syndrome or neurofibromatosis type I. We only had gene mutation analysis data for two patients, because most patients were diagnosed in the 1990's. Patient 1 had *NRAS* mutation and Patient 7 had *PTPN11*.

At initial diagnosis, chromosomal analyses were available in all the 15 children and showed a normal karyotype in 12 children, monosomy 7 in one child and other aberrations in two children.

Three patients had blast crisis during observation without treatment, nine patients were treated with 6-mercaptopurine (6MP) before blast crisis and three patients had blast crisis following relapse after HSCT. The pre-blast crisis status of 12 patients was non-CR, whereas all three patients who received HSCT had achieved CR.

Table II shows the characteristics of the patients at blast crisis. The median time interval between diagnosis and blast crisis was 15 months (range, 1–48 months). The median time interval between the time of initial treatment and blast crisis was 5 months (range, 1–44 months) in the 6MP treatment group, whereas the time interval between HSCT and blast crisis was 8, 19 and 23 months, respectively.

A karyotypic abnormality was detected in 10 out of the 15 children at the time of blast crisis. The majority of patients had a complex karyotype and/or monosomy 7. When the French-American-British (FAB) classification was applied to classify the leukaemic cells at blast crisis, 2 were M2, 1 was M4, 3 were M6 and 2 were unclassified.

The treatment and outcome of these patients after blast crisis are summarized in Table II. Only two of 12 patients given therapy other than HSCT (acute myeloid leukaemia (AML) type chemotherapy: $n = 10$, low dose chemotherapy: $n = 1$, donor leucocyte infusion: $n = 1$) achieved CR. Seven patients received HSCT after the blast crisis and four of them had long-term survival. Ultimately, 11 out of 15 patients died after blast crisis, the main cause of death was the progression of their disease ($n = 10$). One patient died due to transplant-related complication after HSCT. At the time this report was written, four patients were alive in CR at a median of 9 years after blast crisis.

Finally, we compared the characteristics of the patients who developed blast crisis ($n = 15$) and those who did not ($n = 138$; Table III). Among several risk factors, the median

Table II. Characteristics at blast crisis, treatment and outcome.

Pt	BM blast (%)	Interval after Dx (months)	Interval after initial Tx (months)	Karyotype	Treatment after blast crisis			Outcome (months after blast crisis)	Cause of death
					Induction Tx	Response to Tx	Donor source		
1	75	35	17 months after 6MP	44, XX, der(6) (6;15) (q1?;q1?), −7, −15	AML type	No	UBMT	Dead (11)	TRM
2	>20	14	–	45, XY, −7	Etoposide, mPSL, Ara-C	No	None	Dead (1)	Progression
3	30	15	15 months after chemotherapy	50, XY, 19, +21, +X, +Y	AML type + irradiation	No	None	Dead (5)	Progression
4	83	44	44 months after 6MP	45, XY, del(6) (q21), −7, del(p12)	AML type	Yes	None	Dead (8)	Relapse
5	31	26	26 months after 6MP	45, XY, −7	AML type	No	None	Dead (17)	Progression
6	36	3	2 months after 6MP	46, XY, 5q−, 17p+	AML type	No	None	Dead (15)	Progression
7	75	16	3 months after 6MP	45, XX, t(4;15) (q2?; q2?), −7	AML type	No	None	Dead (13)	Progression
8	75	2	–	46, XY	AML type	No	CBT	Alive (>111)	
9	47	1	–	46, XX	AML type	Yes	Related PBSCT	Alive (>182)	
10	37	5	5 months after 6MP	46, XY, −2, −7, +der (9) t(2;9) (q13; q22),+19	–	–	CBT	Dead (5)	Progression
11	40	1	1 months after 6MP	46, XY	AML type	ND	UBMT	Alive (>83)	
12	44	25	8 months after HSCT	−7 and complex karyotype*	AML type	No	None	Dead (31)	Progression
13	26	2	1 months after 6MP	45, XY, −7	–	–	CBT	Dead (4)	Progression
14	30	48	19 months after HSCT	46, XX	DLI	No	None	Dead (4)	Progression
15	20	29	23 months after HSCT	46, XX	–	–	Related BMT	Alive (>74)	

BM, bone marrow; Dx, diagnosis; Tx, therapy; 6MP, 6-mercaptopurine; AML, acute myeloid leukaemia; DLI, donor lymphocyte infusion; mPSL, methylprednisolone; Ara-C, cytarabine; ND, no data; UBMT, unrelated bone marrow transplant; HSCT, haematopoietic stem cell transplantation; CBT, cord blood transplant; PBSCT, peripheral blood stem cell transplant; BMT, bone marrow transplant; TRM, transplant-related mortality.
*Complex karyotype: 44, X, −Y, t(1;3) (p10;p10), del(6) (8;21), −7 and 45, Y, del(X) (q22), add(3) (p25), −6, del(6) (p21), −7, +mar.

Table III. Summary of the characteristics of the patients who progressed to blast crisis.

	With blast crisis	No blast crisis
Number (%)	15 (9·8)	138 (90·2)
Median age at initial Dx, years	2·6 (range: 0·3–7·6)	1·1 (range: 0–7·4)
Median WBC at initial DX, × 10^9/l	38 (range: 8·9–376·2)	30·250 (range: 5·5–185)
Median Plt at initial Dx, × 10^9/l	4·3 (range: 1·3–10·2)	4·6 (range: 0·1–99·7)
Median HbF at initial Dx, %	31·9* (range: 1·1–69·5)	18·8* (range: 0–66·3)
Monosomy 7 at Dx	1 out of 15 patients	9 out of 131 patients
Monosomy7 and/or complex karyotype at blast crisis	9 out of 15 patients (8 including monosomy 7)	No data
Alive	4 out of 15 patients (median 9 years)	75 out of 138 patients

Dx, diagnosis; WBC, white blood cell count; Plt, platelet count; HbF, fetal haemoglobin.
*P = 0·047, P values were calculated using the Mann–Whitney U test for comparisons between medians and ranges.

level of fetal haemoglobin (HbF) was higher in the former group compared to that in the latter (P = 0·047, Mann–Whitney U test).

Discussion

In this retrospective study, 15 (9·8%) of the 153 JMML patients had progressed to blast crisis and this rate was similar to a previous report (Luna-Fineman *et al*, 1999). There have been only a few other reports regarding blast crisis in JMML. Blast crisis was not described in a retrospective analysis of 110 cases with JMML (Niemeyer *et al*, 1997). However, in the 1980's, Castro-Malaspina *et al* (1984) reported that one-third of children with JMML developed blastic transformation.

In the present study, the median time interval between diagnosis and blast crisis was 15 months (range, 1–48 months). Three patients had blast crisis during observation and nine patients were treated with 6MP before the blast crisis, furthermore, three patients underwent a transformation to blast crisis after HSCT.

According to the European Working Group of MDS in Childhood, older children, reduced platelet count and increased percentage of HbF correlate with a poor outcome (Niemeyer *et al*, 1997). Regarding the risk factors for blast crisis, a higher level of HbF was associated with the occurrence of blast crisis and this should be confirmed in a different cohort.

In this study, only two of the 11 patients who received intensive chemotherapy including AML type, entered remission. Therefore, we conclude that JMML blast crisis is very resistant to chemotherapy. Indeed, the main cause of death was disease progression.

It is important to note that monosomy 7 and/or complex karyotype were observed in the majority of patients at the time of blast crisis. It seems that these cytogenetic aberrations play a critical role in the progression. Five patients with a normal karyotype at blast crisis may have had underlying unknown genetic events. Matsuda *et al* (2006) reported that some JMML patients, who initially had a normal karyotype, acquired chromosomal abnormalities including monosomy 7 during treatment with 6MP. They hypothesized that a minor population with an aberrant karyotype already existed at the onset in these patients. In our study, nine patients were treated with 6MP before blast crisis and 7 of them acquired additional chromosomal abnormalities after 6MP treatment. It was possible that 6MP might select resistant clones. Accumulation of molecular changes may also contribute to disease progression in JMML. Kato *et al* (2013) reported a case where a JMML patient with a heterozygous *KRAS* mutation developed aggressive transformation during 6MP therapy after acquisition of homozygous *KRAS* mutation through uniparental disomy mechanism (Kato *et al*, 2013). Very recently, Sakaguchi *et al* (2013) reported that the coexistence of several gene mutations, including *SETBP1* and *JAK3*, in addition to RAS-pathway abnormalities was related with poorer outcome in patients with JMML. Mutations of such genes may be relevant to the occurrence of blast crisis. However, the mechanism underlying the blast crisis was uncertain in most JMML cases. It is possible that 6MP might have induced the second hit and accelerated the disease as 6MP maintenance therapy is reported to be correlated with secondary malignant neoplasm (Schmiegelow *et al*, 2009).

It was possible that some patients developed second malignancy rather than blast crisis. However, in this study, none of the patients who did not receive HSCT achieved remission before presentation of blast crisis. Therefore, we assume that those patients had progression of the disease or blast crisis rather than second malignancy. Of note, Patient 12 had completely different chromosomal abnormalities at diagnosis and blast crisis, which it may indicate a second malignancy.

A recent report suggested that the pathogenesis of JMML is the dysregulation of granulocyte-macrophage colony-stimulating factor (GM-CSF)/RAS signal transduction and mutations in *RAS*, *NF1*, *PTPN11* and *CBL* genes interfering with the downstream components of this pathway (Side *et al*, 1998; Tartaglia *et al*, 2003; Flotho *et al*, 2007; Loh *et al*, 2009). These mutations can be identified in approximately 80% of JMML cases and are associated with clinical features and prognosis in JMML (Yoshida *et al*, 2012). Yoshida *et al* (2009) evaluated 71 children with JMML and they concluded that JMML with *PTPN11* mutation might have a poor

outcome. On the other hand, some patients with *KRAS* or homozygous *CBL* mutation have improved spontaneously (Matsuda *et al*, 2007; Loh, 2011). In our report, gene mutation analysis was performed only in two cases, because most of the patients were diagnosed with JMML in the 1990's. A prospective study with mutational analyses of the above genes in the RAS pathway and search for second molecular events will be needed to elucidate the pathogenesis of blast crisis in JMML.

In the last 10 years HSCT has become the only curative treatment for JMML and, in Japan, it was also proposed as a treatment for JMML after 1999 (Manabe *et al*, 2002). In our present report, 11 (15%) out of 74 patients progressed to blast crisis before 1999; on the other hand, there were only four cases (5%) with blast crisis among 79 patients diagnosed after 2000. It is possible that the frequency of patients with blast crisis decreased because HSCT was administered early on in the course of treatment in these cases. In fact, the number of JMML patients who received HSCT before and after 1999 were 43 out of 74 (58%) patients and 60 out of 79 (76%) patients, respectively. On the other hand, recent advances in genotyping of JMML may lead to a decrease in the indication for HSCT in patients with JMML. For example, children with *RAS* or *CBL* mutations may not need HSCT. In addition, it has been reported that JMML patients with AML-type expression profile had a worse prognosis (Bresolin *et al*, 2010). In their report, monosomy 7 was observed in six of 20 patients with an AML-type profile, whereas it was observed in only one of 20 patients with a non-AML type profile. It is possible that children presenting blast crisis may have the AML-type expression profile. Taken together, the number of patients who need HSCT may decrease as more precise prognostic factors become available. Further studies including genotyping of JMML are necessary to identify those patients who may survive without HSCT and those who require HSCT. Those JMML patients identified as requiring HSCT, should receive HSCT as soon as possible to avoid blast crisis.

We recommend that for JMML cases, karyotype should be carefully monitored using fluorescence *in situ* hybridization to detect monosomy 7 or any other types of karyotypic abnormalities, especially if the patient does not undergo HSCT.

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Authorship

Conception, design and writing the manuscript: YH, AM. Pathological diagnosis: MT, YZ, AMan, AY, SK, MI, AMas. Collection and assembly of data: YH, AK, AMan. Data analysis and interpretation: YH, TN, AMan. Final approval of manuscript: All authors.

Disclosures

The authors declare no competing financial interests.

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bjh research paper

Clinical characteristics and treatment outcome in 65 cases with refractory cytopenia of childhood defined according to the WHO 2008 classification

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Summary

This study analysed 65 children who were prospectively registered between 1999 and 2008 and fulfilled the World Health Organization 2008 criteria of refractory cytopenia of childhood (RCC). First-line therapy was determined by the treating physicians: 25 patients received immunosuppressive therapy (IST), 12 patients received haematopoietic stem cell transplantation (HSCT) and one patient received intensive chemotherapy. The remaining 27 patients were followed without treatment for more than 2 years (watch and wait; WW). In the WW group, 18 patients had stable disease without further intervention. Thirteen of 29 patients (45%) who ended up receiving IST showed response. The combination of ciclosporin and antithymocyte globulin was not shown to be superior to ciclosporin alone with regard to response rate or survival. Of 28 patients who ended up undergoing HSCT, 17 patients are alive in complete remission, whereas nine patients died mostly due to transplantation-related mortality. The 5-year overall survival for all patients was 82 ± 5%. Eight patients suffered from disease progression. Patients with monosomy 7 or multilineage-dysplasia had a significantly higher incidence of disease progression. This analysis revealed heterogeneity in the clinical course of RCC, varying from those who remained stable for long periods to those who progressed to advanced disease.

Keywords: myelodysplastic syndrome, refractory cytopenia of childhood, immunosuppressive therapy, haematopoietic stem cell transplantation, disease progression.

Myelodysplastic syndrome (MDS) without excess of blast in peripheral blood (PB) or bone marrow (BM), also known as low-grade MDS, is the most common subtype in childhood (Passmore *et al*, 2003; Hasle *et al*, 2004; Niemeyer & Kratz, 2008), and some important differences from adult form of the disease have been identified (Sasaki *et al*, 2001; Hasle *et al*, 2003; Kardos *et al*, 2003; Hasegawa *et al*, 2009). Consequently, the World Health Organization (WHO) Working Group introduced refractory cytopenia of childhood (RCC) as a provisional entity in the 2008 classification in order to address those differences (Baumann *et al*, 2008). In this classification, RCC is defined as a childhood MDS characterized by persistent cytopenia with <5% blasts in the BM, <2% blasts in the PB and dysplastic changes in 2 or 3 lineages or exceeding 10% in one single lineage. This classification, however, does not specify the importance of the number of lineages involved in the dysplasia, which has been associated with prognosis in adults with low-grade MDS (Germing

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et al, 2006; Malcovati *et al*, 2007; Matsuda *et al*, 2007; Verburgh *et al*, 2007). It is recommended that children who meet the criteria for refractory cytopenia with multilineage dysplasia (RCMD) be considered as RCC until the prognostic significance of a multilineage presentation is further clarified in children (Baumann *et al*, 2008).

Haematopoietic stem cell transplantation (HSCT) is currently considered to be the only curative therapy for children with MDS (Niemeyer & Kratz, 2008), however it is associated with severe complications, mortality and late sequelae. A number of reports indicated that a subset of adults with low-grade MDS respond to immunosuppressive therapy (IST; (Sloand & Rezvani, 2008). Although the efficacy of IST has also been shown in childhood MDS (Yoshimi *et al*, 2007; Hasegawa *et al*, 2009), it still remains to be elucidated which subpopulation of RCC is most likely to benefit from IST. In addition, it is also unknown how and when to treat children with RCC who do not present with transfusion dependency or infections due to neutropenia.

Because there is no comprehensive study reported to date on RCC that addresses the issue of optimal management of patients, we analysed the clinical characteristics and outcomes of 65 children with RCC who were prospectively registered through the Japanese Society of Paediatric Haematology/Oncology (JSPHO) over a 9-year period.

Methods

Patients

From July 1999 to February 2008, 618 children who were suspected of having MDS were prospectively registered into the JSPHO database and 150 were diagnosed as having MDS (excluding 72 juvenile myelomonocytic leukaemia) based on the central review of morphology of PB and BM by three independent investigators (Hasegawa *et al*, 2009). Of these 150 patients, we excluded 54 patients with secondary MDS who had undergone previous chemotherapy or radiotherapy, or patients with a history of inherited BM failure syndrome (IBMFS) or acquired aplastic anaemia (AA). Of these 96 primary MDS patients, 65 were compatible with criteria of RCC according to the WHO 2008 classification (Baumann *et al*, 2008). A subset of these patients has been reported previously (Hasegawa *et al*, 2009). BM cellularity was determined in 37 patients (57%) whose biopsy specimens were available. Cytogenetic analysis of marrow cells was available from 63 patients (97%). Treatment options were determined by each physician based on the recommendations provided by the Japanese Childhood MDS Study Group MDS99 protocol as follows (Hasegawa *et al*, 2009); patients who were transfusion-dependent and had a matched sibling donor were advised to undergo HSCT, whereas patients who did not have a suitable family donor were candidates for IST, consisting of horse antithymocyte globulin (ATG; Lymphoglobuline; Merieux, Lyon, France; 15 mg/kg/d × 5 d) and ciclosporin (6 mg/kg; Kojima *et al*, 2000; Hasegawa *et al*, 2009). If patients were not transfusion-dependent or neutropenic, physicians were allowed to observe without therapeutic intervention and routine BM examination every 6–8 weeks was recommended. In this analysis, we also subdivided these 65 patients into two subgroups that we tentatively defined according to the number of lineages with dysplasia (Cantù Rajnoldi *et al*, 2005) observed in the BM; patients with dysplasia confined to one single lineage were defined as RCC with unilineage dysplasia (RCC-UD, $n = 40$; 62%), and those showing dysplastic features recognized in ≥2 lineages were defined as RCC with multilineage dysplasia (RCC-MD, $n = 25$; 38%). The study was approved by the institutional review board of the participating institutions. Informed consents were obtained from patients or guardians of the patients according to the Declaration of Helsinki.

Definition

Cytopenia was defined as a haemoglobin (Hb) level less than 100 g/l, an absolute neutrophil count (ANC) $<1{\cdot}5 \times 10^9$/l, or a platelet count $<100 \times 10^9$/l. Severity of cytopenia was evaluated according to the criteria used for patients with AA, i.e., very severe, severe and non-severe (Kojima *et al*, 2000). Response to IST was evaluated at 6 months. Complete response (CR) was defined as an ANC $>1{\cdot}5 \times 10^9$/l, platelet count $>100 \times 10^9$/l and Hb level > 110 g/l. Partial response (PR) was ANC $>0{\cdot}5 \times 10^9$/l, platelet count $>20 \times 10^9$/l and Hb level >80 g/l. When neither CR nor PR criteria were met at 6 months after initiation of IST, a patient was considered as having no response (NR) to IST. Relapse was defined as reversion to NR from CR or PR. Patients with either emergence of new chromosomal abnormality or progression to advanced MDS or acute myeloid leukaemia (AML) were considered to have undergone disease progression. Patients receiving HSCT were considered to have achieved remission when normal haematopoiesis was recovered without blasts or chromosomal abnormality after HSCT. Acute and chronic graft-versus-host disease (aGVHD and cGVHD) were diagnosed and graded by each physician according to the consensus criteria (Przepiorka *et al*, 1995; Filipovich *et al*, 2005). The incidence of cGVHD was evaluated in patients surviving more than 100 d after HSCT. In this analysis, one patient who died of aGVHD on day 104 was also excluded from the analysis of cGVHD incidence. Myeloablative conditioning (MAC) regimens and reduced intensity conditioning (RIC) regimens were defined as previously described (Luger *et al*, 2012).

Statistical analysis

The Kaplan-Meier method was used to produce survival and cumulative incidence curves. Differences were tested

with the log-rank test. Overall survival (OS) was defined as the time between diagnosis or intervention (IST or HSCT) and death or last follow-up. In patients who underwent HSCT, event-free survival (EFS) was defined as the time between HSCT and death or any event consisting of relapse, second malignancy and graft failure. Failure-free survival (FFS) was evaluated in the analyses of patients who were observed for 2 years or more without therapy (watch and wait; WW) or those who received IST. FFS was defined as the time between diagnosis and death, any therapeutic intervention or disease progression in the analysis of the WW group. For the analysis of the IST group, FFS was the time between the initiation of IST and any treatment failure as follows; NR, a second course of IST, HSCT, relapse, disease progression or death. To calculate the cumulative incidence of disease progression, patients who underwent HSCT or died before disease progression were censored at the time of HSCT or death. Continuous variables were compared using the Mann-Whitney *U* test and categorical variables were compared using chi-square test or Fisher exact test. A two-sided *P* value of <0·05 was considered as statistical significant. All analyses were performed using JMP ver.9.0.2 (SAS Institute Inc. Cary, North Carolina) as of October 2012.

Results

Patient characteristics

The median follow-up period for the entire patient population was 5·5 years (range, 0·6–13·3 years) after diagnosis. The median age at diagnosis of the 65 patients (34 boys and

Table I. Patient characteristics according to the severity of dysplasia.

	Overall (n = 65)		RCC-MD (n = 25)		RCC-UD (n = 40)		*P value*
Age at diagnosis (years)*	7·8	(0·3–16·3)	6·6	(0·3–15·7)	9·7	(1·3–16·3)	0·17
Gender							
Male	34	52%	10	(40%)	24	(60%)	0·12
Female	31	48%	15	(60%)	16	(40%)	
Neutrophil count ($\times 10^9$/l)*	0·90	(0·05–8·11)	1·08	(0·14–7·08)	0·81	(0·05–8·11)	0·38
Haemoglobin (g/l)*	76	(36–141)	71	(36–117)	77	(38–141)	0·71
Platelet count ($\times 10^9$/l)*	35·0	(3·0–403·0)	53·0	(4·0–287·0)	34·0	(3·0–403·0)	0·094
MCV (fl)*	101·2	(76·8–121·7)	102·8	(80·7–121·7)	98·2	(76·8–119)	0·13
Reticulocyte count ($\times 10^9$/l)*	49·0	(4·92–333·0)	43·3	(12·3–131·0)	60·0	(4·92–333·0)	0·21
Severity of cytopenia†							
Very severe	3	5%	0	0%	3	8%	0·38
Severe	6	10%	2	9%	4	10%	
Non-severe	53	85%	21	91%	32	82%	
Lineages with cytopenia (n)‡							
3	33	51%	11	44%	22	55%	0·31
2	24	37%	9	36%	15	38%	
1	8	12%	5	20%	3	8%	
Karyotype							
Normal	41	65%	11	46%	30	77%	0·039
Monosomy 7	5	8%	4	17%	1	3%	
Trisomy 8	8	13%	3	13%	5	13%	
Trisomy 1q	4	6%	4	17%	0	0%	
Others§	5	8%	2	8%	3	8%	
Cellularity¶							
Hypercellular	8	22%	4	40%	4	15%	0·072
Normocellular	15	40%	5	50%	10	37%	
Hypocellular	14	38%	1	10%	13	48%	
Follow-up period (years)*	5·5	(0·6–13·3)	4·8	(0·6–11·5)	6·1	(2·1–13·3)	0·33

MCV mean corpuscular volume, RCC-MD refractory cytopenia of childhood with multilineage-dysplasia, RCC-UD refractory cytopenia of childhood with unilineage-dysplasia.

*Median (range).

†62 patients were evaluable. Very severe: neutrophils <0·2 $\times 10^9$/l and 'severe' criteria, severe: ≥2 of the following; neutrophils <0·5 $\times 10^9$/l or reticulocytes <20·0 $\times 10^9$/l or platelets <20·0 $\times 10^9$/l, non-severe: neither 'very severe' nor 'severe'.

‡Cytopenia was defined as follows; haemoglobin level less than 100 g/l, an absolute neutrophil count <1·5 $\times 10^9$/l, or a platelet count <100 $\times 10^9$/l.

§del(20q) n = 2, complex karyotype n = 1, i(8)(q10) n = 1, 46,XX,der(21)t(1;21)(q21;p1?)ins(21;?)(p1?;?) n = 1.

¶Biopsy specimens were available in 37 patients (57% of all patients).

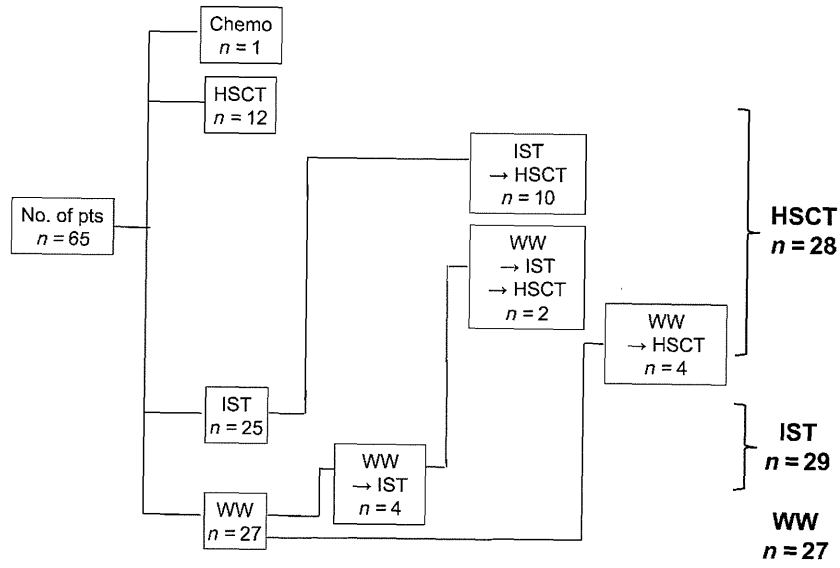


Fig 1. Therapeutic strategies for 65 patients with refractory cytopenia of childhood. Pts, patients; Chemo, chemotherapy; HSCT, haematopoietic stem cell transplantation; IST, immunosuppressive therapy; WW, 'watch and wait'.

31 girls) was 7·8 years (range, 0·3–16·3 years). The majority of the patients showed cytopenia in multiple lineages, whereas cytopenia in single lineage was found in only 12%. According to the criteria of severity used in AA, most patients (85%, n = 53) met the definition for 'non-severe'. Sixty-five percent (n = 41) of the patients whose karyotype was successfully analysed showed a normal karyotype. Of 37 patients who underwent BM biopsy, 38% (n = 14), 40% (n = 15) and 22% (n = 8) was determined as hypocellular, normocellular and hypercellular, respectively. The frequency of cytogenetic abnormality was higher in patients with RCC-MD as compared to those with RCC-UD (Table I). In particular, four of five patients with monosomy 7 and all four patients with trisomy 1q had RCC-MD, whereas the frequency of trisomy 8 appeared similar (Table I).

Overall outcome and initial treatment strategy

The overall therapeutic strategy is described in Fig 1. Twenty-seven patients were followed without therapeutic intervention for 2 years or more (WW). The initial treatment of the remaining 38 patients included IST (n = 25), HSCT (n = 12), and AML-type chemotherapy (n = 1). The patient who received chemotherapy died due to haemophagocytic syndrome that developed after chemotherapy. Among the IST group, 10 patients underwent HSCT due to treatment failure. After ≥2 years follow-up of the WW group, four underwent HSCT and four received IST, of whom two underwent HSCT due to non-response to the IST.

Clinical characteristics according to the initial treatment strategy are shown in Table SI. As expected, the WW group showed mild neutropenia and anaemia. Patients with RCC-MD were more likely to be transplanted as initial treatment compared to those with RCC-UD. The 5-year OS in the whole population was 82 ± 5% (Fig 2).

Watch and wait

Of 27 patients in the WW group, 18 patients maintained stable disease with a median duration of 4·6 years (range, 2·1–13·3 years). Three patients suffered from disease progression; two were successfully treated by HSCT and one is currently preparing for HSCT. Six patients received intervention (two HSCT and four IST) due to deterioration of cytopenia occurring a median of 3·8 years after diagnosis (range, 2·7–4·7 years). The 5-year FFS was 56 ± 12% (Fig 3). As most patients who failed the WW strategy were successfully salvaged by IST or HSCT, the 5-year OS in this group was 87 ± 9% (Fig 3). Variables such as gender, age at diagnosis, blood count and karyotype were not associated with treatment failure in the WW group, but patients with RCC-MD fared significantly worse compared to those with RCC-UD (5-year FFS was 25 ± 20% and 70 ± 13% respectively P = 0·013).

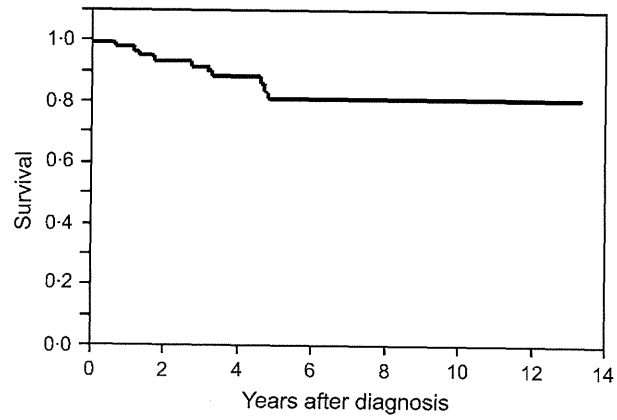


Fig 2. Overall survival in the whole population of patients with refractory cytopenia of childhood (n = 65).

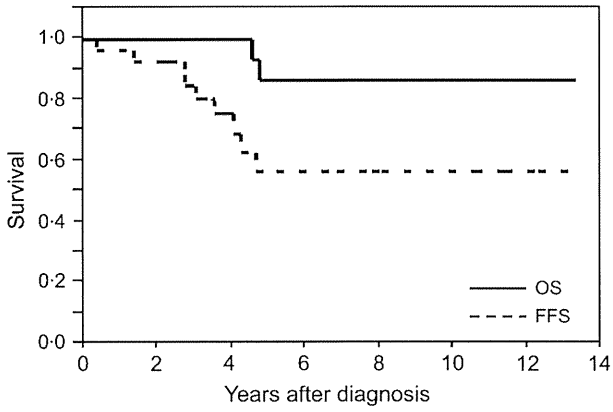


Fig 3. Outcome of patients who were followed without therapy for the first 2 years (watch and wait group; $n = 27$). Overall survival (OS, solid line) and failure-free survival (FFS, dashed line).

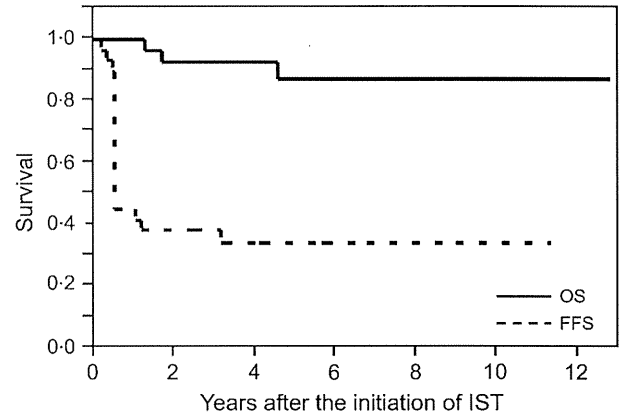


Fig 4. Outcome of patients who received immunosuppressive therapy (IST, $n = 29$). Overall survival (OS, solid line) and failure-free survival (FFS, dashed line).

Immunosuppressive therapy

Twenty-five patients received IST as initial treatment and four after failure of the WW strategy (Fig 1). Median duration between diagnosis and initiation of IST was 95 d (range, 0–1702 d). The IST regimen was decided by the treating physicians; ATG and ciclosporin were administered in 16 patients as previously reported (Kojima *et al*, 2000; Hasegawa *et al*, 2009), whereas 13 patients received only ciclosporin. Patients who received ciclosporin monotherapy showed milder cytopenia than those who received combination regimen, however, there were no differences in cytogenetic abnormalities and the severity of dysplasia between the two groups (Table SII). No severe adverse event was reported after either ciclosporin monotherapy or combination therapy.

Thirteen of 29 patients (45%) showed response at 6 months after initiation of IST (CR, four patients; PR, nine patients). Of 13 responders, 11 still remained transfusion-free, but two relapsed, one of whom suffered disease progression after relapse. Males and those who showed lower reticulocyte counts responded to IST, but other characteristics and variables, such as IST regimen and time from diagnosis to IST, were not significantly different between responders and non-responders (Table SIII).

Eighteen patients were reported as treatment failure (16 non-responders and two relapsed). Five-year FFS was calculated as 34 ± 9% (Fig 4). Of those, 12 underwent HSCT, three received second-line IST, two showed late responses (at 10 months and 25 months respectively), and one was lost to follow-up. Seven patients remained in remission after second-line HSCT, whereas three died due to transplant-related mortality (TRM) and two suffered from secondary graft failure. Five-year OS of 29 patients receiving IST was 87 ± 7% (Fig 4). Statistical differences were not detected between ciclosporin and ATG combination and ciclosporin monotherapy in either FFS (44 ± 12% vs. 23 ± 12%. $P = .40$) or OS (84 ± 10% vs. 92 ± 7%. $P = .75$).

Haematopoietic stem cell transplantation

A total of 28 patients underwent HSCT, 12 as initial treatment, four after WW and 12 after failure of IST (Fig 1). Median duration between diagnosis and HSCT was 668 d (range, 123–2674 d). BM from an unrelated donor was the stem cell source most commonly used ($n = 15$). MAC with total-body irradiation (TBI) regimen was given to eight patients, MAC with busulfan regimen was given to seven patients and 13 patients received RIC. Grade II-IV aGVHD was observed in 10 patients (37%). Of 23 evaluable patients, seven patients had cGVHD. Seventeen patients remained in remission after HSCT. Secondary graft failure was observed in two patients who received HSCT following RIC after failure of IST. The remaining nine patients died due to various causes including TRM (two thrombotic microangiopathy, one bronchiolitis obliterans, one acute respiratory distress syndrome, one Epstein-Barr virus-associated lymphoproliferative disorder, one GVHD, and one fungal infection), relapse ($n = 1$), and secondary brain tumour ($n = 1$). TRM was observed in children who received MAC with TBI ($n = 2$), MAC with busulfan ($n = 2$), and RIC ($n = 3$). Five-year EFS and OS calculated among HSCT patients was 58 ± 10% and 66 ± 9% respectively (Fig 5). No patient characteristics or transplant-related variables showed a remarkable impact on outcome after HSCT (Table SIV).

Disease progression

Of 65 patients, eight suffered from disease progression (Table SV). Two patients acquired monosomy 7 without an increase in blasts, three acquired other abnormal karyotypes followed by progression to advanced disease and three experienced an increase in blasts and progressed to advanced disease. Seven patients did not receive IST or HSCT before disease progression, whereas the remaining patient acquired monosomy 7 after IST. Median time from diagnosis to disease progression was 488 d (range, 28–985 d). Five-year

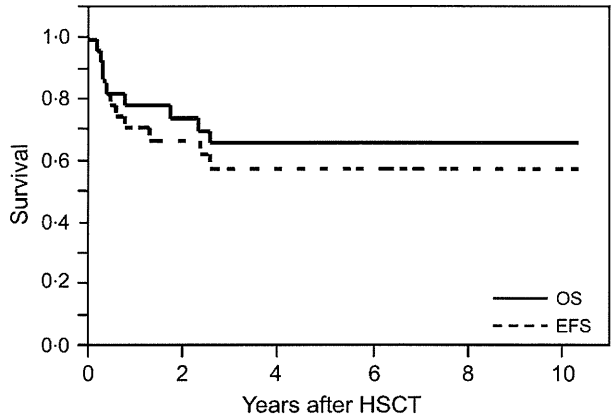
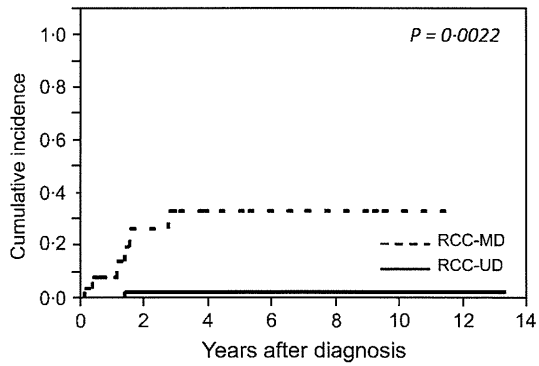


Fig 5. Outcome of patients who underwent haematopoietic stem cell transplantation (HSCT, n = 28). Overall survival (OS, solid line) and event-free survival (EFS, dashed line).

cumulative incidence of disease progression was 13 ± 5%. Patients with monosomy 7 were more likely to experience disease progression (47 ± 25% vs. 12 ± 6% in normal karyotype, P = 0·003). In addition, RCC-MD had significantly higher probabilities of disease progression than RCC-UD (33 ± 12% vs. 3 ± 3%. P = 0·002; Fig 6). Of seven patients underwent HSCT after disease progression, two patients died of TRM. One remaining patient is preparing for HSCT at present. Overall survival of patients who suffered from disease progression was comparable to that of patients who did not, 73 ± 17% and 83 ± 6% respectively (P = 0·55).

Discussion

Although 6 years have passed since the WHO Working Group proposed RCC as an entity that represents childhood



Numbers of patients at risk

RCC-MD	24	12	7	5	4	2	0
RCC-UD	40	33	20	15	9	7	2

Fig 6. Cumulative incidence of disease progression according to severity of dysplasia. RCC-UD, refractory cytopenia of childhood with unilineage-dysplasia (n = 40: solid line); RCC-MD, refractory cytopenia of childhood with multilineage-dysplasia (n = 24: dashed line).

MDS with a low blast count (Baumann *et al*, 2008), the clinical characteristics of RCC are still unclear and an appropriate therapeutic strategy remains to be established. Furthermore, the importance of lineage involvement of dysplasia was not mentioned in the WHO 2008 classification. In order to address these issues, we analysed the clinical characteristics and treatment outcomes in 65 patients with RCC defined according to the WHO 2008 classification. Clinical characteristics of RCC demonstrated in this study were similar to those reported in a previous study, such as age at diagnosis and multilineage cytopenia (Kardos *et al*, 2003). In our study, two-thirds of patients showed normal karyotype, whereas monosomy 7 was observed only in 8%. Of interest, trisomy 1q was documented in four patients (6%). +1/1q+ accounts for 3% of clonal cytogenetic abnormalities in adult MDS and was identified to have good prognostic impact (Haase *et al*, 2007). Gain of 1q has also been shown to be one of the most commonly acquired chromosomal aberrations in children with Fanconi anaemia, but it does not appear to predict progression to MDS or AML (Mehta *et al*, 2010). Of four patients with trisomy 1q in our study, three are alive whereas one died after intensive chemotherapy. The prognostic significance of this aberration in childhood MDS remains to be elucidated in larger cohorts.

Surprisingly, 18 patients (67% of 27 patients in the WW group and 28% of the entire cohort) maintained stable disease without IST or HSCT. This benign nature may resemble non-severe AA. In fact, overlapping immunological features have been reported between AA and low-grade MDS in children (de Vries *et al*, 2008). However, more than half of children with non-severe AA experience deterioration of cytopenia unless IST or HSCT is given, and progression-free survival curves do not reach a plateau, even as late as more than 10 years after diagnosis (Howard *et al*, 2004; Nishio *et al*, 2009). The difference in clinical course between non-severe AA and RCC requires further clarification in a prospective study that is based on a common diagnostic platform (Baumann *et al*, 2012). To address this, we initiated a prospective registration system in 2009, employing histopathological central review for children with both MDS and AA.

Given our observation that time from diagnosis to IST or HSCT did not appear to be associated with outcome, WW seems justifiable when patients with RCC show mild cytopenia. Also, in adult MDS patients considered as low-risk defined according to IPSS, delayed but prior-to-leukaemic transformation HSCT was associated with better outcome (Cutler *et al*, 2004). Our results suggest that patients with RCC who showed multilineage dysplasia may require an intervention because this subgroup of the WW group was more likely to fail.

In adults, hypoplastic MDS is a good candidate for IST because an immune-mediated damage to haematopoiesis is reported to contribute to pathogenesis (Sloand & Rezvani, 2008), which has also been suggested in a subset of RCC (de Vries *et al*, 2008). Hypoplastic MDS, accounting for 10% of

adult MDS, has overlapping characteristics with RCC such as young age, being less anaemic but more neutropenic and thrombocytopenic, and lower frequency of abnormal karyotype (Sloand, 2009; Calado, 2011). The response rate of IST was reported as 30–80% in adult MDS (Molldrem *et al*, 1997; Jonásova *et al*, 1998; Shimamoto *et al*, 2003; Sloand *et al*, 2008) and 60–70% in childhood MDS (Yoshimi *et al*, 2007; Hasegawa *et al*, 2009). Compared to these previous reports, the response rate of IST was lower in the present analysis. We did not identify prognostic factors predictive of response to IST, except that boys and those who had lower reticulocyte counts showed better response rates. The relationship between IST regimen and response rate remains inconclusive (Sloand & Rezvani, 2008). In a clinical trial conducted by the National Institutes of Health, ciclosporin alone showed inferior response rates in comparison to ciclosporin and ATG combination (Sloand *et al*, 2008). Because the sample size of our study was limited, further clinical trials randomizing ciclosporin alone versus combination therapy is warranted in order to examine whether ciclosporin monotherapy is an attractive option for children who require therapeutic intervention but lack a suitable donor.

Only HSCT is considered as a curative therapy for MDS, but is associated with potentially severe complications, mortality and late sequelae. In fact, nine of 10 deaths in our series were reported in children who underwent HSCT. Strahm *et al* (2007) reported that HSCT following RIC is an effective transplantation strategy for children with hypocellular RCC in the absence of chromosomal abnormality. Given that TRM was also noted in those children who received RIC in our study, it remains unclear whether RIC will overcome death due to acute toxicity.

Disease progression is a major problem in treating patients with MDS. Monosomy 7 was reported to be associated with an increased risk of progression to advanced disease in children with refractory anaemia (RA; Kardos *et al*, 2003). In our analysis, eight children suffered from disease progression. We found monosomy 7 and multilineage dysplasia to be risk factors for disease progression. In the WHO 2001 classification, RA according to the French-American-British classification was re-classified into RCMD or RA based on the severity of dysplasia (Vardiman *et al*, 2002). Germing *et al* (2006) analysed 1095 adults with MDS according to the WHO classification and found that patients with RCMD were more likely to evolve to AML and showed shorter survival than RA. They concluded that the distinction between unilineage and multilineage dysplasia in the RA group was highly informative. The significance of lineage involvement of dysplasia, especially in low-grade MDS, was also confirmed by several groups (Malcovati *et al*, 2007; Matsuda *et al*, 2007; Verburgh *et al*, 2007). In the present study, RCC-MD was associated with an abnormal karyotype at diagnosis, inferior FFS after WW, and increased risk of disease progression in comparison with RCC-UD. These results suggest that sub-classification of low-grade MDS according to severity of dysplasia might be important in treatment decisions among children as well as adults. Biological changes underlying morphological differences between RCC with unilineage and multilineage dysplasia should be explored in further studies. A previous study of adults with MDS showed that IST non-responders had a high risk of disease progression (Molldrem *et al*, 2002). In our analysis, most children experienced disease progression during the observation period whereas only one child acquired monosomy 7 after combination IST with ciclosporin and ATG. Thus, our results do not support the finding that IST non-responders are at risk of disease progression.

Although our study can be considered uniquely large for a rare disease such as RCC, we recognize the potential limitations of this study. First, the observational nature of the study allowed physicians to decide treatment strategy, IST regimen and conditioning regimen of SCT. Currently, a prospective clinical trial to evaluate the efficacy of each modality of treatment is under consideration in Japan. Second, BM biopsy specimens were available only in 37 patients (57%). Baumann *et al* (2012) emphasized the importance of histomorphological work-up to differentiate AA and RCC. We compared the characteristics of children with and without biopsy specimens in this study and found no difference. In fact, we observed RCC-MD both in patients who underwent biopsy and in those who did not. Since 2008, all children suspected of having RCC undergo BM biopsy in Japan. Third, genetic and immunological evaluations were not performed prospectively. Under-diagnosis of cryptic IBMFS might be relevant to the unexpectedly high incidence of TRM in our analysis. In our current registration system, the telomere length and paroxysmal nocturnal haemoglobinuria-type cell is investigated in a prospective fashion, and chromosome breakage tests are recommended in all children. Fourth, certain analyses may have lacked sufficient statistical power to detect significant associations. Thus, the confirmation of results in additional studies is important.

In conclusion, our results show that RCC comprises a heterogeneous population that represents a wide spectrum of diseases, ranging from indolent cytopenia to a more progressive nature. This study also clearly shows that mulilineage dysplasia is associated with cytogenetic abnormality and disease progression in children with RCC. Given that a quarter of the entire patient series in this study had an indolent course, patients with mild cytopenia and a normal karyotype can be observed carefully without SCT or IST. However, patients who are transfusion-dependent or endangered by a low neutrophil count should receive intervention. IST may be an acceptable option for those patients who do not have a matched-sibling donor. Patients who have unfavourable chromosomal abnormalities, such as monosomy 7 and complex karyotype, should undergo HSCT even if a matched-sibling is not available. Further studies are needed to explore appropriate IST and HSCT conditioning regimens.

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Authorship contributions

D.H. collected and analysed the data and wrote the manuscript; C.X., S.H. and SW collected and analysed the data; Y.I. analysed the data and wrote the manuscript; Y.Z., M.T., A.Masunaga, A.H. and M.I. performed the central review of specimens; A.Y. performed the central review of specimens and wrote the manuscript; S.K. and T.N. designed the study and performed the central review of specimens; A.Manabe designed the study, performed the central review of specimens, analysed the data and wrote the manuscript.

Conflict of interest disclosures

The authors reported no potential conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table SI. Comparison of characteristics according to the initial treatment strategy.

Table SII. Comparison of characteristics of patients who received IST (n = 29) according to the IST regimen.

Table SIII. Comparison of characteristics between responder and non-responder to IST at 6 months.

Table SIV. Univariate analyses of the probability of 5-year event-free survival (EFS) after HSCT.

Table SV. Summary of eight cases who suffered from disease progression.

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Changes in the Clinical Impact of High-Risk Human Leukocyte Antigen Allele Mismatch Combinations on the Outcome of Unrelated Bone Marrow Transplantation

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A B S T R A C T

Several high-risk HLA allele mismatch combinations (HR-MMs) for severe acute graft-versus-host disease (GVHD) have been identified by analyzing transplantation outcomes in Japanese unrelated hematopoietic stem cell transplant recipients. In this study, we analyzed the effects of HR-MMs in 3 transplantation time periods. We confirmed that the incidence of grade III to IV acute GVHD in the HR-MM group was significantly higher than that in the low-risk (LR) MM group (hazard ratio [HR], 2.74; $P < .0001$) in the early time period (1993 to 2001). However, the difference in the incidence of grade III to IV acute GVHD between the HR-MM and LR-MM groups was not statistically significant (HR, 1.06; $P = .85$ and HR, .40; $P = .21$, respectively) in the mid (2002 to 2007) and late (2008 to 2011) time periods. Similarly, survival in the HR-MM group was significantly inferior to that in the LR-MM group (HR, 1.46; $P = .019$) in the early time period, whereas the difference in survival between the 2 groups was not statistically significant in the mid and late time periods (HR, 1.06; $P = .75$ and HR, .82; $P = .58$, respectively). In conclusion, the adverse impact of HR-MM has become less significant over time. Unrelated transplantation with a single HR-MM could be a viable option in the absence of a matched unrelated donor or an unrelated donor with a single LR-MM.

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) from an unrelated donor has been established as an effective treatment option for patients with hematological diseases who lack a human leukocyte antigen (HLA)–matched related donor. However, an HLA mismatch at the genetic level (allele mismatch) may be observed even in HSCT from a serologically HLA-matched donor (antigen match), and the presence of an allele mismatch adversely affects the incidence of severe acute graft-versus-host disease (GVHD) and survival [1-4]. We recently showed that the presence of single HLA allele mismatches at the HLA-A, -B, -C, or -DRB1 loci equivalently affect the outcome of HSCT, although a previous study from Japan reported that an HLA-A or -B allele mismatch impairs overall survival more strongly than an HLA-C or -DRB1 allele mismatch [4,5]. These findings suggest that the

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Table 1
Patient Characteristics

Characteristic	Match n = 2504			Low-Risk Mismatch n = 1057			High-Risk Mismatch n = 157		
	Early	Mid	Late	Early	Mid	Late	Early	Mid	Late
	802	814	888	412	351	294	64	71	22
Age (recipient)									
Median	32	38	43	31	38	43	33	39	41
Age (donor)									
Median	34	34	36	33	34	37	35	36	37
Sex (recipient)									
Female	292	305	378	162	165	123	27	27	9
Male	510	509	510	250	186	171	37	44	13
Sex (donor)									
Female	286	262	266	164	158	107	20	28	5
Male	512	548	622	247	190	187	43	43	17
N.A.	4	4	0	1	3	0	1	0	0
Sex mismatch									
Match	507	537	512	238	209	166	35	40	14
Male to female	148	158	244	85	72	72	17	15	6
Female to male	143	115	132	88	67	56	11	16	2
N.A.	4	4	0	1	3	0	1	0	0
ABO blood type									
Match	454	462	500	167	151	121	33	31	9
Minor mismatch	154	162	175	112	84	81	15	18	3
Major mismatch	125	114	142	82	67	61	9	18	4
Bidirectional mismatch	58	70	71	45	46	31	7	4	6
N.A.	11	6	0	6	3	0	0	0	0
Disease									
AML	269	415	495	134	168	170	15	29	12
ALL	229	229	249	116	96	76	11	23	8
CML	237	84	29	125	42	14	30	3	0
MDS	67	86	115	37	45	34	8	16	2
Disease risk									
Low	552	533	607	265	219	181	40	38	12
High	230	239	280	135	116	113	21	28	10
Others	20	42	1	12	16	0	3	5	0
Cell dose (cells/kg)									
Median	3.0	2.7	2.7	3.0	2.6	2.6	3.1	2.8	2.6
GVHD prophylaxis									
CSA-based	545	306	185	267	114	47	45	21	2
TAC-based	240	499	689	135	227	240	19	50	20
N.A.	17	9	14	10	10	7	0	0	0
Conditioning regimen									
TBI regimen	760	639	560	394	272	194	59	53	15
Non-TBI regimen	30	114	328	17	52	100	3	11	7
N.A.	12	61	0	1	27	0	2	7	0

N.A. indicates not available; AML, acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; GVHD, graft-versus-host disease; CSA, cyclosporine; TAC, tacrolimus; TBI, total body irradiation.

clinical impact of an HLA mismatch may have changed over time periods.

Some investigators have tried to identify specific donor-recipient allele combinations that may be associated with a higher risk of severe acute GVHD [6,7]. Kawase et al. found 16 high-risk HLA allele mismatch combinations (HR-MMs) for severe acute GVHD [7]. They also showed that the number of HR-MMs was associated with severe GVHD and poor survival, whereas the presence of mismatch combinations other than HR-MMs (low-risk mismatch combinations, LR-MMs) did not affect the outcome of HSCT. However, their study included a variety of benign and malignant hematological diseases. In addition, they included donor-recipient pairs with more than 1 HLA mismatch. The impact of each specific mismatch combination was evaluated after adjusting for the number of HLA mismatches in other loci in a multivariate model, but the possible presence of HR-MMs in other loci or the interaction between HLA mismatch combinations could not be appropriately treated in their model. At that time, the study design was inevitable, because the number of each HLA mismatch combination was limited. However, several years have passed and the amount of unrelated HSCT data in the Transplant Registry Unified Management Program (TRUMP) has increased to more than 13,500 donor-recipient pairs. Therefore, in this study, we reanalyzed the impact of HR-MMs, excluding HSCT with multiple HLA mismatches in patients with relatively homogeneous background diseases. In addition, we evaluated the impact of HLA mismatch on transplantation outcomes considering the period effect, because the impact of HR-MM mismatch might have changed over time periods, as we previously reported in an analysis of single HLA allele mismatches at the HLA-A, -B, -C, and -DRB1 loci [5].

METHODS

Patients

Patients aged at least 16 years with acute myeloblastic leukemia, acute lymphoblastic leukemia, myelodysplastic syndrome, or chronic myelogenous leukemia (CML) who underwent a first HSCT from a serologically HLA-A, -B, and -DR matched unrelated donors between 1993 and 2011, and who had full HLA-A, -B, -C, and -DRB1 allele data, were included in this study. Bone marrow was exclusively used as a stem cell source. Clinical data for

Table 2
Multivariate Analysis to Evaluate the Impact of Single HLA Allele Mismatches on the Incidence of Grade III to IV Acute GVHD Stratified according to the Transplantation Time Period

Year	Factor		Hazard Ratio	*P* Value
1993-2001				
	Donor age		1.02 (1.00-1.03)	.082
	Donor sex	Female	1.00	
		Male	1.65 (1.05-2.60)	.031
	Female to male transplantation	No	1.00	
		Yes	1.52 (.91-2.55)	.11
	Disease	AML	1.00	
		ALL	1.15 (.79-1.68)	.47
		CML	1.62 (1.11-2.36)	.012
		MDS	.65 (.32-1.35)	.25
	Disease risk	Low	1.00	
		High	1.30 (.93-1.83)	.13
		Others	.80 (.23-2.85)	.74
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.83 (.61-1.14)	.25
	HLA	Low-risk mismatch	1.00	
		Match	.89 (.65-1.21)	.44
		High-risk mismatch	2.74 (1.73-4.32)	<.0001
2002-2007				
	Donor age		1.03 (1.01-1.05)	.0028
	Donor sex	Female	1.00	
		Male	1.50 (.96-2.33)	.076
	Female to male transplantation	No	1.00	
		Yes	1.53 (.89-2.64)	.13
	Disease	AML	1.00	
		ALL	1.36 (.95-1.96)	.094
		CML	1.27 (.74-2.20)	.38
		MDS	1.25 (.77-2.02)	.37
	Disease risk	Low	1.00	
		High	1.76 (1.25-2.48)	.0011
		Others	1.65 (.82-3.34)	.16
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.86 (.63-1.19)	.37
	HLA	Low-risk mismatch	1.00	
		Match	.64 (.46-.89)	.008
		High-risk mismatch	1.06 (.58-1.93)	.85
2008-2011				
	Donor age		1.03 (1.01-1.06)	.0016
	Donor sex	Female	1.00	
		Male	1.28 (.78-2.12)	.33
	Female to male transplantation	No	1.00	
		Yes	.98 (.52-1.88)	.96
	Disease	AML	1.00	
		ALL	1.18 (.80-1.74)	.42
		CML	1.53 (.69-3.37)	.3
		MDS	.66 (.36-1.20)	.17
	Disease risk	Low	1.00	
		High	1.53 (1.08-2.17)	.018
		Others	NA (NA-NA)	NA
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.82 (.55-1.24)	.34
	HLA	Low-risk mismatch	1.00	
		Match	.56 (.39-.80)	.0014
		High-risk mismatch	.40 (.10-1.64)	.21

AML indicates acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; GVHD, graft-versus-host disease; CSA, cyclosporine; TAC, tacrolimus.

these patients were obtained from the TRUMP [8]. We excluded patients who lacked data on survival status, those with more than 1 allele or antigen mismatch, those who received a reduced-intensity conditioning regimen, and those who received ex vivo or in vivo T cell depletion, such as antithymocyte globulin or alemtuzumab. Finally, 3718 patients were included in the main part of this study. As a post hoc analysis, 415 patients with 2 LR-MMs and 66 patients with 2 allele mismatches including at least 1 HR-MM were added to compare the impact of 1 HR-MM and 2 LR-MMs and to analyze the statistical interaction between HR-MM and the presence of an additional allele mismatch. The study was approved by the data management committee of TRUMP and by the institutional review board of Saitama Medical Center, Jichi Medical University.

Histocompatibility

Histocompatibility data for serological and genetic typing for the HLA-A, HLA-B, HLA-C, and HLA-DR loci were obtained from the TRUMP database, which includes HLA allele data determined retrospectively by the Japan Marrow Donor Program using frozen samples [7,9]. In this study, the following donor-recipient HLA-mismatch combinations were regarded as HR-MMs: A*02:06-A*02:01, A*02:06-A*02:07, A*26:02-A*26:01, A*26:03-A*26:01, B*15:01-B*15:07, C*03:03-C*15:02, C*03:04-C*08:01, C*04:01-C*03:03, C*08:01-C*03:03, C*14:02-C*03:04, C*15:02-C*03:04, C*15:02-C*14:02, DR*04:05-DR*04:03, and DR*14:03-DR*-DR1401, as we did not have enough data on HLA-DP and -DQ [7]. In HR-MM pairs, the donor and the recipient must have the HLA allele as shown above, and at the same time, these donor and recipient HLA alleles should not be shared by the recipient and the donor, respectively. For example, if the donor has HLA-A*02:06/02:06 and the recipient has HLA-A*02:01/02:06, this pair was not regarded as HR-MM pair, as the donor's HLA-A*02:06 was shared by the recipient. Other HLA mismatch pairs were regarded as LR-MM pairs. Only the HLA-C mismatch group included HLA mismatch at a serological (antigen) level.

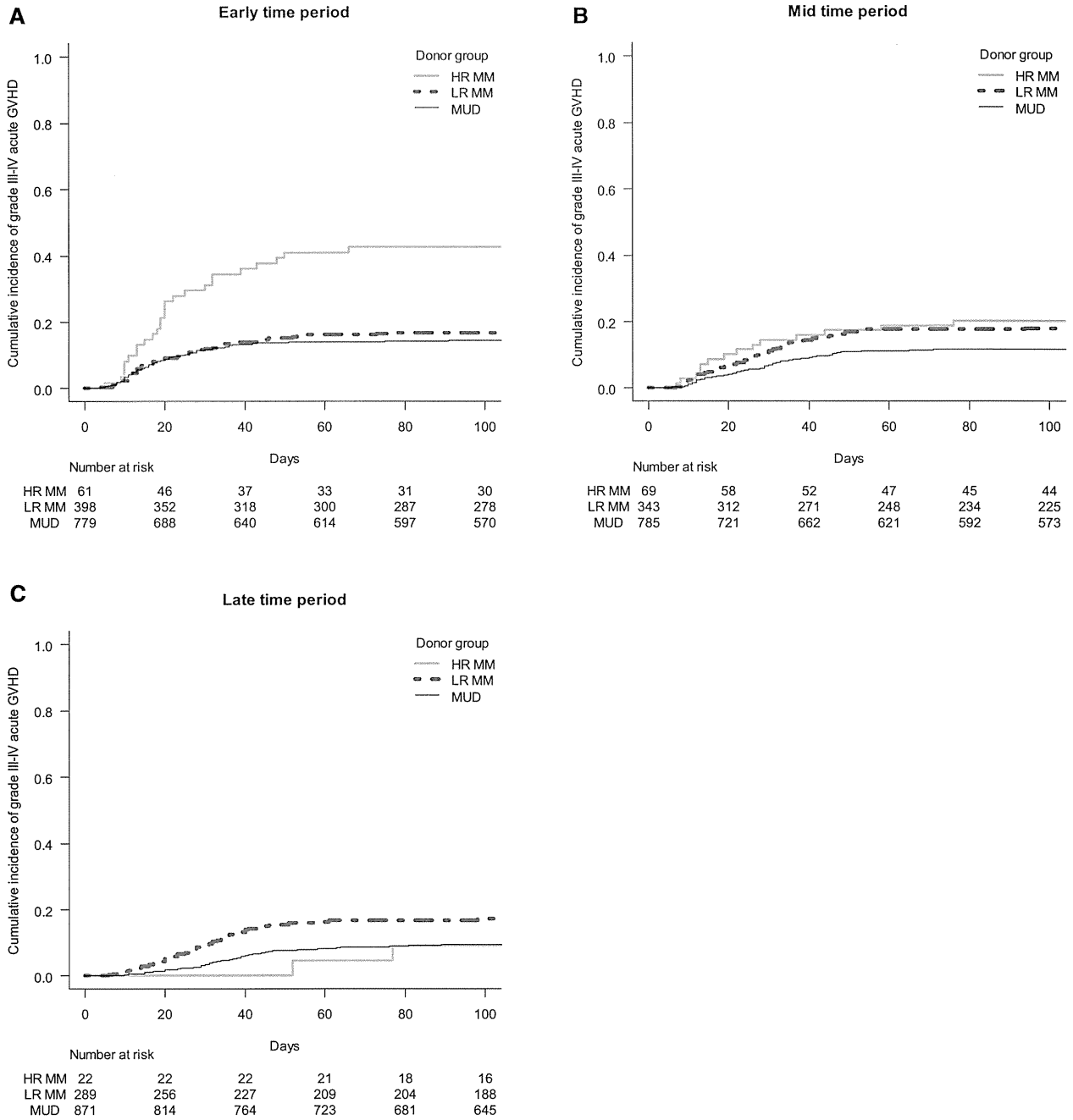

Figure 1. The cumulative incidence of grade III to IV acute GVHD grouped according to the HLA mismatch between the donor and recipient in the early (A), mid (B), and late time periods (C). HR-MM indicates high-risk mismatch; LR-MM, low-risk mismatch; MUD, matched unrelated donor.

Statistical Analyses

We divided the patients into 3 groups according to the time period when HSCT was performed to evaluate whether the impact of HR-MM changed over time periods: the early, mid, and late groups included HSCT performed from 1993 through 2001, 2002 through 2007, and 2008 through 2011, respectively. The break points among groups were determined to make the number of patients in each group equivalent (n = 1278, 1236, and 1204, respectively). To avoid making misleading conclusions by arbitrary grouping, we confirmed that there was a statistically significant interaction between the presence of HR-MMs and transplantation year as a continuous variable, both for overall survival ($P = .0098$) and the incidence of grade III to IV acute GVHD ($P < .001$). The following analyses were performed separately in each group. However, in post hoc analyses to evaluate the impact of HR-MMs at each locus and to compare 1 HR-MM and 2 LR-MMs, the mid and late groups were combined to increase the statistical power, after confirming that similar results were obtained in the 2 groups.

The primary endpoint was the incidence of grade III to IV acute GVHD. Overall survival was evaluated as a secondary endpoint. The chi-square test or Fisher exact test was used to compare categorical variables and Student *t*-test or an analysis of variance test was used for continuous variables to evaluate the homogeneity of background characteristics of the HR-MM, LR-MM, and HLA-matched (MUD) groups. *P* values were adjusted using the Bonferroni's method and Tukey's method for multiple comparisons between each pair. Overall survival was estimated according to the Kaplan-Meier method, and compared among groups with the log-rank test. The incidence of acute GVHD was calculated treating death without GVHD as a competing event, and it was compared using Gray's test [10].

The impact of HR-MMs was evaluated using multivariate models: the Cox proportional hazards model was used for overall survival and Fine and Gray's proportional hazards model was used for acute GVHD [11]. The LR-MM group was regarded as the reference group. Potential confounding factors that were considered in these analyses included recipient/donor age, recipient/donor sex, sex mismatch, ABO major/minor mismatch, the use of

Table 3
Multivariate Analysis to Evaluate the Impact of Single High-Risk Allele Mismatches on Overall Survival Stratified According to the Transplantation Time Period

Year	Factor		Hazard Ratio	*P* Value
1993-2001				
	Age		1.02 (1.01-1.03)	<.0001
	Sex	Female	1.00	
		Male	1.06 (.90-1.23)	.51
	Disease	AML	1.00	
		ALL	1.20 (.99-1.45)	.065
		CML	.89 (.72-1.10)	.29
		MDS	.61 (.45-.83)	.0015
	Disease risk	Low	1.00	
		High	2.72 (2.30-3.23)	<.0001
		Others	2.03 (1.27-3.23)	.0029
	ABO major mismatch	Absent	1.00	
		Present	1.25 (1.06-1.47)	.0092
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.85 (.72-1.00)	.049
	HLA	Low-risk mismatch	1.00	
		Match	.86 (.73-1.01)	.063
		High-risk mismatch	1.46 (1.06-2.01)	.019
2002-2007				
	Age		1.01 (1.00-1.02)	.0025
	Sex	Female	1.00	
		Male	1.20 (1.02-1.41)	.0027
	Disease	AML	1.00	
		ALL	1.16 (.96-1.39)	.13
		CML	.84 (.62-1.12)	.23
		MDS	.56 (.43-.73)	<.0001
	Disease risk	Low	1.00	
		High	2.87 (2.41-3.40)	<.0001
		Others	2.23 (1.58-3.15)	<.0001
	ABO major mismatch	Absent	1.00	
		Present	.97 (.81-1.16)	.77
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.97 (.83-1.15)	.76
	HLA	Low-risk mismatch	1.00	
		Match	.83 (.69-.98)	.032
		High-risk mismatch	1.06 (.75-1.48)	.75
2008-2011				
	Age		1.02 (1.01-1.03)	<.0001
	Sex	Female	1.00	
		Male	1.08 (.89-1.31)	.42
	Disease	AML	1.00	
		ALL	.97 (.76-1.25)	.83
		CML	.97 (.57-1.64)	.9
		MDS	.65 (.48-.87)	.004
	Disease risk	Low	1.00	
		High	2.73 (2.23-3.35)	<.0001
		Others	NA (NA-NA)	NA
	ABO major mismatch	Absent	1.00	
		Present	1.14 (.92-1.41)	.22
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.95 (.75-1.21)	.69
	HLA	Low-risk mismatch	1.00	
		Match	.86 (.69-1.06)	.15
		High-risk mismatch	.82 (.42-1.62)	.58

AML indicates acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; GVHD, graft-versus-host disease; CSA, cyclosporine; TAC, tacrolimus.

total body irradiation in the conditioning regimen, cell dose in the bone marrow graft, the use of cyclosporine or tacrolimus as GVHD prophylaxis, background disease, and disease risk. Acute leukemia in first or second remission, CML in first or second chronic phase, CML in accelerated phase, and myelodysplastic syndrome of refractory anemia or refractory anemia with excess blasts were considered low-risk diseases, and other conditions were considered high-risk diseases. All of these potential confounding factors were included in the multivariate analyses and then deleted in a stepwise fashion from the model to exclude factors with a *P* value of .05 or higher. Finally, HLA mismatch was added to the model. Different multivariate models were compared using the likelihood ratio test. The quantity of interest was the deviance difference between the 2 models, under the null hypothesis that 2 models fit the data equally well and the deviance difference has an approximate chi-square distribution with degrees of freedom equal to the difference in the number of independent variables between the compared models.

All *P* values were 2 sided and *P* values of .05 or less were considered statistically significant. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University) [12], which is a graphical user interface for R (The R Foundation for Statistical Computing). More precisely, it is a modified version of R commander that was designed to add statistical functions frequently used in biostatistics.

RESULTS

Patients

The patient characteristics are summarized in Table 1. HR-MMs were observed in 64 of 1278, 71 of 1236, and 22 of 1204 donor-recipient pairs in the early, mid, and late time periods, respectively. On the other hand, 412, 351, and 294 pairs had LR-MMs, respectively. With regard to the

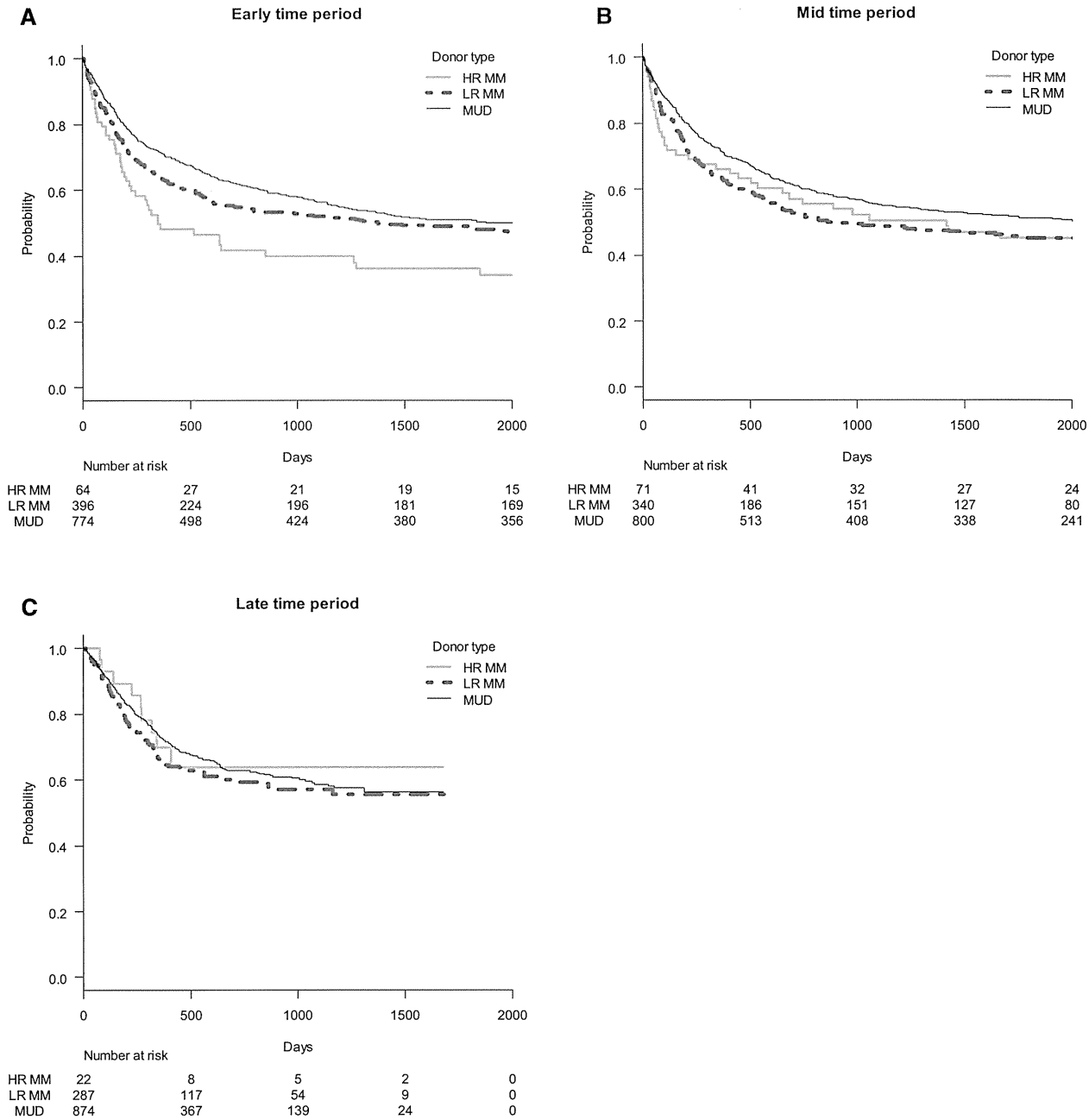

Figure 2. Overall survival grouped according to the HLA mismatch between the donor and recipient in the early (A), mid (B), and late time periods (C). The survival curves were adjusted for other significant factors by the mean of covariates method, in which average values of covariates are entered into the Cox proportional hazards model. HR-MM, high-risk mismatch; LR-MM, low-risk mismatch; MUD, matched unrelated donor.

differences among transplantation time periods, the numbers of LR-MMs and HR-MMs decreased in the late time periods, ie, after the introduction of routine typing for HLA-C and the publication of a paper about HR-MMs [7]. The proportion of HSCTs for CML also dramatically decreased over time periods (30.7%, 10.4%, and 3.6% in the early, mid, and late periods, respectively). With regard to the difference among HLA mismatch groups, the proportion of patients with high-risk underlying disease in the MUD group (29.9%) was significantly lower than those in the HR-MM (37.6%) and LR-MM groups (34.4%). In addition, the proportion of HSCTs for CML was significantly higher in the HR-MM group in the early time period (29.6%, 30.3%, and 46.9% in the MUD, LR-MM, and HR-MM groups, respectively).

Incidence of Grade III to IV Acute GVHD

To adjust the impact of HLA mismatch for possible confounding factors, we identified the following independently significant factors for the incidence of grade III to IV acute GVHD: donor age, donor sex, sex mismatch, disease, disease risk, and GVHD prophylaxis. After we adjusted for these factors, we confirmed that the incidence of grade III to IV acute GVHD in the HR-MM group was significantly higher than that in the LR-MM group (hazard ratio [HR], 2.74; 95% confidence interval [CI], 1.73 to 4.32; $P < .0001$) in the early time period, whereas the difference between the MUD and LR-MM groups was not significant (HR, .89; 95% CI, .65 to 1.21; $P = .44$) (Table 2, Figure 1). On the other hand, in the mid and late time periods, the difference in the incidence of

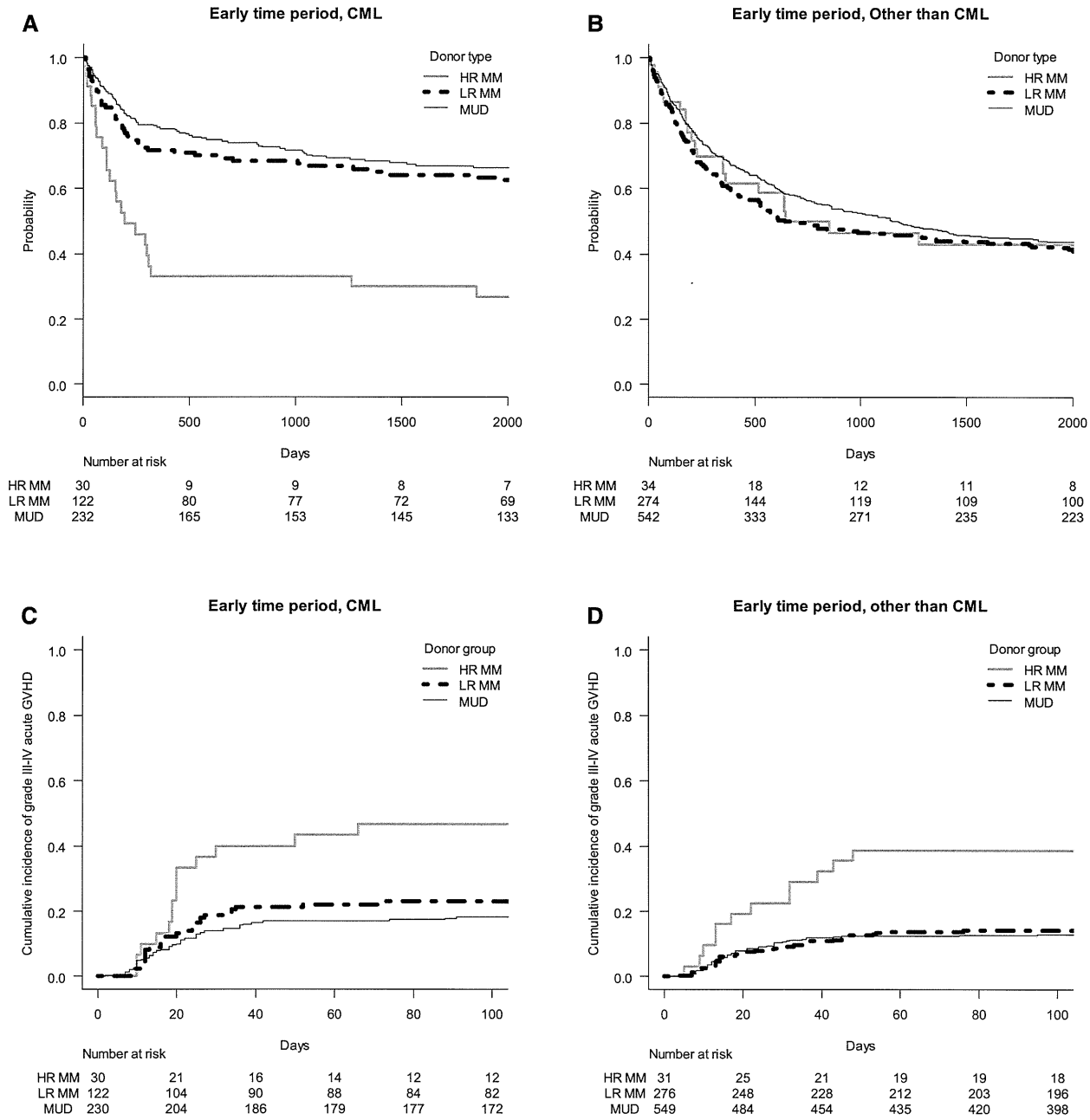

Figure 3. Adjusted overall survival (A,B) and the cumulative incidence of grade III to IV acute GVHD (C,D) grouped according to the underlying disease in the early time period. CML, chronic myelogenous leukemia; HR-MM, high-risk mismatch; LR-MM, low-risk mismatch; MUD, matched unrelated donor.

grade III to IV acute GVHD between the HR-MM and LR-MM groups was not statistically significant (HR, 1.06; 95% CI, .58 to 1.93; $P = .85$ and HR, .40; 95% CI; .10 to 1.64; $P = .21$, respectively). The presence of LR-MM significantly adversely affected the incidence of grade III to IV acute GVHD in the mid and late periods (HR, .64; 95% CI, .46 to .89; $P = .008$ and HR, .56; 95% CI, .39 to .80; $P = .0014$, respectively, for the MUD group).

Similarly, the presence of HR-MM significantly affected the incidence of grade II to IV acute GVHD compared with LR-MM only in the early time period (HR, 1.53; 95% CI, 1.05 to 2.24; $P = .028$), and not in the mid and late periods (HR, .92; 95% CI, .61 to 1.37; $P = .67$ and HR, .79; 95% CI, .40 to 1.58; $P = .51$, respectively).

Overall Survival

After adjusting for recipient age, recipient sex, presence of ABO-major mismatch, disease, disease risk, and GVHD prophylaxis, we again confirmed that survival in the HR-MM group was significantly inferior to that in the LR-MM group (HR, 1.46; 95% CI, 1.06 to 2.01; $P = .019$) in the early time period, whereas there was no significant difference between the MUD and LR-MM groups (HR, .86; 95% CI, .73 to 1.01; $P = .063$) (Table 3). On the other hand, the difference in survival between the HR-MM and LR-MM groups was not statistically significant in the mid and late time periods (HR, 1.06; 95% CI, .75 to 1.48; $P = .75$ and HR, .82; 95% CI, .42 to 1.62; $P = .58$, respectively). The difference in survival between the MUD and LR-MM groups was consistent among