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ORIGINAL ARTICLE

Expression of myeloperoxidase and gene mutations in AML patients with normal karyotype: double *CEBPA* mutations are associated with high percentage of MPO positivity in leukemic blasts

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Abstract The percentage of myeloperoxidase (MPO)-positive blast cells is a simple and highly significant prognostic factor in AML patients. It has been reported that the high MPO group (MPO-H), in which >50% of blasts are MPO activity positive, is associated with favorable karyotypes, while the low MPO group (≤50% of blasts are MPO activity positive, MPO-L) is associated with adverse karyotypes. The MPO-H group shows better survival even when restricted to patients belonging to the intermediate chromosomal risk group or those with a normal karyotype. It has recently been shown that genotypes defined by the mutational status of *NPM1*, *FLT3*, and *CEBPA* are associated with treatment outcome in patients with cytogenetically normal AML. In this study, we aimed to evaluate the relationship between MPO positivity and gene mutations found in normal karyotypes. Sixty AML patients with normal karyotypes were included in this study. Blast cell MPO positivity was assessed in bone marrow smears stained for MPO. Associated genetic lesions (the *NPM1*, *FLT3*-ITD, and *CEBPA* mutations) were studied using nucleotide sequencing. Thirty-two patients were in the MPO-L group, and 28 patients in the MPO-H group. *FLT3*-ITD was found in 11 patients (18.3%), *NPM1* mutations were found in 19 patients (31.7%), and *CEBPA* mutations were found in 11 patients (18.3%). In patients with *CEBPA* mutations, the carrying two simultaneous mutations ($CEBPA^{\text{double-mut}}$) was associated with high MPO expression, while the mutant *NPM1* without *FLT3*-ITD genotype was not associated with MPO activity. Both higher MPO expression and the $CEBPA^{\text{double-mut}}$ genotype appeared to be associated with improved overall survival after intensive chemotherapy. Further studies are required to determine the importance of blast MPO activity as a prognostic factor, especially in *CEBPA* wild-type patients with a normal karyotype.

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1 Introduction

The AML87, -89, and -92 studies conducted by Japan Adult Leukemia Study Group (JALSG) revealed that patient age, ECOG performance status, leukocyte count, FAB subclass, the number of induction courses required to achieve complete remission (CR), the presence of good prognostic chromosomal abnormalities [t(8;21) or inv(16)], and percentage of myeloperoxidase (MPO)-stained positive blast cells at diagnosis were significant risk factors for overall survival (OS) of patients with acute myeloid leukemia (AML) [1]. In more recent AML201 study, it was shown that significant unfavorable prognostic features for OS were adverse cytogenetic risk group [2], age of more than 50 years, WBC more than 20×10^9/L, FAB classification of either M0, M6, or M7, and MPO-positive blasts less than 50% [3]. These observations imply that the percentage of MPO-positive blast cells is one of the important prognostic markers along with cytogenetics and molecular genetic information.

MPO, a microbicidal protein, is considered to be a golden marker for the diagnosis of AML in the French–American–British (FAB) and WHO classifications [4, 5]. In our previous reports [6–8], AML patients with a high percentage of MPO-positive blasts (>50% of blasts are MPO activity positive, MPO-H) had a significantly better complete remission (CR) rate, disease-free survival, and overall survival compared with the low MPO activity positive blast group (≤50% of blasts are MPO activity positive, MPO-L). Most patients with a favorable chromosomal risk profile were in the MPO-H group, and most of the patients with an adverse chromosomal risk profile were in the MPO-L group. The difference in OS between the low and high MPO groups was still observed in a cohort of patients with normal karyotypes, suggesting that MPO is highly expressed in the leukemic blasts of AML patients with a favorable prognosis. To fully understand this phenomenon, it would be important to analyze genetic factors associated with MPO expression, especially in patients with a normal karyotype.

In the WHO classification, mutations of *FLT3*, *NPM1* and *CEBPA* have been emphasized to have prognostic significance in AML patients with normal karyotype. The nucleophosmin 1 gene (*NPM1*) has been shown to be mutated in 45–64% of AML cases with a normal karyotype [9, 10], and *NPM1* mutations are associated with a favorable prognosis in the absence of the internal tandem duplication (ITD) type of fms-related tyrosine kinase-3 gene (*FLT3*) mutation, a known adverse prognostic factor [11]. The CCAAT/enhancer binding protein-alpha gene (*CEBPA*) is another gene that has been shown to be mutated in AML patients with a normal karyotype [12, 13]. Mutations in the *CEBPA* gene are found in 5–14% of all AML cases and are associated with a relatively favorable outcome, and hence, have gained interest as a prognostic marker [14]. Recently, it has been shown that most AML patients with *CEBPA* mutations carry 2 simultaneous mutations ($CEBPA^{\text{double-mut}}$), whereas single mutations ($CEBPA^{\text{single-mut}}$) are less common. In addition it was found that the $CEBPA^{\text{double-mut}}$ genotype is associated with a favorable overall and event-free survival [15, 16]. It is still unclear why $CEBPA^{\text{double-mut}}$ AML patients have better outcomes than those with a single heterozygous mutation.

In this study, we retrospectively examined 60 de novo adult AML patients with normal karyotypes in order to obtain a better insight into the relationships between MPO positivity and other prognostic factors (*NPM1*, *FLT3*, and *CEBPA* mutations). In line with previous reports, both high MPO positivity in AML blasts and the $CEBPA^{\text{double-mut}}$ genotype appeared to be associated with a favorable outcome, and it appeared that it was the $CEBPA^{\text{double-mut}}$ genotype that associated with high blast MPO activity.

2 Materials and methods

2.1 Patients and treatments

The study population included 60 patients with newly diagnosed de novo AML that had been treated at the Department of Internal Medicine, Nagasaki National Medical Center, between 1990 and 2010. All patients had normal karyotype AML. AML was diagnosed according to the FAB classification. Two members independently assessed the percentage of MPO-positive blast cells in MPO-stained bone marrow smears. The main biological and clinical features of the patients are shown in Table 1. Excluding the 25 patients who did not receive conventional induction chemotherapy, all patients were treated according to the Japan Adult Leukemia Study Group (JALSG) protocols (AML89, -92, -95, -97, and -201 studies) [3, 17–19]. CR was determined as when blasts accounted for less than 5% of the cells in normocellular bone marrow with normal peripheral neutrophil and platelet counts. This study was approved by the Ethical Committees of the participating hospitals.

2.2 Analysis of the *FLT3*, *NPM1*, and *CEBPA* genes

High molecular weight genomic DNA was extracted from bone marrow and peripheral blood samples after Ficoll

Table 1 Characteristics of de novo AML patients with a normal karyotype

	All patients ($n = 60$)	Patients who received intensive chemotherapy ($n = 36$)
Median age (range) (year)	59.5 (15–81)	49 (15–67)
Male/female	32/28	18/18
FAB type		
M0	5	3
M1	10	5
M2	21	14
M4	18	11
M5	3	1
M6	3	2
M7	0	0
WBC ($\times 10^9$/L), median (range)	14.9 (0.7–556)	13.0 (0.7–246)
Performance status		
0–2	55	34
3–4	5	2
LDH (IU/L), median (range)	296 (120–5,325)	291 (140–2,606)
MPO		
Low (≤50%)	32	20
High (>50%)	28	16

FAB French–American–British, *WBC* white blood cells, *LDH* lactate dehydrogenase, *MPO* myeloperoxidase

separation of mononucleated cells (35 and 4 patients, respectively) using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). In addition, we isolated genomic DNA from the BM smears of the AML patients (21 samples) using the QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany).

Mutations in the *FLT3*, *NPM1*, and *CEBPA* genes were detected by genomic DNA PCR and direct sequencing. Exons 14 and 15 and the intervening intron of the *FLT3* gene were amplified from DNA using the previously described primers FLT3-11F and FLT3-12R [20]. PCR for NPM1 exon 12 was performed with genomic DNA, the same reagent, and the published primer molecules NPM1-F and NPM1-R [21]. PCR for *CEBPA* was performed using 2 overlapping primer pairs: CEBPA-CT3F (5′-TGCCGGGTATAAAA-GCT GGG-3′) and CT3R (5′-CTCGTTGCTGTTCTTGTCCA -3′), CEBPA-PP2F (5′-TGCCGGGT-ATAAAAGCTGG G-3′) and PP2R (5′-CACGGTCTGGGCAAGCCTCG AGAT-3′). The PCR reactions were run in a final volume of 50 μL containing 10 ng DNA, 5× buffer, 0.2 mmol/L of each deoxynucleotide triphosphate, primers (0.3 μmol/L of each), nucleotides (0.2 mmol/L of each), and 1 U of KOD-Plus-Neo polymerase (TOYOBO, Osaka, Japan). The mixture was initially heated at 94°C for 2 min, before being subjected to 35 cycles of denaturation at 94°C for 10 s and annealing and extension at 68°C for 1 min. The amplified products were cut out from a 1.2% agarose gel and purified with the MinElute Gel extraction kit (QIAGEN, Germany). To screen for mutations, the PCR products were sequenced in both directions with the following primers: FLT3-11F, FLT3-12R, NPM1-F, NPM-R, CEBPA-CT1F, CEBPA-1R, CEBPA-PP2F, CEBPA-PP2R, CEBPA-2F (5′-GCTG GGCGGCATCTGCG-A-3′), and CEBPA-1R (5′-TGT-GC TGGAACAGGTCGGCCA-3′) using a BigDye Terminator v3.1 Cycle Sequencing Kit and the ABI Prism 3100 ×1 Genetic Analyzer (Applied Biosystems, CA, USA). In the case of *NPM1* and *CEBPA* genes, when heterozygous data were identified by sequence screening, mutations were confirmed by cloning with the StrataClone Blunt PCR Cloning Kit (Stratagene, CA, USA) according to the manufacturer's recommendations. Four to ten recombinant colonies were chosen and cultured in LB medium. Plasmid DNA was prepared using a QIAprep spin plasmid miniprep kit (Qiagen, Hilden, Germany), and both strands were sequenced using the T3 and T7 primers and the CEBPA-2F and CEBPA-1R primers.

2.3 Statistical methods

To evaluate the relationship between the frequency of mutations status and clinical characteristics, the following variables were included in the analysis: age, FAB classification, peripheral WBC count, MPO-positivity rate, JALSG score [1], and CR achievement. A comparison of frequencies was performed using Fisher's exact test. Differences in percentage of MPO-positive blasts among patients with different mutational status of genes were compared using the non-parametric Kruskal–Wallis test and followed by Dunn's multiple comparison post-test. Overall survival (OS) was calculated using the Kaplan–Meier method [22], and the group differences were compared using the log-rank test. Thirteen patients who underwent allogeneic or autologous hematopoietic stem cell transplantation were not censored at the time of transplantation. For all analyses, statistical significance was considered at the level of two-tailed 0.05.

3 Results

3.1 Patients' characteristics

As shown in Table 1, the series included 60 patients. Their median age was 59.5 (15–81 years), and there were 32 males (53.3%) and 28 females (46.7%). All patients had normal cytogenetics. Using the percentage of MPO-positive leukemic blasts, as judged from bone marrow slides, the cases

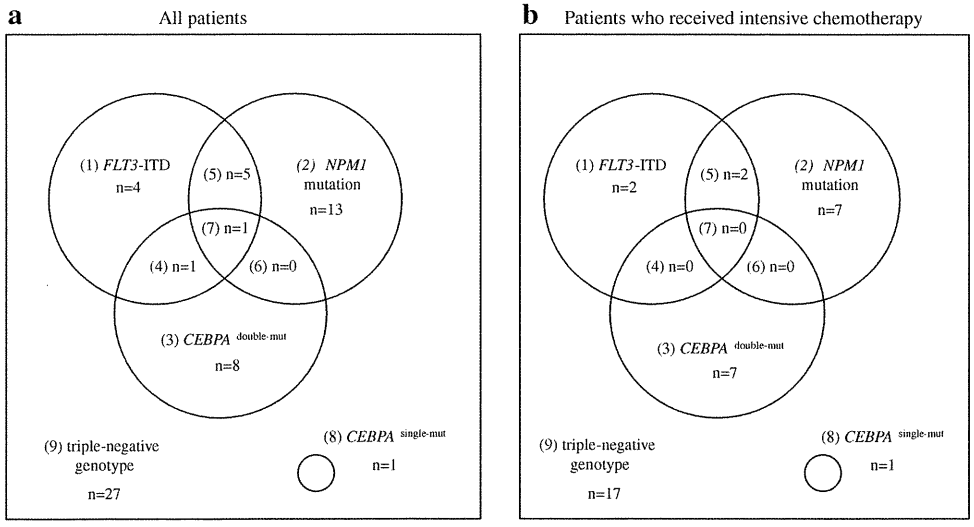

Fig. 1 Frequency and overlapping patterns of AML patients with a normal karyotype. Data are shown for all patients (**a**) and for patients who received intensive chemotherapy (**b**). **a** (*1*) *FLT3*-ITD + wt *NPM1* + wt *CEBPA* ($n = 4$, 6.7%), (*2*) wt *FLT3* + *NPM1* mutation + wt *CEBPA* ($n = 13$, 21.7%), (*3*) wt *FLT3* + wt *NPM1* + $CEBPA^{\text{double-mut}}$ ($n = 8$, 13.3%), (*4*) *FLT3*-ITD + wt *NPM1* + $CEBPA^{\text{double-mut}}$ ($n = 1$, 1.7%), (*5*) *FLT3*-ITD + *NPM1* mutation + wt *CEBPA* ($n = 5$, 8.3%), (*6*) wt *FLT3* + *NPM1* mutation + $CEBPA^{\text{double-mut}}$ ($n = 0$, 0%), (*7*) *FLT3*-ITD + *NPM1* mutation + $CEBPA^{\text{double-mut}}$ ($n = 1$, 1.7%), (*8*) wt *FLT3* + wt *NPM1* + $CEBPA^{\text{single-mut}}$ ($n = 1$, 1.7%), (*9*) triple-negative genotype ($n = 27$, 45%). **b** (*1*) *FLT3*-ITD + wt *NPM1* + wt *CEBPA* ($n = 2$, 5.6%), (2) wt *FLT3* + *NPM1* mutation + wt *CEBPA* ($n = 7$, 19.4%), (*3*) wt *FLT3* + wt *NPM1* + $CEBPA^{\text{double-mut}}$ ($n = 7$, 19.4%), (*4*) *FLT3*-ITD + wt *NPM1* + $CEBPA^{\text{double-mut}}$ ($n = 0$, 0%), (*5*) *FLT3*-ITD + *NPM1* mutation + wt *CEBPA* ($n = 2$, 5.6%), (*6*) wt *FLT3* + *NPM1* mutation + $CEBPA^{\text{double-mut}}$ ($n = 0$, 0%), (*7*) *FLT3*-ITD + *NPM1* mutation + $CEBPA^{\text{double-mut}}$ ($n = 0$, 0%), (*8*) wt *FLT3* + wt *NPM1* + $CEBPA^{\text{single-mut}}$ ($n = 0$, 0%), (*9*) triple-negative genotype ($n = 17$, 47.2%). *wt* wild-type

were divided into the High group (MPO-positive blasts > 50%) and Low group (MPO-positive blasts ≤ 50%). Thirty-two patients were classified into the Low group, and 28 patients were classified into the High group.

3.2 Mutational analysis

FLT3-ITD was found in 11 patients (18.3%), *NPM1* mutations were found in 19 patients (31.7%), and *CEBPA* mutations were found in 11 patients (18.3%). Frequency and an overlapping pattern of mutations are shown in Fig. 1. Among the patients with *CEBPA* mutations, approximately 90% (10 of 11 patients) of the patients had two *CEBPA* mutations ($CEBPA^{\text{double-mut}}$), whereas 10% (1 of 11 patients) had a single mutation. As previously reported, the mutations in the $CEBPA^{\text{double-mut}}$ patients were clustered in the N- and C-terminal hotspots (Table 2; Fig. 2). *FLT3*-ITD mutation was associated with a higher WBC at the time of diagnosis, as reported previously. Neither *NPM1* nor *CEBPA* mutation status displayed a significant association with age, PS, WBC, FAB subtype, JALSG score, or CR achievement (Table 3).

3.3 Clinical outcome

OS was analyzed only in patients who received intensive chemotherapy ($n = 36$). They received chemotherapy based on the treatment protocol described in the JALSG AML89, -92, -95, -97, and -201 studies. As reported previously [6], we observed an association between the percentage of MPO-positive blasts and the survival rate in the normal karyotype patients treated with intensive chemotherapy, although the significance in this cohort was rather low ($P = 0.10$) (Fig. 3). Figure 4 shows Kaplan–Meier curves according to genotype. 'Other genotypes' included the *FLT3*-ITD genotype, the $CEBPA^{\text{single-mut}}$ genotype, and the triple-negative genotype consisting of the wild-type *NPM1* and *CEBPA* genotypes without *FLT3*-ITD. In line with previous reports [14], the patients with the $CEBPA^{\text{double-mut}}$ genotype tended to show higher survival rate compared with patients displaying other genotypes ($P = 0.07$). In this study, the mutant *NPM1* without *FLT3*-ITD genotype was not significantly associated with treatment outcome, possibly due to the small number of patients.

3.4 Difference of MPO-positivity rate by gene mutation status

Figure 5 shows the level of the percentage of MPO-positive blasts by gene mutational status of the *CEBPA*, *FLT3*-ITD, and *NPM1*. The MPO-positivity rate was very high, over 50% (median 96, range 71–100), in all $CEBPA^{\text{double-mut}}$ cases, but it was 20% in one case displaying the

Table 2 Genetic findings of the patients with *CEBPA* mutations

Patient	Category	Nucleotide changes	Amino acid changes	Comments
4	Double	218_219insC	P23fsX107	Produces N-terminal stop codon
		1129_1130insATGTGGAGACGCAGCAGAAGGTGCTGGAGCTG ACCAGTGACAATGACCGCCTGCGCAAGC	K326_327insHVETQQKVLELTSDNDRLRKR	In-frame insertion in bZIP
6	Double	200_218delinsCT	S16fsX101	Produces N-terminal stop codon
		1087_1089dup	K313dup	In-frame duplication in bZIP
7	Double	368_369insA	A72fsX107	Produces N-terminal stop codon
		1080_1082del	T310_Q311del	In-frame deletion in bZIP
13	Double	303_316del	P50fsX102	Produces N-terminal stop codon
		1062_1063insTTG	K304_Q305insV	In-frame insertion in bZIP
19	Double	215_225del	P21fsX103	Produces N-terminal stop codon
		1101_1102insCAGCGCAACGTGGAGACGCAGCAGA AGGTGCTGGAGCTG	L317_T318insQRNVETQQKVLEL	In-frame insertion in bZIP
22	Double	213del	P22fsX159	Produces N-terminal stop codon
		1064_1129dup	K304_Q305insQRNVETQQKVLELTSDNDRLRKR	In-frame insertion in bZIP
27	Double	324_328dup	E59fsX161	Produces N-terminal stop codon
		1062_1063insTTG	K304_Q305insV	In-frame insertion in bZIP
39	Double	213del	P22fsX159	Produces N-terminal stop codon
		1081_1086dup	Q311_Q312dup	In-frame duplication in bZIP
47	Double	397del	F82fsX159	Produces N-terminal stop codon
		1101_1102insCAGCGCAACGTGGAGACGCAGCA GAAGGTGCTGGAGCTG	L317_T318insQRNVETQQKVLEL	In-frame insertion in bZIP
49	Double	297_304del	A48fsX104	Produces N-terminal stop codon
		758del	A202fsX317	Frameshift between TAD2 and bZIP; produces stop codon in bZIP
35	Single	1087_1089dup	K313dup	In-frame duplication in bZIP

Nucleotide numbering was performed according to NCBI Entrez accession no. XM_009180.3, in which the major translational start codon starts at nucleotide position 151. The locations of functional domains are derived from Mueller and Pabst.1

bZIP basic leucine zipper region, *TAD2* second transactivation domain

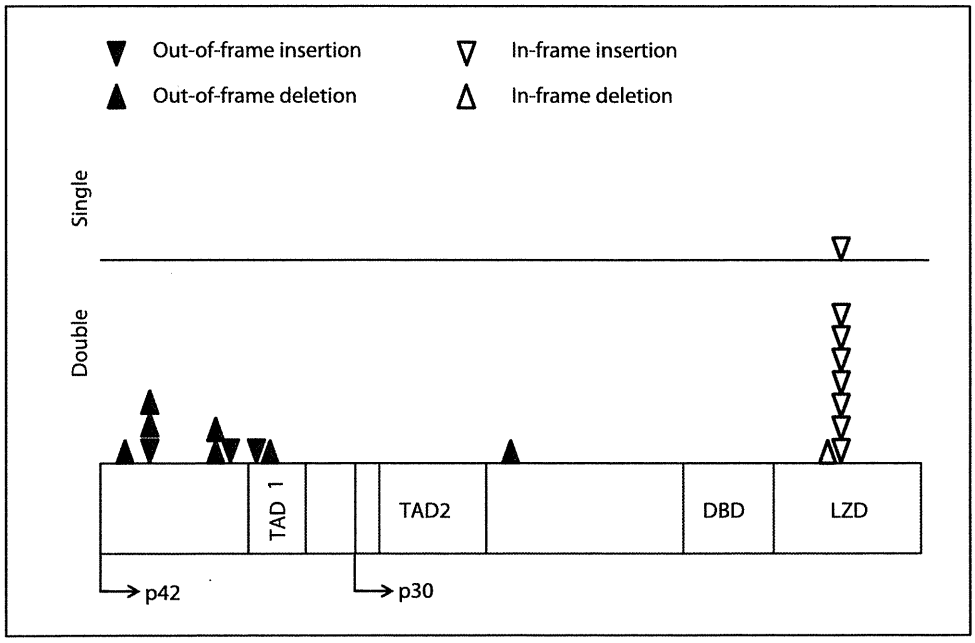

Fig. 2 Location of mutations detected in the *CEBPA*$^{single\text{-}mut}$ and *CEBPA*$^{double\text{-}mut}$ patients. Transactivation domain (TAD) 1, amino acids (AA) 70–97; p30 ATG, AA120; TAD2, AA 126–200; DNA-binding domain (DBD), AA 278–306; leucine zipper domain (LZD), AA 307–358

CEBPA$^{single\text{-}mut}$ genotype (data not shown). The MPO-positivity rate was widely distributed in patients who had mutant *NPM1* without *FLT3*-ITD genotype (median 26, range 0–100) and other genotypes (median 31, range 0–100). Kruskal–Wallis test showed that a significant difference of the MPO-positivity rate among three groups ($P = 0.005$). When comparing the individual groups by Dunn's Multiple Comparisons post hoc test for each group, there was a significant difference only for patients with *CEBPA*$^{double\text{-}mut}$ versus patients with other genotypes.

4 Discussion

While cytogenetic group is considered to be the primary prognostic indicator in AML, the percentage of MPO-positive blast cells could be used to predict the prognosis of patients with normal karyotypes [6]. In this study, we found that *CEBPA* gene mutational status has impact on the frequency of MPO expression: the patients with the *CEBPA* mutation genotype displayed a significantly higher percentage of cells expressing MPO than those with other genotypes ($P < 0.01$). The association was even more significant when analyzed without the *CEBPA*$^{single\text{-}mut}$ carrying patient, suggesting that high blast MPO activity is related to double *CEBPA* mutations. Although the mutant *NPM1* without *FLT3*-ITD genotype has been reported to be associated with a favorable prognosis in AML patients, there was no relationship between this type of mutation and the percentage of blasts showing MPO expression.

It is not clear how the *CEBPA*$^{double\text{-}mut}$ genotype enhances MPO activity in AML blasts. It has been shown that the MPO enhancer contains a *CEBPA* site contributing to its functional activity [23, 24], suggesting that the MPO gene is a major target of C/EBPα. Since it has been shown that both N-terminal frame-shift mutant and C-terminal mutant do not show transcriptional activity [25], we first speculated that mutations of the *CEBPA* gene might lead to decreased MPO activity, which turned out to be wrong. AML1 is another gene that has been reported to participate in up-regulation of MPO gene [26]. An AML1 site was identified in upstream enhancer of the human MPO gene, which appears to be necessary for maximal stimulation of MPO promoter activity. In patients with AML with t(8;21), the translocation results in an in-frame fusion between 5 exons of the AM1 gene and essentially all of the ETO gene producing a chimeric protein [27]. This protein, AML1-ETO, acts as a negative dominant inhibitor of wild-type AML1 [28], which theoretically could lead to down-regulation of AML1 target genes, such as MPO gene. However, blasts with t(8;21) have been shown to display higher levels of MPO expression both in clinical samples and in vitro experiments [29, 30], suggesting that the transcriptional alterations caused by these mutations are complex. The upregulation of blast MPO activity seen in CEBP/α$^{double\text{-}mut}$ cases may be due to alterations in the gene expression profile, rather than a simple dominant negative effect of mutated CEBP/α. Further experiments including investigation of transactivation potential of CEBP/α mutants on MPO promoter is necessary to clarify this mechanism.

CEBPA mutations are associated with a relatively favorable outcome, and it was recently shown in a multivariable analysis including cytogenetic risk and the

Table 3 Frequency *of FLT3*-ITD, *NPM1*, and *CEBPA* mutations by clinical characteristics in de novo AML cases with a normal karyotype

	FLT3		*P*	*NPM1*		*P*	*CEBPA*		*P*
	ITD ($n = 11$)	Other type ($n = 49$)		Mutation without *FLT3*-ITD ($n = 13$)	Other type ($n = 47$)		Double mutation without *FLT3*-ITD ($n = 8$)	Other type ($n = 52$)	
Age			0.08			0.74			0.10
≤50	1	19		5	15		5	15	
>50	10	30		8	32		3	37	
PS			1.00			0.20			0.52
0–2	10	45		11	45		7	48	
3–4	1	4		2	2		1	4	
WBC			0.02			1.00			1.00
≤20,000	2	30		7	25		4	28	
>20,000	9	19		6	22		4	24	
FAB subtype			0.33			0.18			0.58
M1, M2, M4, M5	11	41		13	39		8	44	
M0, M6, M7	0	8		0	8		0	8	
JALSG score[a]			0.79			0.72			0.09
Favorable	0	5		0	5		2	3	
Intermediate	2	18		5	15		5	15	
Adverse	2	9		2	9		0	11	
CR[a]			1.00			0.56			0.56
Achievement	4	27		7	24		7	24	
Failure	0	5		0	5		0	5	

[a] Analysis was carried in 36 patients with intensive chemotherapy

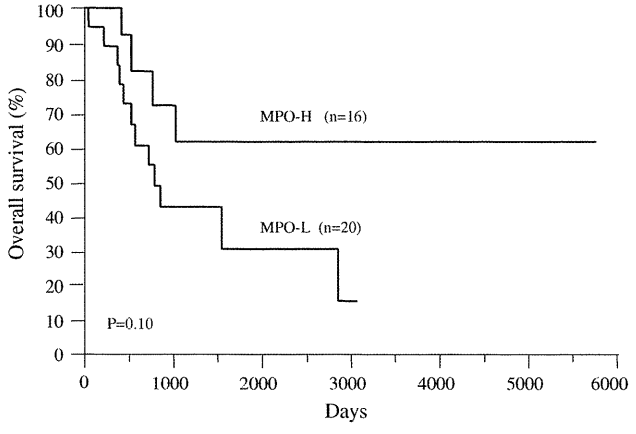

Fig. 3 Kaplan–Meier estimates of the probability of overall survival in 36 patients who received intensive chemotherapy, according to the percentage of myeloperoxidase-positive blasts. MPO-H (MPO-positive blasts: >50%) tended to have a positive effect on overall survival compared with MPO-L (MPO-positive blasts: ≤50%), although the difference was not statistically significant. The statistical significance of differences was evaluated with the log-rank test

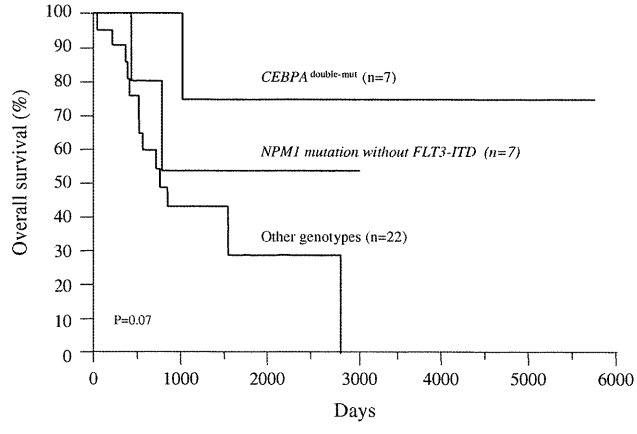

Fig. 4 Overall survival according to genotype in patients administered intensive chemotherapy. 'Other genotypes' was defined as the *FLT3*-ITD genotype, the $CEBPA^{\text{single-mut}}$ genotype, and the triple-negative genotype consisting of the wild-type *NPM1* and *CEBPA* genotypes without *FLT3*-ITD. The patients with the $CEBPA^{\text{double-mut}}$ genotype tended to show higher overall survival compared with the patients with 'other genotypes' ($P = 0.07$)

FLT3-ITD and *NPM1* mutations that the $CEBPA^{\text{double-mut}}$ genotype is associated with favorable overall and event-free survival [15, 16]. In a cohort of 60 cases of adult de novo AML, we identified 1 $CEBPA^{\text{single-mut}}$ case and 10 $CEBPA^{\text{double-mut}}$ cases, and in line with previous reports, our study tended to show better overall survival in $CEBPA^{\text{double-mut}}$ cases compared to cases with wild-type *CEBPA* in patients treated with intensive chemotherapy. We failed to find a prognostic effect in relation to the $CEBPA^{\text{double-mut}}$ in patients treated with low dose

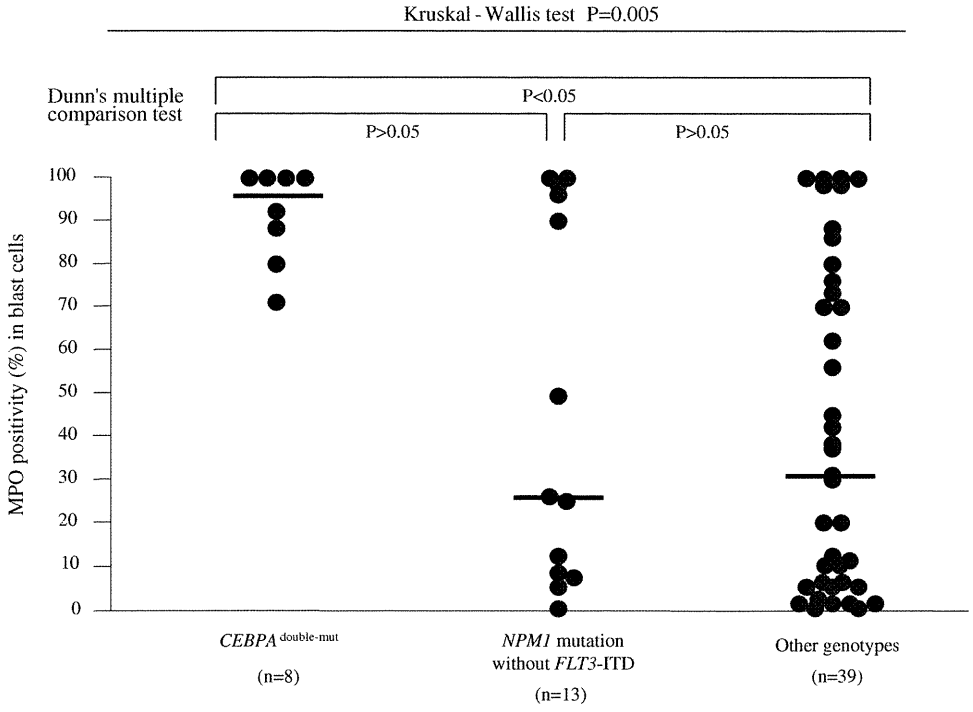

Fig. 5 MPO-positivity rate in blast according to genetic abnormalities in de novo AML patients with a normal karyotype. 'Other genotypes' was defined as the *FLT3*-ITD genotype, the *CEBPA*$^{\text{single-mut}}$ genotype, and the triple-negative genotype consisting of the wild-type *NPM1* and *CEBPA* genotypes without *FLT3*-ITD. The median MPO-positivity rate (*horizontal line*) was significantly different between the *CEBPA*$^{\text{double-mut}}$ genotype and 'other genotypes' (Kruskal–Wallis test followed by Dunn's multiple comparisons test: $P < 0.05$)

chemotherapy (data not shown), suggesting that the standard chemotherapy dose is necessary to improve the outcome of *CEBPA*$^{\text{double-mut}}$ cases.

It is unclear why *CEBPA*$^{\text{double-mut}}$ AML patients have a better outcome than those with *CEBPA* wild-type AML. One explanation is that high MPO expression leads to increased sensitivity to chemotherapeutic agents, such as to Ara-C [8]. To test this hypothesis, we also examined the association between blast MPO positivity and overall survival in *CEBPA* wild-type cases. Unexpectedly, when the patients were treated with intensive chemotherapy, the percentage of MPO-positive blasts was not significantly associated with overall survival in this group (data not shown), suggesting that the level of MPO expression itself is not responsible for the improvement in overall survival. However, as this analysis only involved 28 cases, we need to increase the number of cases in order to draw a definitive conclusion.

In summary, the data presented here suggested that the *CEBPA*$^{\text{double-mut}}$ genotype was associated with high MPO blast activity in patients with a normal karyotype. Although the results were obtained from a single institution, the presence of *CEBPA*$^{\text{double-mut}}$ genotype in high MPO group could explain, at least in part, why high MPO blast activity is associated with better overall survival. Further studies in a larger cohort of patients are necessary to assess blast MPO activity as a prognostic factor, especially in *CEBPA* wild-type patients with a normal karyotype.

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Conflict of interest All authors have no conflict of interest to report.

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CASE REPORT

Successful treatment of a chronic-phase T-315I-mutated chronic myelogenous leukemia patient with a combination of imatinib and interferon-alfa

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Abstract The T315I BCR-ABL mutation in chronic myelogenous leukemia (CML) patients is responsible for up to 20% of all clinically observed resistance. This mutation confers resistance not only to imatinib, but also to second-generation BCR-ABL tyrosine kinases, such as nilotinib and dasatinib. A number of strategies have been implemented to overcome this resistance, but allogeneic stem cell transplantation remains the only established therapeutic option for a cure. A 61-year-old male was diagnosed with Philadelphia chromosome-positive chronic-phase CML in 2002. He was initially treated with imatinib and complete cytogenetic response (CCyR) was achieved 12 months later. However, after 18 months, a loss of CCyR was observed and a molecular study at 24 months revealed a T315I mutation of the BCR-ABL gene. At 30 months, imatinib/interferon-alfa (IFNα) combination therapy was initiated in an effort to overcome the resistance. Thirty months later, he re-achieved CCyR, and the T315I BCR-ABL mutation disappeared at 51 months. To our knowledge, this is the first case report showing the effectiveness of imatinib/IFNα combination therapy for CML patients bearing the T315I BCR-ABL mutation.

Keywords Chronic myelogenous leukemia · Imatinib · Interferon · T315I

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Introduction

Chronic myelogenous leukemia (CML) is a clonal disease of the hematopoietic stem cell, which is characterized by an increased growth of predominantly myeloid cells in the bone marrow. The disease is associated with the Philadelphia chromosome, which arises by a reciprocal translocation between chromosomes 9 and 22 and harbors the BCR-ABL fusion oncogene [1]. Small molecules that specifically target the BCR-ABL gene product provide a successful treatment approach which can lead to a reduction in BCR-ABL transcripts below detectable levels. The drug imatinib, a rationally designed tyrosine kinase inhibitor (TKI), showed a superior response rate, improved progression-free survival, and overall survival, as compared with the previous standard therapy with IFNα [2–4].

Although high response rates are observed in patients who receive imatinib treatment, a small percentage of chronic-phase (CP) CML patients are refractory to the therapy [2]. Patients develop imatinib resistance via

multiple mechanisms, with some being BCR-ABL dependent and others BCR-ABL independent. To overcome the failure of imatinib, multiple strategies are under investigation. These strategies include a dose escalation of imatinib and switching to second-generation TKIs. Nilotinib and dasatinib are currently approved for the treatment of patients with CML who have developed resistance or intolerance to imatinib [5, 6].

The development of a T315I BCR-ABL mutation (threonine to isoleucine mutation at amino acid 315) is of particular concern as it confers resistance to all available TKIs [7–10]. The only established salvage option for patients harboring the T315I BCR-ABL mutation is allogeneic hematopoietic stem cell transplantation (allo-HSCT) [11–13]. However, allo-HSCT can be performed only in eligible patients [14]. For patients who could not receive allo-HSCT, new agents with activity against the T315I BCR-ABL mutation, such as danusertib and omacetaxin, have been developed [15, 16]. However, they are still in the clinical trial stage and it will take years before these agents can be put into use. Hence, patients harboring the T315I BCR-ABL mutation, who are not eligible for allo-HSCT, require treatment with combinations of already approved drugs.

We report the successful treatment of a CML patient harboring the T315I BCR-ABL mutation with a combination of imatinib and IFNα.

Materials and methods

Total RNA extraction and cDNA synthesis

Total leukocytes in bone marrow and peripheral blood samples were isolated by centrifugation following red blood cell lysis and total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA). cDNA was synthesized using oligo-dT primers and Super Script III Reverse Transcriptase (Invitrogen).

TaqMan quantitative reverse transcriptase-polymerase chain reaction

Quantitative reverse transcriptase-polymerase chain reaction (RQ-PCR) for BCR-ABL transcript levels were performed using the LightCycler (Roche Diagnostics, Mannheim, Germany) and LightCycler TaqMan Master (Roche Diagnostics). Primers and TaqMan probe sequences published in the EAC network protocol were used for RQ-PCR [17]. The amount of the fusion gene in the original sample was calculated by means of a standard curve (created with the BCR-ABL fusion gene or the ABL gene cloned in plasmids) and expressed as the BCR-ABL/ABL ratio.

Direct sequencing of ABL kinase domain

A nested PCR sequencing approach was used for direct sequencing of the ABL kinase domain, with a first-round amplification of the BCR-ABL transcript followed by two separate PCR reactions. For the nested PCR, the primers were used as described previously [18, 19]. To screen for mutations, the PCR products were sequenced in both the directions with the following: ABL-1F (5′-ACAGGATCAACACTGCT TCTGA-3′); ABL-1R (5′-TGGCTGACGAGATCTGAGTG-3′); ABL-2F (5′-ATGGCCACTCAGATCTCGTC-3′); and ABL-2R (5′-GATACTGGATTCCTGGAACA-3′) using a BigDye Terminator v3.1 Cycle Sequencing Kit and the ABI Prism 3100xl Genetic Analyzer (Applied Biosystems, CA, USA).

Quantitative T315I BCR-ABL mutational analysis by pyrosequencing

Quantitation of T315I BCR-ABL and un-mutated BCR-ABL transcript levels were performed using the PyroMark ID Pyrosequencing system (QIAGEN). First-round PCR was carried out followed by second-round PCR for T315I BCR-ABL mutation including one biotin-labeled primer. Primers and PCR conditions were used as described previously [20]. The linearity of quantitative T315I BCR-ABL mutation by pyrosequencing was confirmed by subjecting cDNA generated from graded mixes of Ba/F3 cell lines (RIKEN Cell Bank, Tsukuba, Japan) transfected with BCR-ABL cDNAs containing either the un-mutated BCR-ABL sequence or the T315I BCR-ABL mutation.

Case report

A 61-year-old male was referred to our hospital due to leukocytosis, thrombocytosis, and hepatosplenomegaly (hypochondrial spleen size 8 cm) in October 2002. Complete blood cell analysis showed that the white blood cell count was 138,900/μl, with 36% neutrophils, 3% myeloblasts, 5% promyelocytes, 5% myelocytes, 14% metamyelocytes, 6% lymphocytes, 5% monocytes, 5% basophils, and 3% eosinophils; hemoglobin concentration was 11.2 g/dl; and the platelet count was 122.1 × 10^4/μl. Bone marrow analysis showed hypercellularity with significant myeloid hyperplasia with 3.0% myeloblasts. Chromosomal analysis (G-banding) revealed that there were no additional chromosomal abnormalities other than t(9;22)(q34;q11). No BCR-ABL kinase domain mutation was detected by direct sequencing (Fig. 1a) and also by pyrosequencing. He was diagnosed with CP-CML. The Sokal score was 1.94, indicating high risk.

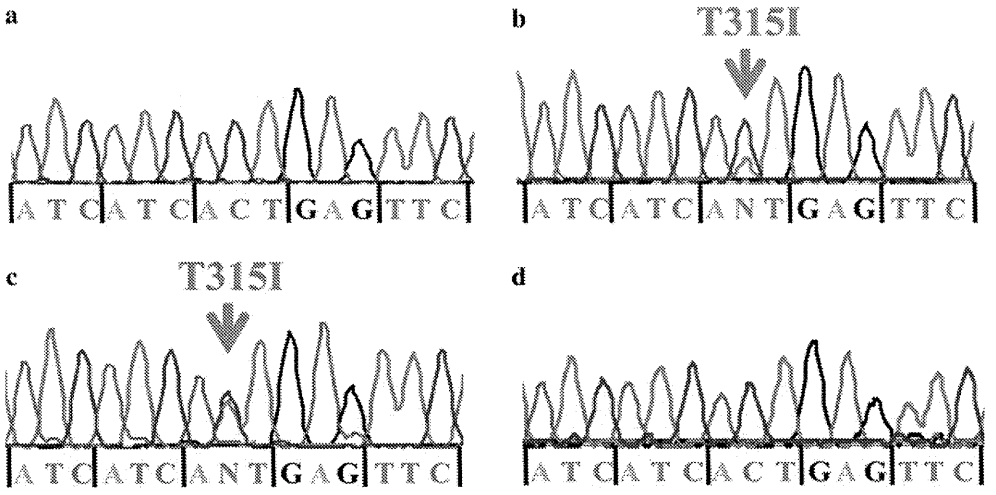

Fig. 1 T315I BCR-ABL mutation by direct sequencing: **a** at diagnosis, **b** at 18 months after starting imatinib, **c** at 24 months after starting imatinib, **d** at 51 months after starting the combination therapy

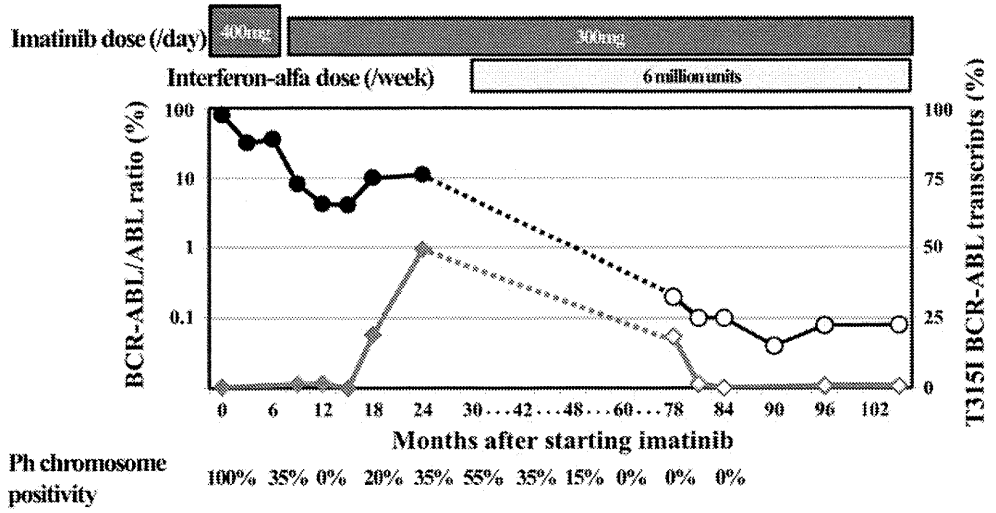

Fig. 2 Clinical course of total and T315I BCR-ABL mutant transcript levels. The figure shows total BCR-ABL transcript levels (*solid line*) measured by RQ-PCR and the relative size of T315I BCR-ABL mutant transcript levels (*dotted line*) by pyrosequencing. The *filled circle* and *filled square* represent samples from bone marrow, and the *open circle* and *open square* represent samples from peripheral blood. Ph chromosome positivity (%) represents the ratio of Ph-positive cells in bone marrow cells determined by G-band chromosomal analysis

He was registered in the clinical trial (Japan Adult Leukemia Study Group, CML202 study) and imatinib was initiated with a dose of 400 mg/day in October 2002. A dose reduction (300 mg/day) was necessary after 6 months due to muscle cramp, which was considered to be a side effect. Complete hematologic response (CHR) and complete cytogenetic response (CCyR) were achieved within 1 and 12 months of treatment, respectively. However, after 18 months of imatinib treatment, a loss of CCyR was observed and a direct sequencing study at 24 months revealed a T315I mutation of the BCR-ABL gene (Fig. 1b). The earlier samples (at 18 months) were then analyzed retrospectively and the mutation was also identified. Even though pyrosequencing revealed that T315I transcripts increased over 2.5-fold during the 18- to 24-month period (Fig. 1c), total BCR-ABL transcripts measured by a RQ-PCR remained unchanged: ratios of BCR-ABL to ABL were 10.1% at 18 months and 11.1% at 24 months, respectively. Because a loss of the major cytogenetic response occurred at 30 months, a combination therapy which consisted of imatinib and IFNα was initiated. IFNα was administrated at a dose of 6 million Units/week. Thirty months after the initiation of the imatinib/IFNα combination therapy, he re-achieved CCyR. Forty-eight months after, the T315I BCR-ABL mutation remained detectable although CCyR was maintained. After 51 months, RQ-PCR revealed a reduction of BCR-ABL transcripts by 3 or more logs [i.e., major molecular response (MMR)], and the T315I BCR-ABL mutation was not detected by direct sequencing and pyrosequencing (Fig. 1d). The MMR was still maintained at 75 months after the initiation of the imatinib/IFNα combination therapy without any signs of a recurrence of the T315I BCR-ABL mutation (Fig. 2). Although he experienced grade 2 anemia, grade 1 neutropenia, and thrombocytopenia according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0, it was possible to continue the imatinib/IFNα combination therapy with no dose reduction.

Discussion

The current treatment algorithm for patients with CML suggests that if the patient develops a T315I BCR-ABL mutation, allo-HSCT or participation in clinical trials should be considered (new agents against the T315I BCR-ABL mutation [15, 16, 21–24] are still in trials). In our case, the imatinib/IFNα combination therapy used resulted in MMR, suggesting its effectiveness in patients harboring the T315I BCR-ABL mutation. De Lavallade et al. [25] have reported the clinical outcome for a CML patient who acquired the T315 BCR-ABL mutation while on imatinib, that was treated successfully with IFNα alone. In their report, while the level of T315I BCR-ABL mutant transcripts decreased with the interferon therapy, the total amount of BCR-ABL transcripts was relatively stable, suggesting that the CML clone harboring an un-mutated BCR-ABL was expanding during that period. To prevent this phenomenon, we chose a combination therapy with imatinib and IFNα. This therapy theoretically seemed reasonable because it would inhibit both the T315I-mutated and the un-mutated BCR-ABL clone, and as shown in this report, it was quite successful. Determining whether or not the T315I BCR-ABL mutated clone is more susceptible to IFNα than an un-mutated clone would be of interest.

In conclusion, although our experience is limited to one patient, imatinib/IFNα combination therapy could be a viable treatment option for CP-CML patients with a T315I BCR-ABL mutation. Further studies are necessary to confirm the efficacy and applicability of imatinib/IFNα combination therapy.

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Late effect of Atomic bomb radiation on myeloid disorders: leukemia and myelodysplastic syndromes

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Abstract Leukemia was the first malignancy linked to radiation exposure in atomic bomb survivors. Clear evidence of the dose-dependent excess risk of three major types of leukemia (acute lymphocytic leukemia, acute myeloid leukemia [AML], and chronic myeloid leukemia) was found, especially in people exposed at young ages. Such leukemia risks were at their highest in the late 1950s, and declined gradually thereafter over the past 50 years. Findings from recent risk analyses, however, suggest the persistence of AML risk even after 1990, and evidence of increased risk of myelodysplastic syndromes (MDS) due to atomic bomb radiation has recently been shown. High-risk MDS and forms involving complex chromosomal aberrations were found to be much more frequent in people exposed to higher radiation doses. These lines of epidemiological evidence suggest that the risk of radiation-induced hematological malignancies has persisted for six decades since the initial exposure.

Keywords Atomic bomb survivors · Ionizing radiation · Leukemia · Myelodysplastic syndromes · Genomic instability

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Introduction

Ionizing radiation is a well-established human carcinogen. Numerous population studies have clearly showed the association between the higher-dose radiation and the higher incidence of various malignancies. The effects of ionizing radiation on human body can be separated into two phases: "early effects" such as acute radiation syndrome, and "late effects" that occurred latter following acute phase. One of the major problems of the "late effects" is the long-lasting effect on the development of malignancies.

Much of what we have known about the long-term carcinogenic effects of radiation exposure comes from studies of Hiroshima and Nagasaki atomic bomb survivors. Nearly 3 years after the atomic bombings in 1945, local doctors in Hiroshima and Nagasaki for the first time noticed the increased number of patients with leukemia in survivors. This early observations led to the establishment of a multidisciplinary collaborative project named "Open City Study (OCS)" in 1950, which was a population-based leukemia registry system including other hematological malignancies in both cities. The first epidemiological paper reporting a clear evidence of an excess risk of leukemia in survivors was published in 1952 [1]. Then, in 1958, a cohort of Life Span Study (LSS) in the Radiation Effects Research Foundation (RERF) was established to investigate the long-term effects of radiation exposure on the development of all malignancies by linkage of Nagasaki and Hiroshima tumor registries. The LSS cohort included about 120,000 people, of whom about 93,000 were in Hiroshima or Nagasaki City at the time of bombing. With OCS and LSS studies, cases of leukemia were captured well in both cities. Now these systems grew larger by linked to the regional tumor registries of both cities, and

cases with a variety of malignant diseases have been accumulated. Since then, considerable scientific evidence has published for over 60 years in collaboration with a variety of investigators, including epidemiologists and statisticians at RERF, and pathologists and hematologists at Hiroshima and Nagasaki universities.

As mentioned above, leukemia was the first malignancy to be associated with radiation exposure in atomic bomb survivors. The incidence of leukemia began to increase rapidly in the early 1950s and declined gradually, whereas the incidence of solid cancers began to increase in the early 1970s and have been still increasing. The excess risk of leukemia due to radiation exposure was the extremely highest among various malignancies in atomic bomb survivors. Therefore, leukemia is considered as a predominant type of radiation-induced malignancies in this population. Moreover, recent our work showed the clear dose–response increase in the incidence of myelodysplastic syndromes (MDS), a leukemia-related hematological malignancy, among Nagasaki survivors even after 40 years from exposure, suggesting that the risk of hematological diseases would last life-long. Based on these epidemiological evidence, this review would like to focus on hematopoietic late effects of radiation observed in Hiroshima and Nagasaki survivors.

Leukemia

Overview

The earliest reports of a dose–response increased risk of leukemia in the LSS cohort of atomic bomb survivors was published in 1971 and 1978 [2, 3]. These reports also showed that for the first time there was a significant relationship between the risk of leukemia and age at bombing and time from the exposure. Since then, a series of reports regarding the risk of leukemia was published until the early 1980s. Although the dose–response risk was tried to analyze separately by acute granulocytic leukemia, acute lymphocytic leukemia, and chronic granulocytic leukemia, the type of leukemia in those days was based on the classic classification, which was not exactly identical for modern leukemia classification.

In 1976, a new morphological classification of leukemia, especially of acute leukemia, was established by the French-American-British (FAB) group [4]. It was immediately used world-wide, and could be applied for leukemia diagnosed among survivors between 1950 and 1980, which were 339 cases in the LSS cohort and 766 cases in the OCS cohort. In the mid-1980s, three hematologists reclassified over 60% of the cases of leukemia using the FAB classification [5, 6].

In the LSS cohort, 180 cases were reclassified among 193 cases of leukemia available for peripheral blood smear and/or bone marrow smear [5]. Of those, there were 17 cases with acute lymphoblastic leukemia (ALL), 88 cases of acute myeloid leukemia (AML), 18 cases of chronic myeloid leukemia (CML), and only 3 cases of chronic lymphocytic leukemia (CLL). Because Adult T-cell leukemia/lymphoma (ATL) had already recognized as a distinct disease entity caused by human T-cell-leukemia virus type-1 (HTLV-1) in the middle 1970s in Japan, 30 cases of ATL was also reclassified. Most of the ATL cases among survivors were found in Nagasaki, and it later showed that there was no relationship between radiation dose and the risk of ATL. As well known, Nagasaki City is located in the western part of the island of Kyushu is one of the endemic area of HTLV-1 infection.

In the OCS cohort, 493 cases were reclassified based on the FAB classification into 66 (13.4%) of ALL, 195 (39.9%) of AML, 110 (22.4%) of CML, 4 (0.8%) of CLL, and 42 (8.6%) of ATL. Moreover, in AML, a variety of subtype was determined; M1, a myeloblastic type without maturation of leukemia cells, M2 with maturation of leukemia cells, M3 of promyelocytic, M4 and M5 with the involvement of monocytic lineage, M6 of erythroleukemia, and M7, a megakaryoblastic type. The number of each subtype is shown in Table 1 [6]. So far, there is no data to show whether or not there is any difference in the frequency of subtypes of AML between atomic bomb survivors and general population. Adjuvant radiation therapy for patients with malignancies may increase the development of secondary leukemia or therapy-related leukemia, however, the distribution of FAB subtypes is identical between therapy-related leukemia and leukemia in atomic bomb survivors. There is a report to show that AML patients who were exposed more than 1 Gy of atomic bomb radiation had heavily complex karyotype in AML cells [7]. Therefore, karyotypic analysis of AML would help to solve the issue above, although there was no karyotypic technique until 1960s.

Table 1 FAB subtype of AML among survivors (data from reference [5])

FAB subtype	Number of cases (%)
M1	20 (24.7)
M2	24 (29.6)
M3	13 (16)
M4	10 (12.4)
M5	7 (8.6)
M6	6 (7.4)
M7	1 (1.2)
Total	81

Based on the re-classification of leukemia using FAB criteria, dose–response and time-dependent trend were performed for four major subtypes of leukemia. Tomonaga et al. [6] analyzed the leukemia subtype data reclassified cases for the period from 1945 to 1980 in the OCS cohort in 1993, suggesting that relative risks for ALL and CML are greater than those for AML and that there is no evidence of an excess risk for ATL or CLL. This analysis also gave an important information on a difference in the risk with by subtypes of leukemia, suggesting that ALL and CML rise and reached the peak in the excess risk rapidly after exposure and then declines, whereas the peak in the excess risk of AML occurred later than ALL and CML. Preston and Tomonaga et al. [8] analyzed the leukemia subtype data reclassified cases for the period from 1950 to 1987 in the LSS cohort in 1994. In this report, 237 cases of leukemia with estimated dose by DS86 were used to calculate excess absolute risk (EAR) and excess relative risk (ERR), considering the effect of age at exposure, gender, time after exposure (attained age). EAR was described as excess the number of leukemia patient per 10,000 person-year per Sv. ERR is expressed as relative risk (RR) minus one described as relative risk per Sv when the risk of minimally exposed cases was set as 1. Since the 1994 report is the latest analysis for the incidence of leukemia in atomic bomb survivors, we summarize this report below.

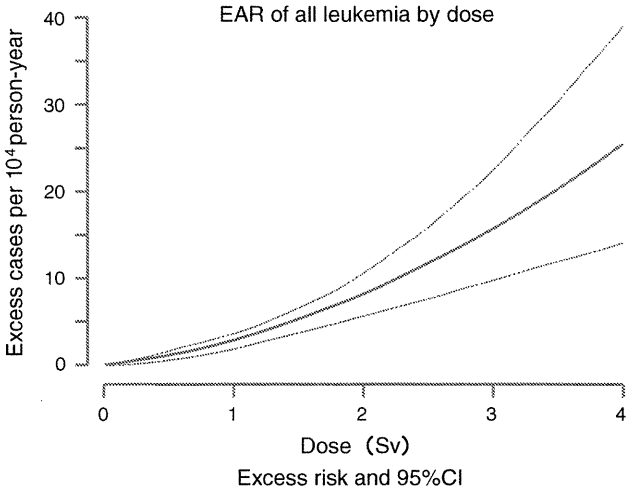

Fig. 1 EAR of all leukemia by dose. The relationship between radiation dose and the excess absolute risk of whole leukemia is shown. *Solid line* is the fitted curve, and the *dotted lines* show the 95% confidence interval (data from reference [8])

Dose–response risk of whole leukemia

If all types of leukemia cases are combined, there is a statistically significant dose response (Fig. 1, $P < 0.001$), which seems non-linear. The dose–response curve is convex downward. The risk itself decreased along with time after exposure. EAR of all types of leukemia was significantly related with gender, age at exposure, and attained age (Fig. 2). Young male had high EARs in the period from 5 to 10 years after exposure, and old men did not have as high risk as young. However, the risk declines more slowly than young. The risk for women was generally lower than men, but that for older female did not seem to decline along with time, suggesting that it may not be so simple as risk of leukemia goes away after certain duration. A model of leukemia risk fitted better when age at exposure and an interaction between age at exposure and time are considered. Adding the interaction of sex and time also gave a better fit for the model than sex alone as a factor. With this model, risk for women decreased slowly with time than for men. The EAR did not differ by city. These results showed that the risk of leukemia differs by sex, age at exposure, attained age, and dose, suggesting a complex mechanism of leukemogenesis by radiation.

Dose–response risk of leukemia by subtype

In this study, risk of leukemia was tested by major subtypes of leukemia: ALL, AML, CML, and CLL. However,

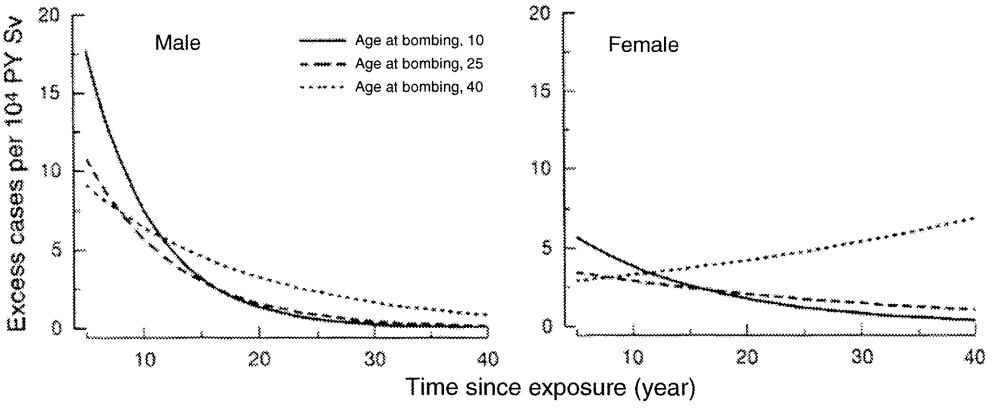

Fig. 2 EAR of all types of leukemia by gender, age at exposure, and attained age. The effect of sex and age at exposure for EAR of whole leukemia is shown. These are estimated using a fitted model that was exposed at 1 Gy. *Left* male, and *right* figure for female (data from reference [8])

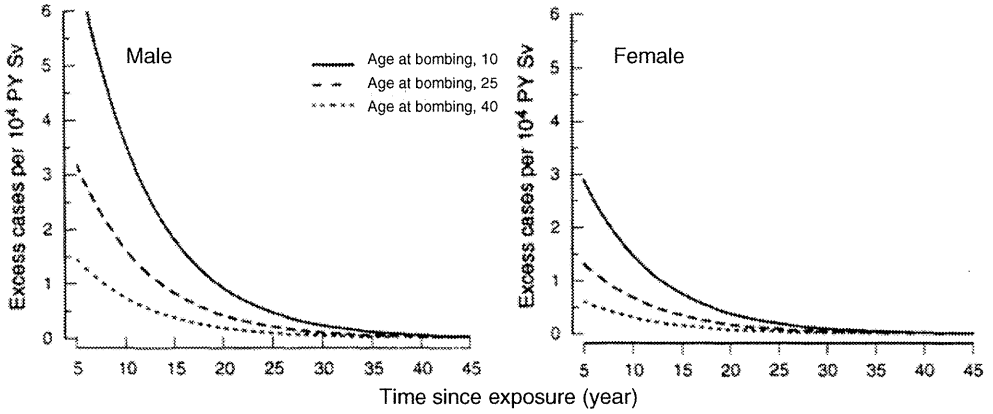

Fig. 3 EAR of ALL by gender, age at exposure, and attained age. EAR of ALL that was affected by sex, age at exposure, and time since exposure is shown. These curves are fitted model for those received 1 Gy. *Left panel* for male, and *right panel* for female (data from reference [8])

because of the small number of CLL cases, risk was not examined well for CLL.

ALL

A dose response of ALL risk was highly significant ($P < 0.001$). Using a linear response model, the EAR decreased with time since exposure and the time-average EAR decreased with increasing age at exposure. Children exposed under age 10 had the highest excess risk, and old female had the lowest risk. Figure 3 demonstrated the EAR for male and female that exposed 1 Sv at the ages of 10, 25 and 40 years. The model-based risk estimates the EAR as 0.62 per 10^4 person-year.

AML

There was a strongly significant dose–response between risk of AML and radiation ($P < 0.001$), which was not a linear pattern. Age at exposure had significant effect on EAR with younger survivors had higher average EAR. Contrast to ALL, sex did not have a significant effect on EAR for AML. Although the EAR for the young decreased along with time, those more than 20-year-old showed constant or increasing risk with time, which also differed from ALL (Fig. 4). Because of the number of cases, risk of each FAB subtype was not tested.

CML

For the EAR for CML, a linear dose–response pattern fitted well. The background rate of CML was significantly different between two cities. Hiroshima male had the highest back ground, and Nagasaki female had the lowest background rate. The EAR for CML decreased rapidly with time. Although age at exposure did not influence the risk,

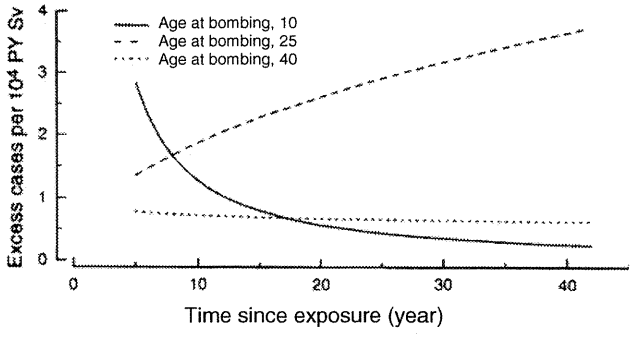

Fig. 4 EAR of AML by age at exposure and attained age. EAR of AML by age at exposure and attained age is shown (models that received 1 Gy). There is no significant effect of sex for EAR of AML, which is different from for ALL (data from reference [8])

there was a significant difference between the EARs for Hiroshima and Nagasaki ($P = 0.005$). The magnitude of the city effect seemed to be explained by differences of the background rates in both cities.

Other leukemias

ATL did not show dose–response, and it is confirmed that radiation exposure did not increase the risk of viral induced leukemia/lymphoma by HTLV-1. The number of CLL cases was too small to analyze.

Leukemia risk after 1990

The results above were based on the data until 1987. So far in three major subtypes of leukemia, ALL, AML and CML, EARs roughly decreased along with time. It seems that the decrease will also continue after 1990, and that it will be soon at the background level or it had been. Although the analyses using an extended follow-up period are in progress, the mortality of leukemia was calculated using LSS data until 2000 [9]. In this report, AML showed a distinct

pattern than other leukemias such as ALL and CML. From 1950 to 2000, there were 124 cases of AML-related death, 58 of CML-related and 19 of ALL-related death with dosimetry system 02 (DS02) dose estimation. ERRs of mortality at 1 Gy were 2.81 for AML, 6.39 for CML and 3.70 for ALL. The ERRs showed clear increase around 10 years after exposure, then decreased rapidly, however, when AML analyzed separately, it showed clear increase after 55 years from exposure (Fig. 5). We do not know what it means so far, but the incidence analysis will be very important, especially for AML. Such effort is under progress.

Myelodysplastic syndromes (MDS)

MDS was clearly defined by FAB group in 1982 [10]. Although several groups of hematologists had already noticed diseases called refractory anemia that did not respond to general treatment, or hematological disorder that proceeded acute leukemia, MDS was not recognized well. It took for a while to be recognized as neoplasm widely. Even "Tumor registry" did not deal MDS as neoplasm until WHO classification defined MDS as neoplasm. These situations had made it very difficult to collect epidemiological data for MDS.

It is well known that chemotherapeutic agents such as alkylating agents can cause MDS and leukemia (mostly AML). These are called therapy-related MDS (AML) or secondary MDS (AML) that are thought to be different from de novo cases. As mentioned above, radiation is a causative agent for leukemia. However, so far, it has not been clear whether radiation causes MDS or not. In the same article describing leukemia among atomic bomb survivors (published in 1994 Ref. [8]), 2 cases of MDS were included, which were too small in number to analyze the relationship between radiation and MDS. To elucidate the relationship between atomic bomb and MDS, "Open City cohort" cases and the LSS cohort were evaluated in Nagasaki area [11]. Among 64,058 survivors who were alive in 1985, 151 cases of MDS were confirmed in the Nagasaki "Open City cohort" (NOC) from 1985 to 2004. These cases had an information about the exposure distance from the hypocenter. In the LSS cohort, among those who were alive at 1985 (22,245 cases), 44 cases of MDS were diagnosed by 2004. These cases had estimated bone marrow dose (DS02). As shown in Tables 2 and 3, each data base provided MDS cases in the category by distance (Table 2, NOC) or bone marrow dose (Table 3, LSS cohort). For MDS cases in NOC, the median age at exposure was 18.5 years (range 0.3–43.4 years), and the

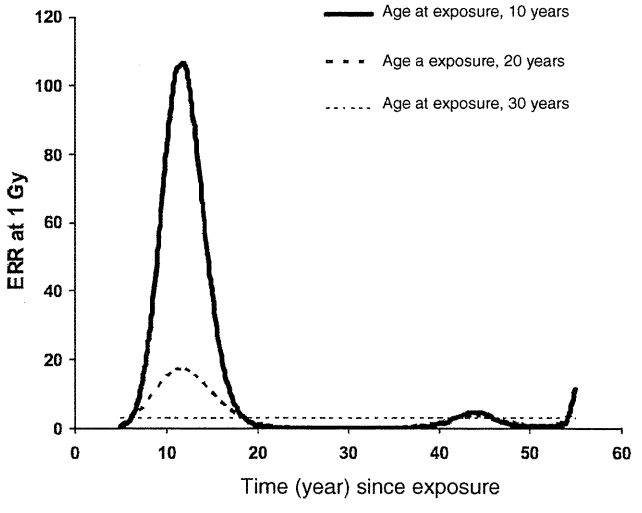

Fig. 5 ERR of mortality by AML at 1 Gy by age at exposure. The excess relative risk of mortality by AML is shown. *Y* axis shows the time after exposure (data from reference [9])

Table 2 MDS cases in the Nagasaki "Open cohort" (data from reference [11])

Exposure distance from the hypocenter (km)	<1.5	1.5–2.999	>3	Total
Sex				
Male	1,693	6,485	16,092	24,270
Female	2,258	10,663	26,835	39,756
Total	3,951	17,148	42,927	64,026
MDS FAB subtypes				
RA	15	28	57	100
RARS	0	1	3	4
RAEB	7	8	14	29
RAEB-t	2	2	2	6
CMML	1	3	4	8
Unclassified	0	2	2	4
Total	25	44	82	151

Table 3 MDS cases in the LSS cohort (data from reference [11])

Bone marrow dose by DS02 (Gy)	>= 1	0.005–0.999	<0.005	Total
Sex				
Male	273	2,665	5,904	8,842
Female	351	4,201	8,851	13,403
Total	624	6,866	14,755	22,245
MDS FAB subtypes				
RA	5	9	20	34
RARS	0	1	0	1
RAEB	2	3	2	7
RAEB-t	1	2	0	3
CMML	0	0	0	0
Unclassified	0	0	2	2
Total	8	15	24	47