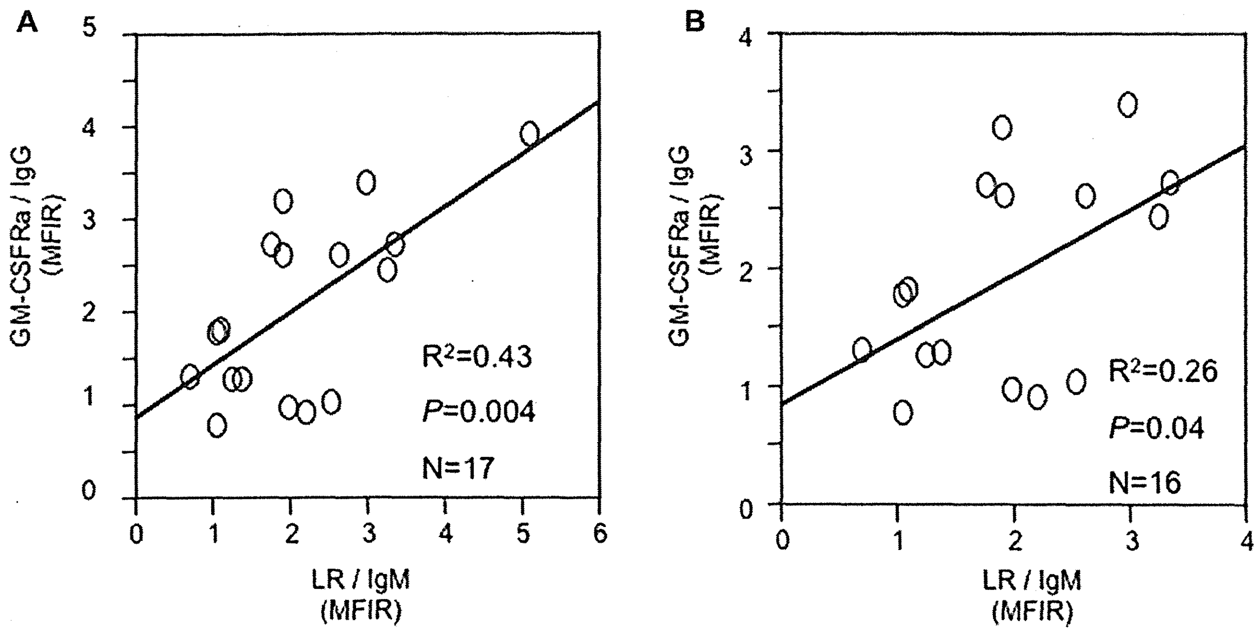

Supplementary Figure E4. Relationship between MFI ratio (MFIR) of LR and GM-CSFRα on AML samples. (**A**) One sample that was plotted at the far right in Figure 4D was removed, then using 17 samples, their relationship was reanalyzed. There was a significant relationship between these factors ($p = 0.004$). (**B**) Reanalysis of MFIR of LR and GM-CSFRα (n = 16) deleting two samples located at the far right and uppermost of Figure 4D. Significance still remained in the relationship between MFIR of LR and GM-CSFRα.

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ORIGINAL REPORT

Risk of Myelodysplastic Syndromes in People Exposed to Ionizing Radiation: A Retrospective Cohort Study of Nagasaki Atomic Bomb Survivors

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A B S T R A C T

Purpose

The risk of myelodysplastic syndromes (MDS) has not been fully investigated among people exposed to ionizing radiation. We investigate MDS risk and radiation dose-response in Japanese atomic bomb survivors.

Patients and Methods

We conducted a retrospective cohort study by using two databases of Nagasaki atomic bomb survivors: 64,026 people with known exposure distance in the database of Nagasaki University Atomic-Bomb Disease Institute (ABDI) and 22,245 people with estimated radiation dose in the Radiation Effects Research Foundation Life Span Study (LSS). Patients with MDS diagnosed from 1985 to 2004 were identified by record linkage between the cohorts and the Nagasaki Prefecture Cancer Registry. Cox and Poisson regression models were used to estimate relationships between exposure distance or dose and MDS risk.

Results

There were 151 patients with MDS in the ABDI cohort and 47 patients with MDS in the LSS cohort. MDS rate increased inversely with exposure distance, with an excess relative risk (ERR) decay per km of 1.2 (95% CI, 0.4 to 3.0; $P < .001$) for ABDI. MDS risk also showed a significant linear response to exposure dose level ($P < .001$) with an ERR per Gy of 4.3 (95% CI, 1.6 to 9.5; $P < .001$). After adjustment for sex, attained age, and birth year, the MDS risk was significantly greater in those exposed when young.

Conclusion

A significant linear radiation dose-response for MDS exists in atomic bomb survivors 40 to 60 years after radiation exposure. Clinicians should perform careful long-term follow-up of irradiated people to detect MDS as early as possible.

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INTRODUCTION

Myelodysplastic syndromes (MDS) are a heterogeneous group of disorders characterized by clonal and ineffective hematopoiesis, morphologic dysplasia, and an increased risk of developing acute myeloid leukemia (AML).[1] MDS can arise de novo or secondary after chemo- and/or radiotherapy (therapy-related MDS).

The pathogenesis and established causative factors remain elusive for most patients with MDS. A widely accepted multistep pathogenesis model involves initial damage to hematopoietic stem cells caused by genotoxic or environmental agents followed by additional genetic or cytogenetic changes, resulting in the expansion of the MDS clone and the subsequent leukemic transformation.[2,3] Ionizing radiation is a well-known environmental carcinogen that induces chromosomal and genetic abnormalities. When an individual's bone marrow is exposed to ionizing radiation, hematopoietic stem cells may be damaged randomly, and some of these changes could induce MDS.

In contrast to the well-documented radiation-induced leukemia,[4-6] there has been no conclusive evidence that radiation exposure plays a significant role in the development of MDS. So far, radiation exposure remains a probable causative factor for MDS.[2] Most review articles have described radiation exposure as a definite causative factor for MDS on the basis of clinical studies of therapy-related MDS/AML. However, the original sources seldom

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evaluated the radiation dose-response relationship for MDS alone. Epidemiologic studies of people exposed to a variety of radiations reported only a small number of cases.[7-10] In a previous study of atomic bomb survivors,[11] a possible radiation dose-response relationship for MDS was suggested, but the analysis included only 12 patients. MDS research among the atomic bomb survivors has been hampered by the fact that case ascertainment was incomplete before publication of the 1982 French-American-British (FAB) classification[1] and that no regional cancer registry officially registered MDS until 2000.

A radiation dose-response relationship for MDS might be predictable from that for AML because of the clinical similarity between the two diseases. However, much data have been accumulated to support that MDS has features that are distinct from AML with regard to latency of onset, genetic and cytogenetic abnormalities, apoptotic activity, and so on.[12,13] These biologic differences between MDS and AML suggest that radiation-induced MDS and AML may have distinct features as a consequence of different damage caused by radiation exposure. Therefore, it is important to evaluate the radiation dose relationship for MDS risk in people exposed to radiation.

In response to the increasing concern about MDS risk in atomic bomb survivors,[14] we initiated a multi-institutional epidemiologic research project. The aim of this study was to assess MDS risk and the radiation dose-response relationship 40 to 60 years after exposure.

PATIENTS AND METHODS

Study Project

This project, begun in April 2004, was a collaboration between the Atomic Bomb Disease Institute (ABDI) of the Nagasaki University Graduate School of Biomedical Sciences, the Nagasaki Prefecture Cancer Registry (NPCR),[15] the hematology departments in five hospitals in Nagasaki City (see Acknowledgment), and the Radiation Effects Research Foundation (RERF). The Institutional Review Boards of Nagasaki University (Research Protocol 16031797) and RERF (Research Protocols 18-66 and 1-75) approved this study.

Patients

We collected clinical information on MDS patients diagnosed in the five hospitals from 1982 to 2004, without regard for exposure status. Skilled hematologists in the hospitals and two authors (M.I. and M.To.) re-evaluated the clinical information, including bone marrow specimens, by using FAB criteria[1] to classify patients as refractory anemia (RA), RA with ringed sideroblasts (RARS), RA with excess blasts (RAEB), RAEB in transformation (RAEB-t), or chronic myelomonocytic leukemia (CMML). We also classified the diagnostic certainty for each patient as either definite, possible, undetermined, or non-MDS by using the criteria listed in Table 1. All reviewed patients were reported to the NPCR to be checked for multiple enrollments, the earliest date of MDS diagnosis, and the presence of malignancies before the MDS diagnosis. MDS patients who received chemotherapy and/or radiation therapy for their earlier malignancy were treated as therapy-related MDS, but those who had only surgery for their earlier malignancy were treated as primary MDS. International Classification of Diseases for Oncology, 3rd Edition (ICD-O-3) codes[16] for MDS were assigned to all patients. We also added information on date of death, date of progression to overt leukemia, if present, and the last recorded follow-up date.

Population

We used two different cohorts of Nagasaki atomic bomb survivors: a cohort defined by the ABDI Data Center and RERF's Life Span Study (LSS) cohort. Although there is some overlap between the cohorts, they were established independently and each has its own strengths and limitations. The ABDI cohort is larger than the LSS cohort but lacks information on individual dose, whereas the LSS cohort has detailed individual dose estimates but fewer Nagasaki survivors. The main reason for using two cohorts in our study was to give more credibility to the LSS dose-response findings by confirming similar distance-response patterns in the two cohorts.

The ABDI database was established in 1977 and consists of data on approximately 120,000 Nagasaki atomic bomb survivors. Available data include information on exposure status, death and migration dates, and the results of medical checkups and cancer screenings conducted at the Nagasaki Atomic Bomb Casualty Council Health Management Center. Details about the ABDI database were given previously.[17]

The LSS database was established in 1950, consisting of approximately 94,000 Hiroshima and Nagasaki atomic bomb survivors and 26,000 nonexposed city residents. Available data include information on exposure status, death and cancer diagnosis dates, and individual organ dose estimates computed by using the Dosimetry System 2002 (DS02).[18] The LSS database includes approximately 32,000 Nagasaki survivors. Details about the LSS database were given previously.[19]

Identification of MDS in Atomic Bomb Survivors

Of the 796 patients with MDS registered in the NPCR, 44 were excluded because of misdiagnosis and 147 were excluded because of residence outside the catchment area. The remaining 605 eligible patients with MDS were linked to the ADBI and LSS databases to identify atomic bomb survivors with MDS (ABDI-MDS and LSS-MDS, respectively). Follow-up for this study began in January 1985 when the FAB classification of MDS had been widely used in Japan. Figure 1 summarizes the patient selection process and provides information on the final cohorts used for analyses.

Statistical Analysis

We performed risk analyses only for those with known exposure distances or dose. Patients were limited to those with a definite or possible level of diagnostic certainty for MDS. Patients of therapy-related MDS (ICD-O-3 code 9987/3) or with an undetermined level of certainty were censored at the date of diagnosis. Follow-up began on January 1, 1985, and continued to the earliest of the date of the primary MDS diagnosis, death, or December 31, 2004. The

Table 1. Criteria for the Level of Diagnostic Certainty for MDS in Case Review

Level	Objective Evidence
Definite	Reaffirmation of dysmegakaryopoiesis and/or dysgranulopoiesis on the bone marrow aspirate smear.
	Bone marrow aspirate smear was not available, but there was a clear description of dysmegakaryopoiesis and/or dysgranulopoiesis on the medical record.
	Bone marrow aspirate smear was not available and there was no clear description of dysmegakaryopoiesis and/or dysgranulopoiesis, but there was a description of the presence of dysplasia in blood cells, myeloblast < 30%, and chromosome aberration on the medical record.
Possible	Morphologic evaluation was not available, but there was a clear clinical course from FAB-refractory anemia or refractory anemia with excess blasts to leukemia on the medical record.
Undetermined	Only the name of MDS was available on the medical record and the death certificate.

Abbreviations: MDS, myelodysplastic syndromes; FAB, French-American-British classification.

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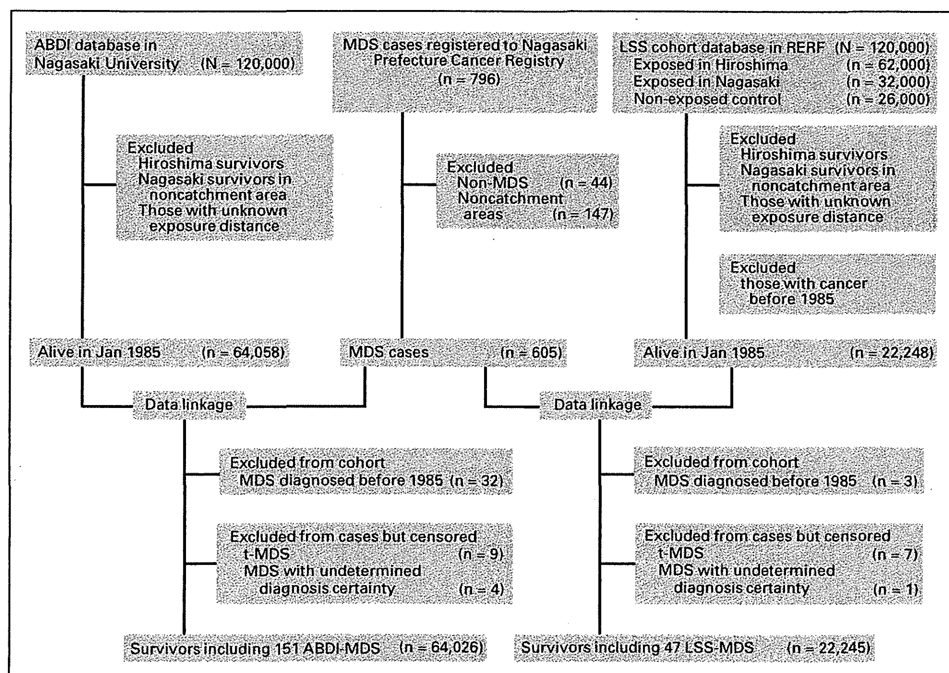

Fig 1. Study profile. ABDI, Atomic Bomb Disease Institute; MDS, myelodysplastic syndromes; LSS, Life Span Study; RERF, Radiation Effects Research Foundation; t-MDS, therapy-related MDS.

person-year calculations took into account date of migration in the ABDI data set, and a migration adjustment was made in the LSS data set. For the LSS data set, we also excluded those with cancer before 1985, and the follow-up was censored at the date of treatment with chemo- or radiotherapy for any cancer, if present, because all LSS cohort members are routinely linked to the NPCR. We treated patients with MDS either together, by FAB category, or by a dichotomized category of low-risk (RA and RARS) and high-risk (RAEB and RAEB-t).[20] We did not include CMML or "not otherwise specified" in the dichotomized category.

We used Cox regression models to estimate the effects of sex, age at exposure, exposure distance, and dose on MDS incidence rates. Relative risk (RR) estimates were computed by using SAS software (version 9.1; SAS Institute, Cary, NC). We used the asymptotic SEs as the basis for hypothesis tests and 95% CIs. Interactions between factors were also tested. We treated age at exposure as two (0 to19 and ≥ 20 years) or three groups (0 to 9, 10 to 19, and ≥ 20 years) or as continuous, as necessary, and exposure distance in km as three groups (< 1.5, 1.5 to 2.99, and 3.0 to 10.0 km) or more detailed categories, and the weighted DS02 bone marrow dose in Gy as three groups (< 0.005, 0.005 to 0.999, and ≥ 1 Gy) or as continuous. The cutoff values for exposure distance or dose were chosen on the basis of data from previous reports.[17,19,21] For categoric data, tests for independence or trend were carried out by using χ^2 or Fisher's exact tests, as appropriate. A two-tailed P value of < 0.05 was judged significant.

We examined linear, linear-quadratic, and other dose-response functions for the LSS data adjusting for sex, age at exposure, and attained age or time since exposure, in a manner similar to earlier leukemia dose-response analyses,[6] and estimated the excess relative risk (ERR) per Gy by using weighted DS02 bone marrow dose. The basic ERR dose-response model can be written as $BR\,[1+\alpha d]$, where BR is the baseline rate described as a parametric function of sex and attained age. We also examined ERR distance-response functions in the ABDI and the LSS cohorts with exposure distance treated as a continuous variable truncated at 3km (r[inf]3k) or with exposure distance categories of < 1.25, 1.25 to1.49, 1.5 to1.74, 1.75 to1.99, 2.0 to 2.49, 2.5 to 2.99, and ≥ 3.0 km. The continuous exposure-distance model can be written as BR $[1 + \gamma \exp(-\beta r$[inf]3k$)]$ where the BRs are modeled as for the dose-response model, β is a distance-decay parameter, and γ is a scaling parameter. The distance-decay parameter value (x) is transformed to the percentage decrease in the ERR per km, which is calculated from the formula, $[1 - \exp(-x)] \times 100\%$. ERR models were fit and likelihood-based P values and CIs were computed by using EPICURE software (Hirosoft International, Seattle, WA).[22]

RESULTS

The ABDI data set consisted of 64,026 Nagasaki atomic bomb survivors with information on exposure distance, including 151 ABDI patients with MDS who were diagnosed from 1985 to 2004. Of those, 147 (97%) were definite MDS patients and 4 (3%) were possible patients. The LSS data set consisted of 22,245 Nagasaki atomic bomb survivors for whom dose estimates were available. The 47 LSS patients with MDS included 45 (96%) definite and two (4%) possible patients. Table 2 presents the frequencies of FAB subtypes in both data sets. The distribution of subtypes in the ABDI and LSS cohorts did not differ ($P = .54$). The distribution characteristics, particularly the high frequency of RA relative to RARS and CMML, were typical for Japanese patients with MDS.[23] Cytogenetics data were available for 107 (71%) of 151 ABDI-MDS patients (Appendix Table A1, online only). The median age at exposure and the median age at diagnosis were 18.5 years (range, 0.3 to 43.4 years) and 71.0 years (range, 42.0 to 96.6 years) for ABDI-MDS, respectively, and 16.5 years (range, 2.5 to 48.8 years) and 72.4 years (range, 48.5 to 94.3 years) for LSS-MDS, respectively. The median time to development of MDS from 1985 was 12.0 years (range, 0.3 to 19.9 years) for ABDI-MDS and 14.5 years (range, 0.9 to 19.5 years) for LSS-MDS.

The total numbers of person-years in the ABDI and LSS cohorts were 947,215 and 270,619, respectively. The crude MDS incidence rates in the ABDI and LSS cohorts were 15.9 and 17.4 patients per 100,000 person-years, respectively. Table 3 summarizes the crude incidence rate and crude RR estimates by exposure status. MDS rates were higher for men than for women and increased with age at exposure. MDS rates also increased with decreasing distance from the hypocenter and with increasing estimated dose.

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Table 2. Distribution of MDS by Exposure Distance or Dose in Two Cohorts of Atomic Bomb Survivors

	Exposure Distance (km) for Nagasaki Atomic Bomb Disease Institute Cohort				DS02 Bone Marrow Weighted Dose (Gy) for Life Span Study-Nagasaki Cohort			
Variable	< 1.5	1.5-2.99	≥ 3.0	Total	≥ 1	0.005-0.999	< 0.005	Total
Sex								
Male	1,693	6,485	16,092	24,270	273	2,665	5,904	8,842
Female	2,258	10,663	26,835	39,756	351	4,201	8,851	13,403
Total	3,951	17,148	42,927	64,026	624	6,866	14,755	22,245
MDS FAB subtypes								
RA	15	28	57	100	5	9	20	34
RARS	0	1	3	4	0	1	0	1
RAEB	7	8	14	29	2	3	2	7
RAEB-t	2	2	2	6	1	2	0	3
CMML	1	3	4	8	0	0	0	0
Unclassified	0	2	2	4	0	0	2	2
Total	25	44	82	151	8	15	24	47

Abbreviations: MDS, myelodysplastic syndromes; DS02, Dosimetry System 2002; FAB, French-American-British classification; RA, refractory anemia; RARS, RA with ringed sideroblasts; RAEB, RA with excess blasts; RABEB-t, RAEB in transformation; CMML, chronic myelomonocytic leukemia.

In Cox analyses for the ABDI cohort with adjustment for sex and age at exposure, the MDS incidence rate was significantly and inversely related to the exposure distance. The RR estimates for those exposed at < 1.5 and 1.5 to 2.99 km from the hypocenter were 2.8 (95% CI, 1.8 to 4.5; $P < .001$) and 1.3 (95% CI, 0.9 to 1.9; $P = .13$), respectively. Analyses of the LSS cohort also revealed that dose was a strong risk factor for MDS. Effects of exposure distance and dose on MDS were observed in both high-risk and low-risk MDS in both cohorts (Figs 2A and 2B). In a joint analysis of the dose and distance effects on MDS rates, there was a suggestion ($P = .08$) of larger radiation effects in high-risk MDS than in low-risk MDS. A significant linear dose association was observed in each risk group ($P < .001$). Effects of exposure distance and dose on MDS were also observed for those exposed before and after age 20 in both cohorts (Figs 2C and 2D). When we adjusted for attained age in 1985 in the ABDI cohort, age-specific MDS risks increased with increasing year of birth, with risks for those born after 1925 being about 1.75 (95% CI, 1.05 to 2.90) times the risks for those born in earlier years. The adjusted MDS risk using exposure dose in the LSS data showed similar results (RR, 1.71; 95% CI, 0.95 to 3.10). After allowing for birth cohort effects on the MDS risk, there was no evidence of a statistically significant interaction between distance or dose and age at exposure in either cohort (ABDI $P = .06$; LSS $P = .36$).

MDS rates decreased significantly with increasing distance for both cohorts ($P < .001$ for both). The fitted ERR curves were similar for the two cohorts. The decay parameters for ABDI and LSS cohorts were 1.2 per km (95% CI, 0.4 to 3.0) and 2.1 per km (95% CI, 0.6 to 4.6), respectively. In other words, the ERR is estimated to decrease by 70% per km (95% CI, 33% to 95%) in the ABDI and 88% per km (95% CI, 43% to 99%) in the LSS cohort. Figure 2E shows the fitted distance-response curves and point estimates of the distance category–specific ERRs with 95% CIs. There was a statistically significant ($P < .001$) linear dose-response for MDS in the LSS cohort with an ERR per Gy estimate of 4.3 (95% CI, 1.6 to 9.5; Fig 2F). A linear-quadratic model that fit the AML[6] did not improve the fit ($P = .46$).

DISCUSSION

To the best of our knowledge, this is the largest study to date evaluating the association between MDS risk and radiation exposure, and the first to provide quantitative estimates of the effect of radiation on MDS risk. We observed a significant ($P < .001$) linear relation between radiation dose and MDS risk among atomic bomb survivors with an ERR per Gy of 4.3. We also observed that the effect of radiation on MDS risk was greater in advanced subtypes of MDS and in those exposed at younger ages.

Our finding of a significant linear dose-response pattern for MDS is in contrast to the significant linear-quadratic dose-response pattern for AML.[6] The fact that the radiation-associated increases of MDS risk still exist 40 or more years after exposure is also in contrast to the risk of radiation-induced leukemia in which the largest dose-related increases were seen in the first 10 to 15 years after the bombings and then decreased slowly with time.[5,6] The linear dose-response pattern and the appearance with a long latency for MDS in atomic bomb survivors seems similar to those seen for radiation-associated solid cancers.[19]

Differences in the dose-response patterns for MDS and AML suggest that the nature of the radiation-induced genetic damages in hematopoietic stem cells may differ for the two diseases. Mutations in the *AML1/RUNX1* gene[24,25] may be one of the genetic damages associated with MDS that occurred in hematopoietic stem cells of atomic bomb survivors because of radiation exposure. Accumulating data on the different characteristics of the molecular and clinical spectrum, including chromosome aberrations between MDS and AML,[12,13,26-29] could shed some light on differences in the role of radiation exposure on these diseases.

Why is radiation-induced MDS seen in atomic bomb survivors more than 40 years after exposure? A primary reason for the long latency of MDS risk could be that atomic bomb survivors, even those exposed early in life, are reaching ages at which MDS rates are increased. In fact, in recent years, hematologists in Nagasaki City have identified an increasing number of MDS occurrences among atomic bomb survivors. Moreover, on the basis of the multistep pathogenesis

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Table 3. Crude Incidence and Crude Relative Risk of Myelodysplastic Syndromes by Exposure Status in Nagasaki Atomic Bomb Survivors

	Nagasaki Atomic Bomb Disease Institute Cohort						Life Span Study-Nagasaki Cohort					
	Exposure Distance (km)						Weighted Bone Marrow Dose (Gy)					
Variable	< 1.5	1.5-2.99	≥ 3.0	Total	Crude RR	95% CI*	≥ 1	0.005-0.999	< 0.005	Total	Crude RR	95% CI*
Sex												
Male												
Population at risk	1,693	6,485	16,092	24,270			273	2,665	5,904	8,842		
No. of patients	12	21	34	67			3	8	10	21		
Person-years	23,071	91,880	233,191	348,144			2,959	29,789	66,102	98,850		
Crude rate†	52.0	22.9	14.6	19.2	1.3	1.0 to 1.9	101.4	26.9	15.1	21.2	1.4	0.8 to 2.5
Female												
Population at risk	2,258	10,663	26,835	39,756			351	4,201	8,851	13,403		
No. of patients	13	23	48	84			5	7	14	26		
Person-years	34,946	158,144	405,980	599,071			4,480	52,926	114,363	171,769		
Crude rate†	37.2	14.5	11.8	14.0	Ref		111.6	13.2	12.2	15.1	Ref	
Age at exposure, years												
0-9												
Population at risk	615	4,770	13,730	19,115			161	2,464	5,064	7,689		
No. of patients	6	9	13	28			3	6	3	12		
Person-years	9,756	77,132	225,071	311,960			1,750	29,274	60,572	91,596		
Crude rate†	61.5	11.7	5.8	9.0	Ref		171.4	20.5	5.0	13.1	Ref	
10-19												
Population at risk	1,950	5,620	13,611	21,181			280	2,256	4,841	7,377		
No. of patients	13	16	29	58			2	5	8	15		
Person-years	31,325	91,011	225,009	347,346			3,532	29,182	63,714	96,428		
Crude rate†	41.5	17.6	12.9	16.7	1.9	1.2 to 3.0	56.6	17.1	12.6	15.6	1.2	0.6 to 2.5
≥ 20												
Population at risk	1,386	6,758	15,586	23,730			183	2,146	4,850	7,179		
No. of patients	6	19	40	65			1	11	8	20		
Person-years	16,937	81,882	189,091	287,909			2,157	24,259	56,179	82,595		
Crude rate†	35.4	23.2	21.2	22.6	2.9	1.9 to 4.5	46.4	45.3	10.7	21.8	1.8	0.9 to 3.8
Total												
Population at risk, n	3,951	17,148	42,927	64,026			624	6,866	14,755	22,245		
No. of patients	25	44	82	151			6	22	19	47		
Person-years	58,018	250,025	639,171	947,215			7,439	82,715	180,465	270,619		
Crude rate†	43.1	17.6	12.8	15.9			80.7	26.6	10.5	17.4		
Crude RR	3.2	1.4	Ref				8.1	1.4	Ref			
95% CI*	2.0 to 5.0	1.0 to 2.0					3.1 to 18.0	0.7 to 2.6				

Abbreviations: RR, relative risk; Ref, reference.
*Analyses were performed using the Cox regression.
†The crude incidence was calculated as the total number of patients divided by person-years accumulated in each row and is presented per 100,000 person-years.

model,[3] we may speculate that hematopoietic stem cells of people exposed to higher radiation doses had more genetic damage than those of people exposed to lower dose or than those of the elderly population in general. However, we feel that the multistep pathogenesis model does not fully explain the recent increased risk of MDS. Chromosomal and genetic instabilities as consequences of targeted and/or nontargeted effects of radiation exposure[30] may play a role in the late development of MDS as well as solid cancers in atomic bomb survivors. In fact, we observed higher frequencies of complex karyotypic abnormalities, including random aneuploidies, among proximally exposed MDS patients in this study (Appendix Table A1). Another possible paradigm is the cancer stem-cell theory, including leukemic stem cells.[31,32] Trosko[33] suggests the role of organ-specific adult stem cells as the target cells for radiation-induced carcinogenesis, and the age-related changes in quality of the injured stem cells could affect cancer risks later in life. This concept may explain the long latency of MDS risk in atomic bomb survivors, although little is known about MDS stem cells.

This study has several limitations. Follow-up is limited and there is no information on MDS risks until 40 years after exposure. It was not possible to determine whether or not the incidence rate of MDS were elevated in the decades immediately after the bombings, since MDS was not recognized as a distinct entity until the mid-1980s. The dose-response analyses were performed for a small number of patients. The distance analyses did not account for variations in shielding among survivors, which would modify their actual doses. Information on dates of prior cancers and other prior chemotherapy or radiotherapy was not available for the ABDI data set.

As of 2007, we confirmed that 42 patients among the 151 ABDI-MDS patients progressed to overt leukemia (data not shown). Further studies are needed to clarify the effect of radiation on leukemic transformation as well as the nature of the radiation-induced MDS and the dose-response pattern. Efforts to expand the study to include MDS occurring among Hiroshima survivors are underway.

In conclusion, this study showed that acute radiation exposure is associated with increased risk of developing MDS later in life. This

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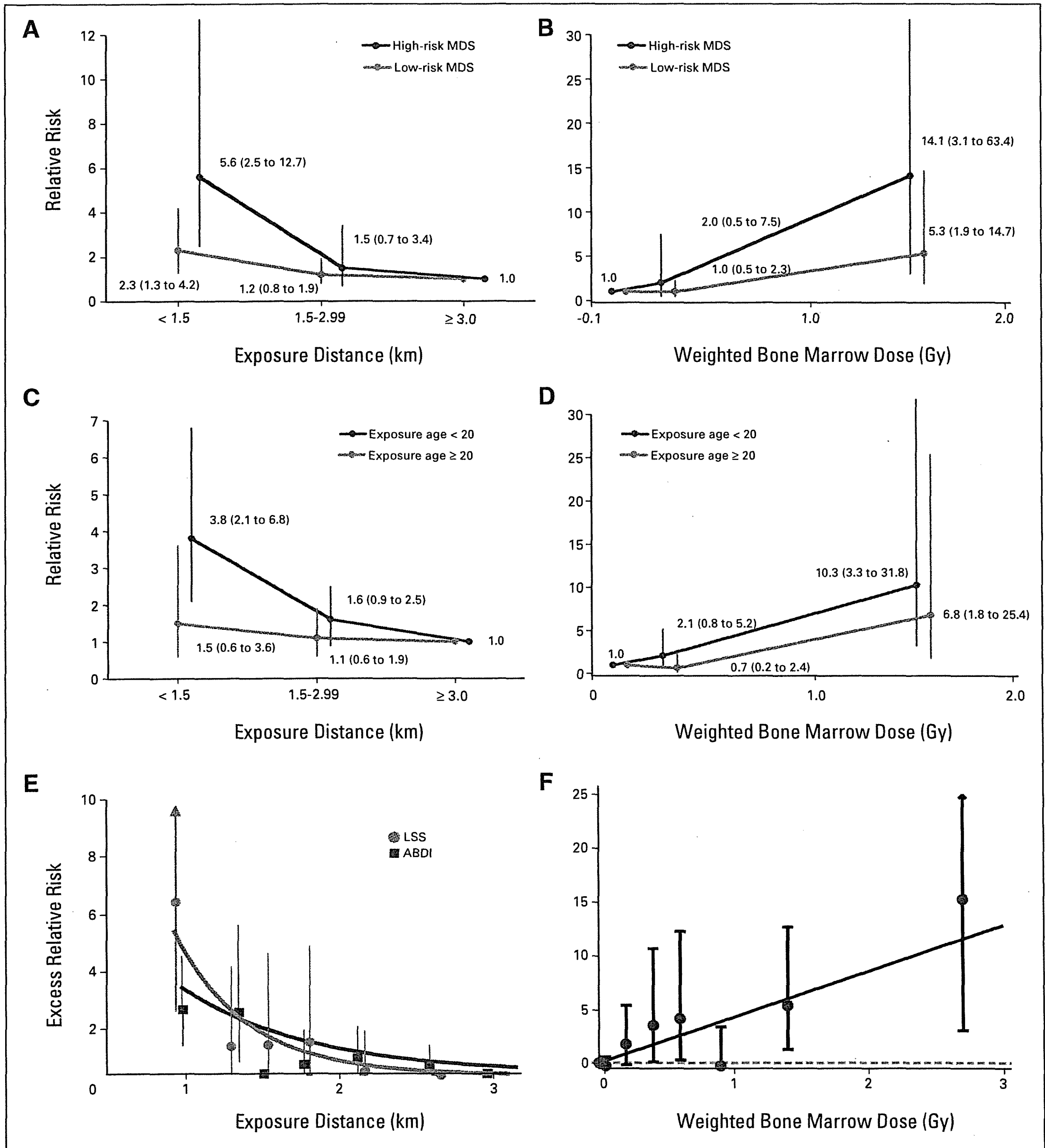

Fig 2. Risk of myelodysplastic syndromes (MDS) by exposure distance and dose. (A) Relative risks of MDS by French-American-British classification subtype in Atomic Bomb Disease Institute cohort, and (B) in Life Span Study-Nagasaki cohort. The high-risk MDS indicates French-American-British classification subtypes of refractive anemia with excess blasts and refractive anemia with excess blasts in transformation, and the low-risk MDS indicates the subtypes of refractive anemia and refractive anemia with ringed sideroblasts. (C) Relative risks of MDS by age at exposure in Atomic Bomb Disease Institute cohort, and (D) in Life Span Study-Nagasaki cohort. (E) Sex- and age-adjusted distance-response for MDS. The lines display the best-fitted excess relative risk curves based on distance category–specific relative risk. (F) Sex- and age-adjusted radiation dose-response for MDS. The line displays the best-fitted linear excess relative risk dose-response without risk modification based on dose category–specific relative risk. The dashed horizontal line represents excess relative risk = 0. Whiskers show the 95% CIs.

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suggests that radiation-induced MDS might involve a different pathogenesis than radiation-induced leukemia. Clinicians should perform careful long-term follow-up of people who have been exposed to radiation to detect MDS as early as possible and reduce the risk of leukemic transformation by using new drugs such as DNA hypomethylating agents.[34]

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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ORIGINAL ARTICLE

Pre-transplant imatinib-based therapy improves the outcome of allogeneic hematopoietic stem cell transplantation for *BCR–ABL*-positive acute lymphoblastic leukemia

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A high complete remission (CR) rate has been reported in newly diagnosed Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph + ALL) following imatinib-based therapy. However, the overall effect of imatinib on the outcomes of allogeneic hematopoietic stem cell transplantation (allo-HSCT) is undetermined. Between 2002 and 2005, 100 newly diagnosed adult patients with Ph + ALL were registered to a phase II study of imatinib-combined chemotherapy (Japan Adult Leukemia Study Group Ph + ALL202 study) and 97 patients achieved CR. We compared clinical outcomes of 51 patients who received allo-HSCT in their first CR (imatinib cohort) with those of 122 historical control patients in the pre-imatinib era (pre-imatinib cohort). The probability of overall survival at 3 years after allo-HSCT was 65% (95% confidence interval (CI), 49–78%) for the imatinib cohort and 44% (95% CI, 35–52%) for the pre-imatinib cohort. Multivariate analysis confirmed that this difference was statistically significant (adjusted hazard ratio, 0.44, *P* = 0.005). Favorable outcomes of the imatinib cohort were also observed for disease-free survival (*P* = 0.007) and relapse (*P* = 0.002), but not for non-relapse mortality (*P* = 0.265). Imatinib-based therapy is a potentially useful strategy for newly diagnosed patients with Ph + ALL, not only providing them more chance to receive allo-HSCT, but also improving the outcome of allo-HSCT.
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Keywords: Philadelphia chromosome-positive acute lymphoblastic leukemia; imatinib; allogeneic hematopoietic stem cell transplantation

Introduction

The Philadelphia chromosome (Ph) presents in 20–25% of adult patients with acute lymphoblastic leukemia (ALL) and is an extremely unfavorable prognostic factor. The outcome of patients with Ph-positive ALL (Ph + ALL) following conventional chemotherapy is dismal, showing <20% long-term survival.[1–4] Although allogeneic hematopoietic stem cell transplantation (allo-HSCT) has offered a curative option in Ph + ALL,[3–5] relatively high rates of relapse and non-relapse mortality (NRM) impair the treatment success even after allo-HSCT. The International Bone Marrow Transplant Registry reported a leukemia-free survival rate of 38% following human leukocyte antigen (HLA)-identical allo-HSCT for Ph + ALL patients transplanted in the first complete remission (CR).[6] Previously, we and others reported that imatinib-based chemotherapy produced very high CR rate, thus allowing a high proportion of patients to prepare for allo-HSCT.[7,8] However, because of the short observation period, the impact of imatinib-based therapy upon the survival outcomes after allo-HSCT remains unclear. To address whether allo-HSCT after imatinib-based therapy is a superior treatment approach to that after conventional chemotherapy, we conducted a retrospective analysis of Ph + ALL patients who underwent allo-HSCT before and after imatinib became available, using data from the Japan Adult Leukemia Study Group (JALSG) Ph + ALL202 study and from the nationwide database of the Japan Society of Hematopoietic Stem-cell Transplantation (JSHCT) and the Japan Marrow Donor Program (JMDP).

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Patients and methods

Data source and patient selection criteria

We compared the transplantation outcome of patients treated by the JALSG Ph + ALL 202 study (imatinib cohort) with those in the historical control data in the pre-imatinib era from the JSHCT and JMDP (pre-imatinib cohort), in which information on patient survival, disease status and long-term complications, including chronic graft-versus-host disease (cGVHD) and second malignancies, is renewed annually using follow-up forms.[9,10] To

attain an adequate level of comparability in terms of allo-HSCT, patients were selected according to the following criteria: (1) patients with *de novo* Ph+ALL; (2) age range of 15–65 years and (3) allo-HSCT during their first CR. A total of 122 patients who received allo-HSCT between January 1995 and December 2001 (before the approval of imatinib by the Japanese government) were selected. This study period of the pre-imatinib cohort included the pioneering period of cord blood transplantation (CBT) when the relevance of cell dose and HLA matching had not yet been recognized. Thus, the subjects were limited to those who received bone marrow (BM) or peripheral blood (PB) as a treatment graft.

Patients

Between September 2002 and May 2005, 100 newly diagnosed patients with Ph+ALL were registered to the JALSG Ph+ALL202 study, and received a phase 2 imatinib-combined chemotherapy as described previously.[7] Ph+ALL was diagnosed by the presence of Ph through chromosome and/or FISH analysis, and positivity for *BCR–ABL* fusion transcripts detection by real-time quantitative polymerase chain reaction (RQ–PCR) analysis.

Of 97 patients who achieved CR, 60 patients received allo-HSCT in their first CR. Of these 60 patients, 9 patients who received unrelated CBT were excluded in this analysis because of the reason as described at the selection criteria for control patients in the pre-imatinib era. Thus, 51 patients transplanted between February 2003 and December 2005 were analyzed. In the JALSG Ph+ALL202 study, allo-HSCT was recommended after achieving CR if an HLA-identical donor was available. The stem cell source for allo-HSCT was chosen in the following order: (1) matched-related allo-HSCT; (2) HLA-A, B and DRB1 allele matched (6/6) or DRB1 one-allele mismatched-unrelated allo-BMT, if patients had no HLA-matched-related donor and (3) unrelated CBT or HLA-mismatched-related allo-HSCT, if they had no donors described in (1) and (2). A prophylaxis for GVHD was determined by each institute, but did not include T-cell depletion. The study was approved by the institutional review board of each participating center and conducted in accordance with the Declaration of Helsinki.

Definition of engraftment and GVHD

Engraftment day was defined as the first day of three consecutive days when the absolute neutrophil count was $\geqslant 0.5 \times 10^9$/l. Graft failure was defined as the lack of any sign of neutrophil recovery. Engraftment that occurred after day 60 was also considered to be a graft failure. Patients who died early (<day 29) were excluded from the analysis of engraftment. Acute GVHD (aGVHD) and chronic GVHD (cGVHD) were defined according to previously described standard criteria.[11]

Quantitation of BCR–ABL transcripts

The copy number of *BCR–ABL* transcripts in BM was determined at a central laboratory using the RQ–PCR as described previously.[7] To minimize the variability in the results because of differences in the efficiency of cDNA synthesis and RNA integrity among the patient samples, the copy number of the *BCR–ABL* transcripts was converted to molecules per microgram RNA after being normalized by means of *GAPDH*. The normalized values of the *BCR–ABL* copies in each sample were reported as *BCR–ABL* number of copies. At least 5.7×10^5 copies/μg RNA *GAPDH* levels were required in a sample to consider a negative PCR result valid; otherwise, the sample was not useful for minimal residual studies. The threshold for quantification was 50 copies/μg RNA. The levels below this threshold were designated as 'not detected' or '<50 copies/μg'. In this study, the former was categorized as PCR negativity.

Minimal residual disease (MRD) at the time of HSCT was evaluated by the result of RQ–PCR within 30 days prior to transplantation.

Statistical considerations

The primary end point of this study was overall survival (OS) after allo-HSCT. Secondary end points included disease-free survival (DFS) and the incidence of aGVHD, cGVHD, NRM and relapse. We defined DFS events as relapse or death, whichever occurred earlier. The observation periods for OS were calculated from the date of transplantation until the date of the event or last known date of follow-up. The probabilities of OS and DFS were estimated using the Kaplan–Meier product limit method. The cumulative incidences of NRM, relapse, aGVHD and cGVHD were estimated as described elsewhere, taking the competing risk into account.[12] In each estimation of the cumulative incidence of an event, death without an event was defined as a competing risk. Risk factors for OS and DFS were evaluated by a combination of uni- and multivariate analyses. The following variables were evaluated for each analysis: imatinib-based therapy prior to HSCT, age group (under 40 versus 40 to 54 versus 55 and older), stem cell source (BM versus PB), HLA disparity (matched (HLA-identical siblings or 6/6 allele matched unrelated) versus mismatched), duration from diagnosis to HSCT and cGVHD as time-varying covariate (yes versus no). Univariate analysis was performed using Cox regression models or log-rank test. Multivariate analysis was performed using Cox proportional hazards regression model or competing risk regression model[13] as appropriate. For the evaluation of time-varying events, such as aGVHD or cGVHD, upon clinical outcomes, we treated these as time-varying covariates. Differences among groups in terms of demographic characteristics were tested using the χ^2 or Mann–Whitney tests as appropriate. All statistical analyses were conducted using STATA 11 (STATA Corp., College Station, TX, USA).

Results

Patient characteristics

In the imatinib cohort, there were 29 males and 22 females, with a median age of 38 years (range, 15–64 years). Regarding transcript types, 36 patients had minor *BCR–ABL* and 15 had major *BCR–ABL*. In 5 patients, pre-treatment cytogenetic data were not available, and of the remaining 46 patients, 8 showed t(9;22) only, 36 had additional chromosome aberrations and 2 showed normal karyotype. Of 48 patients who were evaluable for MRD analysis, 36 patients achieved PCR negativity at the time of HSCT.

Some of the clinical and biological features (such as presence of additional chromosome aberrations, *BCR–ABL* subtype, MRD status at HSCT and performance status at HSCT) were not available in the pre-imatinib cohort and not included in the present analysis.

Table 1 lists the characteristics of patients included in this comparative analysis. Some of the clinical features were significantly different between two cohorts: age distribution at HSCT ($P=0.048$), conditioning regimens ($P<0.001$), GVHD prophylaxis ($P<0.001$) and duration from diagnosis to HSCT ($P=0.041$). The majority of patients received the preparatory

Table 1 Patient characteristics ($N=173$)

Characteristic	*Imatinib cohort*	*Pre-imatinib cohort*	P
No. of transplantations	51	122	
Age, n (%)			0.048
−39	27 (53)	71 (58)	
40–54	17 (33)	49 (40)	
55–	7 (14)	2 (2)	
Median (range)	38 (15–64)	38 (15–57)	
Gender (male/female)	29/22	73/49	0.717
HSCT donor, n *(%)*			0.460
Related	24 (47)	73 (60)	
Unrelated	21 (41)	43 (35)	
HLA-mismatched related	6 (12)	6 (5)	
Hematopoietic cell source, n *(%)*			0.246
Bone marrow	35 (69)	94 (77)	
Peripheral blood	16 (31)	28 (23)	
Conditioning regimen, n *(%)*			<0.001
CY+TBI	24 (47)	26 (22)	
CY+CA+TBI	14 (27)	37 (31)	
CY+VP+TBI	2 (4)	21 (17)	
CY+TESPA+TBI	—	7 (6)	
CY+BU+TBI	—	6 (5)	
Flu+BU	3 (6)	—	
Flu+ LPAM ± TBI	2 (4)	—	
Others	6 (12)	25 (20)	
GVHD prophylaxis, n *(%)*			<0.001
Cyclosporine + sMTX	24 (47)	95 (80)	
Cyclosporine ± other	3 (6)	3 (2)	
Tacrolimus + sMTX	22 (43)	17 (14)	
Tacrolimus + other	—	4 (3)	
Median days from diagnosis to HSCT (range)	162 (67–512)	182 (66–834)	0.041

Abbreviations: BU, oral busulfan; CA, cytarabine; CY, cyclophosphamide; Flu, fuludarabine; GVHD, graft-versus-host disease; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; LPAM, melphalan; sMTX, short-term methotrexate; TBI, total body irradiation; TESPA, tespamine; VP, etoposide.

regimen of total body irradiation followed by cyclophosphamide and/or cytarabine. Five patients aged >55 in the imatinib cohort were given a reduced intensity regimen consisting of fludarabine and melphalan or busulfan. In the pre-imatinib cohort, a combination of cyclosporine (CsA) and short-term methotrexate (sMTX) was mostly used in the prophylaxis of GVHD. On the other hand, both CsA + sMTX and tacrolimus (FK506) + sMTX combinations were commonly used in the imatinib cohort. In both cohorts, none of the patients received imatinib therapy after HSCT in their first CR. In the imatinib cohort, all patients who showed hematologic relapse after HSCT received salvage treatment comprising of imatinib and/or chemotherapy. As for the pre-imatinib cohort, 13 patients relapsed after the approval of imatinib by the Japanese government (beyond December 2001). However, we have no information on how many patients received imatinib-based therapy after their relapse. The median follow-up period for survivors was 2.6 years (range, 1.0–4.6 years) for the imatinib cohort and 6.9 years (range, 0.1–11.4 years) for the pre-imatinib cohort.

Outcome

OS and DFS. In the pre-imatinib cohort, 80 patients died after HSCT: 46 of disease recurrence and 34 of causes other than leukemia. In the imatinib cohort, 35 patients were alive, 32 of them were free of leukemia and 16 patients died after HSCT: 4 of disease recurrence and 12 of causes other than leukemia. The 3-year OS was 65% (95% confidence interval (CI), 49–78%) for the imatinib cohort and significantly higher than 44% (95% CI, 35–52%) for the pre-imatinib cohort ($P=0.0148$; Figure 1a). The 3-year DFS was 58% (95% CI, 41.8–70.9%) for the imatinib cohort and significantly higher than 37% (95% CI, 28.5–45.6%) for the pre-imatinib cohort ($P=0.039$; Figure 1b).

Table 2 shows the result of risk factor analysis for OS and DFS among all 173 patients. In the multivariate analysis, the only variable found to influence OS and DFS was the pre-transplant imatinib-based therapy (hazard ratio (HR) = 0.44 (95% CI, 0.25–0.77); $P=0.004$ and HR = 0.51 (95% CI, 0.31–0.86); $P=0.011$, respectively). The presence of cGVHD showed a tendency of favorable OS and DFS, but did not reach the statistical significance (HR = 0.66 (95% CI, 0.42–1.06); $P=0.085$ and HR = 0.75 (95% CI, 0.47–1.19); $P=0.217$, respectively).

Other outcomes of transplantation

Relapses. In the pre-imatinib cohort, 48 patients relapsed after HSCT with a median of 240 days (range, 42–2302 days).

In the imatinib cohort, 7 patients (3 of 36 with PCR negative and 4 of 12 with PCR positive at HSCT) relapsed after HSCT with a median of 137 days (range, 68–728 days). The estimated cumulative incidence of relapse at 3 years was 15.0% (95% CI, 6.6–26.7%), and significantly lower than that of the pre-imatinib cohort (50.4% at 3 years (95% CI, 39.6–60.2%); $P=0.002$; Figure 1c). Among patients in the imatinib cohort, patients with PCR negative showed significantly lower relapse rate compared with that of PCR positive (10.0% (95% CI, 2.5–23.6%) versus 41.3% (95% CI, 16.9–64.4%) at 3 years, respectively, $P=0.025$).

Non-relapse mortality. In the pre-imatinib cohort, 34 patients died of non-relapse causes at a median of 159 days (range, 5–2094 days) after HSCT. The estimated cumulative incidence of NRM in the pre-imatinib cohort was 28% (95% CI, 20–36) at 3 years (Figure 2a). In the imatinib cohort, 12 patients died of non-relapse causes at a median of 329 days (range, 41–850 days) after HSCT. The 3-year cumulative incidences of NRM were 21% (95% CI, 11–33%; Figure 2a). There were no significant differences between two cohorts ($P=0.265$).

Cause of death. Recurrence of the primary disease was the leading cause of death in both groups: 55% for the pre-imatinib cohort and 25% for the imatinib cohort. In the pre-imatinib cohort, the causes of NRM were organ failure (11%), infection (9%), GVHD (8%), transplantation-associated thrombotic microangiopathy (TMA) (4%), interstitial pneumonia (3%), graft failure (3%) and other causes (6%). In the imatinib cohort, the causes of NRM included infection (19%), bronchiolitis obliterans with organizing pneumonia (13%), TMA (13%), GVHD (13%), organ failure (6%) and other causes (12%).

Graft-versus-host disease. There was no significant difference in the cumulative incidence of Grades 2–4 aGVHD between two cohorts (31% (95% CI, 19–44%) versus 37% (95% CI, 29–46%), $P=0.391$; Figure 2b). The cumulative incidence of cGVHD at 1 year after HSCT was significantly higher in the imatinib cohort than in the pre-imatinib cohort (49% (95% CI, 31–64%) versus 27% (95% CI, 18–37%), $P=0.0261$; Figure 2c).

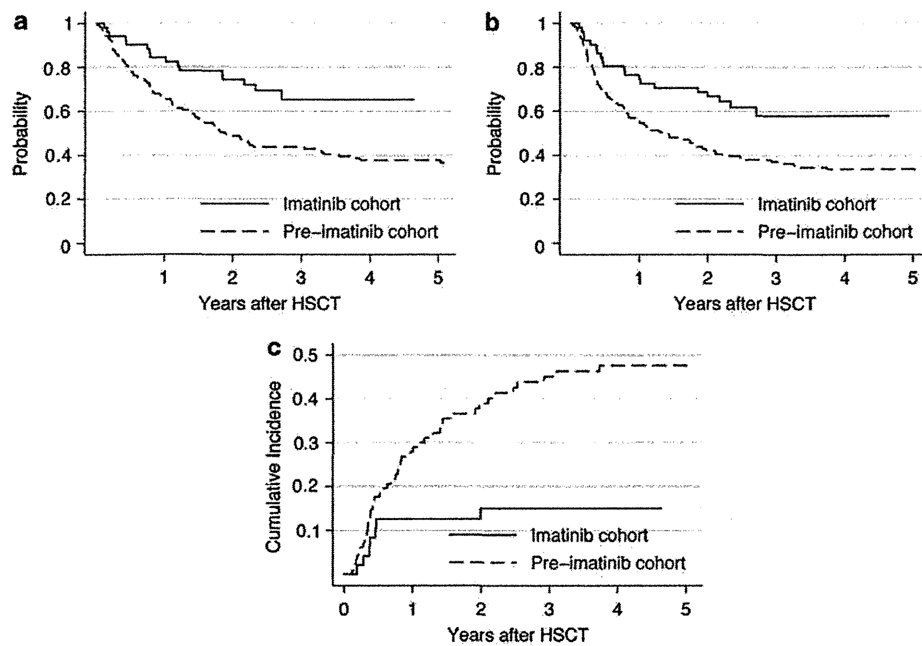

Figure 1 Transplantation outcomes of 51 patients who received imatinib-based therapy and 122 historical patients. (**a**) Overall survival, (**b**) disease-free survival and (**c**) cumulative incidence of relapse.

Table 2 Results of uni- and multivariate analysis of overall survival and disease-free survival among 173 patients with Ph+ALL

Characteristic	*Overall survival*				*Disease-free survival*			
	Univariate analysis		*Multivariate analysis*		*Univariate analysis*		*Multivariate analysis*	
	RR (95% CI)	P	*RR (95% CI)*	P	*RR (95% CI)*	P	*RR (95% CI)*	P
Imatinib-imterim therapy before HSCT	0.45 (0.26–0.77)	0.004	0.44 (0.25–0.77)	0.004	0.51 (0.31–0.83)	0.007	0.51 (0.31–0.86)	0.011
Donor status (RE versus UR)	0.87 (0.57–1.32)	0.521	0.72 (0.40–1.30)	0.275	0.77 (0.51–1.16)	0.211	0.65 (0.37–1.16)	0.147
Age at HSCT (–39 versus 40–55 versus 55–)	1.03 (0.74–1.44)	0.852	1.12 (0.78–1.62)	0.536	0.98 (0.71–1.36)	0.914	1.03 (0.73–1.47)	0.862
HLA-disparity (matched versus mismatched)	0.90 (0.39–2.06)	0.800	0.76 (0.32–1.81)	0.531	1.11 (0.49–2.54)	0.800	1.06 (0.45–2.50)	0.895
Stem-cell source (BM versus PB)	1.15 (0.72–1.82)	0.565	1.23 (0.72–2.10)	0.451	1.30 (0.85–2.00)	0.228	1.34 (0.81–2.20)	0.254
Days from diagnosis to HSCT	1.00 (0.99–1.00)	0.217	1.00 (0.99–1.00)	0.141	1.00 (0.99–1.00)	0.415	1.00 (0.99–1.00)	0.125
cGVHD as time-varying covariate (yes versus no)	0.68 (0.43–1.08)	0.101	0.66 (0.42–1.06)	0.085	0.78 (0.50–1.23)	0.292	0.75 (0.47–1.19)	0.217

Abbreviations: ALL, acute lymphoblastic leukemia; BM, bone marrow; CI, confidence interval; cGVHD, chronic graft-versus-host disease; HLA, human leukocyte antigen; HSCT, hemtopoetic stem cell transplantation; PB, peripheral blood; Ph, Philadelphia chromosome; RE, related; RR, relative risk; UR, unrelated.

However, regarding the cumulative incidence of extensive-type cGVHD, there was no significant difference between two cohorts (22% (95% CI, 10–36%) versus 12% (95% CI, 6–20%), $P=0.119$; Figure 2d).

Association between cGVHD and OS/DFS/relapse. To examine the difference of impacts of cGVHD upon clinical outcome in the pre- and imatinib cohorts, we conducted stratified analysis by cohort, treating cGVHD as a time-varying covariate (Table 3). Multivariate analysis revealed that, in the imatinib cohort, there were no significant associations between cGVHD and OS/DFS/relapse ($P=0.707$, 0.332 and 0.713, respectively). On the other hand, in the pre-imatinib cohort, there was a significant association between cGVHD and OS (HR = 0.59 (95% CI, 0.35–1.00), $P=0.048$), but not between cGVHD and DFS/relapse ($P=0.234$ and 0.338, respectively).

Engraftment. In the pre-imatinib cohort, three patients experienced graft failure. The median periods to reach the neutrophil count of $>0.5\times10^6$/l and platelet count of 50×10^6/l were 15 days (range, 8–49 days) and 25 days (range, 9–120 days), respectively, for evaluable patients. In the imatinib cohort, all 51 patients were engrafted. The median period to reach a neutrophil count of $>0.5\times10^6$/l and platelet count of 50×10^9/l was 15 days (range, 5–41 days) and 25 days (range, 11–504 days), respectively, for evaluable patients. There was no

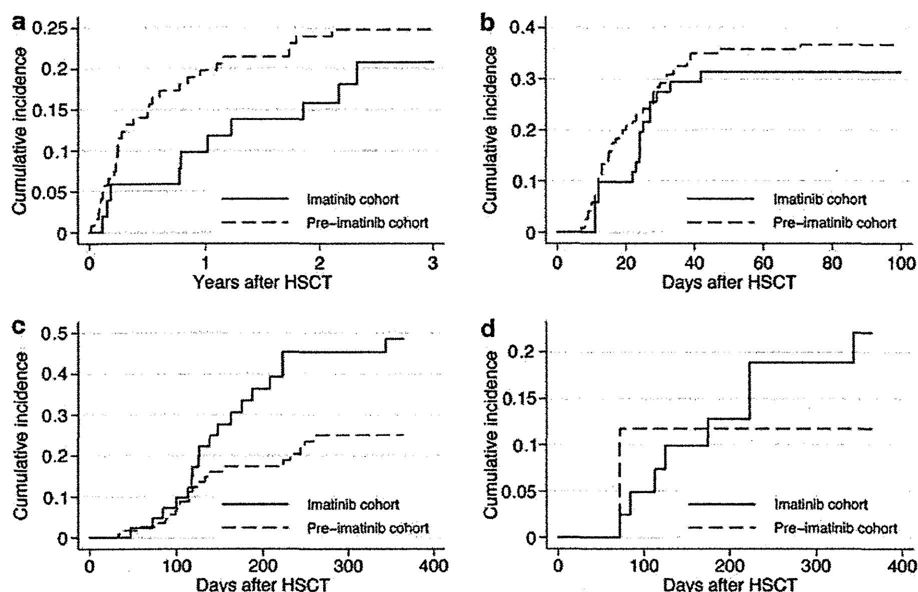

Figure 2 Cumulative incidence of GVHD or NRM. (**a**) Non-relapse mortality, (**b**) Grade 2–4 acute GVHD, (**c**) chronic GVHD and (**d**) extensive-type chronic GVHD.

Table 3 Impact of overall cGVHD on OS, DFS and relapse in multivariate analysis using cGVHD as a time-varying covariate

Cohort	*OS*			*DFS*			*Relapse*		
	Relative risk	*95% CI*	P	*Relative risk*	*95% CI*	P	*Relative risk*	*95% CI*	P
Imatinib cohort	0.80	(0.26–2.51)	0.707	0.59	(0.21–1.71)	0.332	0.74	(0.15–3.67)	0.713
Pre-imatinib cohort	0.59	(0.35–1.00)	0.048	0.73	(0.43–1.23)	0.234	0.75	(0.39–1.44)	0.388

Abbreviations: CI, confidence interval; cGVHD, chronic graft-versus-host disease; DFS, disease-free survival; HLA, human leukocyte antigen; OS, overall survival; PBSC, peripheral blood stem cell.
Data were adjusted for age categories, donors from unrelated subjects, HLA-matching status, PBSC graft and days to transplantation. Cox proportional hazard models were applied to OS and DFS, and a competing risk regression model was applied to relapse.

significant difference in neutrophil and platelet recovery between two cohorts ($P=0.201$ and 0.783, respectively).

Discussion

This study showed that patients with Ph+ALL who achieved CR by imatinib-based therapy and subsequently received allo-HSCT in their first CR showed significantly superior survival outcome to those in the pre-imatinib era. To our knowledge, our current report is the first to describe the superiority of imatinib-based therapy for this disease by analyzing a substantial number of patients with sufficient follow-up period. The treatment of Ph+ALL has changed dramatically since the introduction of imatinib and >90% of patients have achieved CR,[7,14,15] and allows SCT to be performed in a substantial proportion of patients in major or complete molecular remission.[8,16–18] Actually, in the imatinib cohort, 97 of 100 patients (97%) achieved CR and 60 (60%) could receive allo-HSCT in their first CR. Several studies reported improved OS rates compared with that in the pre-imatinib era by incorporation of imatinib-based therapy.[14,15,19,20] However, there had been few reports focusing on the clinical impact of pre-transplant imatinib administration on the outcome of HSCT. Lee *et al.*[8] reported superior outcome of HSCT by imatinib-based therapy compared with the historical control data, in which 29 patients with prior imatinib treatment showed better outcomes in terms of relapse, DFS and OS than the historical control patients. However, their comparative analysis included patients who received HSCT for refractory disease or beyond their first CR (4 of 29 patients in the imatinib group and 16 of 33 patients in the historical group). Several studies showed that remission status at the time of HSCT was one of the most important prognostic factors for outcome.[21,22] Therefore, we contend that it would be better to assess a greater number of patients and exclude patients with advanced stage at HSCT to accurately compare the clinical impact of imatinib-based therapy on the outcome of HSCT. To our knowledge, this study has the largest number of Ph+ALL patients receiving allo-HSCT in their first CR with the longest follow-up duration yet reported.

It is noteworthy from our findings that a lower rate of relapse was found in the imatinib cohort. Our results thus suggest that an imatinib-based therapy provides a survival benefit for newly diagnosed Ph+ALL patients by lowering the rate of subsequent relapse after HSCT. Despite the lack of comparative data of MRD in the pre-imatinib cohort, 75% of patients in the imatinib cohort achieved RQ–PCR negativity for *BCR/ABL* at the time of HSCT. Moreover, the relapse rate was significantly lower among

patients with PCR negative. From these, we believe that a powerful anti-leukemia activity of the imatinib-based therapy mostly contributed to the prevention of subsequent relapse after HSCT in the present analysis. Thinking of the reduced relapse rate after HSCT, impact of cGVHD should also be considered. Several studies in the pre-imatinib era reported beneficial impact of cGVHD on relapse incidence and survival.[23–25] In this study, the incidence of cGVHD was significantly higher in the imatinib cohort compared with that in the pre-imatinib cohort. In the imatinib cohort, more patients received PB as a stem cell source, which might have contributed to the high frequency of cGVHD. Besides, longer leukemia-free survival period in the imatinib cohort might have contributed to the increased frequency of cGVHD, which is a late complication often observed in the recipients of allo-HSCT who had survived without disease for at least 3 months after transplantation. One could argue that this observation could be related to a stronger graft versus leukemia effect and contribute to the lower relapse rate. However, the presence of cGVHD had no significant impact on the OS/DFS/relapse rate in our imatinib cohort by multivariate analysis.

To assist the proper interpretation of our current results, the strengths and limitations need to be considered. As discussed earlier, one of the strengths of this study is the large sample size for the imatinib cohort, which gives us a better estimation of the end points and also adds statistical power to the analyses. In addition, adjustments for potential confounders in the comparisons with the pre-imatinib cohort from a nationwide registry allow unbiased estimates to be made, at least in Japan. Given the evidence for a substantial impact of imatinib in Ph + ALL patients,[7,14–16] it is unrealistic to conduct a prospective study comparing treatments with or without imatinib. Hence, a retrospective cohort design could be suboptimal to address the key questions.

One of the possible limitations of our current analysis could be the presence of residual confounding factors both of known and unknown. Among the known factors, a difference in the conditioning regimens could be noted. The City of Hope National Medical Center reported a favorable result from the use of a fractionated TBI–etoposide regimen in the treatment of Ph + ALL.[26] However, in the comparative analysis, the clinical advantage of this approach seemed to be established mostly among patients transplanted in their second CR.[27] Moreover, this approach was commonly applied in our pre-imatinib cohort rather than in the imatinib cohort (22 and 4%, respectively). Differences in GVHD prophylaxes should also be considered. Tacrolimus was more frequently used in the imatinib cohort than in the pre-imatinib cohort, which reflects the change in practice within the field of allo-HCT in Japan as tacrolimus was widely used for unrelated allo-HSCT since 2000. Nevertheless, the lack of any differences in the incidence of aGVHD between two cohorts indicates that this factor had minimal impact in our analysis.

It may be argued that the improved outcome of the imatinib cohort have been influenced by the pre-transplant chemotherapy in the JALSG Ph + ALL 202 study. Although detailed information on the pre-transplant chemotherapy in the pre-imatinib cohort was not available, it was clear that the majority of patients were most likely treated by the JALSG ALL93 or JALSG ALL97 protocols as pre-transplant chemotherapy,[2] as these were widely used regimens in Japan at the time. The chemotherapeutic regimen in the JALSG Ph + ALL202 study was similar to those used in these protocols. Thus, the effectiveness on Ph + ALL would have been similar between the two cohorts. At least in JALSG, there had been neither remarkable progress in the chemotherapy of Ph + ALL until the clinical introduction of imatinib, nor in other groups including the MD Anderson Cancer Center.[28] Thus, in the present analysis, the influence of pre-transplant chemotherapy appears to be quite limited.

The difference of transplant year between the two cohorts (1995–2001 and 2002–2005, respectively) could have affected the outcome of HSCT, and the improvement of transplantation procedure might have contributed to the favorable outcome in the imatinib cohort. However, Nishiwaki et al.[29] analyzed the clinical outcome of 641 Japanese patients with Ph-negative ALL who had received allo-HSCT in their first CR in 1993–1997, 1998–2002 and 2003–2007, and reported that there was no statistical difference in OS and NRM between three periods. In this study, the incidence of NRM was lower in the imatinib cohort, but did not reach the statistical significance. Therefore, the influence of transplantation year is thought to be limited in this study.

Considering potential benefit by imatinib, the lack of information about post-transplant imatinib use in the pre-imatinib cohort might have led us to underestimate the difference between two cohorts.

In conclusion, we have found that there is a significant improvement in the OS and DFS of Ph + ALL patients who received allo-HSCT following imatinib-based therapy. Although further validation using larger cohorts from different populations is essential to confirm our findings, imatinib-based therapy is likely to be a useful strategy for not only giving patients with Ph + ALL more chance to receive allo-HSCT, but also for improving their outcome after allo-HSCT.

Conflict of interest

Dr Naoe has received research funding and honoraria from Novartis Japan. Dr Ohnishi has received research funding from Novartis Japan. Dr Miyazaki has received honoraria from Novartis Japan. The remaining authors declare no conflict of interest.

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Identification of a novel fusion, SQSTM1-ALK, in ALK-positive large B-cell lymphoma

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ABSTRACT

ALK-positive large B-cell lymphoma is a rare subtype of lymphoma, and most cases follow an aggressive clinical course with a poor prognosis. We examined an ALK-positive large B-cell lymphoma case showing an anti-ALK immunohistochemistry pattern distinct from those of 2 known ALK fusions, CLTC-ALK and NPM-ALK, for the presence of a novel ALK fusion; this led to the identification of SQSTM1-ALK. SQSTM1 is an ubiquitin binding protein that is associated with oxidative stress, cell signaling, and autophagy. We showed transforming activities of SQSTM1-ALK with a focus formation assay and an *in vivo* tumorigenicity assay using 3T3 fibroblasts infected with a recombinant retrovirus encoding SQSTM1-ALK. ALK-inhibitor therapies are promising for treating ALK-positive large B-cell lymphoma, especially for refractory cases. SQSTM1-ALK may be a rare fusion, but our data provide novel biological insights and serve as a key for the accurate diagnosis of this rare lymphoma.

Key words: ALK-positive, large B-cell lymphoma, fusion.

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Introduction

Anaplastic lymphoma kinase-positive large B-cell lymphoma (ALK+LBCL) is a rare subtype of lymphoma that was first described in 1997.[1] Approximately 50 cases have been reported to date,[2] with most cases (60%) following an aggressive clinical course.[3] In well-characterized cases, 3 genes have been reported as a fusion partner of *ALK*: *clathrin* (*CLTC-ALK*),[4-6] *nucleophosmin* (*NPM-ALK*),[7-8] and *SEC31A* (*SEC31A-ALK*).[9] In this paper, we report a case of ALK+LBCL that harbored a novel ALK fusion partner, sequestosome1 (SQSTM1).

Design and Methods

Materials

Biopsied specimens were fixed in 20% neutralized formalin and embedded in paraffin for conventional histopathological examination. We extracted DNA and total RNA from the snap-frozen specimens and subsequently purified the samples. Written informed consent was obtained from the patient. The study was approved by the Institutional Review Board of the Japanese Foundation for Cancer Research.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue was used. For antigen retrieval, we heated the slides for 40 min at 97°C in Target Retrieval Solution (pH 9.0; Dako), and subsequently detected the immune complexes with a dextran polymer reagent (EnVision+DAB system, Dako) and an AutoStainer instrument (Dako).

Isolation of ALK fusion cDNA

To obtain cDNA fragments corresponding to novel ALK fusion genes, we used an inverse reverse transcription-polymerase chain reaction (RT-PCR) method slightly modified from one previously reported.[10] Double-stranded cDNA was synthesized from 2 μg of total RNA with 1 pM of the primer ALKREVex22-23 (5'-TGGTTGAATTTGCTGATGATC-3') and a cDNA Synthesis System (Roche), and was self-ligated by incubation overnight with T4 DNA ligase (TaKaRa Bio). We subjected the resulting circular cDNA to PCR (35 cycles of 94°C for 15 sec, 62°C for 30 sec, and 72°C for 1 min) with primers ALKREV3T (5'-CTGATGGAGGAGGTCTTGCC-3') and ALKFWDex20-21 (5'-ATTCGGGGTCTGGGCCAT-3') in a final volume of 20 μL. We subjected 1 μL of the 1:100 diluted reaction products to a second PCR step (the same settings as above), with primers ALKREV4T (5'-GGTTGTAGTCGGTCATGATGGTC-3') and ALKFWDex21-22 (5'-AGTGGCTGTGAAGACGCTGC-3') in a final volume of 20 μL. The resulting products were purified by gel extraction and directly sequenced in both directions with primers ALKFWDex20-21 and ALKREV4T.

The fusion point of *SQSTM1-ALK* cDNA was amplified by RT-PCR with primers SQSTM1 565F (5'-AAACACGGA-

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Manuscript received on xxxxxxx. Revised version arrived on xxxxxxxxxxxxxxxx. Manuscript accepted on xxxxxxxxx.
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CACTTCGGGT-3') and ALK3078RR (5'-ATCCAGTTCGTCCT-GTTCAGAGC-3').

Full-length *SQSTM1-ALK* cDNA was obtained from the specimen by RT-PCR with primers SQSTM1v1-F90 (5'-CTCGCTATG-GCGTCGCTCACCGTGAA-3') and KA-W-cDNA-out-AS (5'-CCACGGTCTTAGGGATCCCAAGG-3').

Fluorescence in situ *hybridization (FISH)*

We performed FISH analysis of the gene fusion for unstained slides (4 μm thick) with bacterial artificial chromosome (BAC) clone-derived DNA probes for *ALK* (RP11-984I21, RP11-62B19) and *SQSTM1* (RP11-55M16).

Transformation assay for ALK *fusion protein*

We analyzed the transforming activity of SQSTM1-ALK as described previously.[11-13] Briefly, cDNA for SQSTM1-ALK was inserted into the retroviral expression plasmid pMXS.[14] The resulting plasmid and similar pMXS-based expression plasmids for EML4-ALK variant 1 or NPM-ALK were used to generate recombinant ecotropic retroviruses, which were then used to infect mouse 3T3 fibroblasts. We evaluated formation of transformed foci after culturing the cells for 14 days. We subcutaneously injected the same set of 3T3 cells into nu/nu mice and examined tumor formation after 20 days.

PCR for IGH *gene rearrangement*

Genomic PCR was used for amplification of the rearranged *IGH* gene using the primers FR2A 5'-TGG(A/G)TCCG(A/C)CAG (C/G)C(C/T)(C/T)CNGG-3' and LJH 5'-ACCTGAGGAGACG-GTGACC-3'. Several clones were sequenced after subcloning the PCR product into pGEM-T-Easy Vector (Promega).

Results and Discussion

Case presentation

A 67-year old man was admitted with a tumor in the left side of his neck. A systemic workup revealed swelling of cervical, mediastinal, and hilar lymph nodes. Blood counts were within normal ranges. Lactose dehydrogenase was slightly elevated (223 IU/L) in peripheral blood with high IgG (2,425 mg/dL), normal IgA (157 mg/dL) and low IgM (32 mg/dL) levels.

Histopathological examination of the biopsied specimen from the cervical lymph node showed a diffuse infiltrate of tumor cells with a round, vesicular nucleus containing a centrally located large nucleolus. The cytoplasm was abundant (Figure 1A). These features may be consistent with immunoblasts or plasmablasts, but the size of tumor cells was large compared with typical immunoblasts and plasmablasts. Immunophenotypically, the tumor cells were negative for CD3, CD4, CD5, CD10, CD20, CD57, CD79a, and most cytokeratins (CK5/6, CK8, CK19, CK20); focally positive for CD30 and cytokeratins (AE1/AE3, CAM5.2, CK7, CK18) (Figure 1B); weakly positive for PAX5; and positive for CD138 (Figure

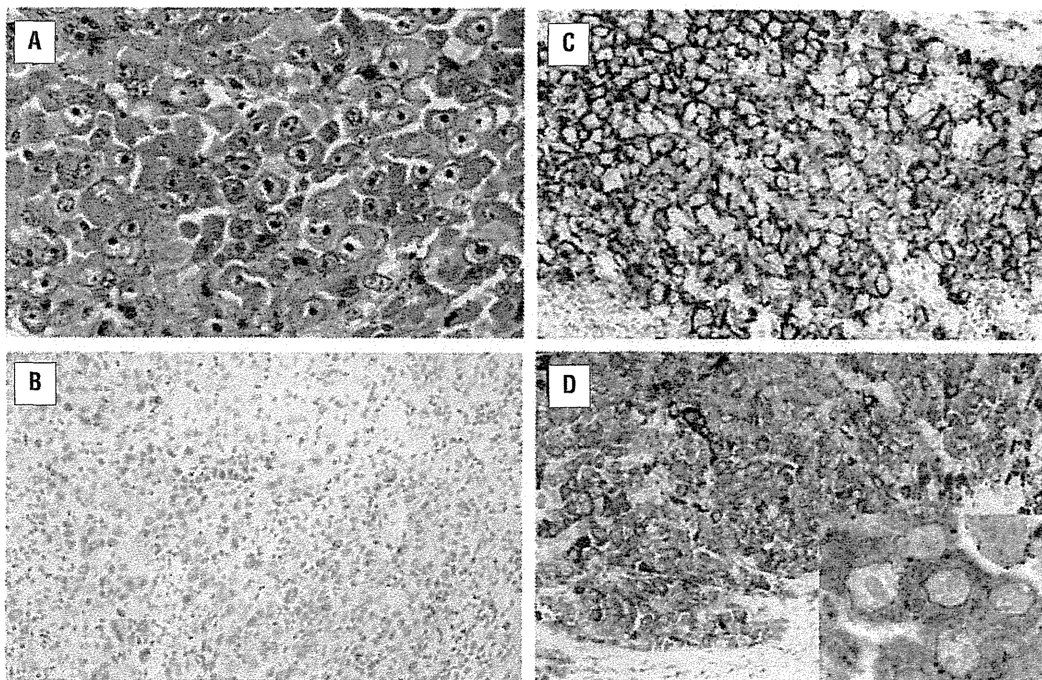

Figure 1. Histopathology of SQSTM1-ALK-positive large B-cell lymphoma. (A) The pattern of tumor infiltration was diffuse. The lymphoma cells were large with abundant cytoplasm and had round, vesicular nuclei, each containing a centrally located large nucleolus. These features may be consistent with immunoblasts or plasmablasts, but the size of tumor cells was extremely large compared with these typical cell types (40× objective). (B) Some lymphoma cells expressed cytokeratin (AE1/AE3) (20× objective). (C) Syndecan1/CD138 was strongly expressed (20× objective). (D) In anti-ALK immunohistochemistry, a diffuse cytoplasmic staining pattern with ill-demarcated spots was clearly shown (20× objective).

1C), EMA, and ALK (Figure 1D). The positivity of focal cytokeratin, which has been reported in a small proportion of ALK+LBCL cases,[15] and the cytomorphology of this case may have led to a misdiagnosis of undifferentiated metastatic carcinoma. The presence of *ALK* translocation was demonstrated by an ALK split FISH assay, which was performed at a commercial laboratory (*data not shown*). The tumor cells were positive for PAX5, which is suggestive of ALK+LBCL. However, we carefully excluded a possibility of metastasis of ALK-positive lung cancer[10] because the tumor cells were positive for some cytokeratins and immunohistochemistry for immunoglobulins was not evaluable due to background staining. Immunohistochemistry for TTF1 was negative; this is usually positive in ALK-positive lung cancers.[16] In addition, PCR and sequencing analyses revealed that *IGH* was monoclonally rearranged and somatically hypermutated (*data not shown*).

The patient was diagnosed as having ALK+LBCL and achieved complete remission after 6 cycles of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) treatment. Four months later, however, he relapsed.

Identification of SQSTM1-ALK

The 2 major ALK fusions in ALK+LBCL are CLTC-ALK and NPM-ALK, and they show a coarse granular cytoplasmic pattern and a nuclear and cytoplasmic pattern in anti-ALK immunohistochemistry, respectively. In the present case, anti-ALK immunohistochemistry showed a diffuse cytoplasmic staining pattern with ill-demarcated spots (Figure 1D), which was different from either of the former 2 patterns. Therefore, we carried out inverse RT-PCR to examine the presence of a novel fusion of *ALK*. We indeed isolated a cDNA containing the exon 5 of *SQSTM1* in-frame fused to the exon 20 of *ALK* (Figure 2A). A separate RT-PCR assay amplified the fusion point of *SQSTM1-ALK* cDNA (*data not shown*). To confirm the chromosome rearrangement, we performed *SQSTM1-ALK* fusion FISH. This result was consistent with the presence of a t(2;5)(p23.1;q35.3) leading to the generation of *SQSTM1-ALK* (Figure 2B). The complete sequences of SQSTM1-ALK are shown in the *Online Supplementary Figure S1*.

SQSTM1 is an ubiquitin binding protein that is associated with oxidative stress, cell signaling, and autophagy.[17-20] Autophagosomal membrane protein LC3/Atg8 binds SQSTM1 and makes SQSTM1-containing protein aggregate to the autophagosome.[21] Mutations within *SQSTM1* are identified in patients with Paget's disease of bone.[22]

SQSTM1 is located very near *NPM*, which is on 5q35.1. Therefore, the cytogenetic findings of the NPM-ALK-positive and the SOSTM1-ALK-positive lymphomas may be similar, and this may mean that SQSTM1-ALK occurrence in lymphoma may be underestimated. As mentioned, however, NPM-ALK and SQSTM1-ALK differ in terms of the anti-ALK immunostaining pattern. NPM has a nuclear transport signal, while SQSTM1 does not. Therefore, NPM-ALK shows a nuclear and cytoplasmic staining pattern while SQSTM1-ALK shows only a cytoplasmic staining pattern. ALK is a representative "promiscuous" molecule because of its various fusion partners. The subcellular localization of ALK fusions depends on the fusion partners. The anti-ALK immunohistochemical staining pattern is, therefore, a simple and useful means to identify the possible partner in a tested case and, in fact, has prompted the identification of many ALK fusion partners, including the present case.

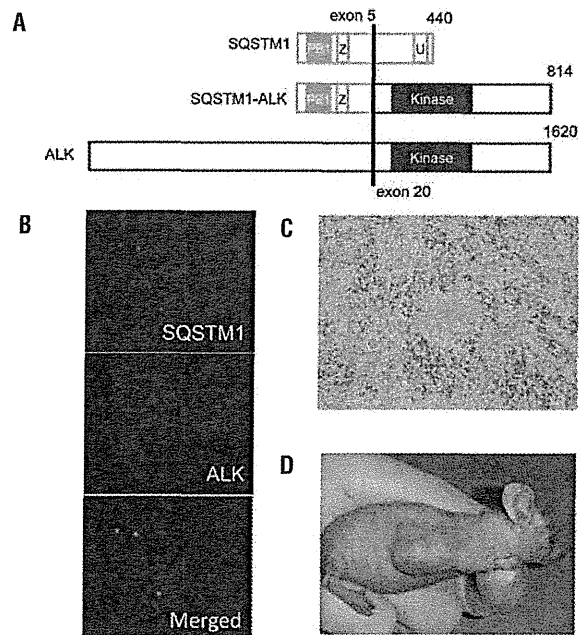

Figure 2. Discovery of *SQSTM-ALK* fusion gene. (A) A chromosome translocation, t(2;5)(p23.1;q35.3), generates a cDNA fusion in which exon 5 of *SQSTM* is joined to the *ALK* cDNA for the intracellular region of its encoded protein (containing the tyrosine kinase domain). Numbers indicate amino acid positions of each protein. PB1: Phox and Bem1p; Z: atypical zinc finger; U: ubiquitin-associated. (B) A section of the specimen for the present case was subjected to FISH with an *SQSTM1-ALK* fusion assay. Nuclei are stained blue with DAPI. (C) Murine 3T3 fibroblasts were infected with retroviruses expressing SQSTM1-ALK. The cells were photographed after culture for 14 days. (D) A nude mouse was injected subcutaneously with 3T3 cells infected as in (C), and tumor formation was examined after 20 days.

Transforming activities of SQSTM1-ALK

We generated a recombinant retrovirus encoding SQSTM1-ALK and used it to infect cultured 3T3 fibroblasts. Infection with the virus, but not with an empty virus, resulted in the formation of multiple transformed foci *in vitro* (Figure 2C). As control experiments for formation, EML4-ALK (variant 1) and NPM-ALK similarly produced transformed foci (*data not shown*). The same 3T3 cells were injected into nude mice for an *in vivo* tumorigenicity assay. As expected, 3T3 cells expressing SQSTM1-ALK developed subcutaneous tumors at all injection sites within an observation period of 20 days (Figure 2D), confirming the transforming potential of the novel fusion kinase, SQSTM1-ALK.

All ALK fusion partners identified so far except moesin (MSN) have a coiled-coil domain(s) in their sequences, and the domain is conserved in its fusion form. The coiled-coil domain allows the protein to homodimerize. The tyrosine kinase domain of the ALK fusions is constitutively phosphorylated and activated through homodimerization via

the coiled-coil domain. It has been speculated that the binding properties of MSN to cell membrane proteins lead to the dimerization of MSN-ALK proteins, enabling the constitutive phosphorylation of the chimeric MSN-ALK protein.[23] SQSTM1 does not harbor a coiled-coil domain and does not bind to membrane proteins. Instead, it has the Phox and Bem1p (PB1) domain in its N-terminus and forms heteromeric and homomeric complexes mediated by this domain.[24] Therefore, SQSTM1-ALK probably homodimerizes through the PB1 domain, leading to constitutive activation of the ALK kinase domain.

In conclusion, we reported a novel ALK fusion, SQSTM1-ALK, and its oncogenicity. ALK+LBCL is an aggressive lymphoma with poor prognosis;[3] ALK inhibitors are promising therapeutic agents for this condition. SQSTM1-ALK may be a rare fusion, but our data provide novel biological insights and may serve as a key to the accurate diagnosis of this rare lymphoma.

Authorship and Disclosures

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Early Release Paper

Overexpression of enhancer of zeste homolog 2 with trimethylation of lysine 27 on histone H3 in adult T-cell leukemia/lymphoma as a target for epigenetic therapy

by Daisuke Sasaki, Yoshitaka Imaizumi, Hiroo Hasegawa, Akemi Osaka, Kunihiro Tsukasaki, Young Lim Choi, Hiroyuki Mano, Victor Marquez, Tomayoshi Hayashi, Katsunori Yanagihara, Yuji Moriwaki, Yasushi Miyazaki, Shimeru Kamihira, and Yasuaki Yamada

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