with FLT3 mutation. *Int J Hematol.* 2006;83:301-308.

21. Speck NA, Gilliland DG. Core-binding factors in haematopoiesis and leukaemia. *Nat Rev Cancer.* 2002;2:502-513.
22. Frohling S, Scholl C, Gilliland DG, Levine RL. Genetics of myeloid malignancies: pathogenetic and clinical implications. *J Clin Oncol.* 2005;23:6285-6295.
23. Renneville A, Roumier C, Biggio V, et al. Cooperating gene mutations in acute myeloid leukemia: a review of the literature. *Leukemia.* 2008;22:915-931.
24. Thomasson MH, Xiang Z, Walgren R, et al. Somatic mutations and germline sequence variants in the expressed tyrosine kinase genes of patients with de novo acute myeloid leukemia. *Blood.* 2008;111:4797-4808.
25. Loriaux MC, Levine RL, Tyner JW, et al. High-throughput sequence analysis of the tyrosine kinome in acute myeloid leukemia. *Blood.* 111: 4788-4796.
26. Zheng R, Levis M, Piloto O, et al. FLT3 ligand causes autocrine signaling in acute myeloid leukemia cells. *Blood.* 2004;103:267-274.
27. Kano Y, Akutsu M, Tsunoda S, et al. In vitro cytotoxic effects of a tyrosine kinase inhibitor STI571 in combination with commonly used antileukemic agents. *Blood.* 2001;97:1999-2007.
28. Minami Y, Yamamoto K, Kiyoi H, Ueda R, Saito H, Naoe T. Different antiapoptotic pathways between wild-type and mutated FLT3: insights into therapeutic targets in leukemia. *Blood.* 2003;102: 2969-2975.
29. Osumi K, Fukui T, Kiyoi H, et al. Rapid screening of leukemia fusion transcripts in acute leukemia by real-time PCR. *Leuk Lymphoma.* 2002;43: 2291-2299.
30. Ninomiya M, Abe A, Katsumi A, et al Homing, proliferation and survival sites of human leukemia cells in vivo in immunodeficient mice. *Leukemia.* 2007;21:136-142.
31. Kiyoi H, Shiotsu Y, Ozeki K, et al. A novel FLT3 inhibitor FI-700 selectively suppresses the growth of leukemia cells with FLT3 mutations. *Clin Cancer Res.* 2007;13:4575-4582.
32. Levis M, Brown P, Smith BD, et al. Plasma inhibitory activity (PIA): a pharmacodynamic assay reveals insights into the basis for cytotoxic response to FLT3 inhibitors. *Blood.* 2006;108:3477-3483.
33. Weisberg E, Boulton C, Kelly LM, et al. Inhibition of mutant FLT3 receptors in leukemia cells by the small molecule tyrosine kinase inhibitor PKC412. *Cancer Cell.* 2002;1:433-443.
34. Harrington EA, Bebbington D, Moore J, et al. VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo. *Nat Med.* 2004;10:262-267.
35. Apperley JF. Part I: mechanisms of resistance to imatinib in chronic myeloid leukaemia. *Lancet Oncol.* 2007;8:1018-1029.
36. Quintas-Cardama A, Kantarjian H, Cortes J. Flying under the radar: the new wave of BCR-ABL inhibitors. *Nat Rev Drug Discov.* 2007;6:834-848.

Epidemiologic study on survival of chronic myeloid leukemia and Ph$^+$ acute lymphoblastic leukemia patients with BCR-ABL T315I mutation

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The BCR–ABL T315I mutation represents a major mechanism of resistance to tyrosine kinase inhibitors (TKIs). The objectives of this retrospective observational study were to estimate overall and progression-free survival for chronic myeloid leukemia in chronic-phase (CP), accelerated-phase (AP), or blastic-phase (BP) and Philadelphia chromosome—positive (Ph)$^+$ acute lymphoblastic leukemia (ALL) patients with T315I mutation. Medical records of 222 patients from 9 countries were reviewed; data were analyzed using log-rank tests and Cox proportional hazard models. Median age at T315I mutation detection was 54 years; 57% cases were men. Median time between TKI treatment initiation and T315I mutation detection was 29.2, 15.4, 5.8, and 9.1 months, respectively, for CP, AP, BP, and Ph$^+$ ALL patients. After T315I mutation detection, second-generation TKIs were used in 56% of cases, hydroxyurea in 39%, imatinib in 35%, cytarabine in 26%, MK-0457 in 11%, stem cell transplantation in 17%, and interferon-α in 6% of cases. Median overall survival from T315I mutation detection was 22.4, 28.4, 4.0, and 4.9 months, and median progression-free survival was 11.5, 22.2, 1.8, and 2.5 months, respectively, for CP, AP, BP, and Ph$^+$ ALL patients. These results confirm that survival of patients harboring a T315I mutation is dependent on disease phase at the time of mutation detection. (Blood. 2009;114:5271-5278)

Introduction

BCR-ABL kinase domain mutations have been identified as the major mechanisms of resistance to tyrosine kinase inhibitors (TKIs) in Philadelphia chromosome—positive (Ph$^+$) leukemias. The BCR-ABL T315I mutation affects a common Abl kinase contact residue and confers high-level cross-resistance to all current approved Abl kinase inhibitors (imatinib, dasatinib, and nilotinib).[1-4] The reported T315I mutation frequency in imatinib-resistant chronic myeloid leukemia (CML) patients ranges between 2% and 20%,[2,5-8] with variability related to detection methods as well as patient cohort characteristics and treatment. T315I mutation frequency appears to be greater in Ph$^+$ acute lymphoblastic leukemia (ALL) patients[2] and likely increases with the continuation of TKI treatment.[3]

Although hematologists are now increasingly aware of the importance of the T315I mutation, published literature focusing on survival information for CML patients with T315I mutation remains very limited. A multicenter French study of 27 T315I$^+$ CML patients suggested that the presence of a T315I mutation is associated with significantly worse survival than other mutations and is more strongly prognostic in chronic phase (CP) compared with accelerated phases (APs) of CML.[8,9] Conversely, a more recent study[10] from the M. D. Anderson Cancer Center, also of 27 patients with the T315I mutation, suggested no statistically significant survival difference between patients with T315I mutation and those with other mutations. Because of limited sample size, neither of these studies had adequate statistical power to query survival information for different phases of CML (CP, AP, or blastic phase [BP]), and no information has been published to date on the survival of Ph$^+$ ALL patients harboring the T315I mutation.

Several investigational agents have been reported to demonstrate activity against the T315I mutation. Giles et al[11] reported 3 patients with T315I$^+$ CML or Ph$^+$ ALL who achieved clinical responses to the aurora kinase inhibitor MK-0457 without associated adverse events, whereas Legros et al[12] reported that the T315I mutation disappeared in a CML patient treated with homoharringtonine. In addition, it has been reported that some CML patients harboring a T315I mutation may have a transient response to second-generation TKIs in vivo.[10] Several other agents have demonstrated activity against T315I mutation-bearing leukemic cells in preclinical models; however, none of these agents has been

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clinically proven to be safe and effective in the treatment of CML and Ph+ ALL patients with T315I mutations.

It can be anticipated that the number of CML and Ph+ ALL patients with T315I mutation will increase as the use of TKIs increases. Given the lack of information regarding the response to treatment and subsequent survival of patients who develop a T315I mutation while on TKIs, we conducted a multicenter epidemiologic study and collected detailed demographic, clinical, treatment, and mutation detection information for patients identified as harboring the BCR-ABL T315I mutation.

Methods

Study population

Eligible patients were those with CML and Ph+ ALL identified as possessing the T315I mutation between the years 1999 and 2008 by the use of any available validated technique. Patients were 18 years of age or older when their T315I mutation was detected, had received treatment with first- (imatinib) or second-generation (dasatinib or nilotinib) TKIs, and had documented hematologic or cytogenetic resistance, either primary or secondary (acquired), as described in the European LeukemiaNet Guidelines.[8,13,14] The minimum follow-up time from the date of T315I mutation detection for patients who were still alive was 3 months; there was no required minimum follow-up time for patients who were deceased. A total of 9 countries from 3 regions participated in this study, including Europe (Denmark, France, Germany, Italy, United Kingdom), Asia (Japan, South Korea, Singapore), and North America (United States). Eligible patients were identified either through a national CML database (ie, French group of CML, Fi-LMC group), central laboratory databases (ie, Italian and German patients), or single hospital-based databases. It is notable that information on a small proportion of patients has been published.[8-10] The study was approved by the institutional review board/ethics review committee for each participating site/country. Written informed consent for voluntary participation in the study was obtained from the patient or his or her legal representative, where required, in accordance with the Declaration of Helsinki.

Data collection

Uniform case report forms (CRFs) were designed to collect detailed demographic, clinical, treatment, mutation detection, and current survival information from medical records and/or clinical databases at each site. The same criteria for different phases of CML and Ph+ ALL, treatment response, disease progression, and TKI resistance were used for all sites. T315I mutation was detected by the use of different methods, including direct sequencing, polymerase chain reaction (PCR)–based methods, and denaturing high-performance liquid chromatography. Detailed information on each mutation test (irrespective of T315I presence or absence) was collected, including dates of sample collection and analysis, biologic sample type, techniques used for mutation detection, and other mutated clones detected. T315I mutation detection date was defined as the sample collection date in which a T315I mutation was first identified, and the predominant clone was defined as the one present in the highest percentage among all detected clones, whenever this information was available.

Survival measurement

Overall survival (OS) and progression-free survival (PFS) were the 2 primary end points for this study. OS was calculated from different starting points (from the date of first TKI treatment initiation, date of first identification of TKI resistance, and date of T315I mutation detection) to date of death or most recent date that the patient was known to be alive, stratified by the corresponding disease phase at different starting points. TKI resistance and treatment failure and disease progression were defined on the basis of the criteria of the European LeukemiaNet[14] and the pivotal International Randomized Study of Interferon Versus STI571 (ie, IRIS) clinical trial.[15]

Quality control

Two levels of quality control were performed to ensure the quality of the data. At each site, a minimum of 25% of patients identified for chart abstraction or at least one medical chart (if $n < 4$) was reviewed by a second, independent staff member at the site. Identification of suboptimal CRF completion resulted in data management retraining and repeat abstraction of data. In addition, site monitors checked the completion of each CRF and performed source data verification on 6 critical data points: date of diagnosis, disease phase at diagnosis, date of first TKI resistance, date of T315I mutation detection, phase at T315I mutation detection, and date of death or most recent date that the patient was known to be alive.

Statistical analysis

Median follow-up time was calculated for all patients. Kaplan-Meier plots and log-rank tests were used for survival analysis for different phases of CML (CP, AP, and BP) and Ph+ ALL patients, starting from the date of first TKI treatment initiation, date of first TKI resistance, and date of T315I mutation detection. Separate Cox proportional hazard models on OS from T315I mutation detection were performed for each phase of CML and Ph+ ALL patients. Candidate covariates include demographics (eg, age, sex, race, country), performance status, and mutation detection (eg, techniques and biologic samples used for T315I mutation detection, whether the T315I BCR-ABL mutation was predominant, whether other mutations were detected before T315I or concurrently with T315I). Because of the retrospective study design and the researchers' inability to collect adequate information on dosing, drug therapies could not be included in the Cox proportional hazard models. If the 2-sided P value for a certain covariate was less than .10 in crude analysis, this covariate was included in the adjusted Cox model for further analysis by the use of backward stepwise methods.[16]

Results

Demographic information

A total of 222 patients were included in this study (Table 1). The median age at time of T315I mutation detection was 54 years (range, 18-84 years). A total of 126 (57%) patients were men. The majority of patients were white (75%), and 22% were Asian, likely the result of disproportionate accrual from 1 site in South Korea. Most of the patients were from France (32%; 13 sites), Italy (21%; 2 sites), South Korea (15%, 1 site), and the United States (12%, 3 sites).

The majority of CML patients were in CP at the time of diagnosis (70%) or TKI initiation (60%); however, at the time of T315I mutation detection, many patients in CP had progressed to AP and BP, such that only 82 patients (37%) remained in CP, whereas 38 (17%) progressed to AP and 56 (25%) progressed to BP, in addition to the 46 (21%) patients who were Ph+ ALL at the time of T315I mutation detection. Of the patients in BP, 16 were lymphoid, 32 were myeloid, and 8 were either biphenotypic or missing phenotype.

Advanced-phase CML (AP and BP) and Ph+ ALL patients were usually treated with TKIs shortly after diagnosis (median time between diagnosis and TKI initiation: AP, 1.1 months; BP, 0.5 months; Ph+ ALL, 0.4 months vs CP, 11.7 months) and developed TKI resistance within a shorter period of time than CP patients (median time between TKI initiation and TKI resistance: AP, 9.4 months; BP, 4.7 months; and Ph+ ALL, 8.8 months vs CP,

Table 1. Demographic characteristics of the patients harboring a T315I mutation (n = 222)

Characteristic	Value
Median age, y (range)	
At diagnosis	49 (16-80)
At first time of TKI resistance	52 (18-81)
At first time of T315I mutation detection	54 (18-84)
CP (n = 82)	60 (21-84)
AP (n = 38)	51 (19-76)
BP (n = 56)*	41 (21-79)
Ph+ ALL (n = 46)†	52 (18-74)
Male/female, n (%)	126/96 (56.8/43.2)
Race, n (%)	
White	166 (74.5)
Asian	49 (22.1)
Other	7 (3.4)
Country/site, n (%)	
France	72 (32.4)
Italy	46 (20.7)
Korea, Republic of	33 (14.9)
United States	27 (12.2)
Germany	17 (7.6)
Singapore	13 (5.9)
Denmark	6 (2.7)
United Kingdom	5 (2.3)
Japan	3 (1.4)
Disease phase at T315I detection, n (%)	
CML CP	82 (36.9)
CML AP	38 (17.1)
CML BP	56 (25.2)
Ph+ ALL	46 (20.7)

ALL indicates acute lymphoblastic leukemia; AP, accelerated phase; BP, blastic phase; CML, chronic myeloid leukemia; CP, chronic phase; and Ph, Philadelphia chromosome.

*Among the 56 CML BP patients, 16 were lymphoid, 32 were myeloid, and 8 were biphenotypic or the immunophenotype was missing.

†A total of 40 (87%) of the 46 Ph+ ALL patients were from France (n = 22) and Italy (n = 18).

17.4 months). The median time between TKI treatment start and T315I mutation detection was 29.2 months, 15.4 months, 5.8 months, and 9.1 months, respectively in CP, AP, BP, and Ph+ ALL patients.

Treatment information

Treatment before TKI initiation. A minority of patients (n = 60, 27%) had TKIs as the first line of treatment after diagnosis, most of whom received imatinib as first-line treatment (56 of 60). A total of 162 (73%) patients had other treatment(s) before TKIs, including hydroxyurea (n = 118, 53%), interferon-α (IFN-α, n = 77, 35%), cytarabine (n = 43, 19%), and stem cell transplantation (n = 14, 6%; including 7 autologous and 7 allogeneic transplantations).

Treatment between TKI initiation and T315I mutation detection. All 222 patients received TKI therapy before T315I detection. Between TKI treatment initiation and T315I mutation detection, 97% of patients received imatinib for a median duration of 13 months (range, 0.3-77.0 months); 50% received a second-generation TKI for a median duration of 3.6 months (range, 0.1-31.2 months); 32% were treated with hydroxyurea (median duration 2.5 months [range, 0.1-49.4 months]); 27% with cytarabine (median duration 0.5 months [range, 0.1-20.9 months]); 10% underwent stem cell transplantation, including 7% allogeneic transplantation; and 8% were treated with IFN-α (median duration 3.8 months [range, 0.4-19.8 months]).

Treatment after T315I mutation detection. Treatment information after T315I detection was available for 216 patients (Table 2); overall, 125 (58%) patients were treated with second-generation TKI (dasatinib or nilotinib), including 58 (27%) who were continuing treatment that had commenced before T315I detection (carry-over); 87 (40%) with hydroxyurea, including 13 (6%) carry-over; 78 (36%) with imatinib, including 50 (23%) carry-over; 57 (26%) with cytarabine (all new treatment, no carry-over); 37 (17%) with stem cell transplantation, including 31 (14%) allogeneic transplantations; 25 (12%) with MK-0457; 26 (12%) with other investigational agent(s); and 14 (6%) with IFN-α.

After the detection of T315I, the first therapy delivered was a second-generation TKI for 88 (41%) patients (81 [38%] as monotherapy), imatinib in 72 (33%) patients (59 [27%] as monotherapy), and hydroxyurea in 38 (18%) patients (18 [8%] as monotherapy). A total of 151 patients received a second-line therapy after T315I detection: 50 (33%) were treated with hydroxyurea, 34 (23%) with second-generation TKI (25 [17%] as monotherapy), and 31 (21%) with cytarabine (4 [3%] as monotherapy). Additional treatment information after T315I detection is summarized in Table 2.

T315I mutation information

Other mutations before T315I mutation detection. Fifty-eight (26%) of 222 patients had a BCR-ABL mutation test before T315I mutation detection, and the median time between the first mutation

Table 2. Treatments information after T315I mutation detection (n = 216)

Treatment	n (%)	Median duration, mo (range)	New treatment, n (%)*	Carry-over, n (%)*	By line of treatment†, n (%)			
					First line (n = 216)	Second line (n = 151)	Third line (n = 97)	Four or more lines (n = 60)
Second-generation TKI	125 (58)	1.3 (0.1-32.1)	67 (31)	58 (27)	88 (41)	34 (23)	15 (15)	23 (38)
Hydroxyurea	87 (40)	1.8 (0.1-34.9)	74 (34)	13 (6)	38 (18)	50 (33)	25 (26)	47 (78)
Imatinib	78 (36)	1.3 (0.1-83.5)	28 (13)	50 (23)	72 (33)	9 (6)	9 (9)	16 (27)
Cytarabine	57 (26)	0.3 (0.1-22.9)	57 (26)	0	13 (6)	31 (21)	18 (19)	37 (62)
Stem cell transplantation	37 (17)	N/A	37 (17)	0	N/A	N/A	N/A	N/A
MK-0457	25 (12)	1.6 (0.1-13.2)	25 (12)	0	1 (0.5)	8 (5)	6 (6)	15 (25)
Other investigational drug	26 (12)	6.8 (0.2-27.6)	24 (11)	2 (1)	5 (2)	9 (6)	6 (6)	15 (25)
Interferon-α	14 (6)	4.1 (0.1-34.9)	12 (5)	2 (1)	2 (1)	4 (3)	6 (6)	3 (5)

*Only the major treatments are summarized in this table. If the start date of the drug is before T315I mutation detection, it is called "carry-over"; if the start date of the drug is after T315I mutation detection, it is called "new treatment."

†A single drug can be used as monotherapy or combined with other drugs and can be used in multiple lines of therapies. Therefore, the total of first- to fourth-line treatments with a drug may be greater than 100%.

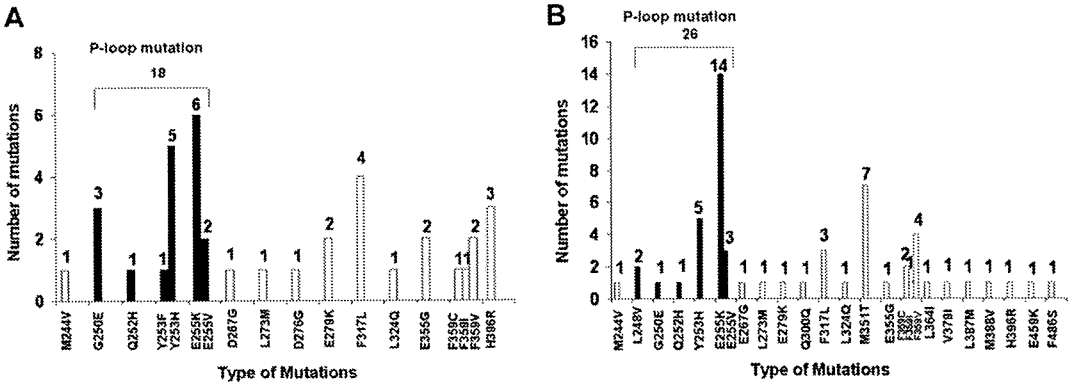

Figure 1. BCR-ABL mutations detected before and concomitantly to the T315I mutation detection. (A) Other BCR-ABL mutations detected before the T315I mutation detection (n = 36; 16%). Two patients had 2 mutations detected in different tests (one with G250E/L273M and one with Y253H/F317L). (B) Other BCR-ABL mutations detected at the time of T315I detection (n = 52; 23%). Five patients had 2 additional mutations detected. *Solid black bars are different types of P-loop mutations.

test and T315I mutation detection was 7 months (range, 0.5-35 months). Before T315I mutation detection, 62% (36/58) of patients had other mutations detected, and 2 patients had 2 mutations detected in different tests (one patient with G250E + L273M and the other patient with Y253H + F317L). A total of 18 P-loop mutations were detected (Figure 1A), with the most frequent being E255K (n = 6) and Y253H (n = 5).

T315I mutation detection. Most patients (n = 162; 73%) had T315I mutation detected after TKI resistance, with a median "lead time" (time from first TKI resistance until collection of sample that first identified a T315I mutation) of 6.4 months (range, 0.1-73.2 months); 14% had simultaneous categorization of TKI resistance and the presence of the T315I mutation; and 13% of patients had the T315I mutation detected before meeting criteria for clinical resistance, with a median "lead time" (time from collection of sample which first identified a T315I mutation until first TKI resistance) of 1.2 months (range, 0.1-20.7 months previously). In addition, within 1 month either before or after TKI resistance, 114 (51%) patients had mutation analysis performed, and 90 (41%) were identified as harboring the T315I.

Other mutations found at the time of T315I mutation detection. A total of 52 (23%) patients had other mutations detected concomitantly with T315I detection. By disease phase, 18% (15/82) of CML CP and 26% (37/140) of non-CML CP patients had other mutations detected in addition to T315I. Five patients had 2 other mutations detected in addition to T315I, including G250E plus F317L, Y253H plus M351T, E255K plus V, L273M plus F317L, and M351T plus L387M, totaling 57 mutations among the 52 patients (Figure 1B). Twenty-five patients (11%) had a P-loop mutation detected, the most frequent being E255K (n = 14).

Clonal predominance at the time of T315I mutation detection. T315I was the predominant clone among 194 (87%) patients when first detected, as reported by each individual participating site. Nine patients had a predominant P-loop mutant clone, including Y253H (n = 1), E255K (n = 6), and E255V (n = 2), and 6 patients had other predominant clones identified, including D267G (n = 1), L273M (n = 1), M351T (n = 2), F359C (n = 1), and F359V (n = 1). Six patients had wild-type BCR-ABL as the predominant clone. The predominant clone could not be determined for 7 patients. Among the 194 patients whose predominant clone was T315I, 30 patients also had other mutations (including 2 patients with 2 other mutations, 1 with G250E + F317L, and 1 with M351T + L387M).

Techniques and biologic samples used for T315I mutation detection. Of the various techniques used to detect the T315I mutation, direct sequencing was the most frequently used method (55% patients underwent the direct sequencing method only and 12% patients underwent both direct sequencing and various PCR-based methods). Other techniques included reverse transcription-PCR/denaturing high-performance liquid chromatography and sequencing (20%), and a PCR-based method only (13%). Among the 12% of patients in whom mutation analysis was done by both direct sequencing and PCR-based method, there was no discordance between the different techniques. Peripheral blood was the most frequently used sample type (73% used blood samples only and 5% used both blood and bone marrow samples). The majority of the T315I mutant samples (n = 172, 77%) were analyzed within 1 month of collection.

OS and PFS

Survival from leukemia diagnosis (OS only), TKI treatment initiation (OS only), first time of TKI resistance (OS and PFS), and T315I mutation detection (OS and PFS) are shown in Figures 2A through D and 3A and B and summarized in Table 3. The results suggest that OS is dependent on disease phase at corresponding starting points and that survival is similar between CP and AP and between BP and Ph^+ ALL. For survival from T315I detection, the median OS was 22.4, 28.4, 4.0, and 4.9 months, and median PFS was 11.5, 22.2, 1.8, and 2.5 months, respectively, for CP, AP, BP, and Ph^+ ALL patients. It should be noted that the sample sizes for AP and BP patients were limited, especially at the time of leukemia diagnosis and TKI treatment initiation.

To further understand the OS from T315I mutation detection for CP patients (n = 82), we classified the CP patients into 4 subgroups: patients who had never progressed to AP or BP during their lifetime (n = 34, median OS = 48.9 months); patients who had progressed to AP or BP before T315I mutation detection while remaining CP from the time of T315I mutation detection (n = 13, median OS = 22.1 months); patients who were constant CP before T315I mutation detection and progressed to AP or BP after T315I mutation detection (n = 28, median OS = 29.5 months); and patients who had progressed to AP or BP both before and after T315I mutation detection, although they were classified as CP at the time of T315I mutation detection (n = 7, median OS = 8.7 months). The results suggested that if CP patients had progressed to AP or BP either before or after T315I mutation, their survival was much worse than those who had never progressed to AP or BP during their lifetime.

In an exploratory analysis combining the 16 lymphoid CML BP

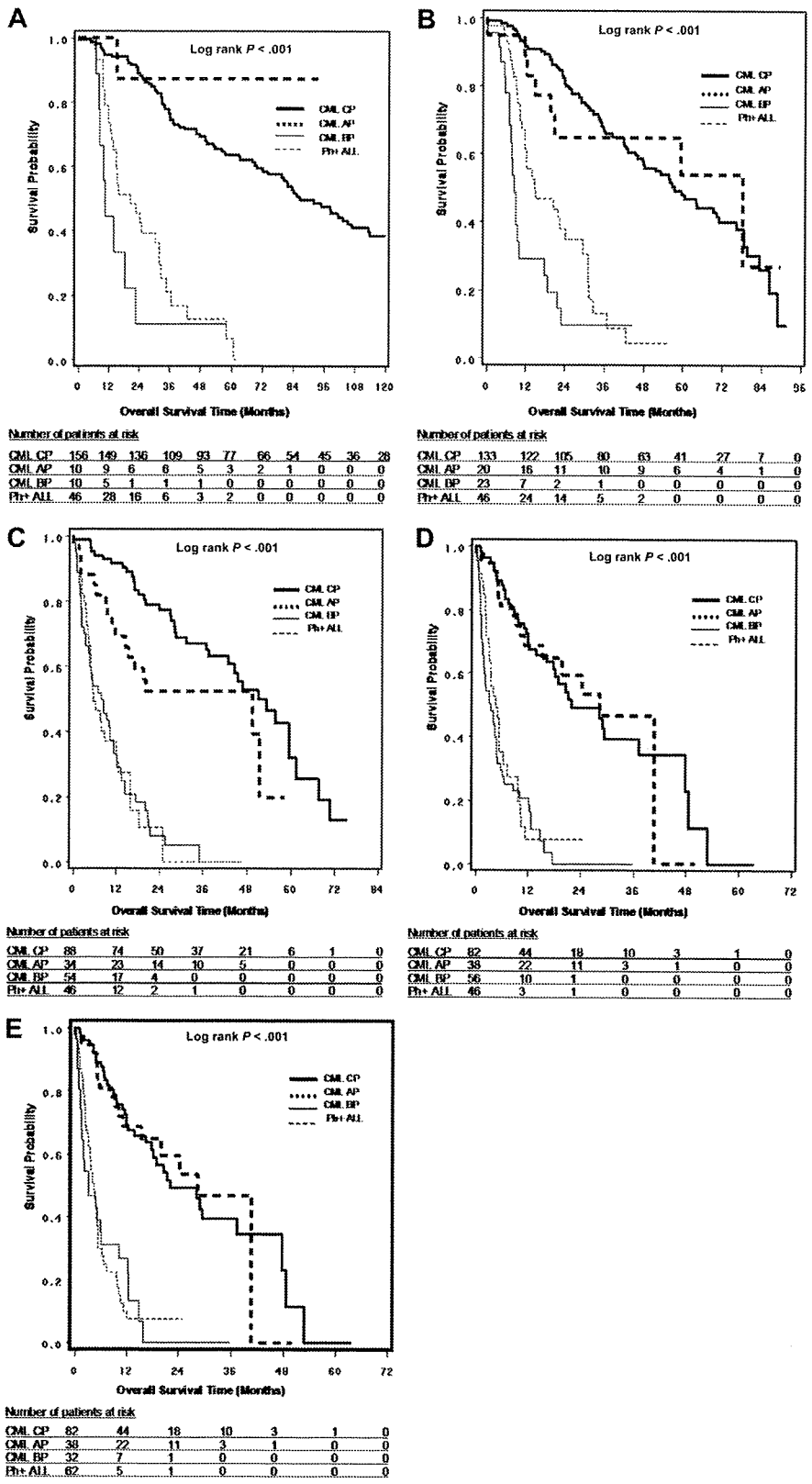

Figure 2. Survival analysis in patients with T315I BCR-ABL mutation. (A) OS from leukemia diagnosis by disease phases at the time of leukemia diagnosis. (B) OS from TKI treatment start by disease phase at the time of TKI treatment start. (C) OS from first TKI resistance by disease phase at the time of first TKI resistance. (D) OS from first T315I mutation detection by disease phase at the time of T315I mutation detection. (E) OS from first T315I mutation detection by disease phase at the time of T315I mutation detection, where CML LBP were combined with Ph+ ALL (n = 214)

(LBP) patients with de novo Ph+ ALL patients, the median OS from the time of T315I mutation detection was 4.8 months (95% confidence interval [CI], 1.6-10.0; n = 32) for CML myeloid blastic phase, and 4.5 months (95% CI, 3.5-5.4; n = 62) for LBP/Ph+ ALL (Figure 2E).

Cox proportional hazard model for OS from T315I mutation detection

Cox proportional hazard models on OS from T315I mutation detection were performed for different phases of CML and Ph+

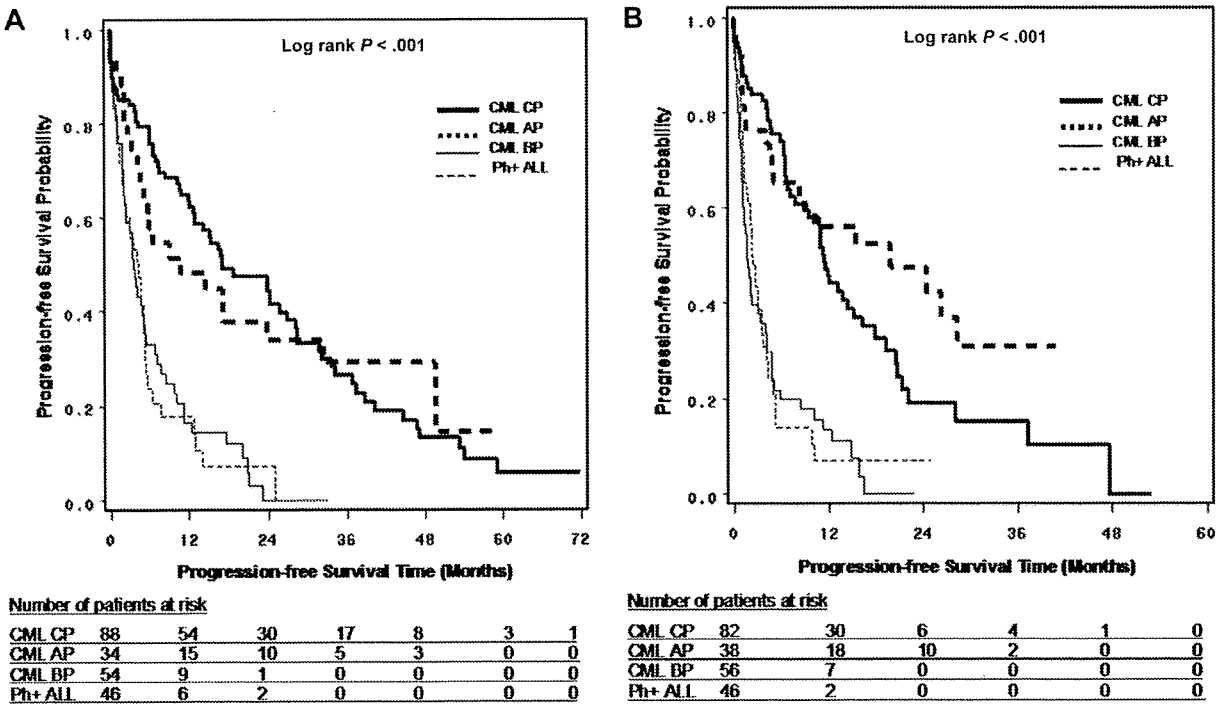

Figure 3. Progression-free survival in patients with T315I BCR-ABL mutation. (A) PFS from first TKI resistance by disease phase at the time of first TKI resistance. (B) PFS from T315I mutation detection by disease phase at the time of T315I mutation detection.

ALL patients. As stated previously, treatment with different drugs were not included in the models because of the retrospective collection of the treatment information. In the crude analysis, older age, female sex, patients from Asian sites, detection of T315I mutation by direct sequencing, detection of T315I mutation by the use of blood samples, worse performance status, and additional clones at the time of T315I mutation detection demonstrated a trend of associating with worse survival across different phases of CML and Ph^+ ALL patients. In the adjusted Cox model, however, only the following covariates were either statistically significantly ($P < .05$) or borderline significantly associated with worse OS (adjusted hazard ratio; 95% CI): older age (by median, 2.30; 1.04-5.09) in Ph^+ ALL patients, female sex in BP (1.73; 0.96-3.10); worse performance status in Ph^+ ALL (1+ vs 0, 2.18; 1.02-4.68); and detection of T315I mutation by direct sequencing (vs other methods) in AP (3.03; 0.89-10.29) and Ph^+ ALL (2.33; 1.06-5.12).

Discussion

We observed a strong link between the emergence of the T315I mutation and disease progression, with a shift in disease phase noted from time of diagnosis/TKI initiation (70%/60% in CP, respectively) to time of T315I mutation identification (37% in CP), with 48% (84 of 176 CML patients) having progressed to AP or BC. In this cohort advanced-phase cases displayed rapid TKI resistance and across all phases the rapidity of identifying a T315I mutation increased incrementally with phase: median time between TKI treatment start and T315I detection was 29.2 months for CP, 15.4 months for AP, 5.8 months for BP, and 9.1 months for Ph^+ ALL. This finding suggests that emergence of kinase domain mutants may be a function of clonal instability and the proliferation rate, both of which increased in advanced disease. In addition,

Table 3. Overall and progression-free survival of CML and Ph^+ ALL patients from TKI resistance or T315I detection

	CML CP	CML AP	CML BP	Ph^+ ALL
Survival since first time of TKI resistance, by phase at time of TKI resistance				
No. of patients	88	34	54	46
Median follow-up, mo	27.7	19.8	6.2	4.8
Median OS, mo (95% CI)	51.4 (44.4-59.5)	49.4 (15.2-52.0)	8.3 (4.6-11.3)	6.3 (5.0-12.2)
1-y OS rate (95% CI)	93% (85%-97%)	72% (54%-85%)	35% (22%-48%)	37% (22%-53%)
Median PFS, mo (95% CI)	17.2 (12.9-27.1)	10.8 (5.3-34.0)	3.6 (2.3-5.4)	4.6 (2.3-5.2)
1-y PFS rate (95% CI)	66% (55%-75%)	48% (30%-64%)	19% (9%-30%)	18% (7%-32%)
Survival since T315I mutation detection, by phase at T315I mutation detection				
No. of patients	82	38	56	46
Median follow-up, mo	12.4	15.2	3.0	3.6
Median OS, mo (95% CI)	22.4 (18.2-48.5)	28.4 (15.9-49.8)	4.0 (2.0-5.0)	4.9 (3.4-7.3)
1-y OS rate (95% CI)	71% (58%-80%)	69 (50%-81%)	23% (13%-36%)	12% (3%-27%)
Median PFS, mo (95% CI)	11.5 (9.2-15.7)	22.2 (9.0-N/A)	1.8 (1.2-4.0)	2.5 (1.8-3.6)
1-y PFS rate (95% CI)	46% (34%-57%)	56% (38%-70%)	16% (7%-27%)	7% (1%-19%)

Median survival and survival rates were calculated according to Kaplan-Meier method.

exposure of Ph+ ALL and CML AP/BP patients to TKI therapy (≤ 1 month for median time between diagnosis and TKI treatment start) was much more rapid than CML CP patients (11.7 months), potentially contributing to emergence of T315I clones.

Regarding the use of TKIs after identification of the T315I mutation, 27% (58 of 125) of patients continued ("carried over") dasatinib or nilotinib, and an additional 31% (the remaining 67 of 125) were switched to dasatinib or nilotinib. Although the use of more potent Abl inhibitors might accentuate T315I clonal selection, this has not been noted to affect survival.[9] An additional proportion of patients was maintained on imatinib (23% carry-over) or switched to imatinib (13%). Outside of currently approved TKIs, 12% of cases were treated after T315I with MK-0457 during its period of investigation (as well as another 12% with other investigational drugs, including homoharringtonine and unspecified drugs), often after multiple (eg, 3 or 4) lines of previous therapy; 40% were moved to hydroxyurea, 26% to cytarabine, and 6% to IFN-α, which is not surprising given the timeframe of the study predates clinical trial options for T315I-targeted agents. Finally, 17% of patients moved on to stem cell transplantation, an option currently still advised given lack of approval or proven efficacy for the BCR-ABL^{T315I} active agents in development.[17]

Regarding mutation detection overall, 62% (36/58) of cases with testing results before T315I detection were found to harbor other kinase domain mutations, consistent with data that initial mutations often are associated with secondary mutations and sequential treatment resistance.[18] T315I was determined to be "dominant" in most (87%) of cases and thus "driving" resistance; whether such cases represented compound mutations in single clones or singular mutations in multiple clones is unknown. In 73% of the cases, T315I mutation was identified after a median of 6 months of TKI resistance. In addition, within 1 month either before or after TKI resistance, 114 (51%) patients had mutation analysis performed, and 90 (41%) were identified as harboring the T315I, suggesting that the time from clinical TKI resistance and identifying the T315I mutation, when it occurs, is rather short.

Cox proportional hazard modeling of the study population was limited in its ability to identify predictors of worse outcome. In cases of Ph+ ALL, older age and poorer performance status (likely influence choice and tolerance of treatment), and for unclear reasons detection of T315I by direct sequencing were all associated with worse outcome. When BP patients were regrouped according to myeloid or lymphoid phenotype (myeloid BP vs lymphoid BP and Ph+ ALL), rates of OS were not different from when cases were divided broadly as either BP or Ph+ ALL, addressing the previously unanswered question of difference in natural history of T315I+ lymphoid and myeloid disease.

The similar survival rates observed in CP and AP likely reflects the difficulty in defining precisely late-CP and true AP patients, rather than a unique biology of T315I-associated progression in the limited number of cases reported as AP, as well as the fact that only a minority of patients in this analysis represent de novo primary imatinib-treated patients with the majority representing post-interferon, "late chronic phase" patients with known inferior response rates to imatinib,[19] and potentially different disease biology with regard to genesis of genetic instability, as evidenced by greater rates of clonal evolution in Ph− cells. Additional analysis suggested that that if CP patients had progressed to AP or BP either before or after T315I mutation, their survival (median OS, 8.7-29.5 months) is much worse than those who had never progressed to AP or BP during their lifetime (median OS, 48.9 months). Therefore, it is very important to collect and understand the whole disease history of this group of patients.

This study represents the first large-scale assessment of the current natural history and implications of the T315I mutation in Ph+ leukemias. Although cases were culled from a broad range of global CML centers/hospitals, one limitation may be the inclusion of a large fraction of Asian patients (22%; 15% of patients from the South Korean site) and that approximately 50% of cases were from France/Italy (including the majority of Ph+ ALL cases) if different patterns of resistance, response, or survival are geographically or ethnically based. Although not observed to any great degree in other studies, there are suggestions of differential ethnic tolerance and efficacy of TKIs.[20,21] Despite its obvious limitations, including retrospective study design and selective populations, this study confirmed that survival of patients harboring a T315I mutation remains dependent on the disease phase at T315I mutation detection, as well as the disease history during patients' lifetime. The fact that we did not observe clear treatment pattern after T315I mutation detection highlights the need for early detection of this mutation in clinically TKI-resistant patients to optimally manage this selective and still elusive resistance pattern with current and emerging therapeutics.

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Authorship

Contribution: F.E.N., M.J.M., G.M., D-W.K., A.H, J.C., J.D.P., S.P, C.R., F.G., and W.Z. designed and performed research; F.E.N., M.J.M., G.M., D-W.K., S.S., M.C.M, A.H., J.C., C.C., I.H.D., J.F.A., F.Y., and C.P. performed research; F.E.N., M.J.M., G.M., D-W K., A.H., J.C., J.F.A., F.Y., J.D.P., S.P., C.R., F.G., J.M.G., and W.Z. analyzed and/or interpreted data; F.E.N., M.J.M., S.S., M.C.M., and W.Z. wrote the manuscript; and G.M., D-W.K., A.H, J.C., C.C., I.H.D., J.F.A., F.Y., J.D.P., S.P, C.R., C.P., F.G., and J.M.G. critically reviewed and approved the manuscript.

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References

1. O'Hare T, Eide CA, Deininger MW. Bcr-Abl kinase domain mutations, drug resistance, and the road to a cure for chronic myeloid leukemia. *Blood.* 2007;110(7):2242-2249.
2. Soverini S, Colarossi S, Gnani A, et al. Contribution of ABL kinase domain mutations to imatinib resistance in different subsets of Philadelphia-positive patients: by the GIMEMA Working Party on Chronic Myeloid Leukemia. *Clin Cancer Res.* 2006;12(24):7374-7379.
3. Soverini S, Iacobucci I, Baccarani M, Martinelli G. Targeted therapy and the T315I mutation in Philadelphia-positive leukemias. *Haematologica.* 2007;92(04):437-439.
4. Soverini S, Colarossi S, Gnani A, et al. Resistance to dasatinib in Philadelphia-positive leukemia patients and the presence or the selection of mutations at residues 315 and 317 in the BCR-ABL kinase domain. *Haematologica.* 2007; 92(04):401-404.
5. Branford S, Rudzki Z, Walsh S, et al. Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. *Blood.* 2003;102(1):276-283.
6. Jabbour E, Kantarjian H, Jones D, et al. Frequency and clinical significance of BCR-ABL mutations in patients with chronic myeloid leukemia treated with imatinib mesylate. *Leukemia.* 2006; 20(10):1767-1773.
7. Willis SG, Lange T, Demehri S, et al. High-sensitivity detection of BCR-ABL kinase domain mutations in imatinib-naive patients: correlation with clonal cytogenetic evolution but not response to therapy. *Blood.* 2005;106(6):2128-2137.
8. Nicolini FE, Corm S, Le QH, et al. Mutation status and clinical outcome of 89 imatinib mesylate-resistant chronic myelogenous leukemia patients: a retrospective analysis from the French intergroup of CML (Fi-LMC GROUP). *Leukemia.* 2006;20(6):1061-1066.
9. Nicolini FE, Hayette S, Corm S, et al. Clinical outcome of 27 imatinib mesylate-resistant chronic myelogenous leukemia patients harboring a T315I BCR-ABL mutation. *Haematologica.* 2007; 92(09):1238-1241.
10. Jabbour E, Kantarjian H, Jones D, et al. Characteristics and outcomes of patients with chronic myeloid leukemia and T315I mutation following failure of imatinib mesylate therapy. *Blood.* 2008; 112(1):53-55.
11. Giles FJ, Cortes J, Jones D, et al. MK-0457, a novel kinase inhibitor, is active in patients with chronic myeloid leukemia or acute lymphocytic leukemia with the T315I BCR-ABL mutation. *Blood.* 2007;109(2):500-502.
12. Legros L, Hayette S, Nicolini FE, et al. BCR-ABL(T315I) transcript disappearance in an imatinib-resistant CML patient treated with homoharringtonine: a new therapeutic challenge? *Leukemia.* 2007;21(10):2204-2206.
13. Hochhaus A, Hughes T. Clinical resistance to imatinib: mechanisms and implications. *Hematol Oncol Clin North Am.* 2004;18: 641-656, ix.
14. Baccarani M, Saglio G, Goldman J, et al. Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. *Blood.* 2006;108(6):1809-1820.
15. Druker BJ, Guilhot F, O'Brien SG, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med.* 2006;355(23): 2408-2417.
16. Hocking RR. The analysis and selection of variables in linear regression. *Biometrics.* 1976; 32(1):1-50.
17. Jabbour E, Cortes J, Kantarjian HM, et al. Allogeneic stem cell transplantation for patients with chronic myeloid leukemia and acute lymphocytic leukemia after Bcr-Abl kinase mutation-related imatinib failure. *Blood.* 2006;108(4):1421-1423.
18. Hughes T, Saglio G, Martinelli G, et al. Responses and disease progression in CML-CP patients treated with nilotinib after imatinib failure appear to be affected by the BCR-ABL mutation status and types [abstract 320]. *Blood.* 2007; 110(11, pt 1):101a.
19. Kantarjian H, O'Brien S, Cortes J, et al. Survival advantage with imatinib mesylate therapy in chronic-phase chronic myelogenous leukemia (CML-CP) after IFN-α failure and in late CML-CP, comparison with historical controls. *Clin Cancer Res.* 2004;10(1):68-75.
20. Park SJ, Choi IK, Seo HY, et al. Reduced dose of imatinib for patients with chronic myeloid leukemia and low body surface area. *Acta Haematol.* 2007;118(4):219-221.
21. Morishima Y, Ogura M, Nishimura M, et al. Efficacy and safety of imatinib mesylate for patients in the first chronic phase of chronic myeloid leukemia: results of a Japanese phase II clinical study. *Int J Hematol.* 2004;80(3):261-266.

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LETTERS

Frequent inactivation of A20 in B-cell lymphomas

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A20 is a negative regulator of the NF-κB pathway and was initially identified as being rapidly induced after tumour-necrosis factor-α stimulation[1]. It has a pivotal role in regulation of the immune response and prevents excessive activation of NF-κB in response to a variety of external stimuli[2–7]; recent genetic studies have disclosed putative associations of polymorphic *A20* (also called *TNFAIP3*) alleles with autoimmune disease risk[8,9]. However, the involvement of A20 in the development of human cancers is unknown. Here we show, using a genome-wide analysis of genetic lesions in 238 B-cell lymphomas, that *A20* is a common genetic target in B-lineage lymphomas. A20 is frequently inactivated by somatic mutations and/or deletions in mucosa-associated tissue lymphoma (18 out of 87; 21.8%) and Hodgkin's lymphoma of nodular sclerosis histology (5 out of 15; 33.3%), and, to a lesser extent, in other B-lineage lymphomas. When re-expressed in a lymphoma-derived cell line with no functional *A20* alleles, wild-type A20, but not mutant A20, resulted in suppression of cell growth and induction of apoptosis, accompanied by downregulation of NF-κB activation. The A20-deficient cells stably generated tumours in immunodeficient mice, whereas the tumorigenicity was effectively suppressed by re-expression of A20. In A20-deficient cells, suppression of both cell growth and NF-κB activity due to re-expression of A20 depended, at least partly, on cell-surface-receptor signalling, including the tumour-necrosis factor receptor. Considering the physiological function of A20 in the negative modulation of NF-κB activation induced by multiple upstream stimuli, our findings indicate that uncontrolled signalling of NF-κB caused by loss of A20 function is involved in the pathogenesis of subsets of B-lineage lymphomas.

Malignant lymphomas of B-cell lineages are mature lymphoid neoplasms that arise from various lymphoid tissues[10,11]. To obtain a comprehensive registry of genetic lesions in B-lineage lymphomas, we performed a single nucleotide polymorphism (SNP) array analysis of 238 primary B-cell lymphoma specimens of different histologies, including 64 samples of diffuse large B-cell lymphomas (DLBCLs), 52 follicular lymphomas, 35 mantle cell lymphomas (MCLs), and 87 mucosa-associated tissue (MALT) lymphomas (Supplementary Table 1). Three Hodgkin's-lymphoma-derived cell lines were also analysed. Interrogating more than 250,000 SNP sites, this platform permitted the identification of copy number changes at an average resolution of less than 12 kilobases (kb). The use of large numbers of SNP-specific probes is a unique feature of this platform, and combined with the CNAG/AsCNAR software, enabled accurate determination of 'allele-specific' copy numbers, and thus allowed for sensitive detection of loss of heterozygosity (LOH) even without apparent copy-number reduction, in the presence of up to 70–80% normal cell contamination[12,13].

Lymphoma genomes underwent a wide range of genetic changes, including numerical chromosomal abnormalities and segmental gains and losses of chromosomal material (Supplementary Fig. 1), as well as copy-number-neutral LOH, or uniparental disomy (Supplementary Fig. 2). Each histology type had a unique genomic signature, indicating a distinctive underlying molecular pathogenesis for different histology types (Fig. 1a and Supplementary Fig. 3). On the basis of the genomic signatures, the initial pathological diagnosis of MCL was re-evaluated and corrected to DLBCL in two cases. Although most copy number changes involved large chromosomal segments, a number of regions showed focal gains and deletions, accelerating identification of their candidate gene targets. After excluding known copy number variations, we identified 46 loci showing focal gains (19 loci) or deletions (27 loci) (Supplementary Tables 2 and 3 and Supplementary Fig. 4).

Genetic lesions on the NF-κB pathway were common in B-cell lymphomas and found in approximately 40% of the cases (Supplementary Table 1), underpinning the importance of aberrant NF-κB activation in lymphomagenesis[11,14] in a genome-wide fashion. They included focal gain/amplification at the *REL* locus (16.4%) (Fig. 1b) and *TRAF6* locus (5.9%), as well as focal deletions at the *PTEN* locus (5.5%) (Supplementary Figs 1 and 4). However, the most striking finding was the common deletion at 6q23.3 involving a 143-kb segment. It exclusively contained the *A20* gene (also called *TNFAIP3*), a negative regulator of NF-κB activation[3–7,15] (Fig. 1b), which was previously reported as a candidate target of 6q23 deletions in ocular lymphoma[16]. LOH involving the *A20* locus was found in 50 cases, of which 12 showed homozygous deletions as determined by the loss of both alleles in an allele-specific copy number analysis (Fig. 1b, Table 1 and Supplementary Table 4). On the basis of this finding, we searched for possible tumour-specific mutations of *A20* by genomic DNA sequencing of entire coding exons of the gene in the same series of lymphoma samples (Supplementary Fig. 5). Because two out of the three Hodgkin's-lymphoma-derived cell lines had biallelic *A20* deletions/mutations (Supplementary Fig. 6), 24 primary samples from Hodgkin's lymphoma were also analysed for mutations, where

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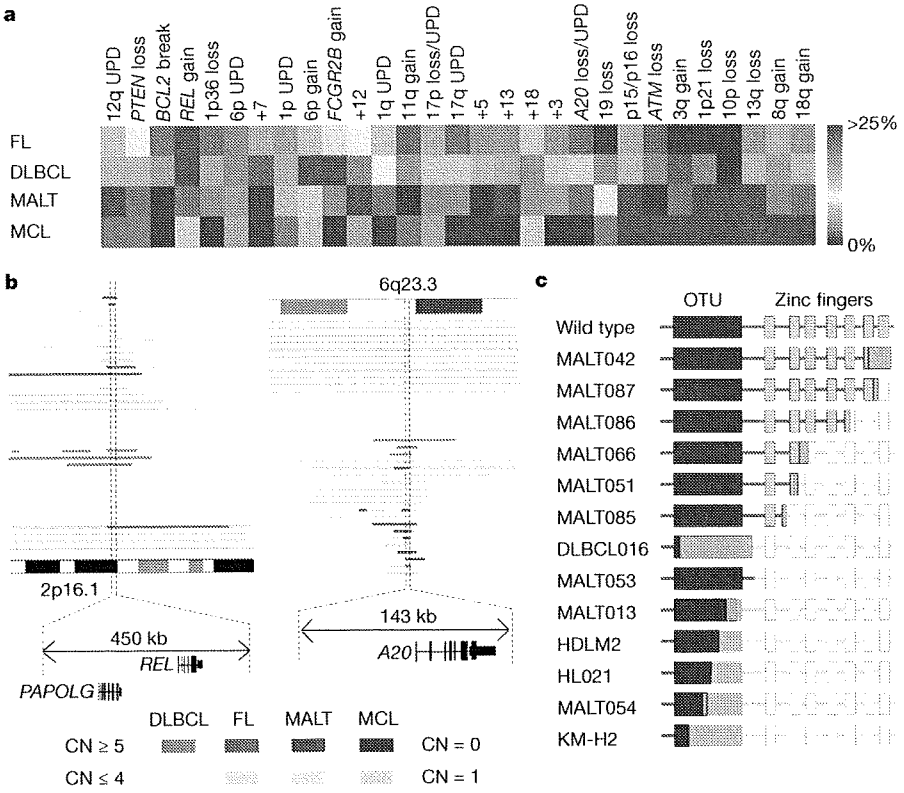

Figure 1 | Genomic signatures of different B-cell lymphomas and common genetic lesions at 2p16-15 and 6q23.3 involving NF-κB pathway genes. **a**, Twenty-nine genetic lesions were found in more than 10% in at least one histology and used for clustering four distinct histology types of B-lineage lymphomas. The frequency of each genetic lesion in each histology type is colour-coded. FL, follicular lymphoma; UPD, uniparental disomy. **b**, Recurrent genetic changes are depicted based on CNAG output of the SNP array analysis of 238 B-lineage lymphoma samples, which include gains at the *REL* locus on 2p16-15 (left panel) and the *A20* locus on 6q23.3 (right panel). Regions showing copy number gain or loss are indicated by horizontal lines. Four histology types are indicated by different colours, where high-grade amplifications and homozygous deletions are shown by darker shades to discriminate from simple gains (copy number ≤4) and losses (copy number = 1) (lighter shades). **c**, Point mutations and small nucleotide insertions and deletions in the *A20* (*TNFAIP3*) gene caused premature truncation of A20 in most cases. Altered amino acids caused by frame shifts are indicated by green bars.

genomic DNA was extracted from 150 microdissected CD30-positive tumour cells (Reed–Sternberg cells) for each sample. *A20* mutations were found in 18 out of 265 lymphoma samples (6.8%) (Table 1), among which 13 mutations, including nonsense mutations (3 cases), frame-shift insertions/deletions (9 cases), and a splicing donor site mutation (1 case) were thought to result in premature termination of translation (Fig. 1c). Four missense mutations and one intronic mutation were identified in five microdissected Hodgkin's lymphoma samples. They were not found in the surrounding normal tissues, and thus, were considered as tumour-specific somatic changes.

In total, biallelic *A20* lesions were found in 31 out of 265 lymphoma samples including 3 Hodgkin's lymphoma cell lines. Quantitative analysis of SNP array data suggested that these *A20* lesions were present in the major tumour fraction within the samples (Supplementary Fig. 7). Inactivation of *A20* was most frequent in MALT lymphoma (18 out of 87) and Hodgkin's lymphoma (7 out of 27), although it was also found in DLBCL (5 out of 64) and follicular lymphoma (1 out of 52) at lower frequencies. In MALT lymphoma, biallelic *A20* lesions were confirmed in 18 out of 24 cases (75.0%) with LOH involving the 6q23.3 segment (Supplementary Fig. 8). Considering the limitation in detecting very small homozygous deletions, *A20* was thought to be the target of 6q23 LOH in MALT lymphoma. On the other hand, the 6q23 LOHs in other histology types tended to be extended into more centromeric regions and less frequently accompanied biallelic *A20* lesions (Supplementary Fig. 8 and Supplementary Table 4), indicating that they might be more heterogeneous with regard to their gene targets. We were unable to analyse Hodgkin's lymphoma samples using SNP arrays owing to insufficient genomic DNA obtained from microdissected samples, and were likely to underestimate the frequency of *A20* inactivation in Hodgkin's lymphoma because we might fail to detect a substantial proportion of cases with homozygous deletions, which explained 50% (12 out of 24) of *A20* inactivation in other histology types. *A20* mutations in Hodgkin's lymphoma were exclusively found in nodular sclerosis classical Hodgkin's lymphoma (5 out of 15) but not in other histology types (0 out of 9), although the possible association requires further confirmation in additional cases.

A20 is a key regulator of NF-κB signalling, negatively modulating NF-κB activation through a wide variety of cell surface receptors and viral proteins, including tumour-necrosis factor (TNF) receptors, toll-like receptors, CD40, as well as Epstein–Barr-virus-associated LMP1 protein[2,5,17,18]. To investigate the role of A20 inactivation in lymphomagenesis, we re-expressed wild-type A20 under a *Tet*-inducible promoter in a lymphoma-derived cell line (KM-H2) that had no functional *A20* alleles (Supplementary Fig. 6), and examined the effect of A20 re-expression on cell proliferation, survival and downstream NF-κB signalling pathways. As shown in Fig. 2a–c and Supplementary Fig. 9, re-expression of wild-type A20 resulted in the suppression of cell proliferation and enhanced apoptosis, and in the concomitant accumulation of IκBβ and IκBε, and downregulation of NF-κB activity. In contrast, re-expression of two lymphoma-derived A20 mutants, $A20^{532Stop}$ or $A20^{750Stop}$, failed to show growth suppression, induction of apoptosis, accumulation of IκBβ and IκBε or downregulation of

Table 1 | Inactivation of A20 in B-lineage lymphomas

Histology	Tissue	Sample	Allele	Uniparental disomy	Exon	Mutation	Biallelic inactivation
DLBCL							5 out of 64 (7.8%)
	Lymph node	DLBCL008	−/−	No	–	–	
	Lymph node	DLBCL016	+/−	No	Ex2	329insA	
	Lymph node	DLBCL022	−/−	No	–	–	
	Lymph node	DLBCL028	−/−	Yes	–	–	
	Lymph node	MCL008*	−/−	Yes	–	–	
Follicular lymphoma							1 out of 52 (1.9%)
	Lymph node	FL024	−/−	No	–	–	
MCL							0 out of 35 (0%)
MALT							18 out of 87 (21.8%)
Stomach							3 out of 23 (13.0%)
	Gastric mucosa	MALT013	+/+	Yes	Ex5	705insG	
	Gastric mucosa	MALT014	+/+	Yes	Ex3	Ex3 donor site>A	
	Gastric mucosa	MALT036	+/−	No	Ex7	delintron6-Ex7†	
Eye							13 out of 43 (30.2%)
	Ocular adnexa	MALT008	−/−	No	–	–	
	Ocular adnexa	MALT017	−/−	No	–	–	
	Ocular adnexa	MALT051	+/−	No	Ex7	1943delTG	
	Ocular adnexa	MALT053	+/+	Yes	Ex6	1016G>A(stop)	
	Ocular adnexa	MALT054	+/−	No	Ex3	502delTC	
	Ocular adnexa	MALT055	−/−	No	–	–	
	Ocular adnexa	MALT066	+/−	No	Ex7	1581insA	
	Ocular adnexa	MALT067	−/−	No	–	–	
	Ocular adnexa	MALT082	−/−	Yes	–	–	
	Ocular adnexa	MALT084	−/−	Yes	–	–	
	Ocular adnexa	MALT085	+/+	Yes	Ex7	1435insG	
	Ocular adnexa	MALT086	+/+	Yes	Ex6	878C>T(stop)	
	Ocular adnexa	MALT087	+/+	Yes	Ex9	2304delGG	
Lung							2 out of 12 (16.7%)
	Lung	MALT042	−/−	No	–	–	
	Lung	MALT047	+/+	Yes	Ex9	2281insT	
Other‡							0 out of 9 (0%)
Hodgkin's lymphoma							7 out of 27 (26.0%)
NSHL	Lymph node	HL10	ND	ND	Ex7	1777G>A(V571I)	
NSHL	Lymph node	HL12	ND	ND	Ex7	1156A>G(R364G)	
NSHL	Lymph node	HL21	ND	ND	Ex4	569G>A(stop)	
NSHL	Lymph node	HL24	ND	ND	Ex3	1487C>A(T474N)	
NSHL	Lymph node	HL23	ND	ND	–	Intron 3§	
	Cell line	KM-H2	−/−	No	–		
	Cell line	HDLM2	+/−	No	Ex4	616ins29bp‖	
Total							31 out of 265 (11.7%)

DLBCL, diffuse large B-cell lymphoma; MALT, MALT lymphoma; MCL, mantle cell lymphoma; ND, not determined because SNP array analysis was not performed; NSHL, nodular sclerosis classical Hodgkin's lymphoma.
* Diagnosis was changed based on the genomic data, which was confirmed by re-examination of pathology.
† Deletion including the boundary of intron 6 and exon 7 (see also Supplementary Fig. 5b).
‡ Including 1 parotid gland, 1 salivary gland, 2 colon and 5 thyroid cases.
§ Insertion of CTC at −19 bases from the beginning of exon 3.
‖ Insertion of TGGCTTCCACAGACACACCCATGGCCCGA.

NF-κB activity (Fig. 2a–c), indicating that these were actually loss-of-function mutations. To investigate the role of A20 inactivation in lymphomagenesis *in vivo*, *A20*- and mock-transduced KM-H2 cells were transplanted in NOD/SCID/γ_c^{null} (NOG) mice[19], and their tumour formation status was examined for 5 weeks with or without induction of wild-type A20 by tetracycline administration. As shown in Fig. 2d, mock-transduced cells developed tumours at the injected sites, whereas the *Tet*-inducible *A20*-transduced cells generated tumours only in the absence of A20 induction (Supplementary Table 5), further supporting the tumour suppressor role of A20 in lymphoma development.

Given the mode of negative regulation of NF-κB signalling, we next investigated the origins of NF-κB activity that was deregulated by A20 loss in KM-H2 cells. The conditioned medium prepared from a 48-h serum-free KM-H2 culture had increased NF-κB upregulatory activity compared with fresh serum-free medium, which was inhibited by re-expression of A20 (Fig. 3a). KM-H2 cells secreted two known ligands for TNF receptor—TNF-α and lymphotoxin-α (Supplementary Fig. 10)[20]—and adding neutralizing antibodies against these cytokines into cultures significantly suppressed their cell growth and NF-κB activity without affecting the levels of their overall suppression after A20 induction (Fig. 3b, d). In addition, recombinant TNF-α and/or lymphotoxin-α added to fresh serum-free medium promoted cell growth and NF-κB activation in KM-H2 culture, which were again suppressed by re-expression of A20 (Fig. 3c, e). Although our data in Fig. 3 also show the presence of factors other than TNF-α and lymphotoxin-α in the KM-H2-conditioned medium—as well as some intrinsic pathways in the cell (Fig. 3a)—that were responsible for the A20-dependent NF-κB activation, these results indicate that both cell growth and NF-κB activity that were upregulated by A20 inactivation depend at least partly on the upstream stimuli that evoked the NF-κB-activating signals.

Aberrant activation of the NF-κB pathway is a hallmark of several subtypes of B-lineage lymphomas, including Hodgkin's lymphoma, MALT lymphoma, and a subset of DLBCL, as well as other lymphoid neoplasms[11,14], where a number of genetic alterations of NF-κB signalling pathway genes[21–25], as well as some viral proteins[26,27], have been implicated in the aberrant activation of the NF-κB pathway[14]. Thus, frequent inactivation of A20 in Hodgkin's lymphoma and MALT and other lymphomas provides a novel insight into the molecular pathogenesis of these subtypes of B-lineage lymphomas through deregulated NF-κB activation. Because A20 provides a

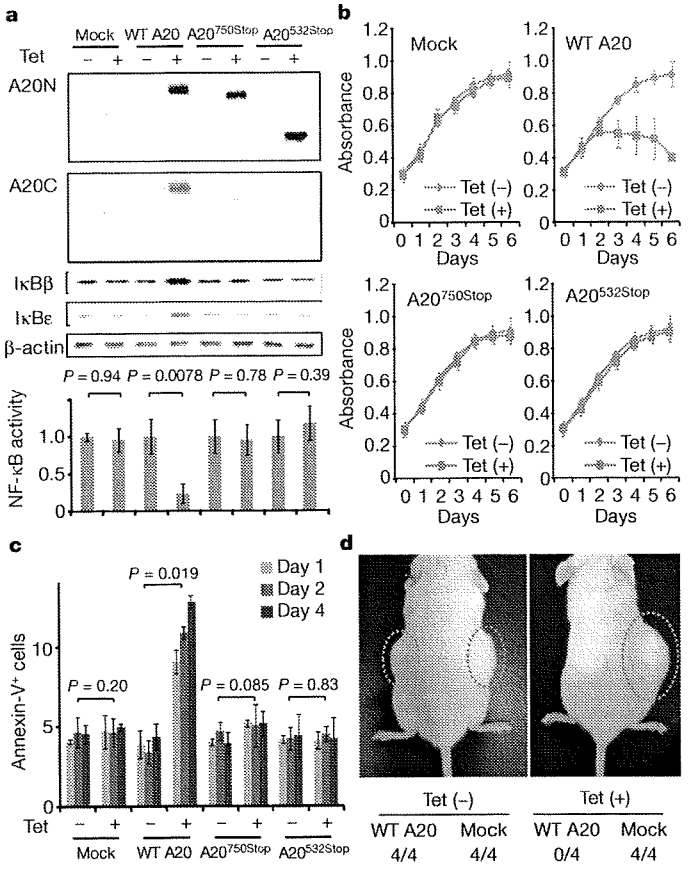

Figure 2 | Effects of wild-type and mutant A20 re-expressed in a lymphoma cell line that lacks the normal *A20* gene. a, Western blot analyses of wild-type (WT) and mutant ($A20^{532Stop}$ and $A20^{750Stop}$) A20, as well as IκBβ and IκBε, in KM-H2 cells, in the presence or absence of tetracycline treatment (top panels). A20N and A20C are polyclonal antisera raised against N-terminal and C-terminal A20 peptides, respectively. β-actin blots are provided as a control. NF-κB activities are expressed as mean absorbance ± s.d. ($n = 6$) in luciferase assays (bottom panel). **b,** Proliferation of KM-H2 cells stably transduced with plasmids for mock and *Tet*-inducible wild-type A20, $A20^{532Stop}$ and $A20^{750Stop}$ was measured using a cell counting kit in the presence (red lines) or absence (blue lines) of tetracycline. Mean absorbance ± s.d. ($n = 5$) is plotted. **c,** The fractions of Annexin-V-positive KM-H2 cells transduced with various *Tet*-inducible A20 constructs were measured by flow cytometry after tetracycline treatment and the mean values (±s.d., $n = 3$) are plotted. **d,** *In vivo* tumorigenicity was assayed by inoculating 7×10^6 KM-H2 cells transduced with mock or *Tet*-inducible wild-type A20 in NOG mice, with (right panel) or without (left panel) tetracycline administration.

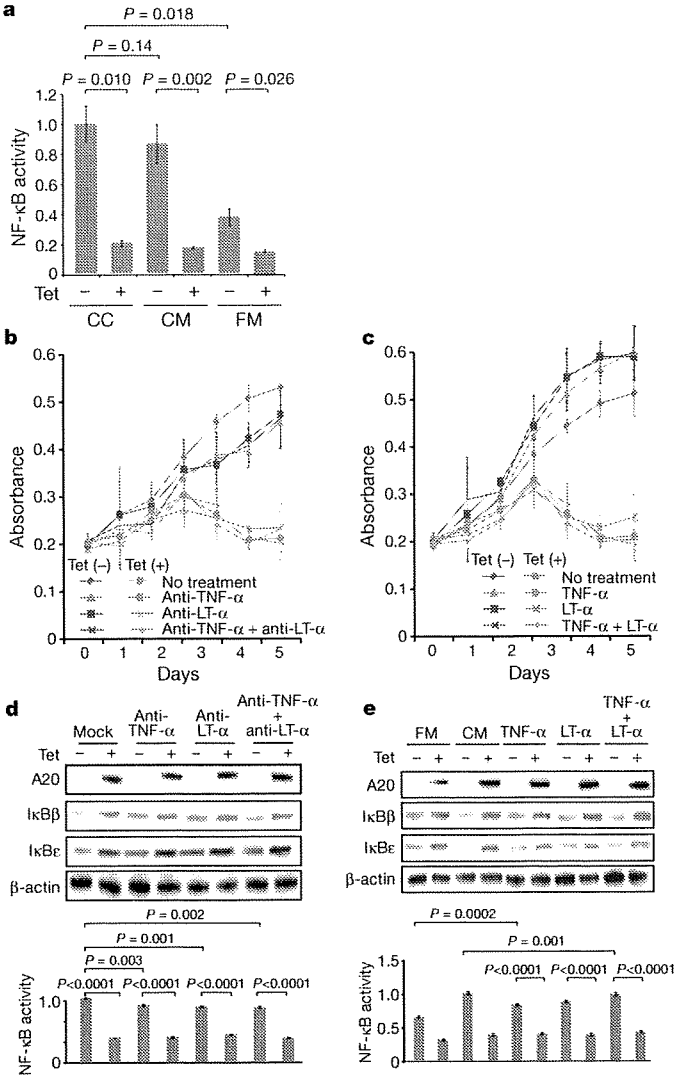

Figure 3 | Tumour suppressor role of A20 under external stimuli. a, NF-κB activity in KM-H2 cells was measured 30 min after cells were inoculated into fresh medium (FM) or KM-H2-conditioned medium (CM) obtained from the 48-h culture of KM-H2, and was compared with the activity after 48 h continuous culture of KM-H2 (CC). A20 was induced 12 h before inoculation in Tet (+) groups. **b, c,** Effects of neutralizing antibodies against TNF-α and lymphotoxin-α (LTα) (**b**) and of recombinant TNF-α and LT-α added to the culture (**c**) on cell growth were evaluated in the presence (Tet (+)) or absence (Tet (−)) of A20 induction. Cell numbers were measured using a cell counting kit and are plotted as their mean absorbance ± s.d. ($n = 6$). **d, e,** Effects of the neutralizing antibodies (**d**) and the recombinant cytokines added to the culture (**e**) on NF-κB activities and the levels of IκBβ and IκBε after 48 h culture with (Tet (+)) or without (Tet (−)) tetracycline treatment. NF-κB activities are expressed as mean absorbance ± s.d. ($n = 6$) in luciferase assays.

negative feedback mechanism in the regulation of NF-κB signalling pathways upon a variety of stimuli, aberrant activation of NF-κB will be a logical consequence of A20 inactivation. However, there is also the possibility that the aberrant NF-κB activity of A20-inactivated lymphoma cells is derived from upstream stimuli, which may be from the cellular environment. In this context, it is intriguing that MALT lymphoma usually arises at the site of chronic inflammation caused by infection or autoimmune disorders and may show spontaneous regression after eradication of infectious organisms[28]; furthermore, Hodgkin's lymphoma frequently shows deregulated cytokine production from Reed–Sternberg cells and/or surrounding reactive cells[29]. Detailed characterization of the NF-κB pathway regulated by A20 in both normal and neoplastic B lymphocytes will promote our understanding of the precise roles of A20 inactivation in the pathogenesis of these lymphoma types. Our finding underscores the importance of genome-wide approaches in the identification of genetic targets in human cancers.

METHODS SUMMARY

Genomic DNA from 238 patients with non-Hodgkin's lymphoma and three Hodgkin's-lymphoma-derived cell lines was analysed using GeneChip SNP genotyping microarrays (Affymetrix). This study was approved by the ethics boards of the University of Tokyo, National Cancer Institute Hospital, Okayama University, and the Cancer Institute of the Japanese Foundation of Cancer Research. After appropriate normalization of mean array intensities, signal ratios between tumours and anonymous normal references were calculated in an allele-specific manner, and allele-specific copy numbers were inferred from the observed signal ratios based on the hidden Markov model using CNAG/AsCNAR software (http://www.genome.umin.jp). *A20* mutations were examined by directly sequencing genomic DNA using a set of primers (Supplementary Table 6). Full-length cDNAs of wild-type and mutant *A20* were introduced into a

lentivirus vector, pLenti4/TO/V5-DEST (Invitrogen), with a *Tet*-inducible promoter. Viral stocks were prepared by transfecting the vector plasmids into 293FT cells (Invitrogen) using the calcium phosphate method and then infected to the KM-H2 cell line. Proliferation of KM-H2 cells was measured using a Cell Counting Kit (Dojindo). Western blot analyses and luciferase assays were performed as previously described. NF-κB activity was measured by luciferase assays in KM-H2 cells stably transduced with a reporter plasmid having an NF-κB response element, pGL4.32 (Promega). Apoptosis of KM-H2 upon A20 induction was evaluated by counting Annexin-V-positive cells by flow cytometry. For *in vivo* tumorigenicity assays, 7×10^6 KM-H2 cells were transduced with the *Tet*-inducible *A20* gene and those with a mock vector were inoculated on the contralateral sides in eight NOG mice[19] and examined for their tumour formation with ($n = 4$) or without ($n = 4$) tetracycline administration. Full copy number data of the 238 lymphoma samples will be accessible from the Gene Expression Omnibus (GEO, http://ncbi.nlm.nih.gov/geo/) with the accession number GSE12906.

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

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1. Dixit, V. M. *et al.* Tumor necrosis factor-α induction of novel gene products in human endothelial cells including a macrophage-specific chemotaxin. *J. Biol. Chem.* **265**, 2973–2978 (1990).
2. Song, H. Y., Rothe, M. & Goeddel, D. V. The tumor necrosis factor-inducible zinc finger protein A20 interacts with TRAF1/TRAF2 and inhibits NF-κB activation. *Proc. Natl Acad. Sci. USA* **93**, 6721–6725 (1996).
3. Lee, E. G. *et al.* Failure to regulate TNF-induced NF-κB and cell death responses in A20-deficient mice. *Science* **289**, 2350–2354 (2000).
4. Boone, D. L. *et al.* The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. *Nature Immunol.* **5**, 1052–1060 (2004).
5. Wang, Y. Y., Li, L., Han, K. J., Zhai, Z. & Shu, H. B. A20 is a potent inhibitor of TLR3- and Sendai virus-induced activation of NF-κB and ISRE and IFN-β promoter. *FEBS Lett.* **576**, 86–90 (2004).
6. Wertz, I. E. *et al.* De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-κB signalling. *Nature* **430**, 694–699 (2004).
7. Heyninck, K. & Beyaert, R. A20 inhibits NF-κB activation by dual ubiquitin-editing functions. *Trends Biochem. Sci.* **30**, 1–4 (2005).
8. Graham, R. R. *et al.* Genetic variants near *TNFAIP3* on 6q23 are associated with systemic lupus erythematosus. *Nature Genet.* **40**, 1059–1061 (2008).
9. Musone, S. L. *et al.* Multiple polymorphisms in the *TNFAIP3* region are independently associated with systemic lupus erythematosus. *Nature Genet.* **40**, 1062–1064 (2008).
10. Jaffe, E. S., Harris, N. L., Stein, H. & Vardiman, J. W. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Hematopoietic and Lymphoid Tissues* (IARC Press, 2001).
11. Klein, U. & Dalla-Favera, R. Germinal centres: role in B-cell physiology and malignancy. *Nature Rev. Immunol.* **8**, 22–33 (2008).
12. Nannya, Y. *et al.* A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. *Cancer Res.* **65**, 6071–6079 (2005).
13. Yamamoto, G. *et al.* Highly sensitive method for genomewide detection of allelic composition in nonpaired, primary tumor specimens by use of affymetrix single-nucleotide-polymorphism genotyping microarrays. *Am. J. Hum. Genet.* **81**, 114–126 (2007).
14. Jost, P. J. & Ruland, J. Aberrant NF-κB signaling in lymphoma: mechanisms, consequences, and therapeutic implications. *Blood* **109**, 2700–2707 (2007).
15. Durkop, H., Hirsch, B., Hahn, C., Foss, H. D. & Stein, H. Differential expression and function of A20 and TRAF1 in Hodgkin lymphoma and anaplastic large cell lymphoma and their induction by CD30 stimulation. *J. Pathol.* **200**, 229–239 (2003).
16. Honma, K. *et al. TNFAIP3* is the target gene of chromosome band 6q23.3-q24.1 loss in ocular adnexal marginal zone B cell lymphoma. *Genes Chromosom. Cancer* **47**, 1–7 (2008).
17. Sarma, V. *et al.* Activation of the B-cell surface receptor CD40 induces A20, a novel zinc finger protein that inhibits apoptosis. *J. Biol. Chem.* **270**, 12343–12346 (1995).
18. Fries, K. L., Miller, W. E. & Raab-Traub, N. The A20 protein interacts with the Epstein-Barr virus latent membrane protein 1 (LMP1) and alters the LMP1/TRAF1/TRADD complex. *Virology* **264**, 159–166 (1999).
19. Hiramatsu, H. *et al.* Complete reconstitution of human lymphocytes from cord blood $CD34^+$ cells using the NOD/SCID/γ^{null} mice model. *Blood* **102**, 873–880 (2003).
20. Hsu, P. L. & Hsu, S. M. Production of tumor necrosis factor-α and lymphotoxin by cells of Hodgkin's neoplastic cell lines HDLM-1 and KM-H2. *Am. J. Pathol.* **135**, 735–745 (1989).
21. Dierlamm, J. *et al.* The apoptosis inhibitor gene *API2* and a novel 18q gene, *MLT*, are recurrently rearranged in the t(11;18)(q21;q21) associated with mucosa-associated lymphoid tissue lymphomas. *Blood* **93**, 3601–3609 (1999).
22. Willis, T. G. *et al.* Bcl10 is involved in t(1;14)(p22;q32) of MALT B cell lymphoma and mutated in multiple tumor types. *Cell* **96**, 35–45 (1999).
23. Joos, S. *et al.* Classical Hodgkin lymphoma is characterized by recurrent copy number gains of the short arm of chromosome 2. *Blood* **99**, 1381–1387 (2002).
24. Martin-Subero, J. I. *et al.* Recurrent involvement of the *REL* and *BCL11A* loci in classical Hodgkin lymphoma. *Blood* **99**, 1474–1477 (2002).
25. Lenz, G. *et al.* Oncogenic *CARD11* mutations in human diffuse large B cell lymphoma. *Science* **319**, 1676–1679 (2008).
26. Deacon, E. M. *et al.* Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells. *J. Exp. Med.* **177**, 339–349 (1993).
27. Yin, M. J. *et al.* HTLV-I Tax protein binds to MEKK1 to stimulate IκB kinase activity and NF-κB activation. *Cell* **93**, 875–884 (1998).
28. Isaacson, P. G. & Du, M. Q. MALT lymphoma: from morphology to molecules. *Nature Rev. Cancer* **4**, 644–653 (2004).
29. Skinnider, B. F. & Mak, T. W. The role of cytokines in classical Hodgkin lymphoma. *Blood* **99**, 4283–4297 (2002).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions M.Ka., K.N. and M.S. performed microarray experiments and subsequent data analyses. M.Ka., Y.C., K.Ta., J.T., J.N., M.I., A.T. and Y.K. performed mutation analysis of *A20*. M.Ka., S.Mu., M.S., Y.C. and Y.Ak. conducted functional assays of mutant A20. Y.S., K.Ta., Y.As., H.M., M.Ku., S.Mo., S.C., Y.K., K.To. and Y.I. prepared tumour specimens. I.K., K.O., A.N., H.N. and T.N. conducted *in vivo* tumorigenicity experiments in NOG/SCID mice. T.I., Y.H., T.Y., Y.K. and S.O. designed overall studies, and S.O. wrote the manuscript. All authors discussed the results and commented on the manuscript.

Author Information The copy number data as well as the raw microarray data will be accessible from the GEO (http://ncbi.nlm.nih.gov/geo/) with the accession number GSE12906. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to S.O. (sogawa-tky@umin.ac.jp) or Y.K. (ykkobaya@ncc.go.jp).

METHODS

Specimens. Primary tumour specimens were obtained from patients who were diagnosed with DLBCL, follicular lymphoma, MCL, MALT lymphoma, or classical Hodgkin's lymphoma. In total, 238 primary lymphoma specimens listed in Supplementary Table 1 were subjected to SNP array analysis. Three Hodgkin's-lymphoma-derived cell lines (KM-H2, HDLM2, L540) were obtained from Hayashibara Biochemical Laboratories, Inc., Fujisaki Cell Center and were also analysed by SNP array analysis.

Microarray analysis. High-molecular-mass DNA was isolated from tumour specimens and subjected to SNP array analysis using GeneChip Mapping 50K and/or 250K arrays (Affymetrix). The scanned array images were processed with Gene Chip Operation software (GCOS), followed by SNP calls using GTYPE. Genome-wide copy number measurements and LOH detection were performed using CNAG/AsCNAR software[12,13].

Mutation analysis. Mutations in the *A20* gene were examined in 265 samples of B-lineage lymphoma, including 62 DLBCLs, 52 follicular lymphomas, 87 MALTs, 37 MCLs and 3 Hodgkin's-lymphoma-derived cell lines and 24 primary Hodgkin's lymphoma samples, by direct sequencing using an ABI PRISM 3130xl Genetic Analyser (Applied Biosystems). To analyse primary Hodgkin's lymphoma samples in which CD30-positive tumour cells (Reed–Sternberg cells) account for only a fraction of the specimen, 150 Reed–Sternberg cells were collected for each 10 μm slice of a formalin-fixed block immunostained for CD30 by laser-capture microdissection (ASLMD6000, Leica), followed by genomic DNA extraction using QIAamp DNA Micro kit (Qiagen). The primer sets used in this study are listed in Supplementary Table 6.

Functional analysis of wild-type and mutant A20. Full-length cDNA for wild-type *A20* was isolated from total RNA extracted from an acute myeloid leukaemia-derived cell line, CTS, and subcloned into a lentivirus vector (pLenti4/TO/V5-DEST, Invitrogen). cDNAs for mutant A20 were generated by PCR amplification using mutagenic primers (Supplementary Table 6), and introduced into the same lentivirus vector. Forty-eight hours after transfection of each plasmid into 293FT cells using the calcium phosphate method, lentivirus stocks were obtained from ultrafiltration using Amicon Ultra (Millipore), and used to infect KM-H2 cells to generate stable transfectants of mock, wild-type and mutant A20. Each KM-H2 derivative cell line was further transduced stably with a reporter plasmid (pGL4.32, Promega) containing a luciferase gene under an NF-κB-responsive element by electroporation using Nucleofector reagents (Amaxa).

Assays for cell proliferation and NF-κB activity. Proliferation of the KM-H2 derivative cell lines was assayed in triplicate using a Cell Counting Kit (Dojindo). The mean absorption of five independent assays was plotted with s.d. for each derivative line. Two independent KM-H2-derived cell lines were used for each experiment. The NF-κB activity in KM-H2 derivatives for A20 mutants was evaluated by luciferase assays using a PiccaGene Luciferase Assay Kit (TOYO B-Net Co.). Each assay was performed in triplicate and the mean absorption of five independent experiments was plotted with s.d.

Western blot analyses. Polyclonal anti-sera against N-terminal (anti-A20N) and C-terminal (anti-A20C) A20 peptides were generated by immunizing rabbits with these peptides (LSNMRKAVKIRERTPEDIC for anti-A20N and CFQFKQMYG for anti-A20C, respectively). Total cell lysates from KM-H2 cells were separated on 7.5% polyacrylamide gel and subjected to western blot analysis using antibodies to A20 (anti-A20N and anti-A20C), IκBα (sc-847), IκBβ (sc-945), IκBγ (sc-7155) and actin (sc-8432) (Santa Cruz Biotechnology).

Functional analyses of wild-type and mutant A20. Each KM-H2 derivative cell line stably transduced with various *Tet*-inducible A20 constructs was cultured in serum-free medium in the presence or absence of A20 induction using 1 μg ml^{-1} of tetracycline, and cell number was counted every day. 1×10^6 cells of each KM-H2 derivative cell line were analysed for their intracellular levels of IκBβ and IκBε and for NF-κB activities by western blot analyses and luciferase assays, respectively, 12 h after the beginning of cell culture. Effects of human recombinant TNF-α and lymphotoxin-α (210-TA and 211-TB, respectively, R&D Systems) on the NF-κB pathway and cell proliferation were evaluated by adding both cytokines into 10 ml of serum-free cell culture at a concentration of 200 pg ml^{-1}. For cell proliferation assays, culture medium was half replaced every 12 h to minimize the side-effects of autocrine cytokines. Intracellular levels of IκBβ, IκBε and NF-κB were examined 12 h after the beginning of the cell culture. To evaluate the effect of neutralizing TNF-α and lymphotoxin-α, 1×10^6 of KM-H2 cells transduced with both *Tet*-inducible A20 and the NF-κB-luciferase reporter were pre-cultured in serum-free media for 36 h, and thereafter neutralizing antibodies against TNF-α (MAB210, R&D Systems) and/or lymphotoxin-α (AF-211-NA, R&D Systems) were added to the media at a concentration of 200 pg ml^{-1}. After the extended culture during 12 h with or without 1 μg ml^{-1} tetracycline, the intracellular levels of IκBβ and IκBε and NF-κB activities were examined by western blot analysis and luciferase assays, respectively. To examine the effects of A20 re-expression on apoptosis, 1×10^6 KM-H2 cells were cultured for 4 days in 10 ml medium with or without *Tet* induction. After staining with phycoerythrin-conjugated anti-Annexin-V (ID556422, Becton Dickinson), Annexin-V-positive cells were counted by flow cytometry at the indicated times.

***In vivo* tumorigenicity assays.** KM-H2 cells transduced with a mock or *Tet*-inducible wild-type *A20* gene were inoculated into NOG mice and their tumorigenicity was examined for 5 weeks with or without tetracycline administration. Injections of 7×10^6 cells of each KM-H2 cell line were administered to two opposite sites in four mice. Tetracycline was administered in drinking water at a concentration of 200 μg ml^{-1}.

ELISA. Concentrations of TNF-α, lymphotoxin-α, IL-1, IL-2, IL-4, IL-6, IL-12, IL-18 and TGF-β in the culture medium were measured after 48 h using ELISA. For those cytokines detectable after 48-h culture (TNFα, LTα, and IL-6), their time course was examined further using the Quantikine ELISA kit (R&D Systems).

Statistical analysis. Significance of the difference in NF-κB activity between two given groups was evaluated using a paired *t*-test, in which the data from each independent luciferase assay were paired to calculate test statistics. To evaluate the effect of A20 re-expression in KM-H2 cells on apoptosis, the difference in the fractions of Annexin-V-positive cells between Tet (+) and Tet (−) groups was also tested by a paired *t*-test for assays, in which the data from the assays performed on the same day were paired.

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ORIGINAL ARTICLE

Phase I/II study of humanized anti-CD33 antibody conjugated with calicheamicin, gemtuzumab ozogamicin, in relapsed or refractory acute myeloid leukemia: final results of Japanese multicenter cooperative study

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Abstract The primary objective of this study was to investigate the tolerability, efficacy and pharmacokinetic profile of gemtuzumab ozogamicin (GO) in patients with relapsed and/or refractory CD33-positive acute myeloid leukemia (AML). Patients received 2-h infusions of GO twice with an interval of approximately 14 days. Tolerability was assessed using the National Cancer Institute Common Toxicity Criteria Version 2.0. Samples for pharmacokinetics were taken on day 1 and day 8 of the first treatment cycle. The dose was increased stepwise and, in each cohort, patients were treated at the same dose. Forty patients, median age 58 years (range 28–68) were treated; 20 and 20 patients were enrolled to the phase I and II parts, respectively. In the phase I part, dose-limiting toxicities (DLTs) were hepatotoxicities, and the recommended dose was established as 9 mg/m^2 given as two intravenous infusions separated by approximately 14 days. The pharmacokinetic study revealed that C_{max} and AUC were equivalent to those of non-Japanese patients. In the phase II

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part, complete remission was observed in 5 patients, and one patient had complete remission without platelet recovery. Four of these 6 in remission and one in the phase I are long-term survivors (alive for at least 44 months). GO is safe and effective as a single agent among Japanese CD33-positive AML patients. Remission lasted longer in a subset of patients than in non-Japanese patients in earlier studies. Further studies of this agent are warranted to establish standard therapy.

Keywords Gemtuzumab ozogamicin · AML · Pharmacokinetic study · Phase I/II study

1 Introduction

Gemtuzumab ozogamicin (GO) (CMA 676) is an immunoconjugate composed of a recombinant humanized murine anti-CD33 antibody linked to calicheamicin, a potent cytotoxic agent [1]. CD33 is expressed on the blast cells of approximately 80–90% of patients with acute myeloid leukemia (AML), but not on stem cell, normal granulocytes, or non-hematopoietic tissues. Leukemia cells rapidly internalize the antibody/calicheamicin complex, leading to cleavage of the toxic calicheamicin, which binds to DNA and induces double-strand breaks and subsequently cell death [2–4].

In a phase I dose escalation study with relapsed or refractory CD33-positive AML patients, eight of 40 (20%) treated with this agent had blast clearance from the blood and marrow [5]. In phase II efficacy and safety trials, a proceeding report indicated that 42 of 142 (30%) patients with CD33-positive AML in first relapse obtained remission after treatment with two doses of GO at 9 mg/m [5, 6]. Although severe myelosuppression was common, mucositis, and severe infections were not. Based on these data, GO was approved in the United States (US) for the treatment of patients with CD33-positive AML in first relapse who are 60 years of age or older and who are not considered candidates for any other cytotoxic chemotherapy.

In Japan, a multicenter study of GO was started in 1999. This is the summary of the treatment of Japanese CD33-positive AML patients. The long-term follow-up demonstrated unexpected longer second remission in a fraction of Japanese patients.

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2 Patients and methods

2.1 Patients

This was a single-arm, multicenter, open-label, phase I/II study. In the phase I part, patients with CD33-positive refractory and/or relapsed AML as well as relapsed AML after hematopoietic stem cell transplantation were eligible. Adequate bone marrow function; platelet level of 20,000/μl or more, neutrophil count of 500/μl or more and independence from transfusion also were required. In the phase II part, patients were limited to first relapse with minimum remission duration of 6 months. In both parts, the patients were required to be at least 18 and no more than 70 years of age and to have Eastern Cooperative Group (ECOG) performance status 0–2, serum creatinine level of 2.0 mg/dl or less, total bilirubin level of 1.5 mg/dl or less, and sterility or adequate contraception. All protocols were approved and monitored by central and institutional review boards, and the studies were conducted in a manner consistent with the Declaration of Helsinki and Good Clinical Practice guidelines. All patients provided a written informed consent before treatment.

2.2 Treatment

Patients received 2 doses of GO, administered as a 2-h intravenous infusion with approximately 14 days between the doses. Acetaminophen at the dose of 400 mg and antihistamines (*d*-chlorpheniramine maleate) at the dose of 2 mg were administered before GO infusion, and two additional doses of acetaminophen were permitted after GO infusion in the phase I part. Corticosteroids (hydrocortisone sodium succinate) at the dose of 100 mg were given before infusion in the phase II part, and two additional doses were allowed after infusion. Before patients received the second dose of GO, they had to have recovered from all reversible, non-hematological toxicities and had no evidence of uncontrolled infection, disease progression, or detectable formation of antibodies to GO.

Patients were evaluated during the treatment period (from the initial dose of GO until 28 days after the last dose). After GO treatment, patients received the AML therapy that was most appropriate, as determined by their individual physicians. Survival data were collected until February 28, 2007.

2.3 Dose escalation

In the phase I part, the initial dose of GO was 2 doses of 6 mg/m^2, which was one level less than the dose described in the product label, where approved [8]. After the initial three patients tolerated this dose, the dose was escalated to 9 mg/m^2, which is the dose described in the product label in the United States. However, the fifth patient treated with this dose experienced sudden death from pulmonary bleeding and accrual was suspended. The Safety Advisory committee recommended that the dose escalation be repeated. Again the starting dose of 6 mg/m^2 was to be given to three patients and, when this dose was demonstrated to be safe, an intermediate dose of 7.5 mg/m^2 was to be given to a second cohort. The dose was to be again escalated if the three patients did not experience toxicities of grade 3 or more. The third cohort was to be given 9 mg/m^2.

Toxicity was graded on a scale of 0–5 using the National Cancer Institute Common Toxicity Criteria Version 2.0. Dose-limiting toxicity (DLT) was defined as grade 3 or more non-hematological toxicity. If the first 3 patients in a cohort had no toxicities, the dose was escalated. If one patient had a DLT, another three patients were enrolled in the same cohort. When two or fewer patients among the six had DLTs, the dose was judged to be tolerable.

2.4 Pharmacokinetics and analysis for antibodies to GO

The plasma was collected at 0, 60, 120, 180, 240 and 360 min, when 0 is starting point of infusion. Maximum plasma concentration (C_{max}), area under the concentration-time curve from 0 to X h (AUC_{0-Xh}), elimination half life ($t_{1/2}$), elimination rate constant in terminal phase (λ_z), distribution volume calculated by λz (Vz), and distribution volume at steady state (Vss) were calculated as previously reported [6, 7]. All patients were screened for antibodies to the hP67.6 monoclonal antibody and the calicheamicin-linker portion of GO using enzyme-linked immunosorbent assays [6, 7].

2.5 Efficacy measures

The primary efficacy measure was complete remission (CR) rate. CR was defined as (1) the absence of leukemic blasts in peripheral blood (2) no more than 5% leukemic blasts in the bone marrow, as measured in bone marrow aspirates or biopsy samples (3) peripheral blood counts with hemoglobin 9 g/dl or more, absolute neutrophil count (ANC) 1,500/μl or more, and platelets 100,000/μl or more and (4) red blood cell transfusion independence for 2 weeks or longer and platelet transfusion independence for 1 week or longer. Determination of remission status was evaluated approximately 28 days after the last dose of GO. All bone marrow slides were evaluated by a central reviewer (S.N.), who was blinded to treatment outcomes.

Some patients had a morphological remission, which was defined as a CR with incomplete platelet recovery (CRp). These patients met all the criteria for CR (including freedom from platelet transfusion for 1 week), but platelet counts were less than 100,000/μl. CRp was included as a secondary efficacy measure in these studies, and the overall remission rate was based on the combined total of patients who had CR or CRp. For patients who had equal to or less than 5% blasts in the bone marrow, no blasts in the peripheral blood, and no extrameningeal leukemia evident, the effect was then defined as blast clearance (BC). Patients who did not meet the criteria for CR, CRp, or BC were categorized as no remission (NR).

To assess the durability of response and relapse-free survival (RFS), overall survival rates also were evaluated. RFS was measured from the first documentation of CR or CRp to the date of recurrence, death, or data cut-off. The overall survival was measured from the date of initial GO administration to the date of death or data cut-off. All survival data were analyzed using Kaplan–Meier estimates.

3 Results

3.1 Patient demographics and baseline clinical characteristics

In the phase I part, a total of 20 patients with CD33-positive relapsed or refractory AML were evaluated (Table 1). The patients' median age was 60 years, ranging from 34 to 68 years. Male/female ratio was 9/11. The median duration of the first CR was 11.8 months ranging from 0 (one patient) to 57 months. All the patients who had achieved CR had been given maintenance therapy. More than 80% of the cells were positive for CD33, which was required by the protocol. The positivity was more than 90% in the majority (19/20 in phase I and 20/20 in phase II part). Subtypes of AML included M0–M5 but no cases of M6 or M7. The number of times of relapse also varied; there were 15 patients with one relapse, 2 patients with two relapses, 1 patient with three relapses and 1 patient with five relapses, respectively.

In the phase II part, a total of 20 patients were evaluated (Table 1). The median age was 58 years, ranging from 28 to 68 years. Male/female ratio was 14/6. The median duration of the first CR was 16.5 months ranging from 6 to 87 months. Subtypes of AML included M1–M6 but no cases of M7. The number of times of relapse also varied; there were 12 patients with one relapse, 4 patients with two

Table 1 Summary of demographic and baseline characteristics

Characteristic	Phase I				Phase II ($N = 20$)
	6 mg/m^2 ($N = 6$)	7.5 mg/m^2 ($N = 3$)	9 mg/m^2 ($N = 11$)	Total ($N = 20$)	
Age					
Median (Min–Max)	57 (34–68)	62 (35–63)	55 (39–67)	60 (34–68)	58 (28–68)
Sex					
Female	4	1	4	9	6
Male	2	2	7	11	14
Number of times of relapse					
0	0	0	1	1	12
1	6	2	7	15	4
2	0	1	1	2	2
3	0	0	1	1	1
4	0	0	0	0	1
5	0	0	1	1	0
Laboratory data before study (median)					
BM Blast (%)	65	80	70	70	75
PB WBC	2900	1700	2900	2800	2000
PB Blast (%)	20	0	21	20	3
Hgb	9.5	9.5	10.6	9.8	11.3
Plt	7.0	7.9	4.5	5.5	5.6
Duration of first remission (months)					
Median				11.8	16.5
Mean	9.3	22.2	16.5	15.5	23.2
Max	21	24	57	57	87
Min	4	20	0	0	6
N	5[a]	3	11	19	20
FAB subtypes (at initial presentation)					
M0	1	1	0	2	0
M1	0	0	1	1	2
M2	2	0	4	6	6
M3	0	0	1	1	2
M4	1	2	4	7	2
M4E0	0	0	1	1	3
M5	2	0	0	2	4
M6	0	0	0	0	1
Prognostic category (at pre-study screening)					
Favorable	0	0	1	1	3
Intermediate	1	2	3	6	7
Poor	0	0	3	3	3
Notavailable	5	1	4	10	7
ECOG PS (at prestudy screening)					
0	4	2	6	12	15
1	1	1	5	7	3
2	1	0	0	1	2

[a] Another case was exempted from analysis object because its remission induction date is unknown

relapses, 2 patients with three relapses, 1 patient with four relapses and 1 patient with five relapses, respectively.

In the phase I part, seven patients did not receive the second infusion: three patients due to progressive disease, two due to toxicities, one due to central nervous system (CNS) invasion and one due to possible infection. In the phase II part, two patients did not receive the second infusion due to persisting toxicities.

3.2 Dose determination

The first dose level of 6 mg/m^2 for the 3 patients was judged to be tolerable based on the absence of toxicities of grade 3 or above. At the second dose level of 9 mg/m^2, one patient died on the day of the infusion due to grade 4 pulmonary hemorrhage. The safety monitoring committee determined that the adverse event was not judged unrelated to the infusion. The adverse event might have occurred due to the coagulopathy related to the disease. Thus, the protocol was amended and doses of 6, 7.5 and 9 mg/m^2 were to be used in the dose escalation. Collection of coagulation data was added for safety monitoring.

At the dose of 6 mg/m^2, 1 patient developed grade 3 toxicity of hyperglycemia, nausea, and infection. Combining with the first three cases at the dose of 6 mg/m^2, only 1 among 6 patients developed grade 3; hence, the next dose of 7.5 mg/m^2 was examined. At the dose of 7.5 mg/m^2, no patients had toxicity of grade 3 or above. At the dose of 9 mg/m^2, of the six patients, two showed toxicities of elevated hepatic enzymes, which were judged to be tolerable. Therefore, the dose level utilized in the subsequent phase II part was 9 mg/m^2.

3.3 Toxicities

The most commonly observed non-hematological toxicities during the treatment period were fever, nausea, vomiting, anorexia, and liver toxicities. Most were mild and below grade 3. In the phase I part, nausea of grade 3 or above was observed in two patients and elevated liver enzymes in two patients. In the phase II part, nausea of grade 3 or above occurred in six patients. In the treatment period of the phase II part, infections of grade 3 or above occurred in three patients, bleeding in 1 patient and elevated liver enzymes in four patients. No patients developed jaundice.

Hematological toxicities were more common during the treatment period. Anemia of grade 3 or above occurred in 13 patients in the phase I part and in 16 patients in the phase II part. In the phase I and II parts, granulocytopenia of grade 3 or above occurred in 16 and 9 patients, respectively; thrombocytopenia of grade 3 or above occurred in 19 patients in each part.

The toxicities that occurred on the day of infusion and the day after following the infusion were distinguished and categorized as infusion-related toxicities (Table 2). They included GI toxicities, which were the same for the other chemotherapeutic agents and not specific to the agents. However, fever, chilliness, headache and coagulopathy were notable and considered to be related to the infusion reactions. The coagulopathy might be related to tumor lysis and occurrence of disseminated intravascular coagulation. Fewer toxicities were observed following the second infusion than subsequent to the first one (tachyphylaxis), although this difference of frequency was not statistically significant. Fever occurred in 16/20 and 8/13 patients following the first and second infusions, respectively, in the phase I part; fever occurred in 15/20 and 5/18 patients in the phase II part, respectively. Nausea also showed tachyphylaxis. In the phase I part, grade 3 or more nausea occurred in two patients during course 1 and in the phase II part, four patients during course 1 and 1 patient during course 2.

3.4 Pharmacokinetics

The plasma concentration of hP67.6, total calicheamicin, and unconjugated calicheamicin was measured (Tables 3, 4). Dose dependence was shown in C_{max} and AUC_{inf} of hP67.6 and total calicheamicin. AUC_{inf} of hP67.6 had tendency to increase; those for the first and second doses were 133 ± 94 and 223 ± 136 mg h/l, respectively. Half lives ($t_{1/2}$) of hP67.6 were variable and no tendency was shown during the subsequent dose periods.

C_{max}, half-lives and AUC_{inf} of total calicheamicin were calculated (Table 4). We found tendency to increase after the second infusion. For 9 mg/m^2, half-lives were 24 ± 16 and 48 ± 13 h for the first and second infusions, respectively. AUCs of total calicheamicin were 2.7 ± 2.0 and 6.2 ± 1.6 mg h/l, for the first and the second infusions, respectively. AUC_{inf} of unconjugated calicheamicin was also higher after the second infusion than after the first infusion.

3.5 Efficacy

In the phase I part, except for one patient who developed pulmonary bleeding, 19 patients were evaluable for efficacy. CR was obtained in two patients who were treated with 9 mg/m^2 (Table 5). Thus, of the patients treated with 9 mg/m^2 GO, 18.2% (2/11) achieved CR.

In the phase II part, among 20 evaluable patients, 5 achieved CR and 1 achieved CRp for an overall remission rate of 30.0% (6/20, 95% confidence interval [CI] 14.0–50.8%). Patients with multiple numbers of relapses or those who were in intermediate or poor prognostic groups achieved CR or CRp (Table 6). No patients who were 60 years of age or older achieved CR or CRp.

In a subset of patients, blasts disappeared in the bone marrow or peripheral blood, but did not fulfill the criteria of CR or CRp. Such responders were observed in 5 of 20 patients, and 3 of 20 patients in the phase I and II parts, respectively. The patients had been heavily pretreated: one patient had received three courses of induction therapy,