Table 3 (*Continued*)

Case#	Karyotype	5/5q	1/7/7q	8	11q	12;21	13q	17p	20q
36 bm	48,XY,−5,+6,+add(8)(p15),+9,del(16)(q12q22),add(17)(p13)[16]/46,XY[4]	41	0	0	0	0	0	46	0
36 pb	NAM	29.5	0	0	0	0	0	29.5	0
37 bm	46,XX,−7,+mar[cp13]/46,XX[2]	0	52.5	0	0	0	0	0	0
37 pb	46,XX,−7,+mar[4]/46,XX[16]	0	22	0	0	0	0	0	0
38 bm	45,XY,−7[19]/46,XY[1]	0	97	0	0	0	0	0	0
38 pb	NAM	0	62	0	0	0	0	0	0
39 bm	46,XX[20]	0	0	0	0	0	0	0	0
39 pb	46,XX[5]	0	0	0	0	0	0	0	0
40 bm	46,XX,del(5)(q14q34)[20]	60.5	0	0	0	0	0	0	0
40 pb	46,XX,del(5)(q14q34)[10]	14.5	0	0	0	0	0	0	0
41 bm	46,XX,del(20)(q11)[18]/46,XX[3]	0	0	0	0	0	0	0	78
41 pb	NAM	0	0	0	0	0	0	0	71.5
42 bm	46,XY[20]	0	0	0	0	0	0	0	0
42 pb	46,XY[5]	0	0	0	0	0	0	0	0
43 bm	46,XY,t(1;14)(p35;q32)[20]	0	0	0	0	0	0	0	0
43 pb	46,XY,t(1;14)(p35;q32)[2]	0	0	0	0	0	0	0	0
44 bm	46,XY[20]	0	0	0	0	0	0	0	0
44 pb	46,XY[20]	0	0	0	0	0	0	0	0
45 bm	47,XX,+8[16]/46,XX[4]	0	0	86.5	0	0	0	0	0
45 pb	47,XX,+8[20]	0	0	90	0	0	0	0	0
46 bm	46,XX,del(5)(q14q34),del(11)(q21q24)[7]/46,XX[18]	6	0	0	5	0	0	0	0
46 pb	46,XX,del(5)(q14q34),del(11)(q21q24)[4]/46,XX[11]	0.5	0	0	0	0	0	0	0
47 bm	46,XY[20]	0	0	0	0	0	0	0	0
47 pb	46,XY[15]	0	0	0	0	0	0	0	0
48 bm	46,XY[20]	0	0	0	0	0	0	0	0
48 pb	46,XY[1]	0	0	0	0	0	0	0	0
49 bm	45,XY,der(2)t(2;3)(p13;p13)ins(2;3)(q33;p?p?),dic(3;20)(p11;q12),del(11)(q14q24)[19]/46,XY[1]	0	0	0	73	0	0	0	79
49 pb	NAM	0	0	0	33	0	0	0	29
50 bm	46,XY[20]	0	0	0	0	0	0	0	0
50 pb	NAM	0	0	0	0	0	0	0	0
51 bm	46,XY[20]	0	0	0	0	0	0	0	0
51 pb	NAM	0	0	0	0	0	0	0	0
52 bm	45,X,−Y[14]/46,XY[6]	0	0	0	0	0	0	0	0
52 pb	NAM	0	0	0	0	0	0	0	0
53 bm	45,XY,−7[20]	0	61	0	0	0	0	0	0
53 pb	NAM	0	34	0	0	0	0	0	0
54 bm	45,X,−Y[3]/46,XY[17]	0	0	0	0	0	0	0	0
54 pb	NAM	0	0	0	0	0	0	0	0
55 bm	47,XX,+20,del(20)(q11.2q13.3)x2[20]	0	0	0	0	0	0	0	55
55 pb	NAM	0	0	0	0	0	0	0	47
56 bm	46,XX[6]	0	0	0	0	0	0	0	0
56 pb	46,XX[3]	0	0	0	0	0	0	0	0
57 bm	48~59,XX,+X,+1,+2,del(5)(q13q33),+9,+10,+11,−13,+15,+16,+19,+21,+22,+22,+2mar[cp20]	76	57.5	0	48.5	38	0	0	0
57 pb	NAM	10	6	0	3	7.5	0	0	0
58 bm	45,X,−Y[20]	0	0	0	0	0	0	0	0
58 pb	NAM	0	0	0	0	0	0	0	0
59 bm	46,XX[20]	0	0	0	0	0	0	0	0
59 pb	NAM	0	0	0	0	0	0	0	0
60 bm	46,XY[20]	0	0	0	0	0	0	0	0
60 pb	46,XY[10]	0	0	0	0	0	0	0	0
61 bm	46,XX[25]	0	0	0	0	0	0	0	0
61 pb	NAM	0	0	0	0	0	0	0	0
62 bm	46,XX[27]	0	0	0	0	0	0	0	0
62 pb	NAM	0	0	0	0	0	0	0	0
63 bm	46,XY,del(5)(q14q33)[2]/46,XY[10]	11	0	0	0	0	0	0	0
63 pb	NAM	11	0	0	0	0	0	0	0
64 bm	45,XY,−7[6]/46,XY[3]	0	12.5	0	0	0	0	0	0
64 pb	NAM	0	11	0	0	0	0	0	0
65 bm	46,XY,+1,der(1;7)(p12;q10)[23]/46,XY[2]	0	19	0	0	0	0	0	0
65 pb	NAM	0	4.5	0	0	0	0	0	0
66 bm	46,XX,del(5)(q13q33)[19]/46,XX[6]	44	0	0	0	0	0	0	0
66 pb	NAM	22.2	0	0	0	0	0	0	0
67 bm	47,XY,+8[30]/46,XY[1]	0	0	77.5	0	0	0	0	0
67 pb	47,XY,+8[8]/46,XY[2]	0	0	30.5	0	0	0	0	0
68 bm	46,XY,+1,der(1;7)(p10;q10)[2]/46,XY[20]	0	11	0	0	0	0	0	0
68 pb	NAM	0	1.5	0	0	0	0	0	0
69 bm	45,XX,del(5)(q13q31),−21[2]/46,XX[6]	43	0	0	0	9	0	0	0
69 pb	46,XX,del(5)(q13q31)[7]/45,idem,−21[2]/46,XX[7]	46	0	0	0	7	0	0	0
70 bm	46,XY,del(5)(q22q31)[2]/46,XY[25]	14	0	0	0	0	0	0	0
70 pb	NAM	0.5	0	0	0	0	0	0	0
71 bm	46,XX[30]	0	0	0	0	0	0	0	0
71 pb	NAM	0	0	0	0	0	0	0	0
72 bm	46,XX,der(5)t(5;17)(q11.2;q11.2),+8,−17[20]/46,XX[20]	60.5	0	52.5	0	0	0	55	0
72 pb	NAM	10	0	3.5	0	0	0	8	0

Table 3 (*Continued*)

Case#	Karyotype	5/5q	1/7/7q	8	11q	12;21	13q	17p	20q
73 bm	46,XX,del(1)(p13p21),del(5)(q21q34)[6]/46,XX[14]	10.5	0	0	0	0	0	0	0
73 pb	NAM	3.5	0	0	0	0	0	0	0
74 bm	46,XY[20]	0	0	0	0	0	0	0	0
74 pb	NAM	0	0	0	0	0	0	0	0
75 bm	46,XX,idic(17)(p11.2)[2]/45,sl,−7[27]/90,sdl1x2[3]	0	87	0	0	0	0	90.5	0
75 pb	46,XX,idic(17)(p11.2)[1]/45,sl,−7[29]	0	93.5	0	0	0	0	85.5	0
76 bm	46,XX,del(5)(q13q33)[19]/46,XX[1]	46	0	0	0	0	0	0	0
76 pb	NAM	50.5	0	0	0	0	0	0	0
77 bm	42~43,X,−Y,−4,−5,−7,−8,−12,add(12)(q24),add(16)(q11.2),−17,−18, +r,+mar1,+mar2,+mar3,+mar4,+mar[cp15]/42~43,sl,−mar4, +mar5,+1~2mar[cp3]/46,XY[2]	54.5	0	32.5	0	64.5	0	62	0
77 pb	42~43,X,−Y,−4,−5,−7,−8,−12,add(12)(q24),add(16)(q11.2),−17,−18, +r,+mar1,+mar2,+mar3,+mar4,+mar[cp12]/42~43,sl,−mar4,+mar5,+1 ~2mar[cp5]/46,XY[3]	64.5	0	42.5	0	71	0	57	0
78 bm	NAM	0	0	0	0	0	0	0	0
78 pb	NAM	0	0	0	0	0	0	0	0
79 bm	46,XX[21]	0	0	0	0	0	0	0	0
79 pb	NAM	0	0	0	0	0	0	0	0
80 bm	44~45,XY,−4,del(5)(q23),−14,+1~2mar[10]/46,XY[11]	89	0	0	0	0	0	0	0
80 pb	44~45,XY,−4,del(5)(q23),−14,+1~2mar[12]	45	0	0	0	0	0	0	0
81 bm	46,XY[21]	0	0	0	0	0	0	0	0
81 pb	NAM	0	0	0	0	0	0	0	0
82 bm	46,XY,del(20)(q11)[10]/47,XY,+20,del(20)(q11)x2[7]/ 48,XY,+9,+20,del(20)(q11)x2[3]	0	0	0	0	0	0	0	79.5
82 pb	46,XY,del(20)(q11)[2]/47,XY,+20,del(20)(q11)x2[1]/ 49,XY,+9,+20,del(20)(q11)x2[5]/46,XY[2]	0	0	0	0	0	0	0	75
83 bm	43~44,X,add(Y)(q12),−5,add(6)(p22),−14,−15,add(17)(p10),−18, +1~2mar[11]/46,XY[10]	76.5	0	0	0	0	0	0	0
83 pb	46,XY[4]	9	0	0	0	0	0	0	0
84 bm	42~47,XY,del(1)(p13),−5,add(17)(p12),+1~5mar[11]/ 39~44,XY,del(1)(p13),−5,add(17)(p12),−18,−20,−21,+1~5mar[11]/ 46,XY[1]	68.5	0	0	0	0	0	0	0
84 pb	42~47,XY,del(1)(p13),−5,add(17)(p12),+1~5mar[12]	10.5	0	0	0	0	0	0	0
85 bm	46,XX[21]	0	0	0	0	0	0	0	0
85 pb	NAM	0	0	0	0	0	0	0	0
86 bm	46,XY[21]	0	0	0	0	0	0	0	0
86 pb	NAM	0	0	0	0	0	0	0	0
87 bm	46,XX,del(5)(q23),del(7)(q21)[21]	74	83	0	0	0	0	0	0
87 pb	46,XX,del(5)(q23),del(7)(q21)[3]	84	83	0	0	0	0	0	0
88 bm	44~45,XY,−3,add(5)(q33),del(5)(q22q33),del(7)(q13), der(7;12)(q10;q10),add(11)(p11),−12,−16,de1(21)(q22), +1~2mar[20]	84.5	86.5	0	29.5	79	0	0	0
88 pb	44~45,XY,−3,add(5)(q33),del(5)(q22q33),del(7)(q13), der(7;12)(q10;q10),add(11)(p11),−12,−16,de1(21)(q22), +1~2mar[20]	81	77	0	24.5	75.5	0	0	0
89 bm	47,XX,+8[23]	0	0	66	0	0	0	0	0
89 pb	47,XX,+8[12]/46,XX[8]	0	0	31	0	0	0	0	0
90 bm	46,XY,+1,der(1;7)(q10;p10)[6]/46,XY[16]	0	40.5	0	0	0	0	0	0
90 pb	46,XY,+1,der(1;7)(q10;p10)[3]/46,XY[3]	0	20.5	0	0	0	0	0	0
91 bm	47,XY,+19[25]	0	0	0	0	85	0	0	0
91 pb	47,XY,+19[18]/46,XY[5]	0	0	0	0	18	0	0	0
92 bm	46,XY,r(7)(p13q11.2)[18]/46,XY[2]	0	86	0	0	0	0	0	0
92 pb	46,XY,r(7)(p13q11.2)[20]	0	41.5	0	0	0	0	0	0
93 bm	45~48,XY,−3,del(5)(q14q33),del(7)(q22),add(7)(q32),+8, del(9)(p22),der(10)?t(3;10)(q12;q22),+11,−15,−18,del(20)(q11), −21,+2~4mar[cp20]/46,XY[1]	94	0	84.5	75	78.5	0	0	9
93 pb	45~48,XY,−3,del(5)(q14q33),del(7)(q22),add(7)(q32),+8, del(9)(p22),der(10)?t(3;10)(q12;q22),+11,−15,−18,del(20)(q11), −21,+2~4mar[cp22]/46,XY[1]	81	0	63	61	72.5	0	0	0
94 bm	45,XY,del(3)(p13),del(5)(q13q33),−7,del(12)(p13),der(12)t(7;12)(q11; p13),der(12)t(12;21)(p13;q22),−21,+mar[8]/44,sl,−Y[8]/43,sdl1, −der(12)[2]/46,XY[3]	62	0	0	0	40	0	0	0
94 pb	NAM	17	0	0	0	27	0	0	0
95 bm	46,XY[24]	0	0	0	0	0	0	0	0
95 pb	NAM	0	0	0	0	0	0	0	0
96 bm	46,XX,del(5)(q13q33)[4]/44,XX,der(5)t(1;5)(p31;q13), −7,dup(8)(q13q24),der(14;16)(q10;p1?)[10]/ 47~48,XX,der(5)t(1;5)(p31;q13),16,+1~2tas,+mar[cp6]/ 46,XX[2]	73.5	13	22	0	0	0	0	0
96 pb	NAM	48	11.5	4	0	0	0	0	0
97 bm	48,XY,+13,+14[2]/46,XY[18]	0	0	0	0	0	32	0	0
97 pb	NAM	0	0	0	0	0	24	0	0

Table 3 (*Continued*)

Case#	Karyotype	5/5q	1/7/7q	8	11q	12;21	13q	17p	20q
98 bm	45,XY,del(5)(q13q33),−17,del(20)(q12.1), der(20)t(17;20)(p11;q12)[3]/44,sl,−16[9]/44,sl,−18[4]/43,sl,−16, −18[3]/45,sl,der(4)t(4;17)(q35;p11)[2]/46,sl,+8[2]	72	0	11.5	0	0	0	16	53
98 pb	NAM	42	0	4	0	0	0	0	36
99 bm	48,XX,+8,+9[13]	0	0	86.5	0	0	0	0	0
99 pb	48,XX,+8,+9[7]	0	0	82	0	0	0	0	0
100 bm	46,XY[20]	0	0	0	0	0	0	0	0
100 pb	NAM	0	0	0	0	0	0	0	0

Table 4
Discordant Cases.

	Discordant cases	Comments
BM CCS vs. PB CCS	3/46 (or 6.5%)	54 PB CCS cases were NAM; 1 BM CCS was NAM
BM FISH vs. PB FISH	3/99 (or 3%)	Low level in BM FISH; 1 PB FISH not done
BM CCS vs. BM FISH	12/99 (or 12%)	Not detectable by FISH panel in 12 cases; 1 BM CCS was NAM
PB CCS vs. PB FISH	8/46 (or 17.3%)	54 PB CCS cases were NAM; 1 PB FISH not done

3.6. *PB: CCS vs. FISH*

Discordant results between PB CCS and FISH were more difficult to troubleshoot, as a large percentage (54%) of PB CCS was unsuccessful. Five cases showed an abnormal PB CCS result but were negative by PB FISH (Cases 1, 8, 12, 43 and 91). In four cases (Cases 1, 8, 43 and 91), abnormalities were not detectable by the FISH panel (*t*(8;10), *t*(14;18), *t*(1;14), +19). Case 12 showed only one trisomy 8 metaphase cell by PB CCS. While BM FISH was positive for trisomy 8 (17.5%), PB FISH was negative. Case 91 had trisomy 19 by BM and PB CCS, but was positive for a 12p/ETV6 deletion (TEL gene) by BM and PB FISH (85% and 18%, respectively).

As mentioned above, Case 25 had a der(1;7) by BM CCS, confirmed by BM FISH in 21.5% of the interphase cells scored, however the PB CCS was NAM and negative by PB FISH. Case 32 also had a der(1;7) by CCS on BM (14% positive by BM FISH), but NAM by PB CCS and negative by PB FISH (Table 3).

Three cases showed abnormal FISH findings with normal PB CCS (Cases 26, 29 and 83). In all three cases, the abnormal FISH findings are concordant with their BM CCS and FISH. In total there are 23 cases with NAM by CCS on PB, but abnormal PB FISH. All of the cases with NAM by PB CCS but abnormal PB FISH had BM CCS and BM FISH abnormalities, which were concordant with these findings (Table 5).

3.7. *Comparison of clone size by FISH*

A side-by-side comparison was made of the percent positive cells detected by FISH in both the BM and the PB for each probe combination (Table 3; Supplemental Data, Table 3). In general, for each probe set used, the percentage of FISH positive cells was typically higher in the BM than it was in the PB. Coefficients of correlation were calculated for each probe set, with the exception of the 13q probe set, as only one case had abnormalities of chromosome #13 (trisomy 13). The data show that all probe sets have a strong association between the BM FISH results and the PB FISH results (all regressions are significant at the 0.05 level), with the exception of the 5/5q probe set (Table 6).

A Student's *t*-test was also performed on the differences between BM and PB FISH for the different probe sets. This revealed that for the 5/5q, 1/7/7q, +8 and MLL probe sets, there was a significant difference ($p < 0.01$), while the p values for the t12;21, 17p and 20q probe sets were not statistically significant (Table 7). There did not appear to be any correlation with MDS classification, percent blasts, age, gender or previous treatment. Other clinical data were not available.

There were 24 cases with −5/5q− positive FISH (Supplemental Data, Fig. 1a). Cases 46 and 70, both positive by BM FISH (6% and 14%), were also called positive on PB FISH by the participating laboratories, however they were scored at 0.5% (or 1/200 nuclei analyzed). The majority of participating laboratories would not have called these results positive. However, as stated earlier, laboratories were to make their own cut-off value calls.

There were 16 cases, which were FISH positive for the 1/7/7q probe set (Supplemental Data, Fig. 1b). Cases 25 and 32 both had positive BM FISH (21.5% and 14%, respectively), but were negative on PB FISH (Table 3).

Table 5
Cases with NAM PB CCS and concordant BM and PB FISH.

Case #	Probe set	% Abn. BM FISH	% Abn. PB FISH
21	MLL	70	36.5
23	20q	92.5	72.5
35	5/5q	62.5	70
36	5/5q	41	29.5
	17p	46	29.5
38	1/7/7q	97	62
41	20q	78	71.5
49	MLL	73	33
	20q	79	29
53	1/7/7q	61	34
55	20q	55	47
57	5/5q	76	10
	1/7/7q	57.5	6
	MLL	48.5	3
	12;21	38	7.5
63	5/5q	11	11
64	1/7/7q	12.5	11
65	1/7/7q	19	4.5
66	5/5q	44	22.2
68	1/7/7q	11	1.5
70	5/5q	14	0.5
72	5/5q	60.5	10
	+8	52.5	3.5
	17p	55	8
73	5/5q	10.5	3.5
76	5/5q	46	50.5
94	5/5q	62	17
	12;21	40	27
96	5/5q	73.5	48
	1/7/7q	13	11.5
	+8	22	4
97	13q	32	24
98	5/5q	72	42
	+8	11.5	4
	17p	16	0
	20q	53	36

Table 6
Coefficient of correlation for each probe set comparing BM and PB FISH.

Probe set	Number of cases	Coefficient of correlation
5/5q	24	0.553
1/7/7q	16	0.876*
+8	15	0.832*
MLL	9	0.878*
12;21	9	0.655*
13q	1	Not done
17p	5	0.882*
20q	9	0.804*

* Regression is significant at the 0.05 level Graphs are shown in Supplemental Data.

Table 7
BM vs. PB FISH, *t*-test for significant differences.

Probe set	*p* value	Mean BM FISH	Mean PB FISH
5/5q	<0.01*	54.7	32.2
1/7/7q	<0.01*	46.8	29.3
+8	<0.01*	52.6	35.2
MLL	<0.01*	53	31
t(12;21)	0.02	59.3	39.4
13q	Not done	Not done	Not done
17p	0.04	53.9	36
20q	0.02	60.9	44.3

* Significantly different.

Fifteen cases were FISH positive for the chromosome 8 probe set (Supplemental Data, Fig. 1c). As discussed above, 17.5% of the interphase BM cells in case 12 were positive for trisomy 8 whereas the PB FISH study was negative. Concurrent BM CCS was abnormal (nine of 20 metaphase cells), with only a single metaphase cell observed in the PB, which had trisomy 8.

In all, there were nine cases with FISH positive results for the 11q probe set (Supplemental Data, Fig.1d). Case 34 had a complex karyotype on BM and PB CCS (Table 3), but was FISH positive in 8% of the BM cells and FISH negative by PB FISH (this case was positive by FISH and CCS on both BM and PB for deletion 5q). Lastly, Case 46 was FISH positive in 5% of the BM cells scored, negative by PB FISH and had deletion 5q and 11q by BM and PB CCS.

Nine cases showed a positive FISH result using the 12;21 (TEL(ETV6)/AML1(RUNX1)) dual fusion probe set (Supplemental Data, Fig.1e). This probe set was used to detect monosomy 21/trisomy 21 or deletion 12p/ETV6 (TEL). It is interesting to note that Case 69 had deletion 5q and monosomy 21, both of which were observed on CCS on BM and PB. While the deletion 5q was detectable by FISH in both BM and PB (43% vs. 46%), the monosomy 21 was also detectable, but at a much lower level (9% vs. 7%), consistent with a secondary aberration (Table 3).

There was only one case with FISH positive results using the monosomy 13/deletion 13q probe set (data not shown). One case, Case 97, is positive for trisomy 13 by FISH in both BM and PB (32% vs. 24%).

There were five cases of FISH positive results using the −17/17p− probe set (Supplemental Data, Fig.1f). Case 98 had a complex karyotype on BM CCS. While other probes were informative by FISH on both BM and PB, 16% of cells were positive for loss of D17Z1 (with two copies of P53) by BM FISH, but negative on PB FISH (Table 3).

Finally, there were nine cases with FISH positive results using the deletion 20q probe set (Supplemental Data, Fig.1g). Case 93, also had a complex karyotype on both BM and PB by CCS and was concordant for FISH results using other probe sets (5/5q−, +8, 11q, *t*(12;21)), deletion 20q was observed in 9% of BM cells by FISH, but was negative by PB FISH (Table 3).

4. Discussion

Phase I of this study showed that there was very good agreement between the 15 participating laboratories when studying two samples with known clonal chromosomal abnormalities (Supplemental Data, Tables 1 and 2). Although all participating sites are designated MDS Centers of Excellence and have considerable FISH experience, the goal of the familiarization phase was to determine to what extent the methods, nomenclature, interpretation of the results and data entry aspects may vary among the laboratories. This phase proved the participants used similar procedures for processing and reporting FISH data and provided data entry experience into a secured database; however cut-off value variability and methods used to determine cut-off values resulted in a few minor discrepancies (Supplemental Data, Appendix I). A review of the data to clear up the data entry errors and standardization of the FISH scoring criteria was implemented by distributing FISH scoring diagrams.

Phase II of our study showed good concordance between BM and PB, both by CCS and FISH (Tables 3 and 4). Three discordant results were clear when comparing BM and PB FISH – Cases 12, 25 and 32. All of these discordant cases had a relatively low rate of abnormal cells (17.5%, 21.5% and 14%, respectively) (Table 3). This is in agreement with earlier studies [10–12], which showed that the sensitivity of PB FISH was largely determined by the size of the abnormal clone in the BM, with lower rates of PB FISH positive cells, even when the PB is enriched for CD34+ cells [11].

There have been numerous studies regarding the ability of MDS FISH panels to detect occult or cryptic anomalies in the bone marrow of MDS patients [7,8,13–16]. These studies described abnormalities in 3–18% of cases, however the clinical significance of these cryptic anomalies has never been established. More recently, the effectiveness and efficiency of MDS FISH panels has been questioned, since the vast majority of MDS FISH panel studies do not find occult anomalies when a full analysis of 20 metaphase cells is performed [17–19]. In the second phase of our study, we found one case with an apparently cryptic anomaly detected by our FISH panel – Case 91 (Table 3). Case 91 had trisomy 19 by both BM and PB CCS, but both BM and PB FISH were positive for deletion 12p13/ETV6 in 85% and 18% of cells, respectively. Therefore, our study's rate of cryptic anomalies detected by FISH is 1%.

In 54% of the cases, PB CCS was unsuccessful. This finding was not unexpected, as the tumor burden in MDS is expected to be relatively low in the periphery. In this study, lower grade MDS samples with single abnormalities of trisomy 8, del(20q) and der(1;7) in ≤20% bone marrow cells were not generally detectable in the circulation (Supplemental Data, Table 3). However, abnormalities were detected by PB FISH when the clone was large enough. As shown by Li et al. [10] when studying 60 cases of MDS, the percentage of clonal bone marrow cells and the percentage of blasts (or mean clonal/blast disparity) was very different (50.1% vs. 7.0%, respectively). This disparity was much lower when looking at AML patients, as well as advanced MDS patients. FISH was performed on 22 MDS patients in the Li et al. study. FISH showed that the percentage of clonal cells was not very different when comparing BM and PB on the same patient (49.8% vs. 39.1%, respectively).

When comparing successful PB CCS with negative PB FISH, four of the five discordant cases had abnormalities, which could not be detected by the FISH panel (Cases 1, 8, 43 and 91). The fifth case, Case 12, had only one trisomy 8 metaphase cell by PB CCS, while BM FISH confirmed trisomy 8 in 17.5% of the cells scored, PB FISH was negative. Clearly, low-level clonality in the PB is a major concern in MDS patients. In addition, Case 91 had trisomy 19 by both BM and PB CCS, but both BM and PB FISH showed deletion 12p. As stated earlier, the finding of a hidden or cryptic clone occurred in only one of our cases (or 1%).

As in the limited number of cases in the literature, it appears that the lack of detection of an abnormal clone by PB FISH is mostly correlated with lower grade MDS with single aberrations and the size of the abnormal clone in the BM detected by FISH [10–12]. In most cases, when there are approximately 20% FISH positive cells in the BM, it is possible to detect FISH positive cells in the PB, albeit at a typically reduced rate.

While array comparative genomic hybridization (aCGH) has been used in studying the BM of MDS patients [20–24] and has shown some utility in detecting otherwise cryptic abnormalities, there is no evidence to date that PB could be substituted for BM unless the tumor burden is high enough in the periphery. Low levels of mosaicism may be missed in the PB.

In summary, our study showed that there is very good correlation between BM and PB FISH (3% discordance). Overall, from our study, we conclude that while PB cannot be substituted for BM at diagnosis, it could be used when serial bone marrow samples are not possible to monitor the clinical course when a FISH detectable clone has been established. Following such a clone over time, as in Braulke et al. [11], might also help to determine when to treat and/or the effectiveness of treatment. As well, it would replace the need for serial bone marrows with the ease of a blood draw for clinical follow-up.

Conflict of interest statement

There is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.leukres.2012.03.013.

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Phase I/II study of decitabine in patients with myelodysplastic syndrome: A multi-center study in Japan

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The management of myelodysplastic syndrome (MDS) remains challenging. We performed a phase I/II study to evaluate the safety and efficacy of decitabine in patients with MDS in Japan. Patients with MDS with red cell transfusion dependence or 5–30% blasts in marrow and with an International Prognostic Scoring System score of intermediate-1 or higher were eligible. Patients received intravenous decitabine at 15 or 20 mg/m^2 daily for 5 days every 4 weeks. A total of 37 patients were enrolled. Three patients received 15 mg/m^2 and experienced no dose limiting toxicity during the first cycle. Thirty-four patients received 20 mg/m^2. Grade 3 or greater non-hematologic toxicities included cerebral infarction (n = 1), subdural hematoma (n = 1), elevated blood glucose (n = 1), and pulmonary hypertension (n = 1). At 20 mg/m^2, complete response, partial response, and hematologic improvement were observed in 7 (20.6%), 2 (5.9%), and 7 (20.6%) patients, respectively. Complete cytogenetic response was observed in 30% of evaluable 20 patients. The median number of cycles to clinical response was 4 (range 4–8), and duration of remission was 474+ days (range 294–598+). The 2-year rate of acute myeloid leukemia-free survival was 52%. Correlative studies revealed hypomethylation in multiple genes in peripheral blood cells after treatment. Hypomethylation was generally more profound in CD15 + peripheral blood cells, which reflects myeloid cells, than in peripheral blood mononuclear cells. In summary, decitabine was safe and demonstrated efficacy in Japanese patients with high-risk MDS. This trial was registered at ClinicalTrials.gov (NCT00796003). (*Cancer Sci* 2012; 103: 1839–1847)

Myelodysplastic syndrome (MDS) is a heterogeneous group of hematopoietic stem cell disorders presenting as cytopenias and dysplastic hematopoiesis with or without increased blast cells.[1] The disease is associated with a dismal outcome due to progression of cytopenias or transformation to acute leukemia. Management of MDS varies depending on a patient's age, degree of cytopenias, performance status and estimated risk of disease progression[1] as assessed by, for example, the International Prognostic Scoring System (IPSS) score.[2]

Development of MDS is a multistep event. One important mechanism involved is epigenetic change, such as promoter DNA methylation.[3] Frequent methylation often confers a poor prognosis.[4] In recent years, treatment with DNA methyltransferase inhibitors has been studied extensively in the management of MDS. Two drugs in this class, azacitidine[5] and decitabine,[6–8] have been approved in the management of MDS in multiple countries. However, very little data exist regarding treatment of Asian patients with decitabine.[9] Therefore, we conducted a phase I/II study in Japan to assess the safety and efficacy of decitabine in Japanese patients with high-risk MDS. We also performed correlative methylation analysis using 3-fucosyl-*N*-acetyl-lactosamine (CD15)-positive myeloid cells selected from the peripheral blood of patients undergoing treatment with decitabine.

Materials and Methods

This open-label multicenter phase I/II study of decitabine in patients with MDS was approved by the institutional review boards of each participating institution and was conducted in compliance with the International Conference on Harmonisation Good Clinical Practice guidelines (ClinicalTrials.gov identifier: NCT00796003).

Phase I: The primary objective was to assess the safety of intravenous decitabine at 15 and 20 mg/m^2 administered over 1 h daily for 5 days every 4 weeks. Secondary objectives were pharmacokinetic and pharmacodynamic assessments during the first cycle of treatment.

Phase II: The primary objective was to evaluate patient response (rates of complete response [CR] and partial response [PR]). Secondary objectives were time to response, response duration, time to acute myelogenous leukemia (AML) or death, transfusion dependency, and cytogenetic response.

Patients. Eligibility criteria included the following: diagnosis of MDS based on French-American-British (FAB) morphologic classification, including refractory anemia (RA), RA with ringed sideroblasts (RARS), RA with excess blasts (RAEB), RAEB in transformation and chronic myelomonocytic leukemia (CMML); patients with RA or RARS were to have required red blood cell transfusion more than once every 4 weeks, while patients with CMML were to have a white blood cell count of <13 000/mm^3; IPSS assessment of intermediate or high risk; age 20 years or older; Eastern Cooperative Oncology Group (ECOG) performance status of 0–2; and normal organ functions including creatinine $\leq$176.8 μM (2 mg/dL), bilirubin $\leq$ 25.7 μM (1.5 mg/dL) and aspartate

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aminotransferase and alanine aminotransferase levels $\leq 2 \times$ the upper limit of normal.

Patients were ineligible if they had: bone marrow blast percentage $\geq 30\%$ on local marrow review; prior chemotherapy with cytarabine ≥ 1 g/m^2; or other serious comorbidities, active infectious disease, autoimmune cytopenia, hepatitis B surface antigen positivity, hepatitis C antibody positivity or HIV antibody positivity. Pregnant or lactating female patients were also ineligible.

Treatment. Two decitabine doses and schedules have been recommended in previous studies in MDS, namely 15 mg/m^2 over 3 h every 8 h for 3 days every 6-week cycle[6] and 20 mg/m^2 over 1 h daily for 5 days every 4-week cycle.[7,8] Because the latter method has shown clinical efficacy and convenience in the outpatient setting, we chose the 5-day dosing schedule for this study. We started treatment at 15 mg/m^2 daily, escalating to 20 mg/m^2 after safety was assessed.

Treatment was repeated every 4 weeks in the absence of dose limiting toxicity (DLT, defined below) or disease progression. In order to receive the next cycle of treatment, a patient was required to have normal organ function as defined above. If the patient did not meet these criteria on day 29, a maximum 2-week delay was allowed. Treatment was also delayed if the patient had febrile neutropenia, grade 3 or 4 infection with neutropenia, or $\geq$ grade 2 bleeding. If, by day 43, the patient did not meet the above criteria, the patient was taken off the study.

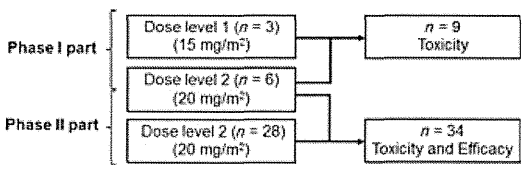

Fig. 1. Study schema. A total of 37 patients were treated in this study. In the phase I part, three received 15 mg/m^2 and six received 20 mg/m^2. In the phase II part, 28 additional patients received 20 mg/m^2. Toxicity was analyzed in all patients. Efficacy was analyzed in all patients who received 20 mg/m^2.

Antiemetics were not routinely given. The use of erythropoietin was not allowed. Granulocyte colony stimulating factor was allowed as clinically indicated, but response was recorded when patients were not receiving growth factor support. Dose reductions of the study drug were not allowed.

Toxicity and response evaluation. Toxicity was evaluated based on Common Terminology Criteria for Adverse Event (CTCAE) version 3.0. DLT was defined as: non-hematologic toxicity $\geq$ grade 3, excluding nausea and vomiting, and neutropenic fever and infection $\geq$ grade 3 that did not improve despite a delay in the initiation of the next cycle of treatment by 2 weeks.

Bone marrow aspiration slides were centrally reviewed and final data analysis was conducted based on central review. Response was evaluated based on International Working Group (IWG) 2000[10] and IWG 2006[11] response criteria in myelodysplasia. Complete cytogenetic response was defined as the disappearance of cytogenetic abnormalities; partial cytogenetic response was defined as a $\geq 50\%$ reduction in cytogenetic abnormalities.[10,11] Response duration was calculated from first evidence of response until disease progression.[10,11] Survival was calculated from start of therapy to death from any cause. Time to AML was calculated from start of therapy to the first date of documented marrow blast percentage $\geq 30\%$.

Statistical considerations. In phase I, three patients were to be accrued at 15 mg/m^2. If DLT was not observed in any patients during the first cycle, six more patients were accrued

Table 1. Baseline characteristics of patients

Patient characteristics	Phase	I		I (*n* = 6) and II (*n* = 28)	Total
	Dose	15 mg/m^2	20 mg/m^2	20 mg/m^2	
n		3	6	34	37
Sex	Male	3 (100.0%)	4 (66.7%)	26 (76.5%)	29 (78.4%)
	Female	0	2 (33.3%)	8 (23.5%)	8 (21.6%)
Age	<65	0	3 (50.0%)	10 (29.4%)	10 (27.0%)
	65–74	3 (100.0%)	1 (16.7%)	17 (50.0%)	20 (54.1%)
	>75	0	2 (33.3%)	7 (20.6%)	7 (18.9%)
	Median	68 (67–71)	66.5 (54–77)	69 (52–81)	69 (52–81)
PS	0	1 (33.3%)	5 (83.3%)	22 (64.7%)	23 (62.2%)
	1	2 (66.7%)	1 (16.7%)	12 (35.3%)	14 (37.8%)
Type	De novo	2 (66.7%)	5 (83.3%)	29 (85.3%)	31 (83.8%)
	Secondary	1 (33.3%)	1 (16.7%)	5 (14.7%)	6 (16.2%)
Hb (g/dL)	Median (range)	7.1 (5.4–8.7)	7.6 (7.1–8.5)	8.1 (4.7–15.2)	8.0 (4.7–15.2)
Neu (/μL)	Median (range)	434 (312–1243)	887 (173–1910)	898 (143–7416)	807 (143–7416)
Plt	Median (range)	1.2 (1.1–3.6)	7.6 (3.1–22.4)	5.25 (0.3–74.2)	4.9 (0.3–74.2)
Serum EPO (mU/mL)	Median (range)	594 (132–728)	615.5 (31.6–1840)	369 (1538–13 600)	393 (15.8–13 600)
Time from diagnosis	Median	1.1 (0.1–4.1)	1.1 (0.1–4.2) 5	0.55 (0.1–12.7)	0.6 (0.1–12.7)
Prior chemotherapy for MDS	Yes	0	1 (16.7%)	6 (17.6%)	6 (16.2%)
RBC transfusion dependent	Yes	2	4 (66.7%)	25 (73.5%)	27 (73.0%)
Platelet transfusion dependent	Yes	0	0	5 (14.7%)	5 (13.5%)
Previous G-CSF	Yes	0	1 (16.7%)	1 (2.9%)	1 (2.7%)
Type of MDS and IPSS	*De novo* – Low	0	0	1 (2.9%)	1 (2.7%)
	De novo – Intermediate-1	1 (33.3%)	1 (16.7%)	9 (26.5%)	10 (27.0%)
	De novo – Intermediate-2	0	2 (33.3%)	7 (20.6%)	7 (18.9%)
	De novo – High	0	1 (16.7%)	11 (32.4%)	11 (29.7%)
	Secondary MDS	1 (33.3%)	1 (16.7%)	5 (14.7%)	6 (16.2%)
	AML	1 (33.3%)	1 (16.7%)	1 (2.9%)	2 (5.4%)

EPO, erythropoietin; G-CSF, granulocyte-colony stimulating factor; IPSS, International Prognostic Scoring System; MDS, myelodysplastic syndrome; RBC, red blood cell.

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Table 2. Baseline characteristics of bone marrow

Bone marrow characteristics	Phase	I		I (n = 6) and II (n = 28)	Total
	Dose	15 mg/m^2	20 mg/m^2	20 mg/m^2	
n		3	6	34	37
Marrow Blast percent	<5	0 (0.0%)	4 (66.7%)	12 (35.3%)	12 (32.4%)
	5–10	2 (66.7%)	0 (0.0%)	4 (11.8%)	6 (16.2%)
	11–20	0 (0.0%)	0 (0.0%)	11 (32.4%)	11 (29.7%)
	21–30	0 (0.0%)	1 (16.7%)	6 (17.6%)	6 (16.2%)
	>30	1 (33.3%)	1 (16.7%)	1 (2.9%)	2 (5.4%)
	Median	9.2 (7.4–37.1)	4.2 (2.2–52.5)	11.1 (1.0–52.5)	11 (1–52.5)
Bone marrow cellularity	Hyper	0 (0.0%)	3 (50.0%)	13 (38.2%)	13 (35.1%)
	Normo	1 (33.3%)	2 (33.3%)	9 (26.5%)	10 (27.0%)
	Hypo	2 (66.7%)	1 (16.7%)	12 (35.3%)	14 (37.8%)
FAB classification	RA	0 (0.0%)	3 (50.0%)	11 (32.4%)	11 (29.7%)
	RARS	0 (0.0%)	1 (16.7%)	1 (2.9%)	1 (2.7%)
	RAEB	2 (66.7%)	0 (0.0%)	14 (41.2%)	16 (43.2%)
	RAEB-T	0 (0.0%)	1 (16.7%)	7 (20.6%)	7 (18.9%)
	CMML	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	AML	1 (33.3%)	1 (16.7%)	1 (2.9%)	2 (5.4%)
WHO classification	RA	0 (0.0%)	0 (0.0%)	3 (8.8%)	3 (8.1%)
	RARS	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	RCMD	0 (0.0%)	3 (50.0%)	7 (20.6%)	7 (18.9%)
	RCMD-RS	0 (0.0%)	1 (16.7%)	1 (2.9%)	1 (2.7%)
	RAEB-1	2 (66.7%)	0 (0.0%)	4 (11.8%)	6 (16.2%)
	RAEB-2	0 (0.0%)	0 (0.0%)	10 (29.4%)	10 (27.0%)
	MDS-U	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	Other	0 (0.0%)	0 (0.0%)	1 (2.9%)	1 (2.7%)
	AML	1 (33.3%)	2 (33.3%)	8 (23.5%)	9 (24.3%)
Chromosome	Any chromosome abnormality	1 (33.3%)	2 (33.3%)	17 (50.0%)	18 (48.6%)
	Good	2 (66.7%)	4 (66.7%)	14 (41.2%)	16 (43.2%)
	Intermediate	0 (0.0%)	0 (0.0%)	3 (8.8%)	3 (8.1%)
	Poor	1 (33.3%)	2 (33.3%)	17 (50.0%)	18 (48.6%)
	Chromosome 7	0 (0.0%)	1 (16.7%)	12 (35.3%)	12 (32.4%)
	Complex	1 (33.3%)	2 (33.3%)	12 (35.3%)	13 (35.1%)

AML, acute myelogenous leukemia; CMML, chronic myelomonocytic leukemia; FAB, French-American-British; MDS, myelodysplastic syndrome; RA, refractory anemia; RARS, RA with ringed sideroblasts; RAEB, RA with excess blasts; RAEB-T, RAEB in transformation; RCMD, refractory cytopenia with multilineage dysplasia; RCMD-RS, RCMD with ringed sideroblast; WHO, World Health Organization.

Table 3. Observed toxicities

	Grade	15 mg/m^2 (n = 3)			20 mg/m^2 (n = 34)		
		1 or 2	3	4	1 or 2	3	4
Hematologic	Leukocytopenia	0	0	3 (100%)	2 (5.9%)	9 (26.5%)	23 (67.6%)
	Neutropenia	0	0	3 (100%)	0	2 (5.9%)	25 (73.5%)
	Thrombocytopenia	0	0	3 (100%)	1 (2.9%)	6 (17.6%)	21 (61.8%)
	Anemia	0	1 (33.3%)	2 (66.7%)	1 (2.9%)	13 (38.2%)	16 (47.1%)
	Lymphocytopenia	0	2 (66.7%)	1 (33.3%)	11 (32.4%)	11 (32.4%)	6 (17.6%)
Infection	Febrile neutropenia	0	0	1 (33.3%)*	0	8 (23.5%)	2 (5.9%)*
	Other infection	1 (33.3%)	1 (33.3%)	0	6 (17.6%)	10 (29.4%)	0
General	Insomnia	1 (33.3%)	0	0	0	0	0
	Abdominal pain	1 (33.3%)	0	0	0	0	0
Dermatologic	Erythema multiforme	1 (33.3%)	0	0	0	0	0
Gastrointestinal	Elevated transaminase	1 (33.3%)	0	0	8 (23.5%)	1 (2.9%)	0
	Elevated bilirubin	1 (33.3%)	0	0	0	0	0
	Elevated alkaline phosphatase	1 (33.3%)	0	0	0	0	0
Endocrine	Hyperglycemia	0	0	0	9 (26.5%)	1 (2.9%)	0
Cardiovascular	Cerebral infarction	0	0	0	0	1 (2.9%)	0
	Pulmonary hypertension	0	0	0	0	1 (2.9%)	0
Hemorrhage	Subdural hematoma	0	0	0	0	1 (2.9%)	0

*Including grade 5 pneumonia (n = 1 each).

Table 4. Response to decitabine treatment

	Doses	15 mg/m^2 (n = 3)	20 mg/m^2 (n = 34)	Total (n = 37)
IWG 2000	CR	0 (0.0%)	7 (20.6%)	7 (18.9%)
	PR	1 (33.3%)	2 (5.9%)	3 (8.1%)
	HI	1 (33.3%)	5 (14.7%)	6 (16.2%)
	SD	1 (33.3%)	4 (11.8%)	5 (13.5%)
	PD	0 (0.0%)	3 (8.8%)	3 (8.1%)
	NE	0 (0.0%)	13 (38.2%)	13 (35.1%)
	CR + PR (%, 95% CI)	1 (33.3% [0.8–90.6%])	9 (26.5% [12.9–44.4%])	10 (27.0% [13.8–44.1%])
	CR + PR + HI (%, 95% CI)	2 (66.7% [9.4–99.2%])	14 (41.2% [24.6–59.3%])	16 (43.2% [27.1–60.5%])
IWG2006	CR	0 (0.0%)	7 (20.6%)	7 (18.9%)
	PR	1 (33.3%)	2 (5.9%)	3 (8.1%)
	mCR	1 (33.3%)	1 (2.9%)	2 (5.4%)
	HI	0 (0.0%)	4 (11.8%)	4 (10.8%)
	SD	1 (33.3%)	4 (11.8%)	5 (13.5%)
	PD	0 (0.0%)	3 (8.8%)	3 (8.1%)
	NE	0 (0.0%)	13 (38.2%)	13 (35.1%)
	CR + PR (%, 95% CI)	1 (33.3% [0.8–90.6%])	9 (26.5% [12.9–44.4%])	10 (27.0% [13.8–44.1%])
	CR + PR + mCR (%, 95% CI)	2 (66.7% [9.4–99.2%])	10 (29.4% [15.1–47.5%])	12 (32.4% [18.0–49.8%])
	CR + PR + mCR+HI (%, 95% CI)	2 (66.7% [9.4–99.2%])	14 (41.2% [24.6–59.3%])	16 (43.2% [27.1–60.5%])

CR, complete response; HI, hematologic improvement; mCR, marrow complete response; NE, not evaluated; PD, progressive disease; PR, partial response; SD, stable disease.

at 20 mg/m^2. If the 20 mg/m^2 dose was not associated with DLT, this population was carried into phase II. Based on prior studies (response rate 17–35%[6–8,12]), the expected response rate was 25%. The drug was considered ineffective if the response rate was $\leq$5%. With $\alpha = 0.05$ and $\beta = 0.2$, 21 patients would be required to evaluate drug efficacy. Estimating that up to 20% of patients might be found ineligible after central pathologic review, a total of at least 26 patients (including six patients from phase I study at the same dose) were to be enrolled.

Correlative analysis. *Pharmacokinetics.* Plasma decitabine levels were analyzed on days 1 and 5 of treatment in patients in phase I. Blood was drawn at 0, 30, 60, 65, 75, 90, 120, 180 and 240 min after initiation of infusion. Samples were immediately stored at 4°C, and plasma decitabine concentrations were determined by liquid chromatography coupled with tandem mass spectrometry (lower limit of quantification: 1.0 ng/mL).[13]

Pharmacodynamics. Preparation of patient samples: In phase I, we conducted DNA methylation analysis using peripheral blood samples from all nine patients. Samples were obtained before treatment (at baseline) and on days 5, 12, and 28 of the first cycle of decitabine treatment. Two separate cell populations were obtained from whole blood: peripheral blood mononuclear cells (PBMCs) were isolated with standard ficoll separation of whole blood and CD15-positive peripheral blood cells (CD15 + PBCs) were isolated using CD15-recognizing antibodies (Dynabeads CD15; Life Technologies, Carlsbad, CA, USA). CD15 + PBCs were chosen because it has been shown that the majority of PBMCs are lymphocytes, which may not be representative of myelodysplastic cells.[14] CD15, however, is expressed on myeloid cells, including neutrophils, eosinophils and monocytes, and this selection enriched the myeloid cell population and eliminated lymphocytes.

Bisulfite-pyrosequencing for DNA methylation analysis: DNA methylation levels were quantitatively measured using bisulfite-pyrosequencing[15–17] (Pyrosequencing AB, Uppsala, Sweden) for *PGR*, *ESR1*, *CDH1*, *CDH13*, and *LINE1*. The list of primers for bisulfite-pyrosequencing is provided in Table S1. *LINE1* is a repetitive component that we used as a surrogate for global methylation. The methylation levels at different CpG sites were averaged to represent the degree of methylation.

Methylated CpG island amplification and microarray analysis: The DNA methylation status of CD15 + PBC during the first cycle of treatment was further analyzed with methylated CpG island amplification and microarray (MCAM) technology as previously reported[18] using microarrays from Agilent Technologies, which analyzes 6157 genes.

Results

Study enrollment. In phase I, the first cycle of treatment was not associated with DLT. Therefore, the study was expanded into phase II. Data from 34 patients treated with 20 mg/m^2 decitabine were analyzed for safety and efficacy (Fig. 1). Patient characteristics and disease characteristics are summarized in Tables 1 and 2, respectively. All patients had MDS based on local hemato-morphological review, among whom two were found to have AML upon central review of marrow (blast count $\geq$ 30%).

Treatment delivery. A total of 298 cycles were given to 37 patients. A median of 6.0 (range 1–17) and 5.5 cycles (1–17) were delivered per patient in the whole group (15 and 20 mg/m^2) and 20 mg/m^2 group, respectively. Drug administration was delayed due to cytopenia and/or infection by longer than 7 days (i.e. interval >5 weeks) for 84 cycles (28%). Eight patients experienced DLT within two cycles of treatment and

Table 5. Cytogenetic response in patients who originally had cytogenetic abnormalities

Patient No.	Karyotype	Chromosome abnormality	Cytogenetic response
11	Poor	Chromosome 7 abnormalities, complex	Major/ Complete
16	Poor	Chromosome 7 abnormalities, complex	Major/ Complete
18	Intermediate	Trisomy 8	Major/ Complete
20	Poor	Complex	Major/ Complete
24	Good	20q-	Major/ Complete
25	Poor	Complex	Minor/Partial
28	Poor	Chromosome 7 abnormalities, complex	Major/ Complete

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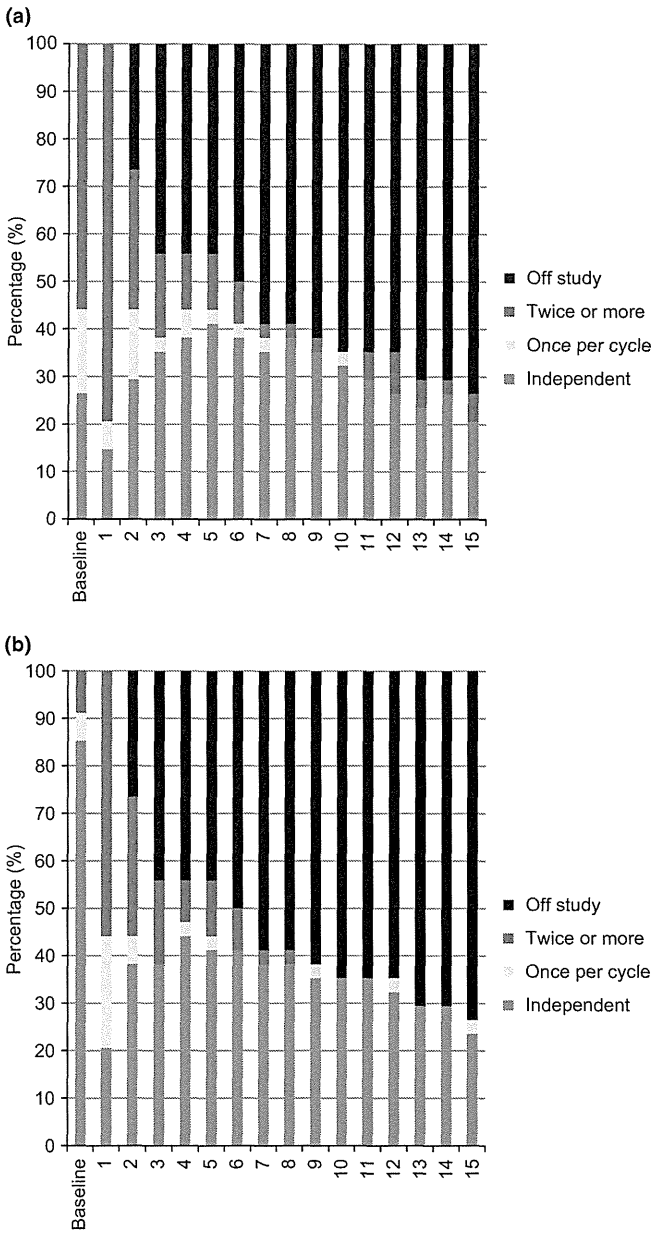

Fig. 2. Transfusion independence. Transfusion independence was calculated using all enrolled patients as the denominator (n = 34). (a) Red cell transfusion independence. The transfusion independence rate increased from 26% (baseline) to 41% after five cycles. (b) Platelet transfusion independence. The color indicates the number of transfusions required per cycle. At baseline 85% of patients were transfusion independent. The majority of patients who continued on treatment past cycle 6 did not require platelet transfusion after that cycle.

did not continue treatment. Reasons for discontinuation included pneumonia associated with neutropenia (n = 5), fungal pneumonia with pulmonary hypertension (n = 1), chronic subdural hematoma (n = 1), and elevated liver enzymes (n = 1). Seven patients were actively receiving decitabine upon trial closure in March 2011 and had received 14–22 cycles of treatment.

Toxicity. Toxicity data are summarized in Table 3. If one patient experienced the same toxicity multiple times, the highest grade is recorded.

15 mg/m^2 group. None of the three patients enrolled in the phase I study experienced DLT during the first cycle of

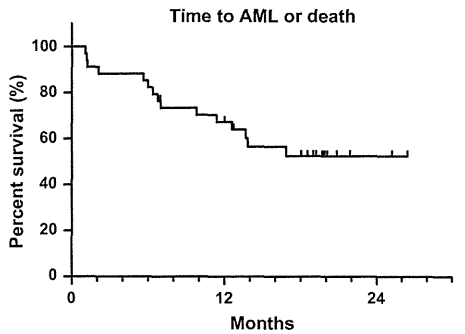

Fig. 3. Time to acute myeloid leukemia or death. Median time to acute myeloid leukemia or death has not been reached. The 2-year rate was 52%.

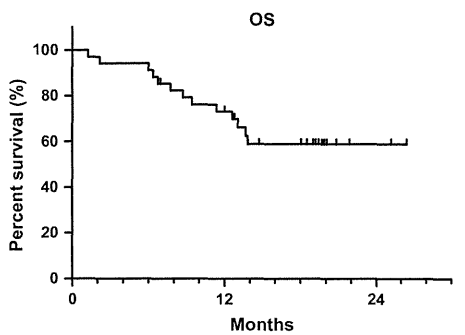

Fig. 4. Overall survival. Median survival has not been reached. The 2-year rate was 56%.

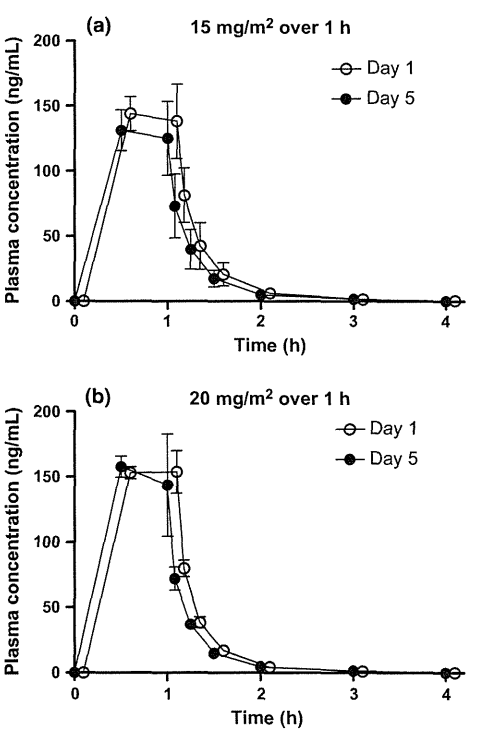

Fig. 5. Plasma concentrations of decitabine – time profile (Mean ± standard error of mean). Mean plasma concentration is plotted with standard error of mean. The data are from: (a) three patients receiving 15 mg/m^2, and (b) five patients receiving 20 mg/m^2 (one patient receiving 20 mg/m^2 in phase I chose not to participate in the pharmacokinetic analysis).

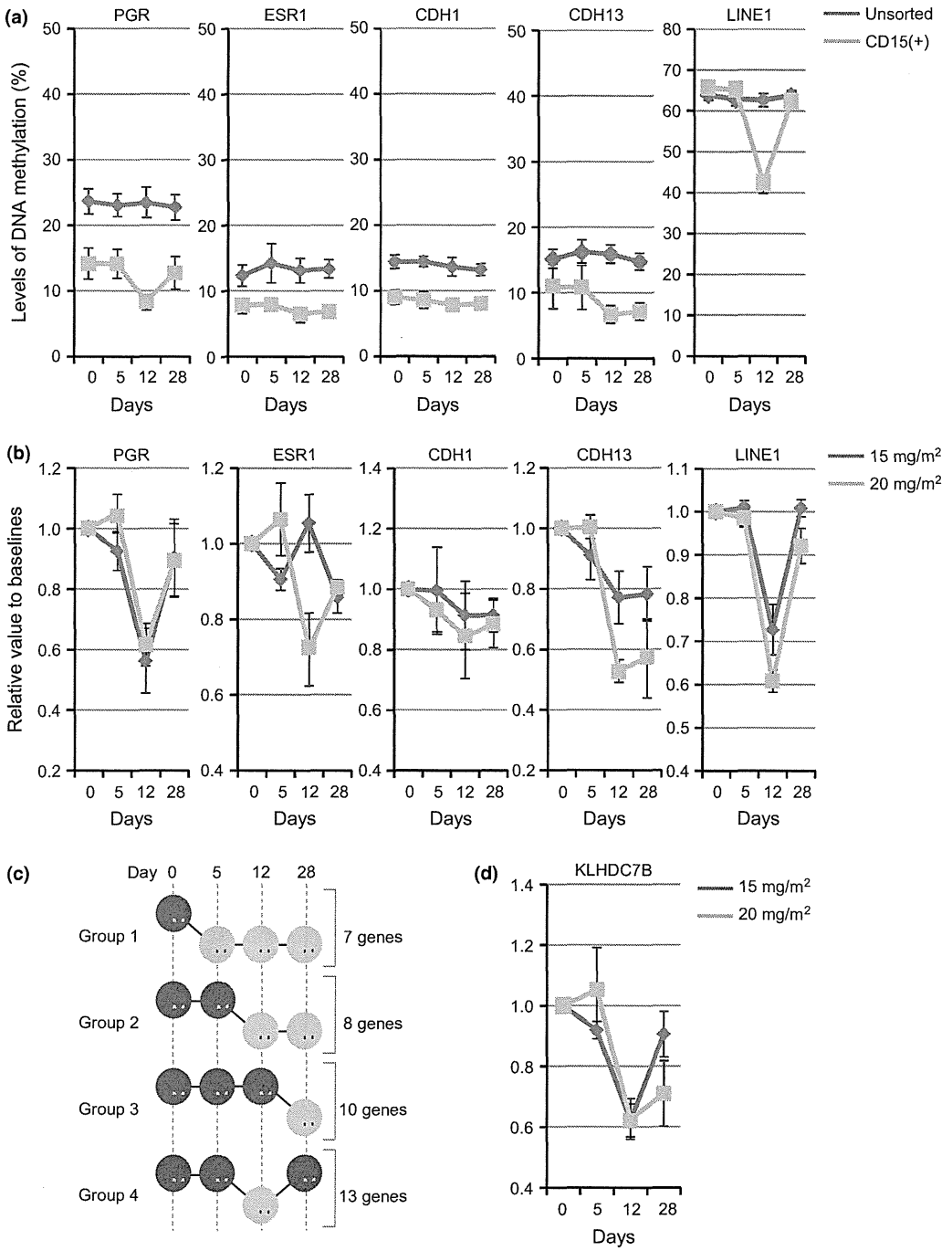

Fig. 6. Changes in the methylation status in five selected genes using peripheral blood mononuclear cells or CD15 + peripheral blood cells (Mean ± standard error of mean). (a) Average methylation changes (0, baseline, and 5, 12 and 28 days after treatment) were compared between peripheral blood mononuclear cells (black lines) and CD15-positive blood cells (gray lines). The *y*-axis shows methylation level (%). (b) Average methylation changes (0, baseline, and 5, 12 and 28 days after treatment) were compared between patients treated with 15 mg/m^2 (black lines) and 20 mg/m^2 (gray lines). The *y*-axis shows relative changes to methylation levels at baseline. (c) DNA demethylation status after decitabine treatment was classified into four groups. (d) Genes in Group 4 show prominent demethylation around 12 days after decitabine treatment. Methylation status of a representative newly identified gene, KLHDC7B, is shown. Methylation changes (at 0, baseline, and 5, 12 and 28 days after treatment) were compared between patients treated with 15 mg/m^2 (black lines) and 20 mg/m^2 (gray lines). The *y*-axis shows relative changes compared to methylation levels at baseline.

treatment, and all continued treatment at this dose. All three patients experienced grade 3 or 4 hematologic toxicities and all received granulocyte-colony stimulating factor (G-CSF). One patient developed pneumonia on day 20 of the 9th course, which progressed rapidly, and this patient died 10 days later. There were no other severe non-hematologic toxicities reported.

20 mg/m^2 group. Thirty-four patients received the 20 mg/m^2 dose. As shown in Table 3, myelosuppression was very common, a similar finding to that of previous studies. A total of 17

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Table 6. Summary of large studies of decitabine and azacitidine

Study	Phase	Drug	Dose	Outcome
CALGB[24]	III	Azacitidine	Best supportive care (n = 92)	CR 0%, PR 0% (IWG2000)
			Crossover allowed	Median OS 14 months
			75 mg/m^2 per day × 7 days (n = 99)	CR 6.1%, PR 10.1%
			CR + 2 more cycles	Median OS 20 months (P = 0.10)
				A land mark analysis eliminating the effect of cross over showed benefit of azacitidine P = 0.03
International[23]	III	Azacitidine	Physician's choice (n = 179)	CR rate 16% (IWG2000)
				Median OS 15.0 months
			75 mg/m^2 per day × 7 days (n = 179)	CR rate 18% (P = 0.80)
			At least four cycles	Median OS 24.4 months (P < 0.01)
US multi institutional[6]	III	Decitabine	Best supportive care (n = 81)	CR 0%, PR 0% (IWG2000)
				Median leukemia free survival 7.8 months
			15 mg/m^2 Q 8 h, nine doses (n = 89)	CR 9%, PR 8%
			Max eight cycles	Median leukemia free survival 12.1 months (P = 0.16)
EORTC[12]	III	Decitabine	Best supportive care (n = 114)	CR 0%, PR 0% (IWG2000)
				Median OS 8.5 months
			15 mg/m^2 Q 8 h, nine doses (n = 119)	CR 13%, PR 6%
			Max eight cycles	Median OS 10.1 months (P = 0.38)
MDACC[7]	II	Decitabine	20 mg/m^2 daily for 5 days and two other arms 100 mg/m^2 per course (n = 95)	CR 34%, mCR 24%, PR 1%, HI 13% (IWG2006)
				Median OS 19 months
ADOPT[8]	II	Decitabine	20 mg/m^2 daily for 5 days (n = 99)	CR 17%, mCR 15%, PR 0%, HI 18% (IWG2006)
				Median OS 19.4%
Japanese Azacitidine	II	Azacitidine	75 mg/m^2 per day × 7 days (n = 53)	CR 15%, mCR 13%, PR 0%, HI 55% (IWG2006)
Our study	II	Decitabine	20 mg/m^2 daily for 5 days (n = 34)	CR 21%, mCR 3%, PR 6% (IWG2006)
				Median OS not reached

ADOPT, Alternative Dosing for Outpatient Treatment Trial; CALGB, Cancer and Leukemia Group B; CR, complete remission; EORTC, European Organization for Research and Treatment of Cancer; HI, hematologic improvement; IWG, Response criteria by International Working Group; mCR, marrow complete remission; MDACC, MD Anderson Cancer Center; OS, overall survival; PR, partial remission.

patients received G-CSF. One patient experienced prolonged grade 4 neutropenia and was taken off the study. Another patient developed pneumonia on day 13 of cycle 2 and died 3 weeks later. Non-hematologic toxicities ≥ grade 3 included cerebral infarction (n = 1, grade 3), subdural hematoma (n = 1, grade 3), elevated blood glucose (n = 1, grade 3), and pulmonary hypertension (n = 1, grade 3).

Response. Response data are summarized in Table 4. Out of 37 patients, CR, PR and hematologic improvement (HI) defined by IWG2000 criteria were observed in 7 (18.9%), 3 (8.1%) and 6 (16.2%), respectively. By IWG2006 criteria, CR, PR, marrow CR and HI were observed in 7 (18.9%), 3 (8.1%) and 2 (5.4%) and 4 (10.8%), respectively. When analysis was limited to the 34 patients who received treatment at 20 mg/m^2, CR and PR were observed in 7 (20.6%) and 2 (5.9%), respectively, both by IWG2000 and IWG2006 criteria. In patients who achieved response (CR + PR, n = 9) at 20 mg/m^2, median time to remission was 130 days (range 67–220), or four cycles (range 4–8), and duration of remission was 474+ days (range 294–598+).

When the analysis was limited to patients with marrow blast percentage <20% (n = 28), the CR rate was 17.9% and CR + PR rate was 25.0% by both IWG2000 and IWG2006 criteria (Table S1).

Cytogenetic response was evaluable in 20 patients who had karyotype abnormalities upon study entry. Six patients (30%) achieved complete cytogenetic response, while one patient (5%) achieved partial cytogenetic response. Cytogenetic changes are shown in detail in Table 5.

Transfusion independence. Transfusion independence over time is shown in Figure 2. Percentages were calculated using all enrolled patients as the denominator (n = 34).

Red cell transfusion independence. At baseline, 26% of patients were red cell transfusion-independent. Although this number decreased to 15% of patients after the first cycle, it increased again after two cycles, hitting a peak of 41% after five cycles. Transfusion independence decreased again after 10 cycles, but many who were transfusion-independent continued treatment. Details are shown in Figure 2(a).

Platelet transfusion independence. At baseline, 85% of patients were platelet transfusion-independent, with that number falling to 21% after the first cycle. However, transfusion independence increased as patients continued on treatment, and most patients who were able to continue treatment through cycle 6 became platelet transfusion-independent after that cycle. Details are shown in Figure 2(b).

Time to acute myelogenous leukemia or death and overall survival. Kaplan–Meier estimates of time to AML or death are plotted in Figure 3. Median time to AML or death has not been reached. The 2-year rate of AML-free survival is 52%. Kaplan–Meier estimates of overall survival are shown in Figure 4. Median survival has not been reached. The 2-year survival rate is 56%. These data are comparable to previously reported data in similar patient populations receiving the same treatment in the United States and Europe.[6–8]

Pharmacokinetics. Plasma concentrations of decitabine were analyzed on days 1 and 5 in three patients receiving 15 mg/m^2 and five patients receiving 20 mg/m^2 (Fig. 5). Plasma decitabine became undetectable within 240 min. No significant difference in plasma decitabine concentrations between days 1 and 5 was observed. In addition, plasma levels at each time point and the calculated area under the curve were not apparently different between the 15 and 20 mg/m^2 groups (data not shown), although this could be due to the limited number of evaluable subjects and considerable individual variation.

Pharmacodynamics. *Changes in DNA methylation in peripheral blood mononuclear cells and CD15 + peripheral blood cells.* Changes in methylation of five selected genes in PBMCs and CD15 + PBCs are summarized in Figure 6(a). In general,

the degree of methylation at baseline was higher in PBMCs than in CD15 + PBCs, although the difference was not statistically significant. Average baseline methylation in PBMCs and CD15 + PBCs was 24% and 14%, respectively, for *PGR* ($P = 0.007$), 12% and 8% for *ESR1* ($P = 0.03$), 14% and 9% for *CDH1* ($P = 0.002$), and 15% and 11% for *CDH13* ($P = 0.28$). The hypomethylating effect of decitabine seemed most prominent on day 12, and was more prominent in CD15 + PBCs than in PBMCs, particularly in *LINE1* ($P < 0.0001$). With this result, and given that CD15 + PBCs represent affected myeloid cells more accurately than PBMCs, we chose to analyze the DNA methylation status of CD15 + PBCs. Changes in methylation status after treatment were more prominent in patients receiving 20 mg/m^2 than in those receiving 15 mg/m^2 (Fig. 6b).

Methylated CpG island amplification and microarray analysis. A variable number of genes were found to be methylated at baseline (average: 548 genes; range: 140–1029 genes) after genome-wide DNA methylation profiling using MCAM.[19] While a majority of these genes sustained detectable levels of DNA methylation after decitabine treatment, 25 genes showed significant hypomethylation. Persistent hypomethylation was observed starting on day 5 (Group 1), day 12 (Group 2), and day 28 (Group 3) in seven, eight, and 10 genes, respectively (Fig. 6c, Table S3). Notably, prominent hypomethylation on day 12 followed by recovery of methylation, as observed most dramatically in *LINE1*, was observed in 13 genes (Group 4). Thus, 21 genes (55%) showed prominent DNA demethylation on day 12. Using MCAM analysis, we identified a kelch domain-containing protein, 7B (KLHDC7B), which warrants further investigation as a surrogate marker for demethylation after decitabine treatment (Fig. 6d). As we did not observe major clinical response in phase I, correlation between methylation and clinical outcome could not be analyzed.

Discussion

Decitabine was well tolerated and induced durable response in this Japanese population. Decitabine has previously been investigated in multiple clinical trials (Table 6). The treatment regimen studied here was investigated in a phase II study at MD Anderson[20] and in a multi-institutional study.[8] Compared to treatment with 15 mg/m^2 over 3 h every 8 h for 3 days, treatment with 20 mg/m^2 over 1 h daily for 5 days is more convenient, can be given in an outpatient setting and has comparable efficacy.

The only grade 3/4 toxicities observed in >10% of patients were hematologic and infectious toxicities, which are characteristic of the disease itself, although treatment with decitabine certainly requires careful blood count monitoring and infection surveillance. Overall, the observed toxicity profile was comparable to that of azacitidine reported in a phase I/II study conducted in Japan.[21] In particular, the incidences of grade 3/4 febrile neutropenia in the present study and the Japanese azacitidine study were similar at 29.4% and 30.2%, respectively.[21]

Randomized phase III studies of decitabine versus best supportive care have not shown significant survival benefit for decitabine.[6,12] This could be due to the fact that decitabine at 15 mg/m^2 over 3 h every 8 h for 3 days, not the dose and schedule studied here, was used in the US and European Organization for Research and Treatment of Cancer (EORTC) studies. In addition, only a limited percentage of patients in these studies continued treatment after experiencing clinical benefit.[6,12] Continuation of treatment in patients who are responding is beneficial, but previous randomized phase III studies set a maximum number of treatment cycles, which affected responders in particular.

There are several differences in the mechanisms of action of azacitidine and decitabine. First, azacitidine is a ribose-based nucleoside and is mostly incorporated into RNA. A small proportion of azacitidine diphosphate is reduced by ribonucleotide reductase to decitabine diphosphate and is eventually incorporated into DNA, which can result in the inhibition of DNA methyltransferase. In contrast, decitabine is incorporated into DNA after phosphorylation and does not require reduction. Second, azacitidine is incorporated into RNA and inhibits DNMT2. This can inhibit methylation of tRNA(Asp)-cytosine 38 and may exert toxicity.[22] Decitabine does not have this effect. Finally, the mechanisms of resistance may be different, as illustrated by the difference in the rate-determining step of phosphorylation of each; deoxycytidine kinase limits the rate of phosphorylation for decitabine while uridine-cytidine kinase limits that of azacitidine. Decreases in the expression or activity of these enzymes may be associated with drug resistance, and thus certain cells may be more sensitive to one drug than the other.

Our study confirmed that hypomethylation is observed after decitabine therapy. Robust methylation analysis also revealed that certain sets of genes were hypomethylated after decitabine therapy, although the significance of these changes should be further evaluated. In this study, methylation status in CD15 + PBCs, rather than PBMCs, was analyzed for the first time. The changes in methylation were generally more prominent in CD15 + PBSs than in PBMCs. As this cell population better reflects myeloid cell behavior, we propose this method be used in future analysis of *in vivo* methylation changes in patients treated with a hypomethylating agent.

In summary, our study has shown the safety and clinical activity of decitabine in Japanese patients with high-risk MDS. Responses and toxicities are comparable to those reported in previous studies. Correlative analysis revealed hypomethylation in a number of genes *in vivo* after decitabine treatment, which warrants further investigation in the setting of epigenetic therapy.

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Disclosure Statement

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Supporting Information

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

Table S1. List of the primers for bisulfite pyrosequencing analysis.

Table S2. Response to decitabine treatment in patients whose marrow blast was <20%.

Table S3. List of hypomethylated genes in response to decitabine treatment.

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ORIGINAL ARTICLE

Differing impacts of pretransplant serum ferritin and C-reactive protein levels on the incidence of chronic graft-versus-host disease after allogeneic hematopoietic stem cell transplantation

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Abstract Studies have suggested an association between pretransplant serum levels of ferritin and C-reactive protein (CRP) and complications of allogeneic hematopoietic stem cell transplantation (HSCT). To evaluate the prognostic impact of these biomarkers on the development of acute and chronic graft-versus-host disease (GVHD), we retrospectively studied 211 patients who underwent allogeneic HSCT for hematologic diseases at our institution. The cumulative incidence rate of chronic GVHD at 3 years was 40.7 %. In the multivariate analysis, elevated CRP levels (≥2 mg/L) were significantly associated with a high incidence of chronic GVHD, whereas high ferritin levels (≥880 ng/mL) showed a tendency, though not statistically significant, to association with a low incidence of chronic GVHD. No significant association was observed between the pretransplant serum ferritin or CRP levels and the incidence of acute GVHD. Multivariate analysis indicated that high pretransplant serum ferritin levels were significantly associated with increases in treatment-related mortality and relapse rates. Overall, an elevated pretransplant serum ferritin level, but not an elevated serum CRP level, is a strong risk factor for overall mortality (hazard ratio, 2.16; $P = 0.002$). Our results also indicate that pretransplant serum CRP levels may be a useful biomarker for predicting the risk of chronic GVHD.

Keywords Ferritin · C-reactive protein · Chronic GVHD · Allogeneic hematopoietic stem cell transplantation

Introduction

Iron overload is frequently observed in patients with hematologic diseases before and after allogeneic hematopoietic stem cell transplantation (HSCT). Many studies have shown that elevated pretransplant serum ferritin levels are associated with lower overall and disease-free survival rates and a higher incidence of treatment-related complications [1–5]. Conversely, several studies have suggested that elevated pretransplant ferritin levels are associated with a lower incidence of chronic graft-versus-host disease (GVHD) [6, 7], and the immunosuppressive effect of iron overload was hypothesized to be an underlying mechanism. However, this association remains controversial because other studies have failed to observe such a relationship [5, 8]. Results are also inconsistent among studies regarding the association between pretransplant serum ferritin levels and the incidence of acute GVHD [4–6, 8].

Serum ferritin is widely used as a surrogate marker of body iron stores. However, ferritin levels are increased by inflammation and iron loading; therefore, it is important to

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adjust for the effect of inflammation on ferritin levels. C-reactive protein (CRP), an acute-phase reactant produced by hepatocytes, has been shown to be a reliable biomarker of systemic inflammation [9, 10]. Several studies have suggested that elevated pretransplant or pre-engraftment serum CRP levels are associated with an increased risk of treatment-related mortality (TRM) after allogeneic HSCT [11–14]. Although studies have reported that elevated CRP levels are associated with a higher incidence rate of acute GVHD [11, 12], this finding is not yet fully confirmed. Moreover, the impact of CRP levels on the incidence of chronic GVHD remains unknown.

We previously demonstrated through a multivariate analysis that elevated pretransplant serum ferritin and CRP levels were significantly associated with the development of bacterial infection after allogeneic HSCT [14]. In the present study, we investigated the association between pretransplant serum ferritin and CRP levels and the incidence of acute and chronic GVHD as well as other clinical outcomes of allogeneic HSCT, to determine whether these events could be predicted using these parameters.

Patients and methods

Study population

We retrospectively reviewed the medical records of adult patients who underwent their first allogeneic HSCT for hematologic diseases at the Kyoto University Hospital from January 2000 to December 2010. A total of 211 patients whose pretransplant serum ferritin and CRP profiles were available were included in the analysis. This study was performed in accordance with the Helsinki Declaration and approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine. Written informed consent to undergo the transplantation protocol was obtained from all of the patients.

Serum analysis

The serum ferritin and CRP levels before the start of the conditioning regimen were measured using the standard laboratory technique (reference ranges: ferritin level $\leq$150 ng/mL; CRP level $\leq$2 mg/L) [14].

Statistical analysis

The primary end point was the impact of the pretransplant serum ferritin and CRP levels on the incidence of grade 2–4 acute GVHD and chronic GVHD. In the analysis of chronic GVHD, patients who survived for at least 100 days after allogeneic HSCT were included. The secondary end point was the impact of these 2 markers on the incidence of TRM and relapse, and the overall survival (OS) rate.

The patients were divided into 2 groups depending on their pretransplant serum ferritin or CRP levels. The cutoff point for the CRP levels was 2 mg/dL, the median value. For the ferritin levels, the median value was 470 ng/mL, which was much lower than the cutoff points used in most other studies; therefore, we used 880 ng/mL, which was the higher tertile value, as the cutoff point. The patient and transplant characteristics between the 2 groups were compared using the Mann–Whitney U test or Chi-square analysis, as appropriate. Standard-risk disease was defined as complete remission in cases of acute myeloid leukemia, acute lymphoblastic leukemia, malignant lymphoma, and plasma cell myeloma; as untreated or complete remission in cases of myelodysplastic syndrome and myeloproliferative disorder; as chronic phase in cases of chronic myeloid leukemia; and as nonmalignant disease. High-risk disease was defined as any other hematologic disease status. The conditioning regimen was categorized as either myeloablative or reduced intensity according to the National Marrow Donor Program and the Center for International Blood and Marrow Transplant Research operational definitions [15].

Acute and chronic GVHD were defined and graded according to conventional criteria [16, 17]. Depending on whether it developed before or after day +100, GVHD was classified as acute or chronic, respectively. To eliminate the effect of a competing risk, the cumulative incidence was assessed using methods described elsewhere [18]. The competing event in the cumulative incidence analyses was defined as death without an event of interest. OS was estimated using the Kaplan–Meier methods. The Cox proportional hazards model was applied.

The following items were added as confounders: the age of the recipient (<50 or $\geq$50 years), the sex of the recipient (male or female), diagnosis (myeloid or lymphoid malignancies, or nonmalignant diseases), risk of disease (standard or high risk), source of stem cells (HLA-matched- or HLA-mismatched-related donor graft, unrelated bone marrow, or unrelated cord blood), conditioning regimen (myeloablative or reduced intensity), and prophylaxis against GVHD (tacrolimus or cyclosporine based). Stepwise backward selection procedures were used with a variable retention criterion of $P < 0.05$ to identify important confounders; these confounders, as well as the serum ferritin and CRP levels, were then included in the final model.

$P < 0.05$ was considered to be statistically significant. All the analyses were conducted using the Stata (version 11; StataCorp LP, College Station, TX, USA) and R version 2.13.0 software (The R Foundation for Statistical Computing, Vienna, Austria).

Results

Characteristics of patients and transplants

The characteristics of the patients and transplants are shown in Table 1 and Supplementary Table S1. The median age of the patients was 48 years (range 17–69 years). The primary diseases were myeloid malignancies, lymphoid malignancies, and nonmalignant diseases in 115, 88, and 8 patients, respectively. A total of 90 patients (43 %) had a high-risk disease. No patient received T cell-depleted grafts. A myeloablative regimen was used in 116 patients (55 %). There was no significant difference in the patient and transplant characteristics between the low- and high-ferritin groups, except in the serum CRP levels ($P = 0.011$). The patients in the high-CRP group were more likely to be male ($P < 0.001$) and have a high-risk disease ($P < 0.001$).

Acute and chronic GVHD

Among all patients, the median follow-up period for the survivors after allogeneic HSCT was 41.2 months (range 1.2–132.6 months). A total of 188 patients survived for 100 days or longer after transplantation.

The cumulative incidence rates of grade 2–4 acute GVHD at 100 days after transplantation were 35.8 % [95 % confidence interval (CI) 27.9–43.7 %] and 32.5 % (95 % CI 22.0–43.5 %) in the low- and high-ferritin groups, respectively, and 34.9 % (95 % CI 26.2–43.7 %) and 34.4 % (95 % CI 25.2–43.8 %) in the low- and high-CRP groups, respectively (Fig. 1, panels a, b). With regard to grade 3 or 4 acute GVHD, the cumulative incidence rates were 7.9 % (95 % CI 4.2–13.1 %) and 14.1 % (95 % CI 7.2–23.3 %) in the low- and high-ferritin groups, respectively, and 10.7 % (95 % CI 5.9–17.3 %) and 9.1 % (95 % CI 4.5–15.8 %) in the low- and high-CRP groups,

Table 1 Characteristics of patients and transplants according to pretransplant serum ferritin and CRP levels

Variables	Low ferritin (<880 ng/mL) $n = 140$	High ferritin (≥880 ng/mL) $n = 71$	P value	Low CRP (<2 mg/L) $n = 112$	High CRP (≥2 mg/L) $n = 99$	P value
Age at transplant						
Median (range), years	47.5 (17–69)	50 (20–66)	0.46	46.5 (17–69)	49 (17–66)	0.26
Sex, *n* (%)			0.15			<0.001
Male	70 (50)	43 (61)		47 (42)	66 (67)	
Female	70 (50)	28 (39)		65 (58)	33 (33)	
Disease, *n* (%)			0.082			0.54
Myeloid malignancies	73 (52)	42 (59)		64 (57)	51 (52)	
Lymphoid malignancies	64 (46)	24 (34)		43 (38)	45 (45)	
Nonmalignant diseases	3 (2)	5 (7)		5 (4)	3 (3)	
Risk of disease, *n* (%)			0.83			<0.001
Standard	81 (58)	40 (56)		80 (71)	41 (41)	
High	59 (42)	31 (44)		32 (29)	58 (59)	
Source of stem cells, *n* (%)			0.72			0.73
HLA[a]-matched related	44 (31)	18 (25)		31 (28)	31 (31)	
HLA[a]-mismatched	14 (10)	10 (14)		11 (10)	13 (13)	
Unrelated bone marrow	62 (44)	33 (46)		54 (48)	41 (41)	
Unrelated cord blood	20 (14)	10 (14)		16 (14)	14 (14)	
Conditioning regimen, *n* (%)			0.76			0.13
Myeloablative intensity	78 (56)	38 (54)		67 (60)	49 (49)	
Reduced intensity	62 (44)	33 (46)		45 (40)	50 (51)	
GVHD prophylaxis, *n* (%)			0.84			0.53
Tacrolimus-based	114 (81)	57 (80)		89 (79)	82 (83)	
Cyclosporine based	26 (19)	14 (20)		23 (21)	17 (17)	
Serum CRP level, *n* (%)			0.011			
<2 mg/L	83 (59)	29 (41)				
≥2 mg/L	57 (41)	42 (59)				

[a] HLA compatibility was defined according to the results of serologic or low-resolution molecular typing for HLA-A, -B, and -DR antigens

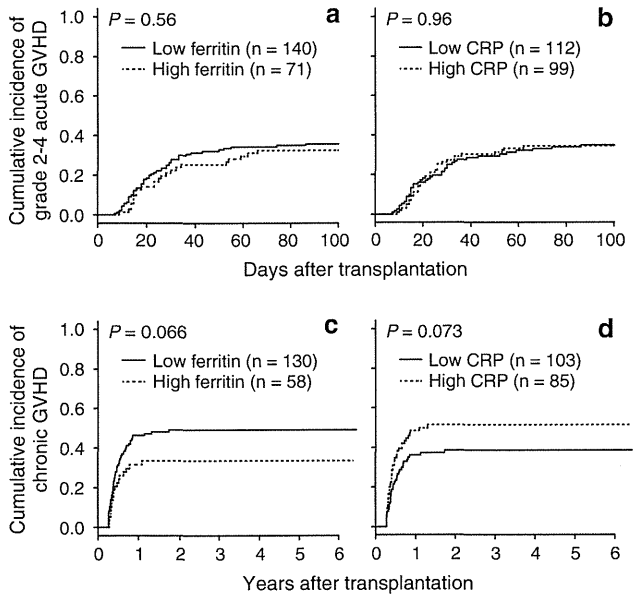

Fig. 1 Cumulative incidence of grade 2–4 acute GVHD according to pretransplant **a** ferritin and **b** CRP levels, and chronic GVHD according to pretransplant **c** ferritin and **d** CRP levels. **a**, **c** *Solid line* low-ferritin group (<880 ng/mL), *dotted line* high-ferritin group (≥880 ng/mL). **b**, **d** *Solid line* low-CRP group (<2 mg/L), *dotted line* high-CRP group (≥2 mg/L). The statistical significance between the 2 groups was calculated using the Gray test

respectively. There was no significant difference in the incidence rates of grade 2–4 and 3 or 4 acute GVHD between the low- and high-ferritin groups or between the low- and high-CRP groups.

The cumulative incidence rates of chronic GVHD at 3 years after transplantation were 49.3 % (95 % CI 40.1–57.8 %) and 33.5 % (95 % CI 21.6–45.8 %) in the low- and high-ferritin groups, respectively, and 38.6 % (95 % CI 29.0–48.2 %) and 51.4 % (95 % CI 40.0–61.7 %) in the low- and high-CRP groups, respectively (Fig. 1, panels c, d). The patients in the high-ferritin group tended to have a low incidence of chronic GVHD, although this association was not significant in the multivariate analysis [≥880 vs. <880 ng/mL; hazard ratio (95 % CI), 0.64 (0.38–1.09); $P = 0.099$; Table 2]. A subgroup analysis showed that the negative effect of the ferritin levels on the incidence of chronic GVHD was significant only in patients with myeloid malignancies ($n = 103$; hazard ratio, 0.46; $P = 0.040$), but not in patients with lymphoid malignancies ($n = 78$; hazard ratio, 1.11; $P = 0.79$). The multivariate analysis showed that the elevated serum CRP levels (≥2 vs. <2 mg/L) were significantly associated with the increased incidence of chronic GVHD [hazard ratio (95 % CI), 1.71 (1.07–2.74); $P = 0.024$; Table 2]. The effect of the CRP levels on the incidence of chronic GVHD was similar regardless of the primary disease (myeloid or lymphoid malignancies). There was no significant difference

Table 2 Multivariate analysis of acute and chronic GVHD

	Hazard ratio (95 % CI)	*P* value
Grade 2–4 acute GVHD		
Conditioning regimen		
Myeloablative intensity	1.00	Reference
Reduced intensity	0.60 (0.37–0.97)	0.037
Serum ferritin level		
<880 ng/mL	1.00	Reference
≥880 ng/mL	0.86 (0.52–1.43)	0.57
Serum CRP level		
<2 mg/L	1.00	Reference
≥2 mg/L	1.13 (0.70–1.81)	0.62
Grade 3–4 acute GVHD		
Risk of disease		
Standard	1.00	Reference
High	3.93 (1.52–10.14)	0.005
Conditioning regimen		
Myeloablative intensity	1.00	Reference
Reduced intensity	0.39 (0.15–1.02)	0.055
Serum ferritin level		
<880 ng/mL	1.00	Reference
≥880 ng/mL	2.10 (0.87–5.09)	0.10
Serum CRP level		
<2 mg/L	1.00	Reference
≥2 mg/L	0.57 (0.22–1.43)	0.23
Chronic GVHD[a]		
Risk of disease		
Standard	1.00	Reference
High	1.48 (0.93–2.37)	0.098
GVHD prophylaxis		
Tacrolimus-based	1.00	Reference
Cyclosporine based	0.52 (0.27–1.01)	0.052
Serum ferritin level		
<880 ng/mL	1.00	Reference
≥880 ng/mL	0.64 (0.38–1.09)	0.099
Serum CRP level		
<2 mg/L	1.00	Reference
≥2 mg/L	1.71 (1.07–2.74)	0.024

[a] Patients who survived for at least 100 days after transplantation were included in the analysis

in the incidence of extensive chronic GVHD between the low- and high-ferritin groups ($P = 0.43$) or between the low- and high-CRP groups ($P = 0.52$).

A history of acute GVHD is known to be an important risk factor for developing chronic GVHD. Therefore, we also conducted an analysis of the association between the incidence of chronic GVHD and the incidence of prior acute GVHD (none or grade 1 vs. grade 2–4) stratified by the pretransplant ferritin and CRP levels. The cumulative incidence rates of chronic GVHD at 3 years were as

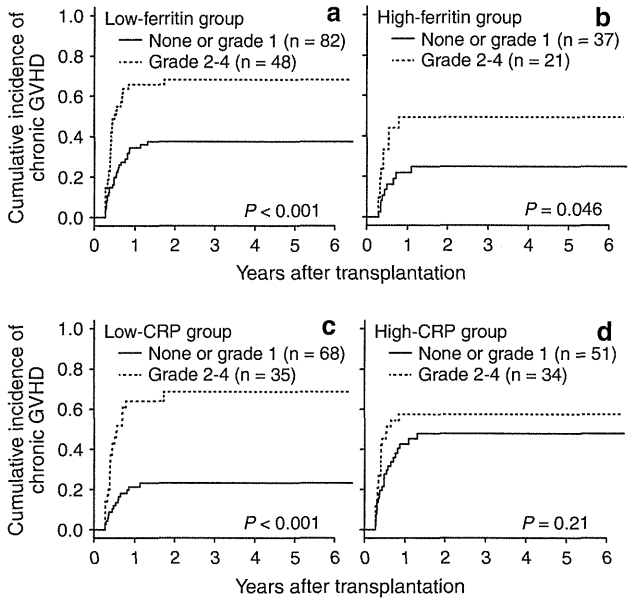

Fig. 2 Cumulative incidence of chronic GVHD according to prior acute GVHD stratified by pretransplant ferritin and CRP levels. **a** Low-ferritin group (<880 ng/mL, $n = 130$), **b** high-ferritin group (≥880 ng/mL, $n = 58$), **c** low-CRP group (<2 mg/L, $n = 103$), and **d** high-CRP group (≥2 mg/L, $n = 85$). *Solid line* no or grade 1 acute GVHD, *dotted line* grade 2–4 acute GVHD. The statistical significance between the 2 groups was calculated using the Gray test

follows: low-ferritin group, 37.8 versus 68.4 %, $P < 0.001$ (Fig. 2, panel a); high-ferritin group, 24.9 versus 49.2 %, $P = 0.046$ (Fig. 2, panel b); low-CRP group, 23.2 versus 68.5 %, $P < 0.001$ (Fig. 2, panel c); and high-CRP group, 47.8 versus 57.5 %, $P = 0.21$ (Fig. 2, panel d). Notably, the patients in the high-CRP group demonstrated a high incidence of chronic GVHD, both in the absence and presence of prior acute GVHD.

TRM, relapse, and OS

At 3 years, the patients in the high-ferritin group had a significantly higher TRM than those in the low-ferritin group (21.5 vs. 9.7 %, $P = 0.026$; Fig. 3, panel a), with the main cause of TRM being infection in both groups [5 of 14 TRM cases in the high-ferritin group ($n = 71$) and 5 of 13 TRM cases in the low-ferritin group ($n = 140$)]. The patients in the high-CRP group had a significantly higher TRM than those in the low-CRP group (20.0 vs. 7.5 %, $P = 0.035$; Fig. 3, panel b). The impact of ferritin on TRM was significant ($P = 0.046$) according to the multivariate analysis, whereas the impact of CRP was not (Table 3).

The cumulative incidence of relapse was similar between the low- and high-ferritin groups and between the low- and high-CRP groups (Fig. 3, panels c, d). However, in the multivariate analysis, the high ferritin levels were significantly associated with the high relapse rate ($P = 0.018$; Table 3).

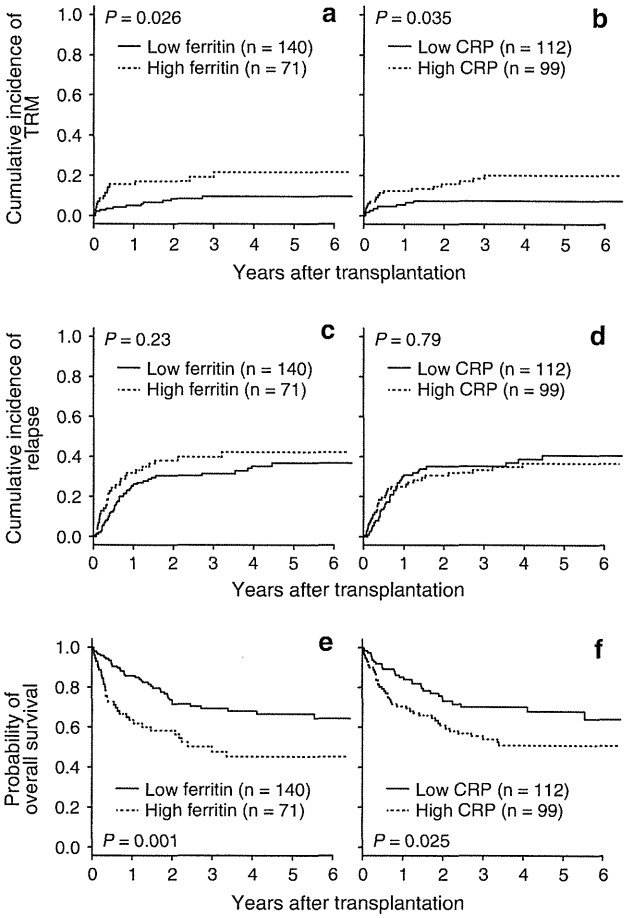

Fig. 3 Cumulative incidence of TRM according to pretransplant **a** ferritin and **b** CRP levels, and relapse according to pretransplant **c** ferritin and **d** CRP levels. The Kaplan–Meier estimate of overall survival according to pretransplant **e** ferritin and **f** CRP levels is also shown. **a**, **c**, **e** *Solid line* low-ferritin group (<880 ng/mL), *dotted line*, high-ferritin group (≥880 ng/mL). **b**, **d**, **f** *Solid line* low-CRP group (<2 mg/L), *dotted line* high-CRP group (≥2 mg/L). The statistical significance between the 2 groups was calculated using the Gray test or the log-rank test. **e**, **f** Note that pretransplant high ferritin levels were significantly associated with inferior overall survival in both univariate ($P = 0.001$) and multivariate ($P = 0.002$) analyses, while high CRP levels were significantly associated with inferior overall survival in only univariate analysis ($P = 0.025$) but not in multivariate analysis ($P = 0.79$; see Table 3)

In the 211 cases, early death within 100 days after SCT occurred in 17 cases, with the cause being TRM in 11 cases and disease relapse in 6 cases. Fifty-seven patients died after day 100, with the cause of death being TRM in 16 cases and disease relapse in 41 cases. At 3 years, the patients in the high-ferritin group had a significantly inferior OS than those in the low-ferritin group (47.7 % vs. 69.5 %, $P = 0.001$; Fig. 3, panel e); the patients in the high-CRP group had a significantly inferior OS than those in the low-CRP group (54.0 % vs. 70.1 %, $P = 0.025$; Fig. 3f). In the multivariate analysis, high ferritin levels ($P = 0.002$), older age ($P = 0.014$), male sex ($P = 0.031$),

Table 3 Multivariate analysis of TRM, relapse, and overall mortality

	Hazard ratio (95 % CI)	*P* value
TRM		
Sex		
Male	1.00	Reference
Female	0.37 (0.15–0.92)	0.034
Serum ferritin level		
<880 ng/mL	1.00	Reference
≥880 ng/mL	2.19 (1.01–4.75)	0.046
Serum CRP level		
<2 mg/L	1.00	Reference
≥2 mg/L	1.67 (0.73–3.82)	0.23
Relapse		
Risk of disease		
Standard	1.00	Reference
High	2.83 (1.74–4.59)	< 0.001
Serum ferritin level		
<880 ng/mL	1.00	Reference
≥880 ng/mL	1.81 (1.11–2.95)	0.018
Serum CRP level		
<2 mg/L	1.00	Reference
≥2 mg/L	0.73 (0.45–1.19)	0.21
Overall mortality		
Age at transplant		
<50 years	1.00	Reference
≥50 years	1.80 (1.13–2.87)	0.014
Sex		
Male	1.00	Reference
Female	0.58 (0.36–0.95)	0.031
Risk of disease		
Standard	1.00	Reference
High	2.51 (1.55–4.07)	<0.001
Serum ferritin level		
<880 ng/mL	1.00	Reference
≥880 ng/mL	2.16 (1.34–3.48)	0.002
Serum CRP level		
<2 mg/L	1.00	Reference
≥2 mg/L	1.07 (0.66–1.73)	0.79

and high-risk disease ($P < 0.001$) were significantly associated with a higher mortality rate (Table 3). The high ferritin levels remained to be associated with a high mortality rate among the patients who survived for 100 days or longer after transplantation ($P = 0.032$).

Discussion

In our cohort of 211 patients with hematologic diseases who underwent allogeneic HSCT, we found that an elevated CRP level before transplantation was a significant risk factor for the development of chronic GVHD. We also observed a tendency toward a lower incidence of chronic GVHD in the high-ferritin group, although a significant association was found only in patients with myeloid malignancies. In contrast, the ferritin and CRP levels were not associated with the development of acute GVHD. Consistent with previous reports, we also confirmed that an elevated ferritin level was an adverse prognostic factor for survival [2–8, 14].

Chronic GVHD is the primary cause of late morbidity and nonrelapse mortality after allogeneic HSCT. Although a history of acute GVHD is one of the strongest predictors for the development of chronic GVHD, successful strategies for reducing acute GVHD with combinations of immunosuppressive agents have not resulted in reduced incidence of chronic GVHD [19–21]. In the present study, we demonstrated that an elevated pretransplant CRP level was significantly associated with a high incidence rate of chronic GVHD, without affecting the incidence of acute GVHD (Fig. 1, panels b, d). In addition to the current study, we have analyzed the association between post-transplant (between day +50 and +99) serum CRP levels and the incidence of chronic GVHD and found no significant association between them. When we divided the 186 cases into low- and high-CRP groups based on the post-transplant serum CRP levels (<2 vs. ≥2 mg/L), the incidence of chronic GVHD was 49.4 % in the low-CRP group ($n = 96$) and 40.0 % in the high-CRP group ($n = 90$, $P = 0.43$). Chronic GVHD is considered an immune-mediated syndrome, and its clinical manifestation often resembles autoimmune and other immunological disorders. However, the pathophysiological mechanism underlying chronic GVHD remains poorly understood and there is no reliable marker for predicting and monitoring chronic GVHD. On the other hand, the pathophysiological mechanism underlying acute GVHD is thought to involve the release of proinflammatory cytokines and chemokines from damaged host tissues that activate host antigen-presenting cells and the infused donor T lymphocytes that proliferate and differentiate in response to the host antigen-presenting cells, resulting in target tissue destruction [22]. The difference between the pathophysiological mechanisms of acute and chronic GVHDs may explain our results, which showed that ferritin or CRP levels had no significant influence on the development of acute GVHD. CRP, a surrogate marker of systemic inflammation, has been shown to be a risk factor for the progression of atherosclerosis and future cardiovascular events [23, 24]. In addition, several studies have suggested that CRP itself has a proatherogenic effect [25, 26]. An elevated pretransplant CRP level may be associated with vascular endothelial damage or immune activation in transplant recipients as a