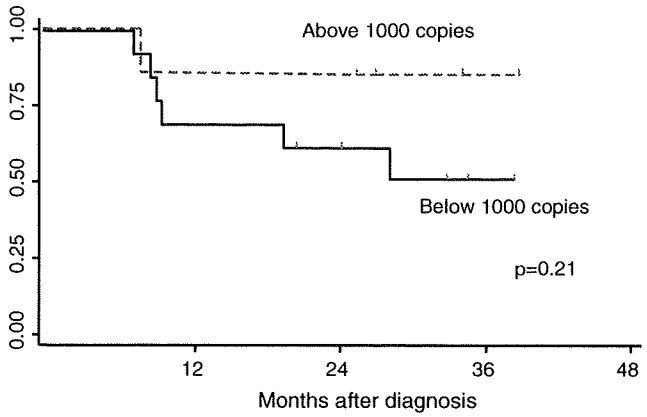

Fig. 2 Relapse-free survival according to the level of minimal residual disease after the first consolidation course. No difference was found between the patients with the *RUNX1/MTG8* level above ($n = 7$) and below 1,000 copies/μg RNA ($n = 13$)

consolidation, we examined the effect of MRD level after the first consolidation on RFS. The copy number of the *RUNX1/MTG8* transcript at this time point was less than 1,000 copies/μg in 13 patients (65%). Six patients (30%) exhibited less than 100 copies/μg. Figure 2 compares RFS according to the MRD level after the first consolidation. Here, a cutoff of 1,000 copies/μg was chosen in accordance with the findings of Tobal et al. [6]. RFS did not differ between the patients with an MRD level below or above 1,000 copies/μg ($P = 0.21$), with respective 2-year rates of 62 and 86%. The results were similar when we used different cutoffs such as 100 copies/μg ($P = 0.74$), the median value of 480 copies/μg ($P = 0.28$) or 3 log reduction from the time of diagnosis ($P = 0.41$). With respect to the MRD level after induction therapy, our data also failed to show any favorable effect of a lower MRD on RFS. Rather, an inferior RFS was observed in patients whose MRD level was less than the median value ($P = 0.03$).

4 Discussion

This is the first report from Japan, which investigated the prognostic value of MRD in t(8;21) AML. The study highlights two principal results. First, an increase in the *RUNX1/MTG8* level strongly predicted a subsequent relapse, and it was observed after the second consolidation or later. Second, unlike previous studies from Western countries [5–9], the MRD data obtained during the early course in CR did not correlate with outcome. A lack of difference in RFS by MRD level in this study might be attributable to the relatively favorable outcome of the patients with a higher *RUNX1/MTG8* level. Although the cutoffs of the MRD level vary from study to study, the RFS rate of 69% at 2 years for the patients with lower MRD level was closely comparable with other studies [5–9]. In contrast, the 2-year RFS rate was 85% for our patients with higher *RUNX1/MTG8* level, which was much better than 10–40% in those reports [5–9]. Although it is not clear why the prognosis of such "poorer responders" was different between the Western reports and ours, this difference might contribute to the more favorable overall outcome observed in Japanese patients with t(8;21) AML [10]. Recent studies have shown that the kinase domain mutations of the *KIT* gene are detected in a substantial proportion of patients with t(8;21) AML and are associated with poor prognosis [13–15]. Further investigations on molecular pathogenesis may therefore provide further insights into this issue. On the other hand, it has been well documented that non-leukemia stem cells in t(8;21) AML patients during CR possess the AML1-MTG8 fusion gene.[16] Therefore, in patients with a high MRD, AML1-MTG8 transcripts might derive from non-leukemia cells. Further basic research on the leukemia genesis of t(8;21) AML are thus warranted.

It should be noted that the patients were not treated with uniform regimens due to the retrospective nature of the study. We therefore restricted the analysis to patients who were given intensive chemotherapy. Accordingly, all but two patients received the same induction therapy consisting of idarubicin and cytarabine, and the other two received daunorubicin and cytarabine, another standard induction regimen for AML. Regarding consolidation therapy, 46% of the patients received high-dose cytarabine, while others received standard-dose cytarabine. However, there was no difference in RFS between these two groups. Notwithstanding, such limitations make it necessary to confirm our results with a larger number of patients in prospective studies.

In conclusion, our data raise an important issue that the clinical significance of MRD in t(8;21) AML may differ between Japanese and Western patients. The MRD level measured at a given time during the early course in CR may not be useful in predicting the outcome of Japanese t(8;21) AML patients. Although this needs to be verified by future studies, clinicians should note the possibility of such potential differences among ethnicities.

Acknowledgments We wish to thank all the staff and resident members of the participating institutions. A complete list of participating institutions appears in the Appendix.

Appendix

This study was conducted at the following institutions: Toyohashi Municipal Hospital, Toyohashi; Yamanashi Prefectural Central Hospital, Kofu; National Hospital Organization Nagoya Medical Center, Nagoya; Komaki

City Hospital, Komaki; Nagoya University Hospital, Nagoya; Yokkaichi Municipal Hospital, Yokkaichi; Okazaki City Hospital, Okazaki; Meitetsu Hospital, Nagoya; Fujita Health University Hospital, Toyoake, Japan.

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Karyotype at diagnosis is the major prognostic factor predicting relapse-free survival for patients with Philadelphia chromosome-positive acute lymphoblastic leukemia treated with imatinib-combined chemotherapy

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ABSTRACT

To identify factors associated with relapse-free survival (RFS), 80 patients with newly diagnosed Philadelphia chromosome-positive acute lymphoblastic leukemia, enrolled in a phase II study of imatinib-combined chemotherapy, were analyzed. The median follow-up of surviving patients was 26.7 months (maximum, 52.5 months). Twenty-eight out of 77 patients who had achieved CR relapsed. The probability of RFS was 50.5% at 2 years. Multivariate analysis revealed that the presence of secondary chromosome aberrations in addition to t(9;22) at diagnosis constitute an independent predictive value for RFS (p=0.027), and increase the risk of treatment failure by 2.8-fold.

Key words: acute lymphoblastic leukemia, Philadelphia chromosome, BCR-ABL, imatinib, karyotype.

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Introduction

The treatment for Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph$^+$ ALL) has changed dramatically since imatinib, a selective inhibitor of the ABL tyrosine kinase, was introduced.[1,2] Combined with chemotherapy, or even as a single agent, it can produce complete remission (CR) rates of 90% or higher in newly diagnosed patients.[3-9] We previously reported the results of a phase II study by the Japan Adult Leukemia Study Group (JALSG) to test the efficacy and feasibility of imatinib-combined chemotherapy for newly diagnosed Ph$^+$ ALL.[6] The rate of CR reached 96%, and that of BCR-ABL negativity in bone marrow 71%. However, despite a relatively short follow-up period, relapse occurred in a subset of the patients who had achieved CR.

On the other hand, remarkable progress is being made with the development of novel tyrosine kinase inhibitors with more potent *in vitro* and *in vivo* activities than imatinib.[10,11] Given this, we investigated factors associated with relapse-free survival (RFS).

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Design and Methods

Patients and treatments

Eligibility criteria included newly diagnosed Ph$^-$ ALL, age between 15 and 64 years, an Eastern Co-operative Oncology Group performance status between 0 and 3, and adequate liver, kidney and heart function. Written informed consent was obtained from all patients prior to registration.

For remission induction therapy, imatinib was administered from day 8 to day 63 in combination with daunorubicin, cyclophosphamide, vincristine (VCR) and prednisolone (PSL). Consolidation therapy consisted of an odd course (C1) comprising high-dose methotrexate, high-dose cytarabine and methylprednisolone, and an even course (C2) with single-agent imatinib for 28 days. C1 and C2 were alternated for 4 cycles each. After completion of the consolidation therapy, patients received maintenance therapy consisting of VCR, PSL and imatinib for up to 2 years from the date CR had been achieved.[6] The daily dose of imatinib used in this study was 600 mg. Allogeneic hematopoietic stem cell transplantation (HSCT) was recommended if a matched sibling donor was available, and was allowed from an alternative donor.

The protocol was reviewed and approved by the institutional review board of each of the participating centers and was conducted in accordance with the Declaration of Helsinki.

Cytogenetic and molecular analysis

At diagnosis, bone marrow samples were examined for cytogenetic abnormalities with standard banding techniques. Karyotypes were classified according to the International System for Human Cytogenetic Nomenclature.[12] The number of BCR-ABL copies in bone marrow was determined at a central laboratory with the real-time quantitative RT-PCR test according to the previously described method.[13]

Statistical analysis

Kaplan-Meier survival analysis was performed to estimate the probabilities of RFS, event-free survival (EFS), and overall survival (OS), with differences between the groups compared by the log-rank test. Cumulative incidences of relapse were calculated with non-relapse mortality considered as a competing risk, and differences between the groups were compared with the Gray's test. For risk factor analysis, a Cox proportional hazards model was constructed. In multivariate analysis, variables with p values of <0.10 determined by univariate analysis were included in the final model. A hazard ratio (HR) was calculated in conjunction with a 95% confidence interval (CI).

Results and Discussion

A total of 80 patients were recruited between September 2002 and January 2005. The median age was 48 years (range 15-63), with 49 males and 31 females. CR was achieved by 77 (96.2%) patients. During a median follow-up of 26.7 months (maximum 52.5 months), 28 patients relapsed. Of the 17 relapses observed during the consolidation therapy, 13 occurred during the imatinib course. The probabilities of EFS and OS were 48.5±5.7% and 58.1±5.7% at 2 years (Figure 1). For patients who had achieved CR, the probability of RFS was 50.5±5.9% at 2 years. Allogeneic HSCT was performed for 60 patients, including 24 from a sibling donor, 1 from a related donor other than a sibling, 25 from an unrelated donor, and 10 from unrelated cord blood. Disease status at the time of transplantation was first CR for 44 patients, second CR for 4 and non-CR for 12. The 2-year RFS for those who had undergone allogeneic HSCT during first CR was 62.6±7.5% and 62.1±12.3% for those who had not undergone allogeneic HSCT. When allogeneic HSCT was considered as a time-dependent covariate, it was shown to have no significant effect on RFS (HR, 1.03; 95% CI, 0.51-2.09; p=0.934). Major and minor BCR-ABLs were detected in 23 and 56 patients respectively. The transcript type of the remaining patient could not be determined because fluorescent *in situ* hybridization analysis was used instead of the PCR test. Neither transcript types nor copy numbers at diagnosis were associated with RFS (p=0.763 and 0.912). Pre-treatment cytogenetic results were not available for 4 patients because analysis was not performed (n=2) or was not successful (n=2). Of the remaining 76 patients, 22 showed only t(9;22) or variant translocations, 51 showed additional chromosome aberrations, and 3 showed normal karyotype. Additional aberrations exceeding a frequency of 10% comprised +der(22)t(9;22) in 17 patients, abnormalities involving the short arm of chromosome 9 [abn(9p)] in 17, monosomy 7 in 10, and trisomy 8 in 10. Figure 2 compares RFS for patients with and without additional chromosome aberrations. The presence of additional aberrations was significantly associated with shorter RFS (p=0.003). The relapse rate was also higher in patients with additional aberrations (41% *vs.* 20% at 2 years, p=0.0414). Analyses of the 4 recurrent abnormalities mentioned above demonstrated a statistically significant negative impact on RFS for +der(22)t(9;22) and abn(9p) (p<0.001 and p=0.005). Even after allogeneic HSCT, patients with additional aberrations appeared to have a trend for shorter RFS than those without (p=0.080), but this might reflect a larger proportion of transplantation beyond first CR in the former (31% *vs.* 17%). In patients allografted during first CR, there was no difference in cumulative incidences of relapse dated from the day of transplantation between the 2 groups

(16.5% *vs.* 12.5% at 2 years, p=0.546). Variables that showed a significant effect on RFS in the univariate Cox model included additional chromosome aberrations (p=0.005), peripheral blood blasts % (p=0.024) and sex (p=0.03). Results of multivariate analysis are shown in Table 1. The presence of additional chromosome aberrations was identified as the only independent prognostic factor for RFS (p=0.027). These updated data strongly support recent reports showing the feasibility and remarkable efficacy of imatinib-combined chemotherapy for newly diagnosed Ph+ ALL.[3-9,14,15] The main objective of this report was to identify factors affecting RFS, an issue of rapidly increasing importance given the development of novel tyrosine kinase inhibitors which are expected to further expand the treatment options for this disease. Our data indicated that additional chromosome aberrations, particularly +der(22)t(9;22) and abn(9p), were associated with shorter RFS. It is well known that additional chromosome aberrations are seen frequently in Ph+ ALL. Before the imatinib era, some groups reported the prognostic relevance of additional aberrations.[16-18] By contrast, from a large series of 204 patients, Moorman *et al.*[19] recently showed no significant effect of specific additional aberrations, including +der(22)t(9;22) and del(9p), on survival. In this study, analyzing patients treated with imatinib-combined chemotherapy, the 2-year RFS rate exceeded 80% for those without additional aberrations, whereas outcomes for those with additional aberrations were relatively unfavorable.

Acquisition of resistance to imatinib is an emerging problem in the treatment of chronic myeloid leukemia. One of the most common mechanisms of resistance is the mutation involving the ABL kinase domain. Although it has not been confirmed whether such mutations compromise the clinical outcome of Ph+ ALL patients treated with imatinib-combined chemotherapy, our observation that most of the early relapses occurred during the consolidation courses consisting of imatinib alone implies possible imatinib resistance. If that is the case, switching from imatinib to other novel tyrosine kinase inhibitors based on the pre-treatment cytogenetic results soon after achieving CR or even earlier could be an alternative treatment approach for further improving outcome in Ph+ ALL. Lack of mutation analysis is a major limitation of this study. Recently, Pfeifer *et al.*[20] studied the ABL kinase domain mutation status in newly diagnosed Ph+ ALL patients who were treated with imatinib-combined chemotherapy, and showed that even before exposure to imatinib, mutations were detected in 38% of patients. Importantly, the frequency of the mutant allele was low in such patients. However, at the time of relapse, the same mutation was present as the dominant clone in 90% of the relapsing cases.[20] Altogether, further insights will be provided by investigating the association between karyotype and mutation status at diagnosis.

Despite such limitations, the analysis of 80 patients entered into a single trial identified karyotype at diagnosis as a significant prognostic factor for RFS in newly diagnosed Ph+ ALL patients treated with imatinib-combined chemotherapy. Although our results need to be confirmed regarding kinase domain mutation status, these findings may play a critical role in the future treatment of Ph+ ALL.

Table 1. **Multivariate analysis of factors associated with relapse-free survival.**

P-value	*HR (95% CI)**	*Factors*	
Additional [chromosome aberrations	0.027	2.84 (1.12-7.19) 1.00	Present Absent
Peripheral blood blasts%	0.051	1.12 (1.00-1.22)	Per 10% increase
Sex	0.148 1.00	1.73 (0.82-3.64) Female	Male

*HR, hazard ratio; 95% CI, 95% confidence interval. *Values higher than unity indicate higher risk for failure.*

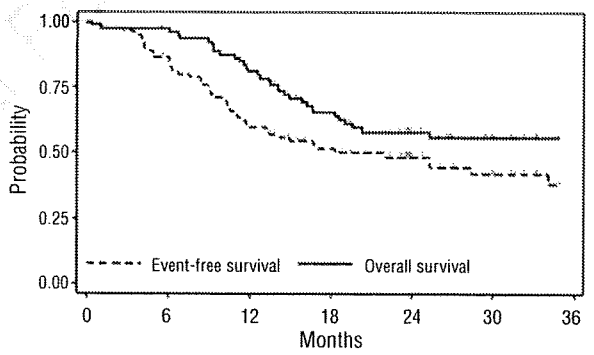

Figure 1. **Kaplan-Meier curves for event-free and overall survival. The probabilities of event-free and overall survival at 2 years were 48.5% and 58.1% respectively (n=80).**

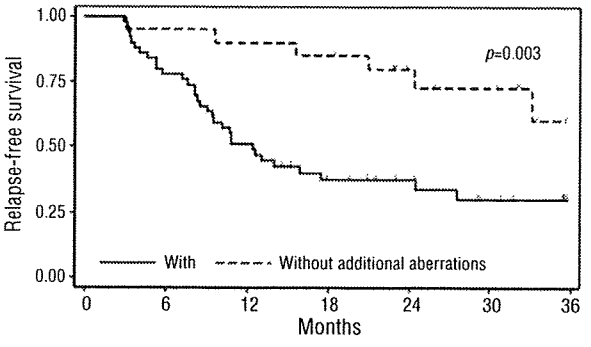

Figure 2. **Relapse-free survival for patients with and without additional cytogenetic aberrations. Patients with additional cytogenetic aberrations (n=50) had significantly shorter relapse-free survival than those without (n=20).**

Authorship and Disclosures

MY designed and co-ordinated the study, analyzed the data, and wrote the paper; JT, NU, FY, SM, and IJ designed the study, and provided patient sample and clinical data; IS, HA, KN, YU, MT, and AM provided patient sample and clinical data; HN co-ordinated the study, and revised the paper. YM provided patient sample and clinical data, and engaged in data management. SO designed the study, provided patient sample and clinical data, and engaged in data management; KM designed the study, and analyzed the data; TN chaired the study group, co-ordinated the study, and revised the paper; RO served as the principal investigator, chaired the study group, and revised the paper. All authors reviewed the paper, interpreted the results, and approved the final version. The authors reported no potential conflicts of interest.

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Characteristics of Dasatinib- and Imatinib-Resistant Chronic Myelogenous Leukemia Cells

Seiichi Okabe, Tetsuzo Tauchi, and Kazuma Ohyashiki

Abstract **Purpose:** Although dual src-family kinase/BCR/ABL inhibitor, dasatinib (BMS-354825), provides therapeutic advantages to imatinib-resistant cells, the mechanism of dasatinib resistance was not fully known.

Experimental Design: We used TF-1 BCR/ABL cells, by introducing the *BCR/ABL* gene into a leukemia cell line, TF-1 and K562, and established dasatinib- (BMS-R) and imatinib-resistant (IM-R) cells. We characterized chronic myelogenous leukemia drug-resistant cells and examined intracellular signaling.

Results: The IC_{50} of dasatinib was 0.75 nmol/L (TF-1 BCR/ABL), 1 nmol/L (K562), 7.5 nmol/L (TF-1 BCR/ABL IM-R), 10 nmol/L (K562 IM-R), 15 μmol/L (TF-1 BCR/ABL BMS-R), and 25 μmol/L (K562 BMS-R). The number of BCR/ABL copies in resistant cell lines was the same as the parental cell line by fluorescence *in situ* hybridization analysis. There was no mutation in Abl kinase. We found that protein levels of BCR/ABL were reduced in dasatinib-resistant cell lines. BCR/ABL protein was increased by treatment of an ubiquitin inhibitor. The Src kinase, Lck, as well as mitogen-activated protein kinase and Akt were activated, but $p21^{WAF}$, phosphatase and tensin homologue was reduced in K562 BMS-R cells. Removal of dasatinib from the culture medium of K562 BMS-R cells led to apoptosis, and activated caspase 3 and poly (ADP-ribose) polymerase.

Conclusion: These results suggest that the expression and protein activation signatures identified in this study provide insight into the mechanism of resistance to dasatinib and imatinib and may be of therapeutic chronic myelogenous leukemia value clinically.

The development of imatinib has redefined the management of chronic myelogenous leukemia (CML; refs. 1, 2). Most newly diagnosed patients with chronic-phase disease, treated with imatinib, achieve durable complete cytogenetic responses (3). An update of the IRIS study after 5 years of follow-up shows a hematologic remission rate of 98%, a major cytogenetic response rate of 92%, a rate of complete cytogenetic response of 87%, and a progression-free survival rate in 84% of the patients (4). However, because only a minority of patients achieves undetectable levels of BCR/ABL transcripts, it is suggested that imatinib could not kill the all BCR/ABL expressing cells (5, 6). Moreover there were some patients who experienced relapse during imatinib treatment, especially in the advanced phase, and were resistant to imatinib. This is clinically problematic (7, 8). Various mechanisms may contribute to imatinib resistance. The leading cause of acquired resistance to imatinib is reactivation of BCR/ABL kinase activity via kinase domain mutations (9–11). Mutations in the kinase domain of BCR/ABL impair binding of imatinib. However, only 35% to 45% of imatinib resistance arises from mutations in the ABL kinase domains (10, 12), suggesting that other mechanisms may also be identified in many cases, although this is not fully evaluated at this time.

Dasatinib (SPRYCEL, formally BMS-354825) is an oral, multitargeted kinase inhibitor of BCR/ABL and Src kinases, and is now used in the treatment for CML patients resistant or intolerant to previous therapy, including imatinib (13). Dasatinib inhibits BCR/ABL kinase activity in the low-nanomolar range and inhibits all clinically relevant imatinib-resistant forms with the exception of the T315I mutation. Dasatinib is also being tried in a phase II clinical study of CML and Philadelphia chromosome–positive acute lymphoblastic leukemia patients (START-A, START-C, START-R, and START-L; refs. 14–17). Although dasatinib, a second generation of tyrosine kinase inhibitor, is another promising new clinical candidate for CML treatment, the mechanism of dasatinib resistance has also not been evaluated.

In this study, we first established the dasatinib- and imatinib-resistant cell lines and examined the intracellular signaling in CML cell lines for insight into the resistance to two BCR/ABL inhibitors. Our results indicate that resistance to dasatinib can

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Translational Relevance

The chimeric *BCR/ABL* gene, originated by the Philadelphia chromosome, encodes a fusion protein, BCR/ABL, bearing unregulated tyrosine kinase activity, the pivotal pathogenetic step of chronic myelogenous leukemia (CML). Targeted therapy with the Abl kinase inhibitor, imatinib, has markedly improved the outlook for patients with CML. Although imatinib is the standard front-line therapy for patients in chronic phase, a substantial number of patients are either primarily refractory or acquire resistance to imatinib particularly in patients with advanced-stage disease. The investigation of therapeutic options postimatinib failure resulted in the development and regulatory approval of dasatinib, a BCR/ABL and Src-family kinase inhibitor. Dasatinib has shown clinical benefit and tolerability in patients in all phases of CML. Although dasatinib has shown remarkable activity against most imatinib-resistant ABL mutants, the mechanism of dasatinib resistance, except for the T315I mutation, was not fully known. In this study, we report dasatinib resistance by degradation of BCR/ABL protein. Our study provides new information regarding the molecular basis of strategy against the dasatinib resistance except for the T315I mutation in CML patients. This study provides a prospect for dasatinib-resistant CML and wider clinical experience is necessary to establish for the development of dasatinib-resistance.

be identified on the basis of intracellular protein levels such as BCR/ABL. Understanding BCR/ABL-mediated resistance to imatinib and dasatinib in these cell lines may lead to additional insights into the treatment of CML.

Materials and Methods

Reagents and antibodies. Imatinib and dasatinib were kindly provided by Novartis Pharmaceuticals and Bristol-Myers Squibb. Stock solutions were dissolved in distilled water or DMSO. Antiphosphotyrosine antibody (Ab) was from Upstate Biotechnology. Antiphospho Akt (Ser473), mitogen-activated protein kinase (Thr202/Tyr204), Src (Tyr416), Lyn (Tyr507), phosphatase tensin homologue, cleaved caspase 3, and poly (ADP-ribose) polymerase (PARP) Abs were from Cell Signaling. Lck and Abl Abs were from Santa Cruz Biotechnology. $p21^{WAF}$ Ab was from Transduction Laboratories. Antibody Microarray was from Lab Vision Corporation. Other reagents were from Sigma.

Cell culture and transfection. The CML cell line, K562, was obtained from American Type Culture Collection. The TF-1 BCR/ABL (p210) cell line was created as described previously (18). K562 and TF-1 BCR/ABL cells were incubated with increasing concentrations of dasatinib and imatinib (starting at 0.1 nmol/L and 0.1 μmol/L), and surviving cells were collected and treated with 2-fold higher concentrations (up to concentrations of 1 and 10 μmol/L). The resistant cells were cloned by limiting dilution in the presence of 1 μmol/L dasatinib.

Cell proliferation assay. Cell proliferation assay was done as described previously (19).

Ab microarray assay. Protein microarray assay was done according to the manufacture protocol. Briefly, total protein was extracted from cells. One hundred micrograms of protein sample was labeled by biotinylation reagent. After blocking, the microarry slide was hybridized to a biotin-labeled protein and was incubated with streptavidin solution. After washing, the slide was incubated with Cy3-conjugated detection Ab and detected on a GenePix4000B (Axon Instruments). Signal intensity was measured for each microarray and the values were normalized for each array. Data were filtered for the genes whose expression level increased or decreased by at minimum of 1.5-fold.

Immunoprecipitation and Western blot analysis. Immunoprecipitation and Western blot analysis were done as described previously (20, 21).

Fluorescence in situ *hybridization analysis.* Interphase nuclei were hybridized with fluorescent-labeled probes for BCR-ABL extra signal following standard procedures. For each slide, >100 nuclei were analyzed by 2 different observers.

Statistics. Data are given as mean ± SD values. Comparisons between two groups were assessed with the Student's *t* test.

Results

Characteristics of dasatinib- and imatinib-resistant CML cell lines. We established dasatinib- and imatinib-resistant cell lines and examined the BCR/ABL expression using fluorescence *in situ* hybridization to analyze interphase nuclei with probes against the *BCR* and *ABL* genes. The resistant cells exhibited the same number of BCR/ABL copies as the parental cell line, K562 (Fig. 1), and we could not find a point mutation in these cell lines (data not shown). Next, we investigated the effect of dasatinib and imatinib by measuring cell viability and apoptosis after incubation with increasing concentrations of the two drugs for 72 hours. The response of K562 and TF-1 BCR/ABL cells and their resistant counterparts (K562-BMS-R, K562-IM-R, TF-1 BCR/ABL BMS-R, and TF-1 BCR/ABL IM-R) to dasatinib and imatinib was compared. After exposure to 0.1 nmol/L to 50 μmol/L of dasatinib, parental K562 cells and TF-1 BCR/ABL cells displayed a reduction in growth (IC_{50}, 0.75-1.0 nmol/L), whereas their dasatinib-resistant counterparts (K562 BMS-R and TF-1 BCR/ABL BMS-R) were minimally affected (Fig. 2*A*). These dasatinib-resistant cells exhibited an IC_{50} for dasatinib that is 1,000- to 2,000-fold greater in value than that of the parental cells. The imatinib-resistant cell lines were treated with several concentrations of dasatinib. These cell lines were sensitive to high concentrations of dasatinib (IC_{50},

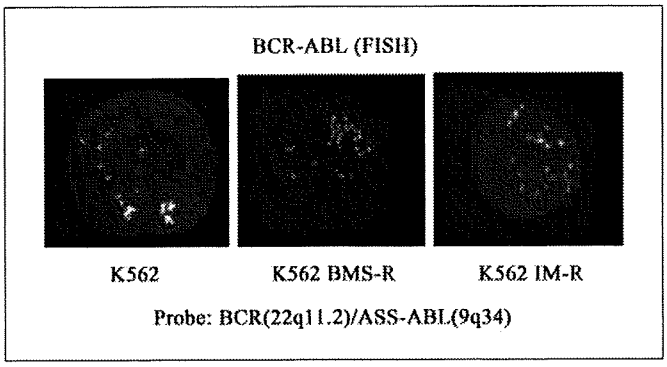

Fig. 1. Fluorescence *in situ* hybridization analysis of dasatinib- and imatinib-resistant K562 cell lines. Interphase fluorescence *in situ* hybridization for fusion of the ABL oncogene and BCR (breakpoint cluster region) of K562, K562 BMS-R, and K562 IM-R cells.

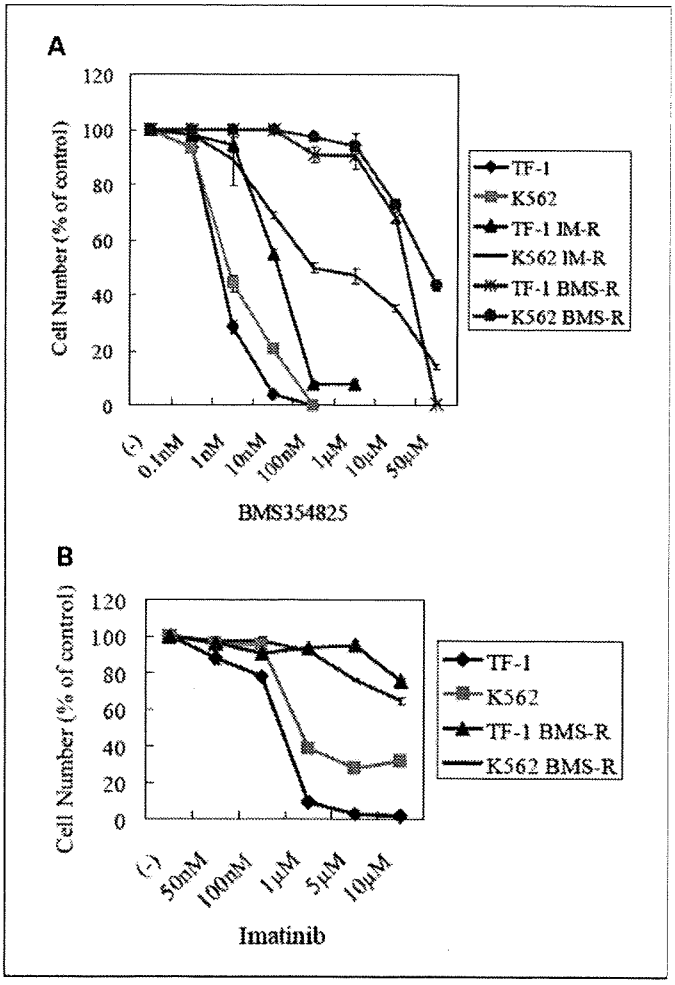

Fig. 2. Proliferation of dasatinib- and imatinib-sensitive and imatinib-resistant cell lines. K562, TF-1 BCR/ABL, K562IM-R, TF-1 BCR/ABL IM-R, K562 BMS-R, and K562 IM-R cells exposed to dasatinib (*A*) or imatinib (*B*) for 72 h were quantified by cell proliferation assay as described in "Materials and Methods." Each result is presented as the mean percentage of proliferation of unexposed control cultures, and three independent experiments are represented. Points, mean; bars, SD.

7.5-10 nmol/L). Thus, both the TF-1 BCR/ABL BMS-R and K562 BMS-R cells displayed marked resistance to imatinib (Fig. 2B).

Intracellular signaling in dasatinib- and imatinib-resistant cell lines. To clearly identify the signaling pathways in these dasatinib- and imatinib-resistant cell lines, we analyzed the protein expression profile in the dasatinib-resistant cell line using Ab microarray. In this experiment, both K562 BMS-R cells and the corresponding parental cells, K562, were cultured in a drug-free medium for 24 hours and total cell extracts were hybridized to Ab microarrays. We summarized the protein expression pattern in Fig. 3. In this study, we cut off the protein expression at the 1.5-fold change. We found that one of the phosphatase, phosphatase tensin homologue (0.33-fold), the DNA repair protein, Ku70 (0.19-fold), and the cell cycle–related protein, $p21^{WAF}$ (0.41-fold), decreased. One of the src kinase families, Lck (1.53-fold), transcription factors, c-jun (1.63-fold) and c-fos (1.54-fold), increased. To confirm the protein expression in resistant cell lines, the cell lysates of parental cell line, TF-1 BCR/ABL and K562, and resistant cell lines (TF-1 BCR/ABL BMS-R, IM-R, K562 BMS-R, and IM-R) were immunoblotted with the indicated Ab. We found that tyrosine phosphorylation was reduced in K562 BMS-R cells (Fig. 4A). We also found that the protein level of BCR/ABL was reduced in dasatinib-resistant two cell lines (Fig. 4A). We next examined whether ubiquitin is involved in the degradation of BCR/ABL in K562 BMS-R cells. After 24 hours of culture with or without 10 ng/mL of lactacystin, cell lysates were immunoprecipitated with Abl Ab and blotted with Abl Ab. Figure 4B and C show that the protein level of BCR/ABL was enhanced after lactacystin

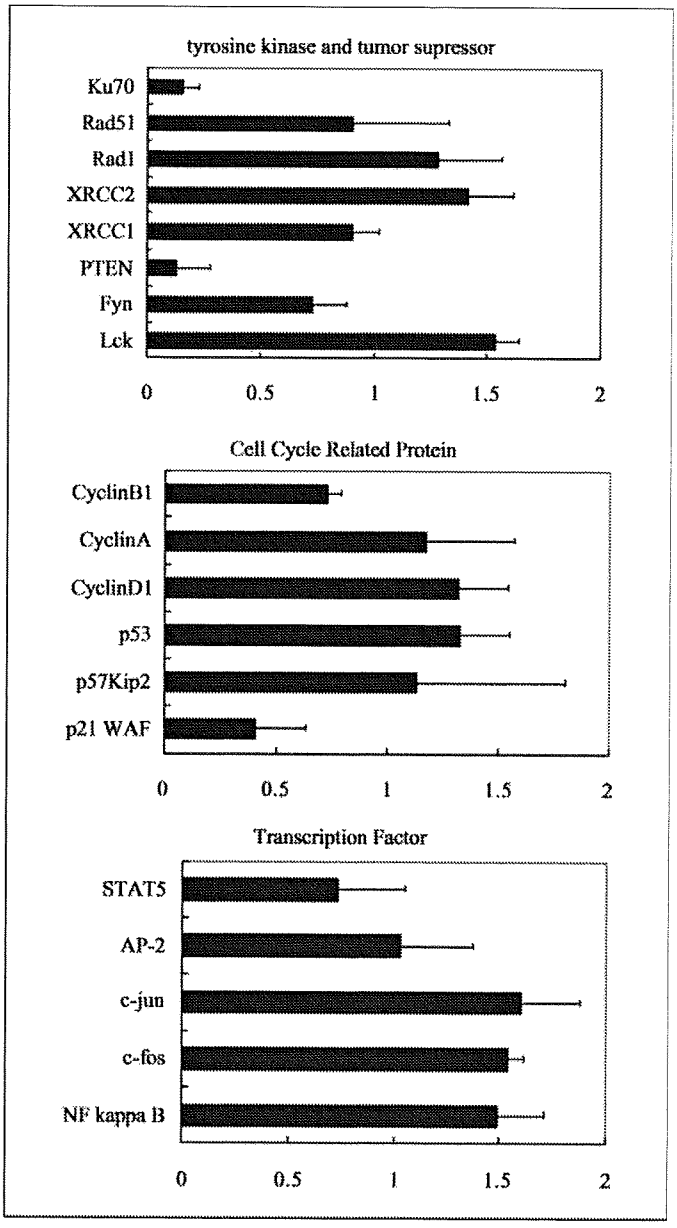

Fig. 3. Protein expression profile in a dasatinib-resistant cell line. Dasatinib-resistant and control cells were cultured in a drug-free medium, and protein expression data from three independent Ab microarray hybridizations were analyzed as described in "Materials and Methods". Tyrosin kinase and a tumor suppressor protein, a cell cycle related protein, and the transcription factors are shown.

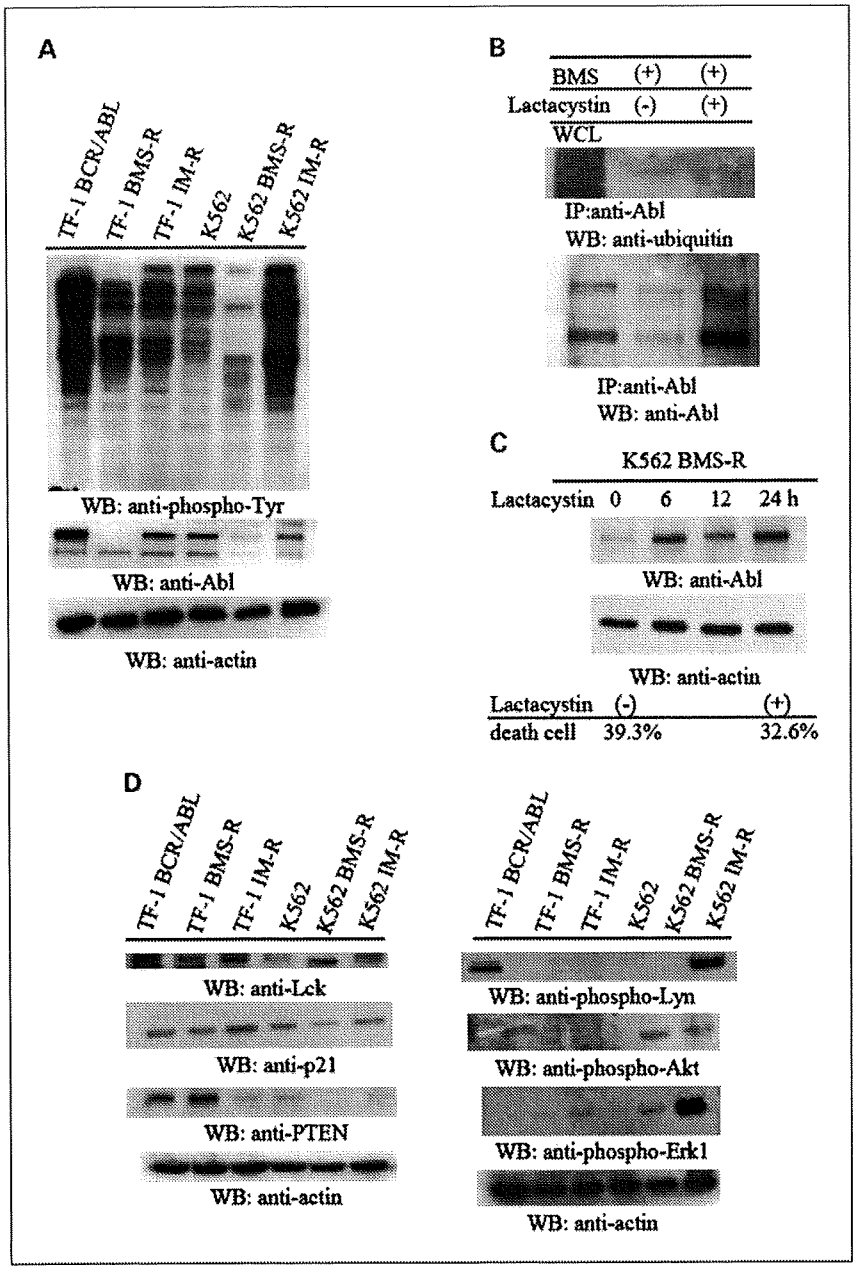

Fig. 4. BCR/ABL expression, tyrosine phosphorylation, and signaling in dasatinib- and imatinib-resistant cell lines and the ubiquitin inhibitor lactacystin modifies the BCR/ABL protein. Protein tyrosine phosphorylation and abl (*A*), phosphorylation of Lyn, Lck, $p21^{WAF}$, Akt, mitogen-activated protein kinase, actin (*D*) levels were analyzed by an immunoblotting protein (30 μg) from cell lysates. *B*, K562 BMS-R cells were treated with or without lactacystin for 24 h. Cell lysates were immunoprecipitated (*IP*) with anti-Abl Ab and immunoblotted (*WB*) with the ubiquitin or Abl Abs. *C*, K562 BMS-R cells were treated lactacystin for indicated hours. Cell viability was evaluated through the trypan blue exclusion and cell lysates were immunoblotted with Abl or actin Abs.

treatment within 24 hours. Moreover, treatment with lactacystin also inhibited the induction of apoptosis in K562 BMS-R cells. These results indicate that the degradation of BCR/ABL was mediated by the ubiquitin-proteasome system. We also found that one of the Src family kinases, Lck, was greatly enhanced in K562 BMS-R cell, but $p21^{WAF}$ and phosphatase tensin homologue were reduced (Fig. 4D). Erk and Akt are activated principally in response to external stimuli and regulate cell growth. We found that Erk and Akt were activated in dasatinib- and imatinib-resistant cells. Although enhanced phosphorylation of Lyn was detected in TF-1 BCR/ABL and K562 IM-R cells (Fig. 4D). Determination of the loading of equal amounts of protein in all lanes was accomplished by stripping and blotting with actin Ab.

Removal of Dasatinib for 24 hours induces apoptosis in K562 BMS-R cells. We examined caspase activation 24 hours after the removal of dasatinib or imatinib in K562 BMS-R cells. Cleaved caspase 3 and cleaved PARP were detected 24 hours after the removal of dasatinib in K562 BMS-R cells (Fig. 5A). We also found that activation of PARP was detected from 6 to 24 hours after the removal of dasatinib in K562 BMS-R (Fig. 5B). These results indicate that apoptosis of K562 BMS-R cells after the removal of dasatinib involves activation of caspases.

Discussion

In this study, we established K562 and TF-1 BCR/ABL cell lines that were resistant to the second generation of the BCR/

ABL kinase inhibitor, dasatinib, in our laboratory. These dasatinib-resistant cell lines are 1,000-fold more resistant than the parental cell lines. These cell lines showed no mutation in the Abl kinase domain, suggesting that other mechanisms were involved in resistance. There are several reports on imatinib resistance, including the amplification of the *BCR/ABL* gene, overexpression of the multidrug resistance P-glycoprotein, and persistence of tyrosine phosphorylation of specific proteins implying compensatory signaling via BCR/ABL-independent pathways (22). It has also been reported that resistance to imatinib in CML often is associated with specific point mutations in the *BCR/ABL* kinase domain (23–26).

In the fluorescence in situ hybridization analysis, the *BCR/ABL* gene was found in K562 BMS-R cells and there was no amplification of BCR/ABL copies compared with the parental cell line, K562. We also showed that BCR/ABL protein expressions were down-regulated in dasatinib-resistant cell lines. These results indicate that inhibition of BCR/ABL occurs in posttranslation. It is known that the ubiquitination-proteasome system is a major tool for extralysosomal cytosolic and nuclear protein degradation in the cell (27). The ubiquitin-proteasome system plays a pivotal role in controlling levels and/or activities of proteins in the cell. Moreover, the ubiquitination-proteasome system functions as a quality control mechanism that selectively removes abnormal and damaged proteins. Proteasome inhibitors represent a powerful tool for detailing the role of the ubiquitination-proteasome system in the cell. We showed that the BCR/ABL protein levels increased in the presence of the proteasome inhibitor, lactacystin, within 24 hours. The treatment with lactacystin also prevent the induction of apoptosis in dasatinib-resistant K562 BMS-R cells. These results indicate that the inhibition of BCR/ABL is induced by protein degradation through the ubiquitination-proteasome system. The removal of dasatinib may affect the activation of the ubiquitination-proteasome pathway in dasatinib-resistant K562 BMS-R cells.

BCR/ABL confers resistance to apoptosis in leukemic cells. Protection from programmed cell death may be mediated, in part, through the activation of STATs, PI3K, Ras, and Src family kinases (28–31). Src kinases are involved in BCR/ABL-mediated leukemogenesis and have been implicated in some cases in imatinib resistance (32). Src family kinases regulate multiple cellular events such as proliferation, differentiation, survival, cytoskeletal organization, adhesion, and migration (33). Using the Ab microarray system, we could show that one of the src family kinases, Lck, was enhanced in K562 BMS-R cells. We also could show that one member of the Src family kinases, Lyn, was activated in imatinib-resistant K562 cells. These results are consistent with previous reports (32). Lyn kinase may regulate survival in these imatinib-resistant cell lines. We also found that one of the PI3-K/Akt phoshatases, phosphatase tensin homologue, was reduced in K562-BMS-R cells, and mitogen-activated protein kinase and Akt were activated in dasatinib- and imatinib-resistant K562 cells. Elevations of Akt kinase and mitogen-activated protein kinase activity were also causes leading to the inhibition of apoptosis.

In this report, we could show that K562 BMS-R cells undergo apoptosis after the removal of dasatinib. We found that the removal of dasatinib induces apoptosis after 6 hours. It has been reported that the removal of imatinib leads to the apoptosis of BCR/ABL-overexpressing leukemic cells via a transient activation of the STAT5/Bcl-xL pathway (34). In this study, the BCR/ABL protein was reduced in dasatinib-resistant cells, suggesting that another mechanism was involved in the apoptotic process.

We first established and investigated the dasatinib-resistant cells. Dasatinib is now clinically available and used in CML patient with imatinib resistance or imatinib intolerance. We have also evaluated the primary leukemia cells from imatinib- or dasatinib-resistant patients. The expression of BCR/ABL protein is very low; therefore, it is hard to evaluate the characteristics of primary leukemia cells (data not shown). In this study, we are the first to report dasatinib resistance by degradation of BCR/ABL protein. Our data provides new information regarding the molecular basis of strategy against the dasatinib resistance in CML.

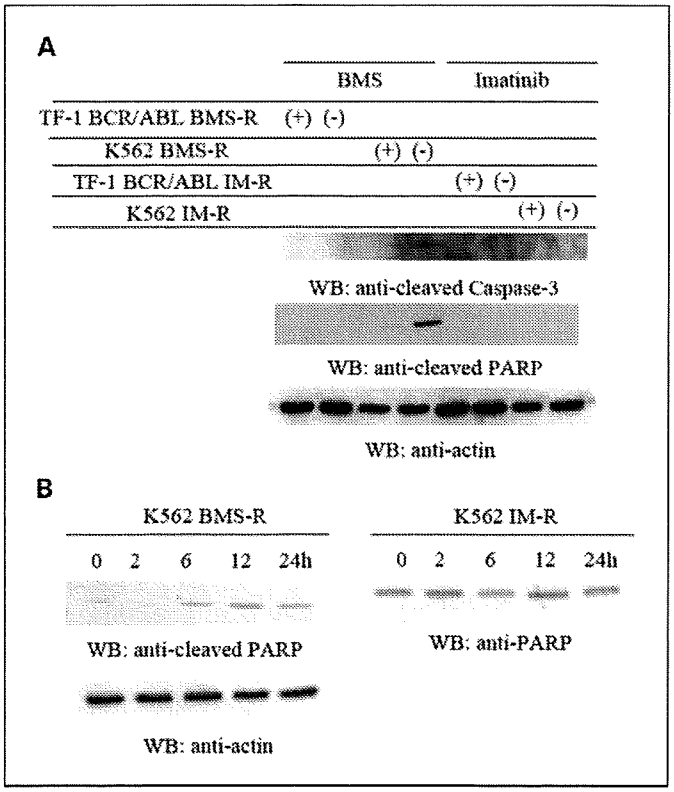

Fig. 5. Dasatinib deprivation induces apoptosis in dasatinib-resistant K562 BMS-R cells. *A*, resistant cell lines were treated with or without dasatinib (1 μmol/L) or imatinib (10 μmol/L) for 24 h and analyzed by immunoblotting with anti-cleaved caspase 3, cleaved PARP, and actin Abs. *B*, K562 BMS-R and K562 IM-R cells were treated without dasatinib (1 μmol/L) or imatinib (10 μmol/L) for the periods indicated and immunoblotted with anti-cleaved PARP or PARP and actin Abs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Activity of a novel Aurora kinase inhibitor against the T315I mutant form of BCR-ABL: *In vitro* and *in vivo* studies

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Despite promising results from clinical studies of ABL kinase inhibitors, a challenging problem that remains is the T315I mutation against which neither nilotinib nor dasatinib show significant activity. In the present study, we investigated the activity of a novel Aurora kinase inhibitor, VE-465, against leukemia cells expressing wild-type BCR-ABL or the T315I mutant form of BCR-ABL. We observed a dose-dependent reduction in the level of BCR-ABL autophosphorylation in VE-465-treated cells. Exposure to the combination of VE-465 and imatinib exerted an enhanced apoptotic effect in K562 cells. Combined treatment with VE-465 and imatinib caused more attenuation of the levels of phospho-AKT and c-Myc in K562 cells. Further, the isobologram indicated the synergistic effect of simultaneous exposure to VE-465 and imatinib in K562 cells. To assess the *in vivo* efficacy of VE-465, athymic nude mice were injected intravenously with BaF3 cells expressing wild-type BCR-ABL or the T315I mutant form. The vehicle-treated mice died of a condition resembling acute leukemia by 28 days; however, nearly all mice treated with VE-465 (75 mg/kg, twice daily; intraperitoneally for 14 days) survived for more than 56 days. Histopathological analysis of vehicle-treated mice revealed infiltration of the spleen. In contrast, histopathological analysis of organs from VE-465-treated mice demonstrated normal tissue architecture. Taken together, the present study shows that VE-465 exhibits a desirable therapeutic index that can reduce the *in vivo* growth of T315I mutant form and wild-type BCR-ABL-expressing cells in an efficacious manner. (*Cancer Sci* 2008; 99: 1251–1257)

The development of imatinib has redefined the management of chronic myeloid leukemia (CML).[1,2] Most newly diagnosed patients with chronic-phase disease, treated with imatinib, achieve durable complete cytogenetic responses.[3] However, a small percentage of these patients and most advanced-phase patients relapse on imatinib therapy.[3–5] Furthermore, only a minority of patients achieve undetectable levels of BCR-ABL transcript.[6] The leading cause of acquired resistance to imatinib is reactivation of BCR-ABL kinase activity via kinase domain mutations that decrease the sensitivity of the kinase to imatinib by 3- to >100-fold.[7–9] Structural analysis has revealed that imatinib binds to a unique, inactive conformation of the ABL kinase domain in which the activation loop is in a closed position that precludes substrate binding.[10] Mutations that confer resistance to imatinib either affect residues involved directly in drug binding, impair the ability of the ABL kinase to undergo the extensive conformational changes required for imatinib binding, or favor the active conformation of the kinase to which imatinib is unable to bind.[10–12] Thus, the need for alternative or additional treatments for imatinib-resistant BCR-ABL-positive leukemia has led to the design of a second generation of targeted therapies, and these efforts have resulted mainly in the development of clinically active small-molecule inhibitors such as nilotinib and dasatinib.[13] However, the challenging problem that remains is the T315I mutation, against which neither nilotinib nor dasatinib show significant activity.

The T315I mutation is one of the most common mutations found in patients undergoing imatinib therapy, and this mutation accounts for approximately 20% of imatinib-resistant cases. Thr315 is located in the center of the imatinib-binding site in ABL.[12,14] The residue at this position is referred to as the 'gatekeeper' residue because it separates the ATP-binding site from an internal cavity that is of variable size in different protein kinases, and the nature of the gatekeeper residue is an important determinant of inhibitory specificity.[15] The compound MK-0457, originally developed as an Aurora kinase inhibitor and currently in clinical trials, has been shown to bind a number of recurring imatinib-resistant mutant forms of BCR-ABL, including those with mutations at the gatekeeper position.[16–18] In contrast to imatinib, which penetrates deeply into the ABL kinase domain, MK-0457 is not fully buried within the kinase domain, and is anchored to it by four hydrogens bound to sequence-invariant elements.[19] Comparison with the structures of the Aurora kinases reveals that all of the essential contacts between MK-0457 and the protein involve highly conserved elements, explaining the broad specificity of this compound.[19]

In the present study, we investigated the activity of the novel Aurora kinase inhibitor VE-465, a compound related to MK-0457, against leukemia cells expressing wild-type or the T315I mutant form of BCR-ABL. We observed a dose-dependent reduction in the level of T315I mutant form and wild-type BCR-ABL kinase activity in VE-465-treated cells. Further, we evaluated the effect of VE-465 on the growth of BaF3 cells expressing wild-type or T315I mutant BCR-ABL *in vivo*. Systemic administration of VE-465 prolonged the survival in mice injected with BaF3 cells expressing wild-type or T315I mutant BCR-ABL. These results demonstrate that VE-465 exhibits a desirable therapeutic index that can reduce the *in vivo* growth of wild-type and T315I mutant BCR-ABL-expressing cells in an efficacious manner.

Materials and Methods

Antibodies and reagents. Anti-ABL antibody[11,20] was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine monoclonal antibody (PY20) was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Anti-Akt and antiphospho-Akt (Ser473) antibodies were purchased from Cell Signaling (Beverly, MA, USA). Anti-c-Myc antibody was purchased from Novus (Littleton, CO, USA). VE-465 was kindly provided by Merck (Blue Bell, PA, USA).

Cells and cell culture. K562 cells were obtained from the American Type Culture Collection (Rockville, MD, USA).

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TF-1BCR-ABL cells were described previously.[21] BaF3 p185 BCR-ABL cells and BaF3 T315I BCR-ABL cells were provided by Dr Martin Ruthardt (Frankfurt University, Frankfurt, Germany). These cell lines were cultured in RPMI-1640 (Life Technologies, Rockville, MD, USA) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT, USA).

Apoptosis assay. The incidence of apoptosis was determined by flow cytometric analysis with the fluorescein isothiocyanate-conjugated APO2.7 monoclonal antibody (clone 2.7), which was raised against a 38-kDa mitochondrial membrane protein (7A6 antigen) expressed by cells undergoing apoptosis.[22]

Immunoblotting and immunoprecipitation. Immunoblotting and immunoprecipitation were carried out as described previously.[23]

Colony-forming assay. K562 cells, BaF3 p185 BCR-ABL cells, primary leukemia cells expressing T315I, and primary bone marrow mononuclear cells were treated with each agent and seeded in triplicate in conditioned MethoCult GF H4434 medium (Stem Cell Technologies, Vancouver, Canada). The leukemic colonies (>50 cells) were scored on day 14. Macroscopic colonies were counted in triplicate dishes on day 14. Primary leukemia cells expressing T315I and primary bone marrow mononuclear cells were obtained with informed consent prior to the study.

Isobologram method. The theoretical basis of the isobologram method and the procedure for making isobolograms have been described previously in detail.[24] K562 cells were suspended to a final concentration of 1×10^5 cells/mL in fresh medium, plated in 24-well dishes, and incubated with VE-465 or imatinib or a combination of the two at 37°C for 72 h. The number of cells in each well was counted by flow cytometry, and the cell numbers were normalized by dividing the number of cells.[20]

Cell cycle analysis. Logarithmically growing K562 cells were incubated with either VE-465 or dimethyl sulfoxide for 48 h. Single-cell suspensions were fixed in 70% ethanol for 15 min, incubated with RNase (1 mg/mL) at 37°C for 30 min, and labeled with 400 μL propidium iodide (50 μg/mL) for 15 min at room temperature. Cell cycle profiles were determined by flow cytometric analysis.[25]

***In vivo* efficacy of VE-465 in a mouse model of BCR-ABL-induced leukemia.** For the *in vivo* assessment of VE-465, 6-week-old female nude mice were injected with 10^6 BaF3 p185 BCR-ABL cells or BaF3 T315I BCR-ABL cells and then assigned randomly to either the vehicle alone or VE-465 treatment groups. At 24 h after the injection, these mice were treated with either vehicle or VE-465 (75 mg/kg b.i.d.; intraperitoneally for 14 days; resting for 14 days). Mice were observed daily, and bodyweight as well as signs of stress (e.g. lethargy, ruffled coat, or ataxia) were used to detect possible toxicities. The average tumor weight per mouse was calculated and used to analyze the group mean tumor weight ± SE ($n = 5$ mice).

Tumor and tissue processing. Tumors were collected at the selected times and fixed in paraformaldehyde. Paraffin-embedded tissues were sectioned and processed for gross histopathology by hematoxylin–eosin staining.

Results

VE-465 inhibits the proliferation of wild-type and T315I mutant BCR-ABL-expressing leukemia cells and inhibits BCR-ABL autophosphorylation. VE-465 is a synthetic small-molecule inhibitor of Aurora kinase (Fig. 1a) with apparent inhibition constant (Ki, app) values of 1.0, 26.0, and 8.7 nM for Aurora-A, Aurora-B, and Aurora-C, respectively. VE-465 inhibited the proliferation of K562 cells, BaF3 p185BCR-ABL cells, and BaF3 T315IBCR-ABL cells with inhibitory concentration (IC_{50}) values ranging from 50 to 500 nM (Fig. 1b–d). We also evaluated its growth-inhibitory effects on several human leukemia cell lines, primary leukemia cells, and primary bone marrow mononuclear cells (Table 1; Fig. 1e). VE-465 inhibited the human leukemia cell lines and primary leukemia cells with IC_{50} values ranging from 50 to 150 nM (Table 1; Fig. 1e). Because the Aurora kinases are essential for proliferation, BaF3 T315IBCR-ABL cells were incubated with the indicated concentrations of VE-465 for 48 h, after which, we examined the effects of VE-465 on the cell cycle profile by flow cytometry (Fig. 1f). VE-465 caused accumulation of cells in G_2/M arrest (Fig. 1f). To assess the inhibition of BCR-ABL kinase activity, BCR-ABL-expressing cells were cultured with the indicated concentrations of VE-465 for 24 h, and autophosphorylation of BCR-ABL was analyzed by immunoblotting (Fig. 2a–c). VE-465 inhibited wild-type and T315I mutant BCR-ABL autophosphorylation with IC_{50} values ranging from 2.0 to 5.0 μM, significantly higher than the results of the cell-proliferation assay (Figs 1,2). These results suggest that the growth-inhibitory effect of VE-465 on BCR-ABL-expressing cells is caused by Aurora kinase inhibition.

Table 1. VE-465 blocks leukemia cell proliferation

Cell line	IC_{50} (nM)
HL-60	50.2
NB4	38.6
U937	110.5
HAL-01	148.2
OM9; 22	98.4
TF-1	112.2
TF-1 BCR-ABL	152.4

IC_{50}, inhibitory concentration.

Cotreatment of VE-465 and imatinib enhances the induction of apoptosis in BCR-ABL-transformed cells. K562 and TF-1 BCR-ABL cells were cultured with the indicated concentrations of VE-465 and imatinib for 72 h, after which the percentage of apoptotic cells was determined using APO2.7 (Fig. 3a,b). When 50 nM VE-465 was combined with imatinib in K562 cells, the increase in apoptotic cells was virtually complete for imatinib concentrations higher than 1 μM (Fig. 3a). Cotreatment of VE-465 and imatinib also enhanced the induction of apoptosis in TF-1 BCR-ABL cells (Fig. 3b). Next, we determined the effect of cotreatment with VE-465 and imatinib on the level of signaling proteins downstream of BCR-ABL (Fig. 4a). Compared with treatment with either agent alone, relative low concentrations of VE-465 (50 nM) and imatinib (100 nM) for 24 h caused more attenuation of the levels of phospho-Akt and c-Myc (Fig. 4a). Corresponding immunoblotting analysis also revealed that the combination of 50 nM VE-465 and 100 nM imatinib resulted in increased caspase-3 and poly (ADP-ribose) polymerase (PARP) degradation in K562 cells (Fig. 4b). Together, these findings indicate that a combination of minimally toxic concentrations of VE-465 and imatinib is effective in inducing apoptosis in wild-type BCR-ABL-expressing cells.

Combined effects of VE-465 and imatinib in BCR-ABL-transformed cells. We determined the colony growth of K562 and BaF3 cells expressing p185 BCR-ABL (Fig. 5a,b). Cotreatment with VE-465 and imatinib caused significantly more inhibition of colony growth than treatment with either agent alone in K562 and BaF3 p185BCR-ABL cells (Fig. 5a,b). Further, we used the isobologram method to determine whether the combined effect of VE-465 and imatinib was additive or synergistic. Figure 5c showed the dose–response curve for VE-465 in combination with imatinib in K562 cells. The isobologram was generated on the dose–response curve (Fig. 5d). The observed data from the isobologram indicated the synergistic effect of simultaneous exposure to VE-465 and imatinib in K562 cells (Fig. 5d).

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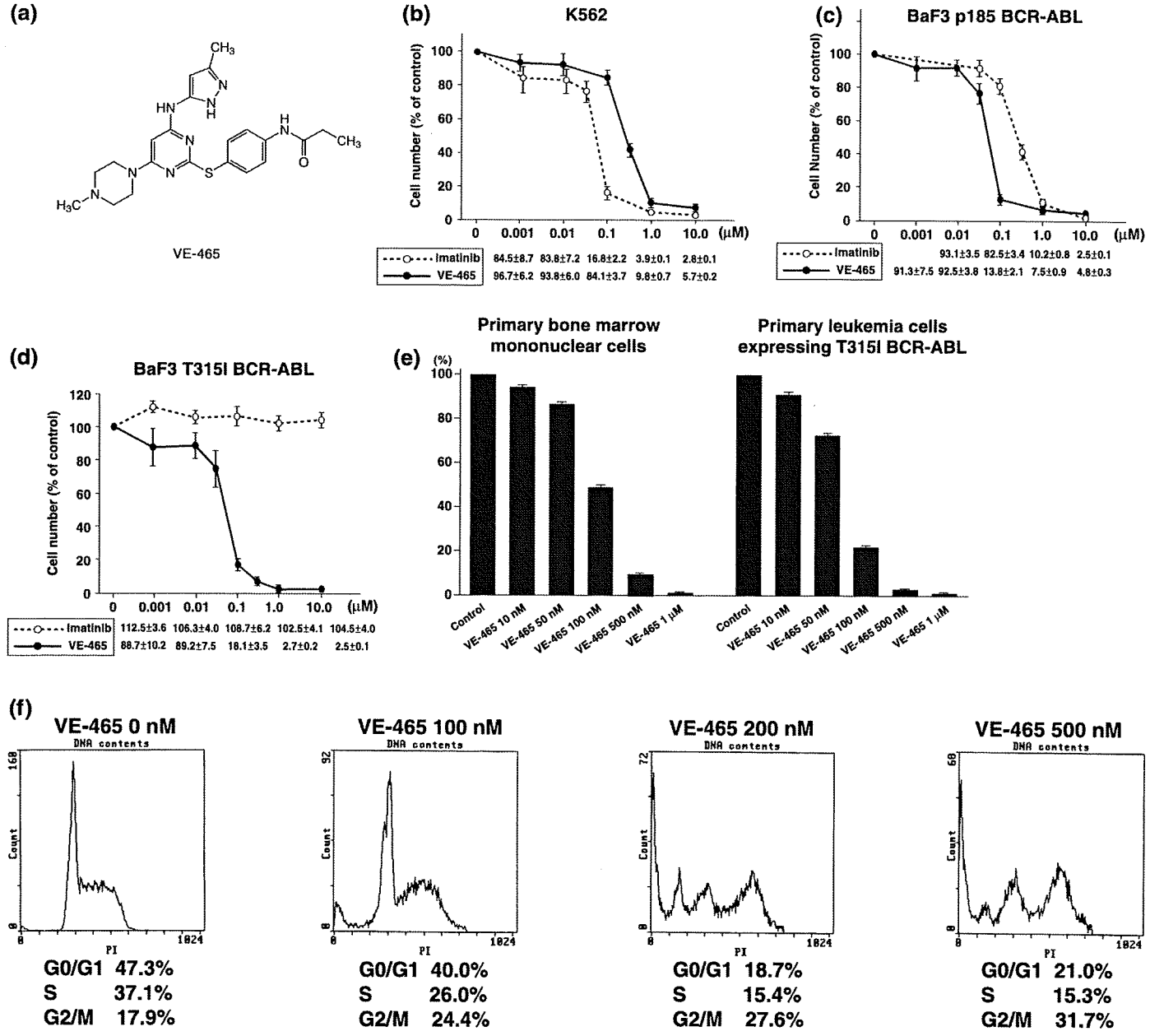

Fig. 1. VE-465 inhibits the growth of wild-type BCR-ABL or T315I mutant form of BCR-ABL-expressing leukemia cells. (a) Chemical structure of VE-465. (b) K562, (c) BaF3 p185BCR-ABL, and (d) BaF3 T315IBCR-ABL cells were cultured with the indicated concentrations of imatinib or VE-465 for 48 h. Bars = SEM, $n = 3$. In all dose-dependent curves, viable cell counts are represented as the percentage of control cells for each compound's dose. (e) Primary bone marrow mononuclear cells and primary leukemia cells expressing T315T obtained with informed consent prior to conducting studies were grown in methylcellulose containing the indicated concentrations of VE-465. Colony counts were assessed on each individual sample at least twice, and results are presented as average ± SD for colonies counted from triplicate plates under each condition. (f) BaF3 T315IBCR-ABL cells were incubated with the indicated concentrations of VE-465 for 48 h, after which we examined the effects of VE-465 on cell-cycle profile of BaF3 T315I BCR-ABL by flow cytometry.

Systemic VE-465 treatment decreases T315I BCR-ABL kinase activity *in vivo* and prolongs survival in a mouse model of BCR-ABL-induced leukemia. To further study the activity of VE-465 on tumor growth *in vivo*, we tested a mouse model of BCR-ABL-induced leukemia (Fig. 6). Intravenous injection of BaF3 cells expressing p185BCR-ABL or T315IBCR-ABL into nude mice resulted in an aggressive malignancy resembling acute leukemia, characterized by splenomegaly, circulating blasts, and invasion of leukemia cells into hematopoietic and non-hematopoietic tissue (Fig. 6a). At 24 h after the injection, the mice were divided into two groups (five mice per group), with each group receiving either vehicle or VE-465 (75 mg/kg, b.i.d.; intraperitoneally for 14 days; resting for 14 days). The vehicle-treated mice died of a condition resembling acute leukemia by 28 days; however, nearly all of the mice treated with VE-465 survived for more than 56 days (Fig. 6b). All VE-465-treated mice demonstrated modest weight loss (less than 10% initial bodyweight). Histopathological analysis of vehicle-treated mice revealed infiltration of the spleen and bone marrow with leukemic blasts (Fig. 6a). In contrast, histopathological analysis of organs from VE-465-treated mice demonstrated normal tissue architecture and no evidence of residual leukemia (Fig. 6a). BCR-ABL kinase