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Genome analysis of myelodysplastic syndromes among atomic bomb survivors in Nagasaki

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ABSTRACT

Ionizing radiation is a risk factor for myeloid neoplasms including myelodysplastic syndromes (MDS), and atomic bomb survivors have been shown to have a significantly higher risk of MDS. Our previous analyses demonstrated that MDS among these survivors had a significantly higher frequency of complex karyotypes and structural alterations of chromosomes 3, 8, and 11. However, there was no difference in the median survival time between MDS among survivors compared with those of *de novo* origin. This suggested that a different pathophysiology may underlie the causative genetic aberrations for those among survivors. In this study, we performed genome analyses of MDS among survivors and found that proximally exposed patients had significantly fewer mutations in genes such as *TET2* along the DNA methylation pathways, and they had a significantly higher rate of 11q deletions. Among the genes located in the deleted portion of chromosome 11, alterations of *ATM* were significantly more frequent in proximally exposed group with mutations identified on the remaining allele in 2 out of 5 cases. *TP53*, which is frequently mutated in therapy-related myeloid neoplasms, was equally affected between proximally and distally exposed patients. These results suggested that the genetic aberration profiles in MDS among atomic bomb survivors differed from those in therapy-related and *de novo* origin. Considering the role of *ATM* in DNA damage response after radiation exposure, further studies are warranted to elucidate how 11q deletion and aberrations of *ATM* contribute to the pathogenesis of MDS after radiation exposure.

Introduction

Myelodysplastic syndromes (MDS) are clonal myeloid disorders characterized by cytopenias related to ineffective hematopoiesis, dysplasia, and progression to acute myeloid leukemia.[1] The pathogenesis of MDS is not yet fully understood; however, recently developed DNA sequencing technologies have clearly demonstrated the important roles of genome alterations. The most frequent mutations observed in *de novo* MDS are in the genes coding splicing factors (e.g. *SF3B1* and *SRSF2*), followed by mutations in the genes for DNA methylation (e.g. *TET2* and *DNMT3A*) and histone modification (e.g. *ASXL1* and *EZH2*).[2-4] Typically, these somatic muta-

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tions are sequentially acquired *de novo* with age, and lead to the development of MDS through the aging-related hematopoietic condition called clonal hematopoiesis of indeterminate potential (CHIP).[5-8]

Chemotherapy and radiotherapy are well-known risk factors for the development of myeloid neoplasms (therapy-related myeloid neoplasms, t-MN) including MDS (t-MDS), and the clinical and genetic features of t-MDS are different from those of *de novo* MDS with some overlap.[9] For example, response to treatment and survival rates are very poor for t-MDS, and the karyotypes of t-MDS frequently show deletions of the long arms or the whole of chromosomes 5 and 7, often associated with complex karyotypes.[10] The most frequently mutated gene in t-MDS is *TP53* followed by *RUNX1*. However, *TET2* mutations are less frequent in t-MDS than in *de novo* cases. Several reports have demonstrated that chemotherapy/radiotherapy provides an opportunity for the proliferation of pre-existing hematopoietic stem cells carrying mutations in genes such as *TP53* and *RUNX1*, which eventually progresses to overt t-MN.[11]

Atomic bomb (A-bomb) radiation has also been reported to be a risk factor for developing MDS, with the degree of risk being associated with radiation dose exposure and distance from the hypocenter.[12] Our previous reports showed an increase in chromosomal aberrations and complex karyotypes in MDS among the proximally exposed survivors. However, the median survival time and time to progression to leukemia did not differ between the proximally and distantly exposed groups.[13] Detailed comparisons of chromosomal aberrations between A-bomb survivors and unexposed patients with MDS demonstrated that structural alterations in chromosomes 3, 8, and 11 were significantly increased in MDS among survivors, while alterations in chromosomes 5 and 7 were equally frequent in both groups.[14]

These observations suggest that MDS among A-bomb survivors may have a different pathogenesis compared with *de novo* and t-MDS cases, which may be reflected by their different patterns of genome alterations. To address these issues, we analyzed MDS among A-bomb survivors using next generation sequencing technologies. We found different profiles of driver mutations among proximally exposed patients, as well as frequent deletion of the long arm of chromosome 11 associated with aberrations of ATM.

Methods

We analyzed 32 patients diagnosed as having MDS, and three patients as idiopathic cytopenia of undetermined significance among A-bomb survivors (*Online Supplementary Table S1*), and we divided them into two groups: patients exposed within 2.7 km of the hypocenter were categorized as the proximally exposed (PE) group, and the others as the distally exposed (DE) group according to the approach adopted by the Radiation Effect Research Foundation[15] (*Online Supplementary Methods*). In this study, we compared clinical/genome data between PE and DE groups because these two groups would have lived in similar circumstances (stayed in Nagasaki after A-bomb under similar environmental circumstances including medical access) except for the dose of A-bomb radiation, more than 5mGy (at 2.7 km) or less, which suggested DE as an appropriate control for PE. Based on our previous epidemiological analysis, excess relative risk (ERR) of MDS among survivors that had been exposed to 5mGy would be 0.02 (ERR, 4.3 / Gy).[12] Several different sequencing methods were applied in this study depending on the amount and the quality of DNA samples (*Online Supplementary Tables S1* and *S2*).

First, we performed whole exome sequencing (WES) with matched germline controls for five patients in the PE group, coded as unbiased-WES (U-WES), then WES without matched germline controls (B-WES-T) for three and eight patients in the PE and DE groups, respectively. Limited number of genes (356 genes) were validated for B-WES-T, which were putative drivers of hematologic malignancies[3,4,16,17] or candidate genes identified through U-WES (*Online Supplementary Table S3*). Targeted capture sequencing (T-S) of 154 genes was performed for another ten and nine patients in the PE and DE groups, respectively, without matched controls. Target genes were selected based on published data[3,4,16,17] and results of the U-WES cases (*Online Supplementary Table S4*).

We also investigated three cases (U-WES-3, 4, and 7) using whole genome sequencing (WGS) with matched germline controls (*Online Supplementary Methods* and *Online Supplementary Table S5*).

The DNA copy number alterations (CNA) were analyzed with a SNP array (CytoScan HD Array, Affymetrix, Santa Clara, CA, USA), and CNA in T-S cases were identified in the sequencing data using the CNACS pipeline,[18] because of the insufficient quality and quantity of DNA for SNP array. Although copy number states of whole chromosomes could not be evaluated, frequently affected regions in MDS, such as the long arms of chromosome 5 (5q), 7q, and 20q, were included among the targets. We also targeted the genes affected by 11q deletion to evaluate the whole arm of 11q because this region was of interest in this study. This study was approved by the ethics committee of Nagasaki University.

Further details of the methods used for this study are available in the *Online Supplementary Methods.*

Results

Clinical features of patients in this study

The major clinical characteristics of the 35 patients who took part in this study are listed in Table 1 with further details provided in *Online Supplementary Table S1*. The median exposure distance from the hypocenter was 1.1 km in the PE group and 3.4 km in the DE group (P<0.001). There were no significant differences in the sex, subtype of MDS, age at diagnosis, or age at the time of the bombing between the two groups. The frequencies of abnormal karyotype and complex karyotype were higher in PE but without statistical significance (Table 1). There was no difference in survival time after diagnosis between two groups (P=0.652) (*Online Supplementary Figure S7*).

Somatic mutations, mutational spectrum, and clonal architecture of myelodysplastic syndromes in the proximally exposed group

Among the five patients (U-WES-3, 4, 5, 7, 8) in the PE group who were analyzed using WGS and/or WES, we identified 5-15 somatic missense and nonsense SNV (mean 9.2 per sample), and 0-2 somatic INDEL (insertions or deletions; mean 1 per sample) on coding exons (Figure 1 and *Online Supplementary Table S6*) per patient. The number of somatic SNV identified using WGS of three patients (U-WES-3, 4 and 7) in the PE group was 1,695, 573, and 756, respectively (*Online Supplementary Tables S7-1, -2, and -3*). The most frequent pattern of nucleotide sub-

Table 1. Patients' characteristics.

Characteristics	PE group (n=18)	DE group (n=17)	P
Sex			
Male	9	5	0.31
Female	9	12	
MDS type (WHO, 2008)			
RCUD	2	1	0.79
RCMD	7	10	
RAEB-1	3	1	
RAEB-2	4	2	
MDS/AML	1	1	
ICUS	1	2	
Age at diagnosis (y.o)			
Median	73	74	0.85
(range)	(57-86)	(53-83)	
Exposure distance (km)			
Median	1.1	3.4*	<0 .001
(range)	(0.5-2.5)	(2.8-7.5)	
Age at exposure (y.o)			
Median	12	12	0.86
(range)	(2-19)	(3-19)	
Number of chromosomal abnormality (G-banding)			
0 (normal karyotype)	4	7	0.31
1-2	8	8	
3 (complex)	6	2	

*The data of three patients were excluded when calculating the exposure distance in the distally exposed (DE) group because they were not present at the time of the bombing but entered within a 2 km radius from the hypocenter within two weeks after the bombing. PE: proximally exposed; RCUD: refractory cytopenia with uni-lineage dysplasia; RCMD: RC with multi-lineage dysplasia; RAEB: refractory anemia with excess blasts; ICUS: idiopathic cytopenia of undermined significance; MDS: myelodysplastic syndromes; AML: acute myeloid leukemia.

stitution was cytosine-to-thymine (C to T) (Figure 2 and *Online Supplementary Figure S3*). Clonal heterogeneities of MDS in these patients were inferred from the analysis of variant allele frequencies of the identified somatic SNV (*Online Supplementary Figure S4*).

Comparison of mutated genes between the proximally exposed and distally exposed groups

Using the U-WES, B-WES-T, and T-S methods, somatic and oncogenic mutations were identified in 16 out of 18 patients (89%) in PE, and 12 out of 17 patients (71%) in DE groups (Figure 3 and *Online Supplementary Tables S6, S8* and *S9*). Among these mutations, in DE group, *TET2* was most frequently affected (5 out of 17 patients, 29%), followed by *SF3B1* (3 out of 17, 18%) and *STAG2* (18%) (Figure 4). However, none of the PE patients had *TET2* or *STAG2* mutations, and the most frequently mutated gene was *SF3B1* (4 out of 18 patients, 22%). There was a statistically significant difference in the frequency of *TET2* mutations between the two groups (P=0.019), but not for *STAG2* (P=0.104). Mutations in *TP53* were identified at very similar frequencies in the two groups: PE: 2 out of 18 patients (11%); DE: 2 out of 17 (12%) (P=1.00). There was also no significant difference in the frequency of *RUNX1* mutations between the two groups (11% and 6% in PE and DE, respectively; P=1.00).

Mutated genes were categorized on the basis of their assumed roles in functional pathways (Figure 5 and *Online Supplementary Table S10*). We found that gene mutations along the DNA methylation pathways were significantly less frequent in the PE group (1 out of 18 patients, 5.6%) than in the DE group (7 out of 17, 41%; P=0.018). Genes coding RNA splicing factors were mutated with equal frequency in both groups. Mutations in genes for transcription factors and the chromatin modification pathway were more frequent in PE than in DE without statistical significance (transcription factors, 39% and 24%, respectively, P=0.47; chromatin modification pathway, 33% and 12%, respectively, P=0.23).

Copy number alterations and affected genes on 11q

Copy number alterations were evaluated by SNP array or T-S as described in the Methods and the *Online Supplementary Methods*, although the T-S data did not cover whole chromosomes. Using these methods, we identified CNA in 11 out of 18 (61%), and 7 out of 17 patients (41%) in the PE and DE groups, respectively (Figure 6A and *Online Supplementary Table S11*). CNA in chromosomes 11 and 20 were more frequent in PE (Figure 6B and *Online Supplementary Figure S5A*). Among the CNA, 11q deletion was identified only in PE with statistically significant difference (33% and 0% in PE and DE, respectively; P=0.019). Copy number loss of chromosome 5q and chromosome 7 were identified with almost equal frequency in both groups [chromosome 5q: PE: 4 out of 18 patients (22%); DE: 2 out of 17 (12%), P=0.66; chromosome 7: PE: 22%; DE: 5 out of 17 (29%), P=0.71] (*Online Supplementary Figure S5B* and *C*).

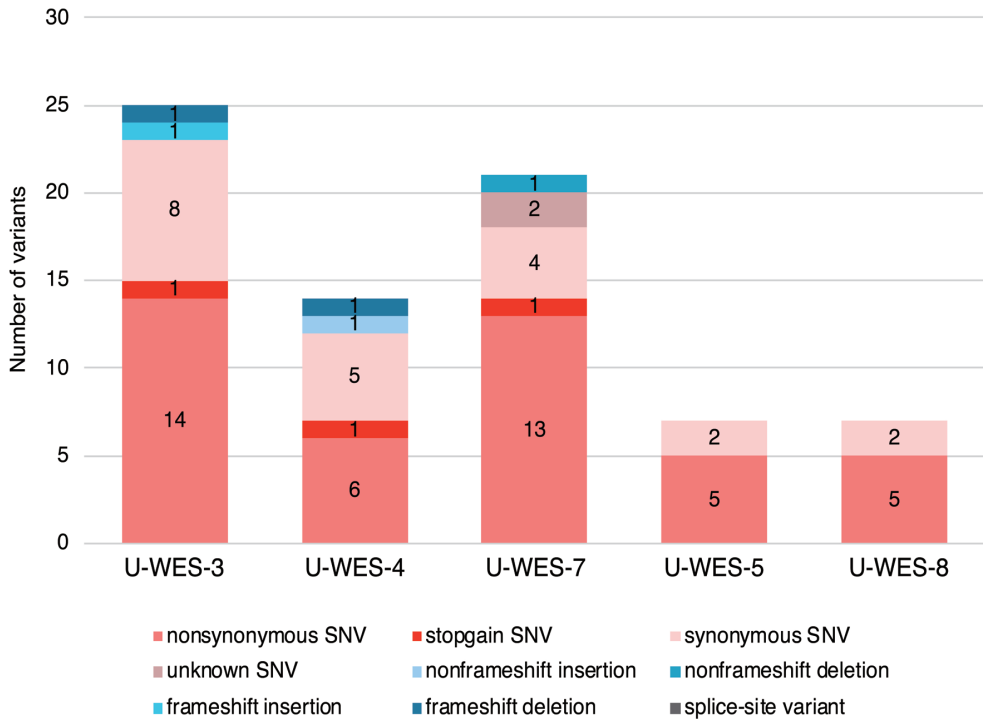


Figure 1. Somatic mutations identified in coding exons of five patients in the proximally exposed group. Each numerical number on the bar charts represents the number of variants of each mutation type identified using whole exome sequencing. No splice site variants were identified among these five patients. U-WES: unbiased-whole exome sequencing.

There are several genes observed to be recurrently affected in MDS and acute myeloid leukemia on 11q, such as *ATM, KMT2A*, and *CBL*.[3,11,16,17] Copy number loss of *ATM, KMT2A,* and *CBL* were identified in five, five, and four patients in the PE group, respectively (Table 2), while copy number gain of *KTM2A* and *CBL* were found in one patient in the DE group (*Online Supplementary Figure S6*). We also identified mutations in *ATM* on the remaining allele in two patients (U-WES-3 and -5) in the PE group; thus, both *ATM* alleles were affected in these patients. Alterations of *ATM* were significantly more frequent in PE than in DE patients (28% and 0%, respectively; *P*=0.046).

Discussion

To better understand how A-bomb radiation contributed to the pathogenesis of MDS among survivors, we analyzed DNA samples from these MDS patients using next generation sequencing and SNP arrays for the first time. We found no apparent increase in the number of SNV among MDS patients proximally exposed to A-bomb radiation compared with those reported for patients with *de novo* or secondary MDS/AML.[5,11] The pattern of nucleotide substitutions was also similar to that observed in *de novo* cases with C-to-T substitution being the most frequent, although this analysis was performed for only three patients in our cohort. We previously reported that the number of chromosomal aberrations was significantly increased in MDS among A-bomb survivors, especially in proximally exposed patients.[13] This finding led us to predict increased nucleotide alterations in MDS among patients in the PE group but this was not the case. In spite of the genotoxic effects of ionizing radiation, it did not apparently contribute to increase the mutational burden in MDS. This may be related to the specific nature of A-bomb radiation: one-off, whole-body exposure that was mostly external. This could explain, at least in part, the reason why there was no difference in survival of MDS patients between the PE and DE groups (*Online Supplementary Figure S7*), and between exposed and unexposed *de novo* MDS, or by the distance from the hypocenter, as we previously reported.[13,14] Considering that our previous study showed cytogenetic risk categories (by revised-International Prognostic Scoring System) significantly divided survival for both MDS among survivors and those unexposed, the same cytogenetic hits seemed to have had a similar influence on their survival.[13,14]

However, we found significant differences in the profile of mutated genes between proximally and distally exposed patients. *TET2* mutations, which are one of the most frequent alterations in myeloid neoplasms including MDS,[3,4] were not detected in the PE group but were observed in the DE group, as frequently as reported for *de novo* MDS (approx. 29%). This was related, at least in part, to the significantly less mutations along with DNA methylation pathways in the PE group, as *TET2* is one of the major genes in this pathway. Mutations in *TP53* and genes coding splicing factors, such as *SF3B1*, were comparable to those in *de novo* cases,[3,4] and were equally frequent in the PE and DE groups. *TP53* is the most frequently mutated gene in t-MN including t-MDS, and it is highly correlated with poor outcome.[11] Our previous work

demonstrated no significant difference in survival time between A-bomb survivors with MDS and unexposed MDS patients,[13,14] which could be partly explained by their similar frequency of *TP53* mutations. Taken together, these findings suggest that the profile of gene mutations in MDS among proximally exposed survivors is different to that of *de novo* MDS patients (reduction or lack of *TET2* mutations in PE cases) and t-MDS patients (fewer *TP53* mutations in PE cases).

A study on mutations of *RUNX1* in MDS among A-bomb survivors (both proximally and distally exposed cases) in Hiroshima noted an increased alteration rate of 46% (6 out of 13 cases) and a missense/frameshift mutation rate of 31% (4 out of 13 cases).[19] In the present study, however, 3 out of 35 cases (8.6%) had *RUNX1* mutations, which was as frequent as reported for *de novo* MDS (approx. 10%). There is no clear explanation for this difference in the frequency of *RUNX1* mutations but the small number of cases examined in each study (13 cases in the Hiroshima study, and 35 cases in this study) might have played a role. It is also possible that the differences in MDS subtypes influenced the results between two studies, as *RUNX1* mutations are enriched in high-risk MDS.[3] In the Hiroshima study, among 13 patients analyzed, there were one patient with refractory anemia with excess blasts (RAEB), eight with RAEB in transformation (RAEB-t), and one AML, sharing 76.9% (10 out of 13) by MDS with increased blasts. Our patient cohort contained

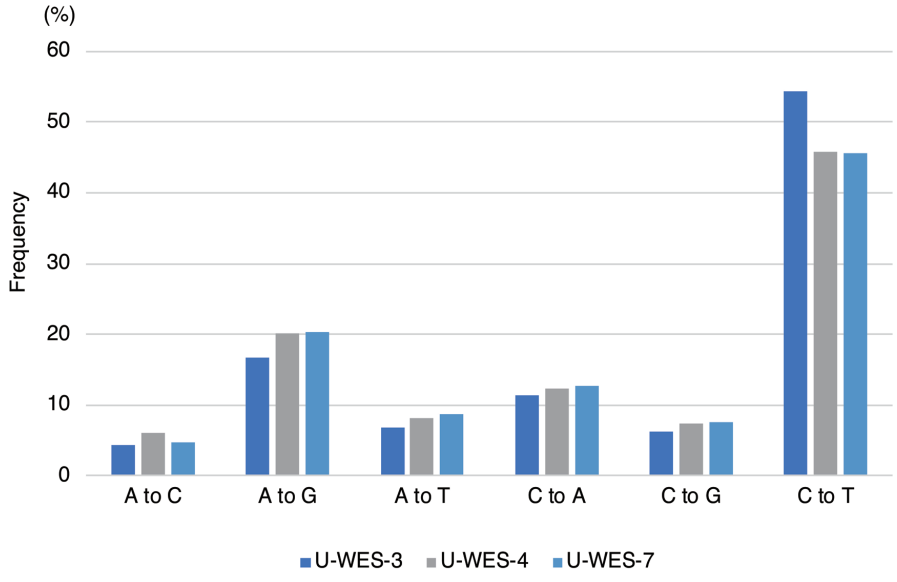


Figure 2. Pattern of nucleotide substitutions in the whole genomes of three patients in the proximally exposed group. The pattern of nucleotide substitution was examined in three patients in the proximally exposed group who were analyzed using whole genome sequencing. Frequencies of each pattern of substitution are represented on the y-axis.

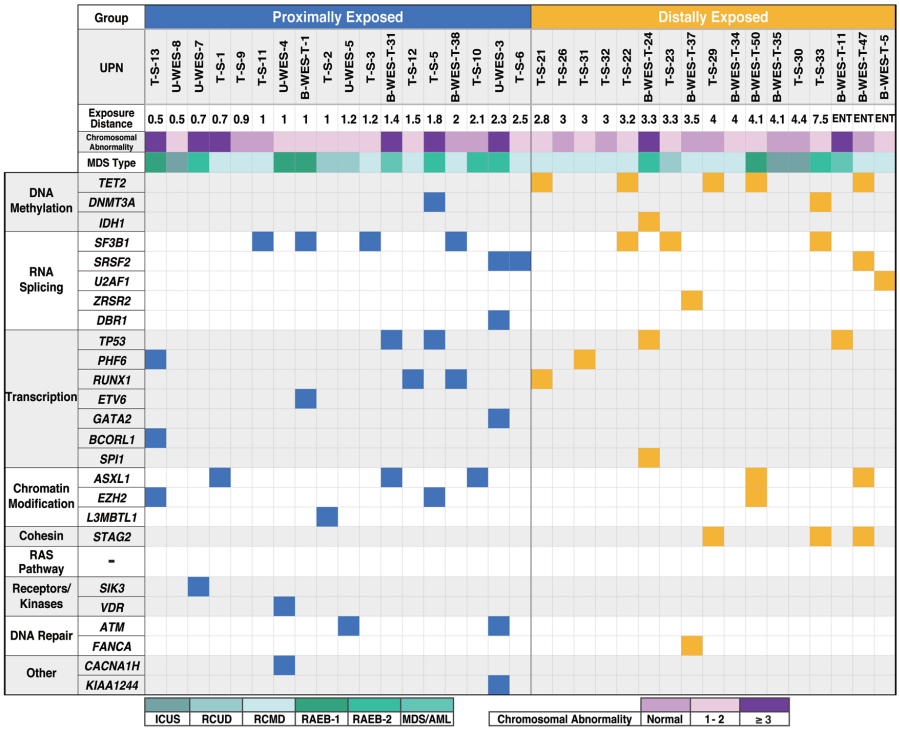


Figure 3. Somatic mutations in myelodysplastic syndromes (MDS) among A-bomb survivors. Each row and column represents a mutated gene and patient, respectively. Identified gene mutations are shown as blue (proximally exposed group) or yellow (distally exposed group) squares. Assumed functional pathways are shown on the far left. UPN: unique patient number; ENT: entered within a 2 km radius from the hypocenter within two weeks after the atomic bombing. RCUD: refractory cytopenia with uni-lineage dysplasia; RCMD: RC with multi-lineage dysplasia; RAEB: refractory anemia with excess blasts; ICUS: idiopathic cytopenia of undetermined significance; AML: acute myeloid leukemia. DE: distally exposed group; PE: proximally exposed group.

12 patients with RAEB or AML, which was 34.3% (12 out of 35) of all participants.

Copy number alterations are frequently found in MDS.[20,21] In this study, we found a significantly higher frequency of copy number loss for 11q in the PE group than in the DE group (P=0.019). Loss of chromosomes 5 or 7, which occurs more frequently in t-MDS (40-50%), and is usually accompanied by a complex karyotype, was observed at an almost equal frequency in the PE and DE groups but less frequently than in t-MDS. Although we did not analyze the entire genes within the commonly deleted region of 11q in the PE group, we detected mutations on the residual *ATM* allele in 2 out of 5 cases (U-WES-3 and U-WES-5) but not in *KMT2A* or *CBL*. The *ATM* mutations, p.D2448V and p.G2891D, were located in the FAT and PI3K domains, respectively. Because of the pathogenic nature of these mutations, U-WES-3 and U-WES-5 appeared to lack expression of functional *ATM* protein. Deletions or mutations of *ATM* have been reported in *de novo* MDS[3,4,7,8,22] and it does not seem to be specific to MDS among A-bomb survivors. However, the significantly higher frequency of 11q deletion and the presence of biallelic alterations of *ATM* strongly suggested its importance in the pathogenesis of MDS among survivors.

Since ionizing radiation induces DNA double-strand breaks (DSB),[23,24] deletions and translocations are frequently observed as a consequence of exposure. Accordingly, our previous study demonstrated that chromosomal translocations were significantly increased in MDS among A-bomb survivors; however, the translocations involving 11q23 where *KMT2A* locates were rare.[14] We observed a significantly higher frequency of 11q aberrations but not translocations among survivors, compared with MDS of unexposed patients.[14] Taken together, these results indicated that aberrations of 11q, especially hemizygous deletion of 11q, could be caused by A-bomb radiation.

Table 2. Copy number alterations and mutations of candidate genes in the frequently affected region on chromosome 11q.

Group	UPN	Candidate genes					
		ATM		KMT2A		CBL	
		CNA	Mutation	CNA	Mutation	CNA	Mutation
PE	U-WES-3	Loss	D2448V	Loss	–	Loss	–
	U-WES-4	Loss	–	Loss	–	Loss	–
	U-WES-5	Loss	G2891D	Loss	–	Loss	–
	U-WES-7	–	–	Loss	–	–	–
	U-WES-8	Loss	–	–	–	–	–
	T-S-3	Loss	–	Loss	–	Loss	–
DE	B-WES-11	–	–	Gain	–	Gain	–
Frequency of alterations (CNA and mutations) in PE- and DE-group		28 % and 0 % (P=0.046)		28 % and 6 % (P=0.18)		22 % and 6 % (P=0.34)	

Minus sign (–) indicates no copy number alterations (CNA) or mutations. PE: proximally exposed group; DE: distally exposed group.

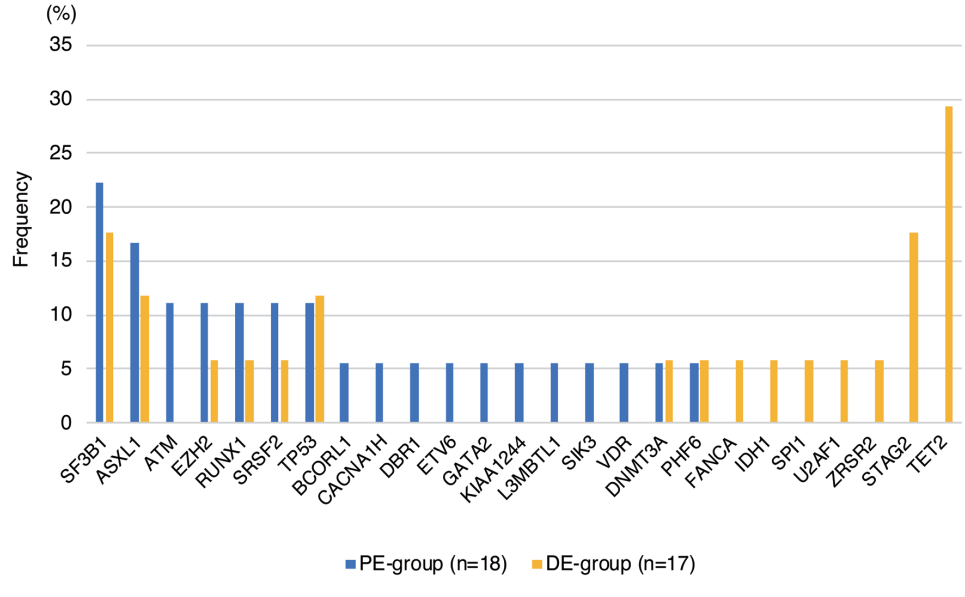


Figure 4. Frequencies of somatic gene mutations in the proximally and distally exposed groups. Frequencies were calculated as the percentage of patients in each group carrying the different mutated genes.

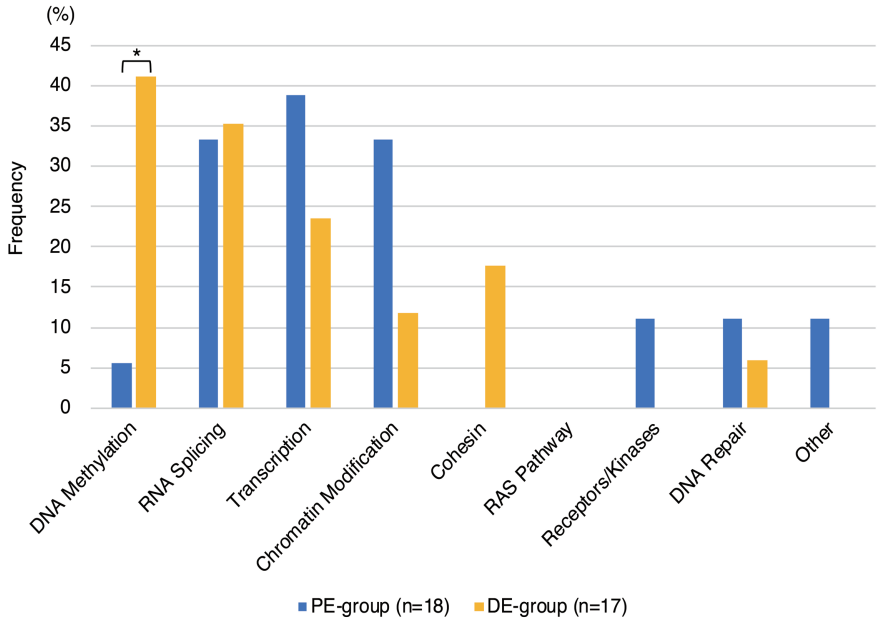
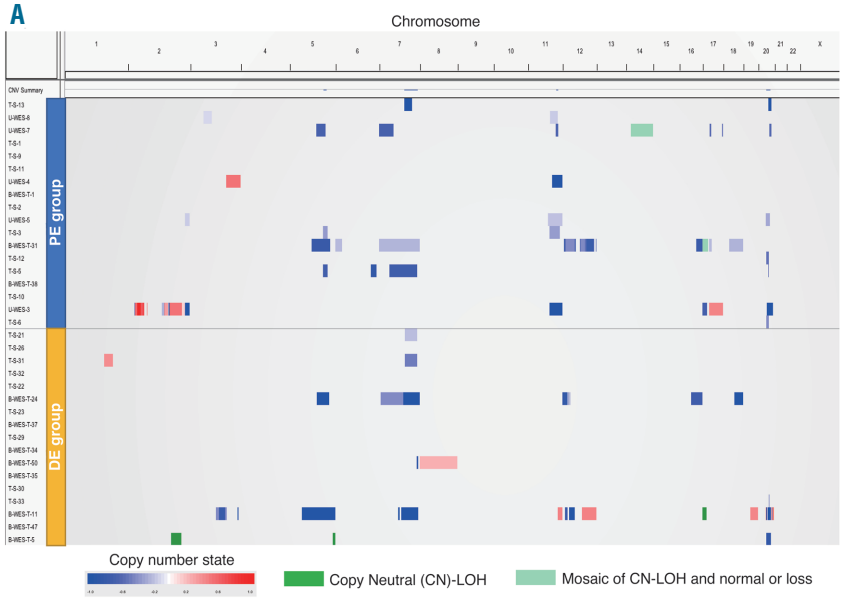


Figure 5. Frequencies of mutated genes categorized by assumed functional pathway. Frequencies were calculated as the percentage of patients in each group carrying mutated genes within the different functional pathways. *P=0.018 using Fisher's exact test. DE: distally exposed group; PE: proximally exposed group.



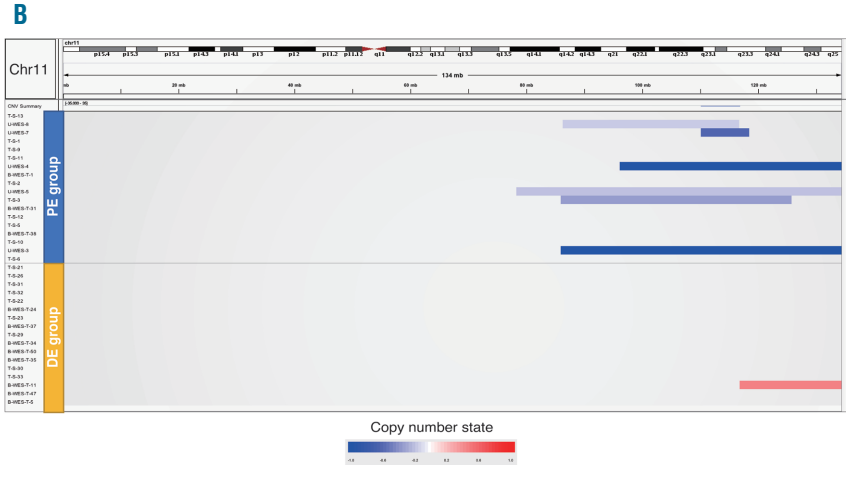


Figure 6. Copy number alterations in myelodysplastic syndromes (MDS) among A-bomb survivors. Each row represents the copy number alterations (CNA) in each patient. The order of patients was in accordance with the exposure distance. (A) CNA on whole chromosomes except for chromosome Y, (B) CNA on chromosome 11. DE: distally exposed group; PE: proximally exposed group.

ATM protein is a key molecule in DNA damage response, in particular, for DSB caused by ionizing radiation,[25,26,27] and it is possible that the loss of one allele of *ATM* was the initial event for clonal selection towards the development of MDS among A-bomb survivors. It is assumed that immature hematopoietic cells that lost *ATM* following A-bomb radiation either responded poorly or incorrectly to other DNA damage generated at the same time. This might also explain why *TET2* mutations, which are common in *de novo* MDS, and are usually thought to be an initiating mutation for *de novo* MDS, were observed at a low frequency in the PE group in this study. Considering the gain-of-function alterations of *KMT2A* and *CBL* in MDS,[28,29,30] the defect in *ATM* function generated by 11q deletion that has also been found in *de novo* MDS[3,22] would have a greater impact on the initiation of MDS among A-bomb survivors. It is necessary to investigate whether alterations in *ATM*, rather than *TET2*, are frequently present in A-bomb survivors who have clonal hematopoiesis of indeterminate potential (CHIP).

In conclusion, we reported a profile of genetic alterations in MDS among survivors exposed to A-bomb radiation, such as fewer mutations in genes along DNA methylation pathways, and frequent 11q deletions and aberrations in *ATM*. Further investigations are warranted to elucidate the role of these genetic alterations in the pathogenesis of MDS after radiation exposure.

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