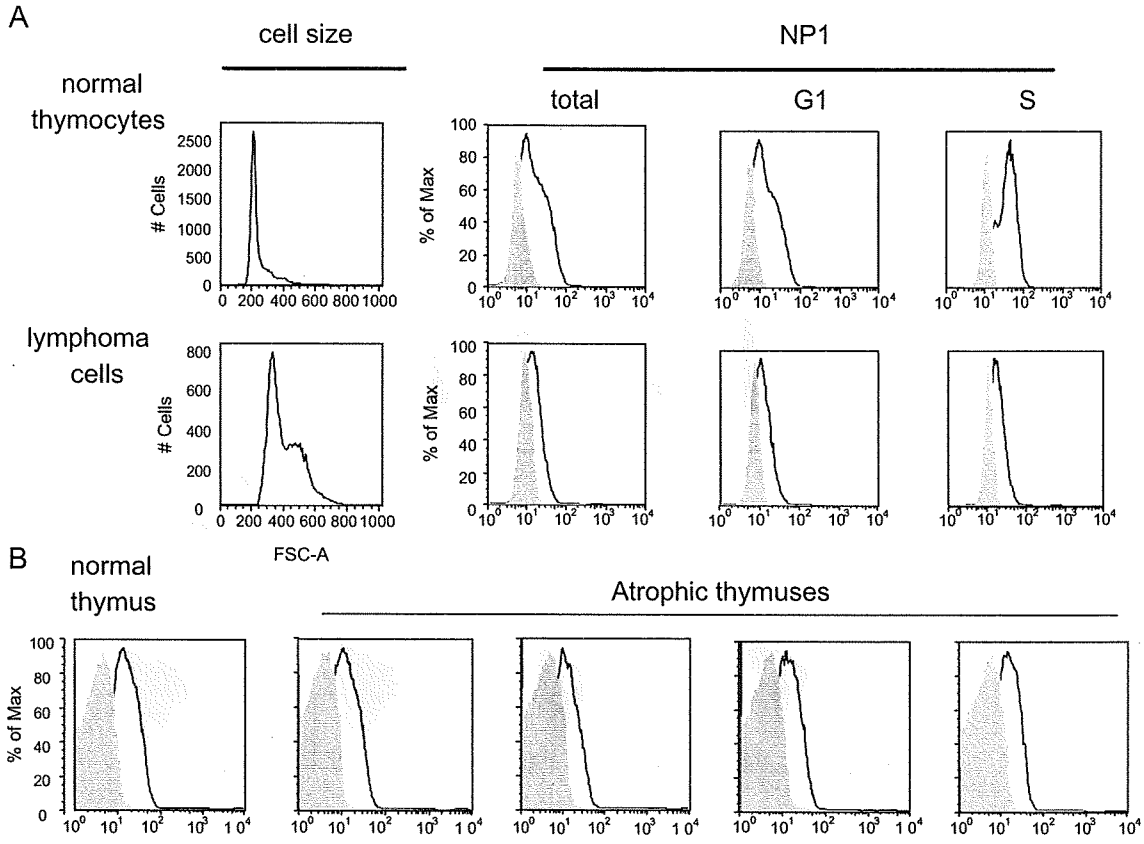

Fig. 3. Flow cytometry of Nrp-1 expression on thymocytes. (A) Profiles of FSC analysis and Nrp-1 staining in total, S phase, and G1 phase cells of unirradiated thymus and thymic lymphoma. Shadowed profiles show control staining without using anti-Nrp-1 antibody. (B) Nrp-1 staining in 40-day atrophic thymuses.

were (Fig. 2B). These findings suggest decrease in the cell number and no marked change in differentiation in atrophic thymus.

Bone marrow cell transfer to irradiated mice 1 week after the last irradiation suppresses the development of thymic lymphomas (1). To confirm this, we examined eight thymic lobes at 60 days after bone marrow cell transfer. As predicted, the cell number was restored to the normal level (3.1×10^7 cells on average), and no C type thymuses were found (not shown).

Nrp-1 expression in atrophic thymuses

Nrp-1 proteins are expressed on the cell surface of both thymocytes and thymic epithelial cells and play a key role in heterocellular adhesions (19, 20). We examined Nrp-1 on thymocytes in normal thymus, thymic lymphomas, and 16 40-day atrophic thymuses using flow cytometry. Levels of Nrp-1 expression on normal thymocytes varied in different phases of the cell cycle, higher in S cells than G1 cells (Fig. 3A). On the other hand, levels on lymphoma cells were lower than those on normal thymocytes and did not much differ between S and G1 cells. Figure 3B shows examples of 40-day atrophic thymuses. Most of them exhibited expression levels similar to that of the control. Neither was any marked difference seen between T type and C type thymus (not shown). These results suggest persistence of the interaction between thymocytes and thymic stroma cells even in atrophic thymuses.

Allelic loss at Bcl11b in C type and T type thymuses

Clonal expansion of thymocytes may result from genetic changes. Hence, we examined allelic loss at *Bcl11b* tumor suppressor gene locus, which was detected at a high frequency in thymic lymphomas (15). *Bcl11b* encodes zinc finger transcription factors involved in the development of $\alpha\beta$ T cells (17). Mice used for this experiment were F_1 hybrids between BALB/c and MSM strains, and hence allelic differences were easily detectable with PCR. Figure 4A shows examples of 40-day thymuses including D-J rearrangement patterns (see Fig. E2A for others). We determined the BALB/c and MSM band ratio in a total of 95 40-day atrophic thymuses and compared the ratios between each atrophic thymus and normal F_1 mouse thymus. When the ratio was >2 or <.50, the thymus was judged as allelic loss-positive (Fig. 4B). The loss was detected in not only C type but also T type thymuses. Nineteen (44%) of the 43 C type thymuses and 17 (33%) of the 52 T type thymuses were allelic loss-positive (Fig. 4C). The high frequency observed in T type thymuses was unexpected. This suggests that clonal expansion of this type proceeds in T type DP thymocytes before β-selection.

Analysis of 80-day thymuses (Fig. E2B) showed that 10 (50%) of the 20 C type thymuses but only 1 (5%) of the 22

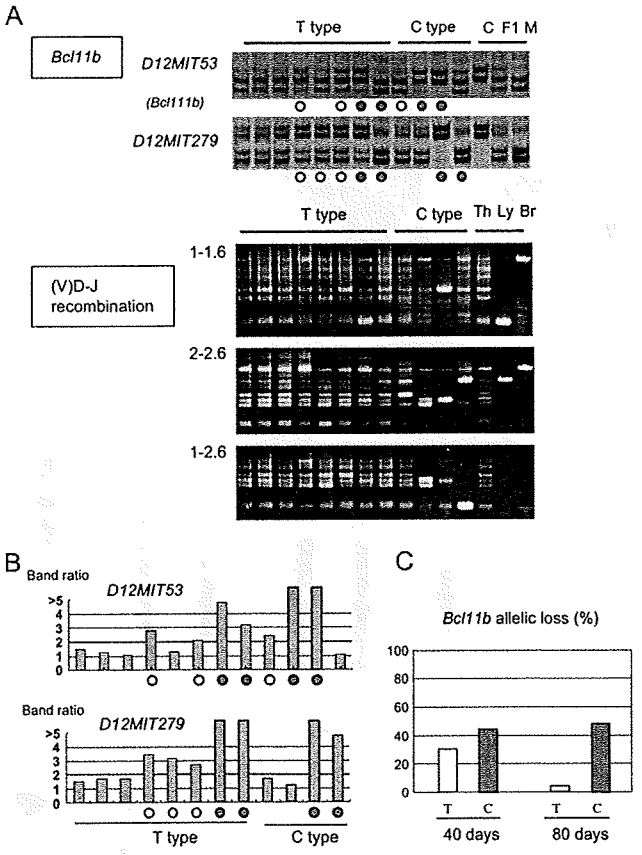

Fig. 4. Allelic losses at the *Bcl11b* locus. (A) Top two panels show polyacrylamide gel electrophoresis for polymerase chain reaction products of *D12Mit53* and *D12Mit279* primer pairs. Chromosomal location of *D12Mit53*, *Bcl11b*, and *D12Mit279* is 108.69 Mb, 109.15–24 Mb, and 109.69 Mb from the centromere, respectively. Bottom three panels show D-J rearrangement patterns, which identifies T type or C type thymus. Closed circles show allelic loss and open circles exhibit allelic imbalance. Thymuses that exhibited allelic loss in at least one of the two loci were decided as allelic loss positive, and this decision was based on three independent polymerase chain reaction experiments. Th = thymus; Ly = lymphomas. (B) Band ratios of BALB/c vs MSM or MSM vs BALB/c are shown relative to that of normal F_1 mouse thymus. Allelic losses are marked by filled circles and allelic imbalances by open circles. (C) Percentage of allelic loss in 40-day and 80-day thymuses that were divided into T type and C type thymus.

T type thymuses exhibited allelic loss. One reason for this rareness relative to 40-day T type thymuses ($p < 0.001$) might be that those T type thymocytes undergo normal differentiation process and hence are not retained within the thymus.

Characteristics of clonally expanded thymocytes

To characterize clonally expanded thymocytes, we examined the cell cycle of the 95 40-day and 42 80-day thymocytes that were isolated from mice 1 h after injection of BrdU (Fig. 5A and B, Fig. E3). We defined the gated region on the FSC vs. SSC dot plot to exclude debris and dead cells. The percentage of the gated region markedly decreased, suggesting enhanced apoptosis. Figure 5B shows BrdU incorporation levels at the vertical axis and DNA contents at the horizontal axis. The DNA content of G1 cells did not differ among unirradiated, irradiated thymocytes, and lymphoma cells, which is consistent with the finding that even thymic lymphoma cells sustain diploidy (21). Figure 5C summarizes the percentage of S phase cells in T type and C type thymuses. Of the 40-day thymuses, no marked difference was found between T and C type thymuses. However, significant increase in the percentage was seen in C type thymus relative to T type thymus in the 80-day thymuses ($p = 0.0034$). Of note is that the high percentage of S cells is a hallmark of thymic lymphomas.

The size of G1 phase cells was measured with flow cytometry (Fig. 5B), because it represents the level of cell cycle progression and metabolic activity. The FSC values of G1 cells depicted a sharp peak in normal thymus, indicating a rather homogeneous cell-size population. The values were much smaller than those of S cells showing a broad peak, as predicted. The FSC analysis of 40-day and 80-day thymuses tended to show the values larger than normal thymus. The cell size of G1 cells in some atrophic thymuses exhibited a broad peak, indicating that those thymuses contained a fraction of larger-sized G1 cells more than normal thymuses did. We designated the cells in this fraction as middle-sized G1 cells because their size was between that of normal G1 and S phase cells. The middle-sized cells may be related to premalignancy because the cell-size enlargement was another characteristic of thymic lymphomas. The middle-sized G1 thymocytes are probably cells that are growing and progressing toward S phase but pausing at the late G1 stage.

Figure 5D summarizes the percentage of middle-sized G1 cells within the thymus. The percentage was determined in each thymus by the criterion whereby the percentage in normal thymus was set to approximately 5% in FSC analysis. The percentage showed a significant difference between T and C type thymuses in 40-day thymuses ($p = 0.014$), and the difference was more prominent in 80-day thymuses ($p = 0.0002$). Half of the 80-day C type thymuses exhibited the percentage more than 40%, whereas only 4 did so in the 21 T type thymuses. Notably, all thymic lymphomas consisted of middle-sized cells, suggesting that the C type middle-sized thymocytes are closer to lymphoma cells. Comparison between 40-day and 80-day thymuses suggests a process of irradiated thymuses toward thymic lymphoma in the order of T type thymus, C type thymus with a low percentage of middle-G1 cells, and C type thymus with a high percentage of middle-G1 cells.

Figure 5E summarizes the percentage of middle-sized G1 cells in *Bcl11b* allelic loss-negative and -positive thymuses. The two groups of 40-day thymuses showed difference in the percentage ($p = 0.041$). The 80-day thymocytes also showed a difference between the two ($p = 0.017$). This suggests the contribution of *Bcl11b*-allelic loss to cell cycle progression. On the other hand, no significant difference in the percentage of S cells was observed between the two groups (not shown).

No marked change in DNA damage checkpoint response

Deoxyribonucleic acid damage checkpoints are activated in premalignant cells and thought to act as barriers against

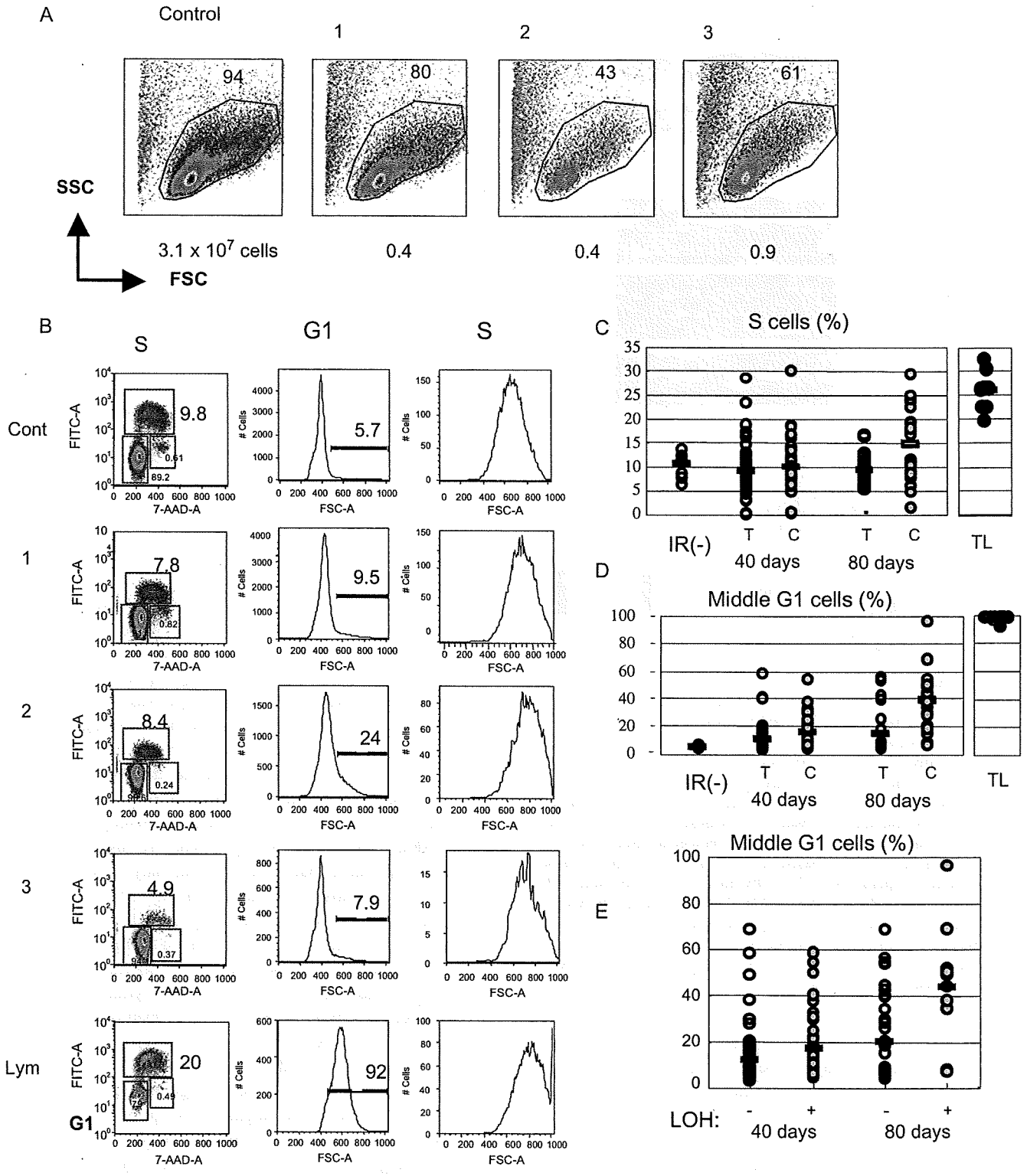

Fig. 5. Hindrance in cell cycle. (A) Flow cytometry of thymocytes in control and irradiated atrophic thymuses. The indicated area is the gated region to exclude dead cells and debris. (B) Flow cytometry of cell cycle and cell size. Left: Vertical axis shows 5-bromo-2-deoxyuridine incorporation levels, and the horizontal axis displays 7-Aminoactinomycin D staining for DNA contents. Middle, Right: Vertical axis shows cell number, and the horizontal axis displays FSC values reflecting the cell size in G1 and S phase thymocytes. The bar in G1 cell analysis shows a fraction of thymocytes of the indicated larger cell sizes, and the number above the bar indicates the percentage of those thymocytes. (C) Percentages of S-phase thymocytes in unirradiated thymus and the atrophic thymuses at 40 days and 80 days after irradiation, which were divided into T type and C type thymus. (D) Percentages of middle-sized G1 cells in the different groups of thymuses as indicated above. (E) Percentages of middle-sized G1 cells in unirradiated thymus and the atrophic thymuses at 40 days and 80 days after irradiation (IR), which were divided into the *Bcl11b* allelic loss-negative and -positive thymus. TL= thymic lymphomas; LOH = loss of heterozygosity.

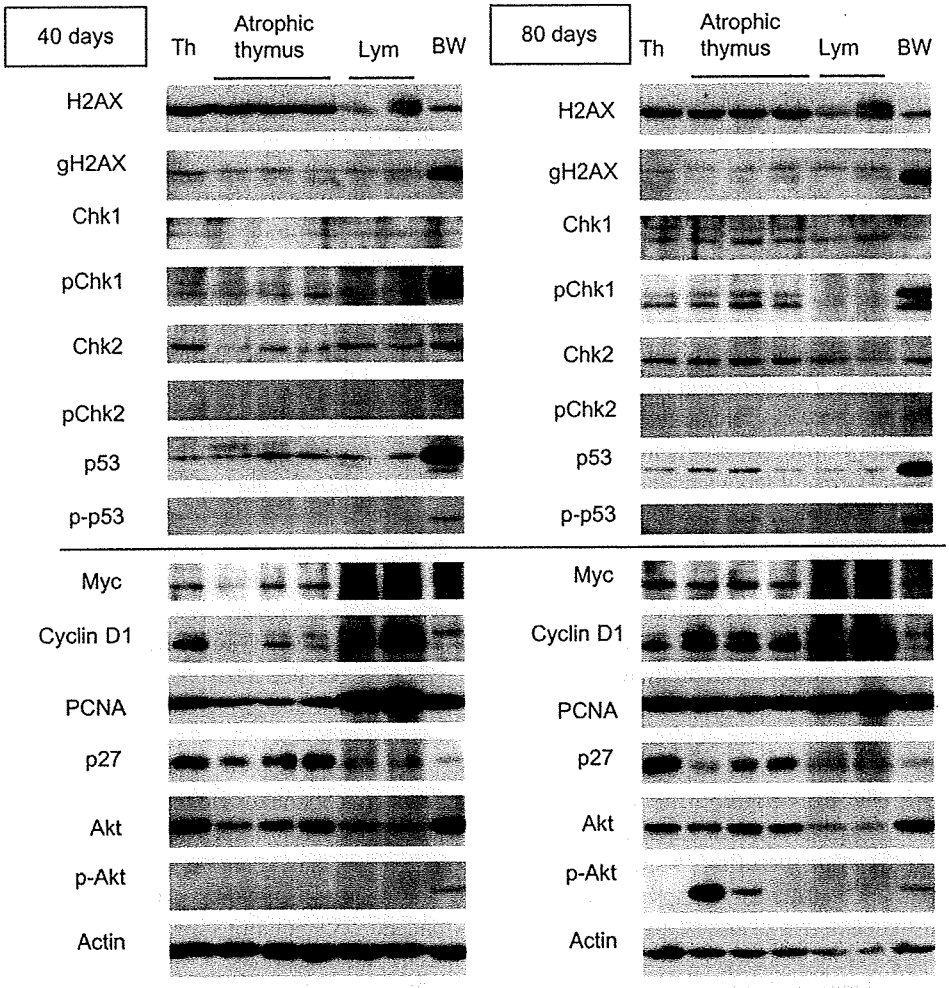

Fig. 6. No marked activation of DNA damage checkpoint genes in C type atrophic thymuses at 40 days and 80 days after irradiation. Western blot analysis includes unirradiated thymus (Th), thymic lymphomas (Lym), and BW5147 mouse T cell lymphoma cell line (BW) for comparison. Antibody used is shown left of each panel. Some BW5147 cell samples were isolated after γ-irradiation. The cell number of the three 40-day thymuses was 4.0, 8.0, and 6.0 × 10^6, respectively, and that of the three 80-day thymuses was 2.4, 2.5, and 1.8 × 10^7, respectively.

cancer development (10–13). To examine the checkpoint status in C type atrophic thymuses, we performed Western blot analysis for proteins involved in the checkpoint responses H2AX, Chk1, Chk2, and p53 (Fig. 6). For comparison, thymic lymphomas and BW5147 mouse T cell lymphoma cell line were analyzed. No difference in their activation was observed between normal thymus and either 40-day or 80-day atrophic thymuses. Another nine samples of 40-day thymuses also showed similar results (not shown). Only p53 amount showed minimal increases in some of the atrophic thymuses. These results indicated no activation of DNA damage checkpoints in C type atrophic thymuses.

Figure 6 includes analysis of Myc, cyclin D1, PCNA, and p27, which are related to cell cycle progression, and Akt, which is related to cell size (22). Expression of cyclin D1 and PCNA was decreased in the 40-day C type atrophic thymuses, whereas expression of p27 cdk-inhibitor also tended to decrease. No activation was noted in Akt. Of the 80-day C type atrophic thymuses, levels of cyclin D1 and PCNA expression increased relative to the 40-day thymuses. On the other hand, the decrease of p27 was more marked, and phosphorylation of Akt was noted in some of the thymuses. These results indicated changes in signaling pathways of cell cycle and cell size in some of the 80-day C type atrophic thymuses.

DISCUSSION

Prelymphoma is assumed to exist in the γ-ray–induced atrophic thymus (1, 2). In this study, we characterized thymocytes in the atrophic thymus and changes in signaling pathways in those thymocytes. Approximately 40% of 40-day thymocytes (harvested 40 days after irradiation) at an early stage during lymphomagenesis showed limited D-J rearrangement patterns at the *TCRβ* locus, indicating clonal expansion of a few parental thymocytes having passed β-selection. Despite their clonal expansion, the C type thymocytes mainly consisted of $CD4^+CD8^+$ DP cells, suggesting retention of the differentiation capability. The percentage of C type thymus in 80-day thymuses was similar to that in 40-day thymuses. This suggests that the generation of C

type thymus is mostly completed until 40 days after γ-irradiation. C type thymocytes, but not thymic lymphomas, maintained the expression of Nrp-1 cell-surface protein at the same level of normal thymocytes. This maintenance in interaction between thymocytes and thymic epithelial cells may affect the cellular fate of those thymocytes. It might also contribute to lymphoma development when thymocytes in irradiated mice are transplanted (1). This speculation is based on the fact that thymocytes formed lymphoma only when transplanted in the thymus, whereas lymphomas could generate lymphoma irrespective of the transplantation sites (2, 5).

Among the 95 40-day atrophic thymuses, 17 were allelic loss-positive T type thymuses. This detection of allelic losses reflects clonal expansion of a given thymocyte before β-selection because D-J rearrangement patterns at the *TCRβ* locus were the same as that of normal thymocytes. It also suggests that the allelic loss of *Bcl11b* contributes to clonal expansion. This is supported by the finding that the *Bcl11b* allelic loss-positive thymocytes were enriched in middle-G1^{high} thymocytes more than the allelic loss-negative thymocytes, because this suggests the elevated stimulation of cell cycle at the G1 phase. However, it is unclear how a *Bcl11b*-allelic loss contributes to clonal expansion. Downregulation of Bcl11b in Jurkat cells by siRNA results in decrease of p27 cell cycle inhibitor (18), and this may support that hypothesis. It is also not known what genetic changes contribute to the formation of C type thymus. The candidate may include *Ikaros*, *Myc*, *Notch1*, and *Pten* (23–25) other than *Bcl11b*, genetic alterations of which were found in thymic lymphomas at high frequencies (15). Taken together, we observed two groups of thymocytes possessing intrinsic self-renewal capability that occurred at different developmental stages before and after β-selection. Both the *Bcl11b* allelic loss-positive T type and the C type thymocytes retain the capability to differentiate. Because the T type thymocytes were similar to normal thymocytes in cell size, they might be a precursor of C type thymocytes, but their relationship remains to be clarified.

The percentage of middle-sized G1 cells was increased in C type thymuses more than in T type thymuses. Those thymocytes may be cells that tend to pause at a late G1 stage before the cell-grown stage entering into S phase. The increase in the fraction of such middle G1 cells may reflect stimulation and/or hindrance of cell cycle progression of thymocytes. This implication is consistent with the decreased expression of both cell cycle activators (cyclin D1) and the inhibitor (p27). Of the 80-day C type thymocytes, on the other hand, approximately half showed increases in not only the percentage of middle G1 cells but also the percentage of S cells. Those thymocytes may be cells that have overcome hindrance(s) giving the pause at a late G1 stage but still failed to increase in the cell number at the level of thymic lymphomas, possibly owing to apoptosis. This is consistent with the finding that the expression level of cyclin D1 and PCNA increased and the level of p27 decreased in the 80-day thymuses relative to the 40-day thymuses.

A feature of the premalignant lesions such as dysplasia is the activation of DNA damage checkpoints, such as Chk1, Chk2, γH2AX, and p53 (10, 11). This DNA damage response is one of the barriers to constrain tumorigenesis, though it is uncertain whether the DNA damage response represents the predominant mode for preventing cancer development at the early stage (14). Analysis of the C type thymocytes revealed no marked activation in Chk1, Chk2, γH2AX, or p53. The observed minimal increases of p53 might be ascribed to increased levels of reactive oxygen species that stabilize p53 mRNA (26). Therefore, the result suggests that the probable prelymphoma cells in atrophic thymus are an exceptional case that does not undergo aberrant stimulation of cell proliferation or DNA replication stress. If this is the case, the C type thymocytes do not undergo selective pressure for inactivation of DNA damage checkpoint genes. Indeed, *p53* mutations were infrequent in γ-ray–induced thymic lymphomas (16).

To summarize, this study characterizes clonally expanding thymocytes in γ-ray–induced atrophic thymus that occurs at two distinct developmental stages before and after β-selection. The thymocytes resemble CML in possessing self-renewal and lineage capacity. Therefore, they can be a candidate of the lymphoma-initiating cells, and the importance of leukemia/lymphoma-initiating cells is pointed out in relapsed acute lymphoblastic leukemia in humans (9). The mouse lymphoma model, including *Bcl11b*-KO and *Bcl11b*-floxed mice, will provide new insights into leukemia/lymphoma-initiating cells, a target of radiation and chemical therapy.

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Effect of *Bcl11b* genotypes and γ-radiation on the development of mouse thymic lymphomas

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ABSTRACT

Bcl11b is a haploinsufficient tumor suppressor gene and expressed in many tissues such as thymus, brain and skin. Irradiated *Bcl11b*$^{+/-}$ heterozygous mice mostly develop thymic lymphomas, but the preference of *Bcl11b* inactivation for thymic lymphomas remains to be addressed. We produced *Bcl11b*$^{+/-}$ heterozygous and *Bcl11b* wild-type mice of *p53*$^{+/-}$ background and compared their incidence of γ-ray induced thymic lymphomas. Majority of the tumors in *p53*$^{+/-}$ mice were skin tumors, and only 5 (36%) of the 14 tumors were thymic lymphomas. In contrast, *Bcl11b*$^{+/-}$*p53*$^{+/-}$ doubly heterozygous mice developed thymic lymphomas at the frequency of 27 (79%) of the 34 tumors developed ($P = 0.008$). This indicates the preference of Bcl11b impairment for thymic lymphoma development. We also analyzed loss of the wild-type alleles in the 27 lymphomas, a predicted consequence given by γ-irradiation. However, the loss frequency was low, only six (22%) for *Bcl11b* and five (19%) for *p53*. The frequencies did not differ from those of spontaneously developed thymic lymphomas in the doubly heterozygous mice, though the latency of lymphoma development markedly differed between them. This suggests that the main contribution of irradiation at least in those mice is not for the tumor initiation by inducing allelic losses but probably for the promotion of thymic lymphoma development.

γ-Radiation is a complete carcinogen able to initiate and promote neoplastic progression. It induces various types of tumors in mice including thymic lymphomas and incidence of each tumor varies among mouse strains and mice of different genetic predispositions [1–3]. The thymic lymphomas are one of the classic models in radiation-induced malignancies [4,5] and genetic analyses have revealed allelic losses and mutations in a variety of genes such as *Bcl11b/Rit1*, *Ikaros*, *Pten*, and *p53* [3,6–10], although those in *p53* are not prevalent. These allelic losses are thought to be a result of the radiation effect, induction of DNA double-strand breaks (DSBs) [1–3]. *Bcl11b*, *Pten*, and *p53* belong to a subset of tumor suppressor genes that are haploinsufficient for tumor suppression. The haploinsufficiency is a situation in which functional loss of only one allele confers a selective advantage for tumor growth [11–16]. One of the consequences given by *Ikaros*, *Pten* or *p53* inactivation is to prevent apoptosis, one of the six hallmarks of cancer cells proposed by Hanahan and Weinberg [17]. However, *Bcl11b* inactivation leads to apoptosis in mouse thymocytes *in vivo* [18] and in Jurkat cells *in vitro* [19,20]. Hence, *Bcl11b* seems to differ from the other three genes in the manner of contribution to lymphomagenesis.

Knockout mice of those four tumor suppressor genes exhibit genetic predispositions to various tumors. *Pten* or *p53* heterozygous mice developed a broad spectrum of tumors including thymic lymphomas [21–25]. On the other hand, *Bcl11b* heterozygous mice of F_1 hybrid between BALB/c and MSM strains mostly developed thymic lymphomas when irradiated and showed its higher incidence and shorter latency than irradiated *Bcl11b* wild-type mice [15–26]. This suggests that inactivation of *Bcl11b* may give a preference for the development of thymic or T-cell lymphomas. However, this is not conclusive because of the high incidence of thymic lymphomas also observed in the irradiated wild-type F_1 hybrid mice [6–10]. Furthermore, Bcl11b is expressed not only in thymus but also in other tissues such as brain and skin and this suggests the possibility of its involvement in tumorigenesis in those expressed tissues [18–27]. So, to elucidate this issue of preference, we produced *Bcl11b*$^{+/-}$ and *Bcl11b*$^{+/+}$ mice of *p53*$^{+/-}$ background and compared the incidence of γ-ray induced thymic lymphomas. We

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also analyzed loss of the wild-type alleles of *Bcl11b* and *p53*. Here we report that the presence of a knockout allele of *Bcl11b* confer the mice propensity to develop thymic lymphomas and that the loss of the wild-type allele was unexpectedly low. The latter suggests less contribution of γ-irradiation to the tumor initiation by inducing allelic losses than previously predicted.

Materials and methods

Mice and lymphoma development. Bcl11b/Rit1$^{+/-}$ mice of BALB/c background were mated with p53$^{-/-}$ mice of MSM background and Bcl11b$^{+/-}$p53$^{+/-}$ doubly heterozygous (DH) or p53$^{+/-}$ heterozygous mice of (BALB/c x MSM) F1 background were produced. Sixty-five of those mice were exposed to a single dose of 3-Gy γ radiation at 4 weeks of age. Development of thymic lymphoma was diagnosed by inspection of labored breathing and palpable induration of thymic tumor. Existence of tumors was confirmed upon autopsy of the mice. Five of the 65 mice died of unknown causes and these mice were excluded in this study. Bcl11b$^{+/-}$ mice and p53$^{-/-}$ mice used in this study were maintained under specific pathogen-free conditions in the animal colony of the Niigata University. All animal experiments comply to the guidelines by the Animal Ethics Committee for animal experimentation of Niigata University. The thymic lymphomas spontaneously developed in DH mice used in this study were previously obtained [15].

DNA isolation and PCR analysis of allelic differences. DNA was isolated from brain and lymphomas using the DNeasy Tissue Kit (Qiagen). Genotyping of *Bcl11b* and *p53* were carried out with PCR as described previously [10,15,26]. Loss of the wild-type allele at *Bcl11b* was analyzed by using *D12 Mit181* marker in the vicinity of *Bcl11b*, and loss of the wild-type allele at *p53* was with the primers used for genotyping. Primers for *Ikaros* and for *Pten* were as described previously [26].

Western blotting. Rabbit anti-*Bcl11b* antibodies were generated as described previously [18]. Thymus and lymphoma cells were suspended in a solution of 40 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 5 mM MgCl2, 25 mM KCl and mixed with an equal volume of lysis buffer, 0.125 M Tris–HCl (pH 6.8), 10% sucrose, 10% SDS, 10% 2-ME, and 0.004% bromophenol blue. The extract (200 μg) was electrophoresed in 8% SDS–PAGE gels and blotted onto Hybond membranes (Amersham Pharmacia Biotech. Piscataway, NJ). Protein bands were visualized using chemiluminescent detection (ECL plus, Amersham Pharmacia Biotech.).

Results

A total of 37 mice of *Bcl11b*$^{+/-}$*p53*$^{+/-}$ doubly heterozygous (DH) genotype and 23 mice of *p53*$^{+/-}$ singly heterozygous genotype were obtained by mating *Bcl11b*$^{+/-}$ BALB/c mice with *p53*$^{-/-}$ MSM mice. Those 60 offspring were subjected to a single whole-body γ-irradiation of 3 Gy. Forty-eight of the mice developed tumors, mostly (32 mice) thymic lymphomas. Of the 37 DH mice, 27 mice developed thymic lymphomas, nine developed other tumors, and the remaining three were free from tumors. On the other hand, only five of the 23 *p53*$^{+/-}$ mice developed thymic lymphomas, nine developed other tumors, and the remaining nine were free from tumors. Table 1 summarizes incidences of various tumors developed. Fig. 1 displays the cumulative tumor incidence of mice of the two different genotypes. The mean latency was 159 days in DH mice, shorter than 169 days of *p53*$^{+/-}$ mice and the incidence of tumors were higher than *p53*$^{+/-}$ mice. These results indicated the incidence of thymic lymphomas in irradiated (BALB/c × MSM) F_1 DH mice higher than in irradiated F_1 *p53*$^{+/-}$ mice ($P = 0.008$). This demonstrated the preference of *Bcl11b* heterozygous genotype for the development of thymic lymphomas in mice of *p53*$^{+/-}$ genotype.

Table 1
Tumor types of *Bcl11b*$^{+/-}$*p53*$^{+/-}$ and *p53*$^{+/-}$ mice

Genotype	Bcl11b$^{+/-}$p53$^{+/-}$	p53$^{+/-}$
No. of mice	37	23
Thymic lymphoma	27	5[a]
Systemic leukemia	1	0
Skin tumor	5[b]	7
Lung tumor	2	0
Liver tumor	1	2
Free from tumors	3	9

[a] The incidence of thymic lymphomas was higher in *Bcl11b*$^{+/-}$*p53*$^{+/-}$ mice than *p53*$^{+/-}$ mice ($P = 0.008$).
[b] Two of the four mice also developed thymic lymphomas.

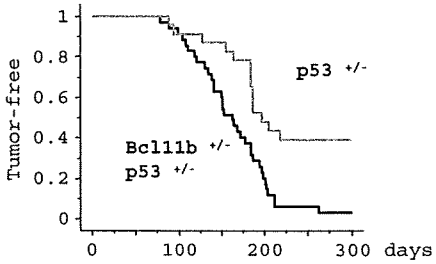

Fig. 1. Kaplan-Meier analysis of tumors in γ-irradiated *Bcl11b*$^{+/-}$*p53*$^{+/-}$ mice (black line) and *p53*$^{+/-}$ mice (gray line). Mice of the *Bcl11b*$^{+/-}$*p53*$^{+/-}$ genotype developed tumors at a higher incidence and a shorter latency than those of the *p53*$^{+/-}$ genotype ($P = 0.0004$ in Mantel-Cox test).

We examined loss of the wild-type allele of *Bcl11b* in the 27 thymic lymphomas of DH mice. Fig. 2A shows examples of PCR analysis. A pair of primers was designed to detect difference between the wild-type MSM allele and the knockout BALB/c allele. Loss of the wild-type allele was detected in only six (22%) of the 27 lymphomas (Table 2). To confirm this at the expression level, Western blot analysis was performed using anti-Bcl11b antibodies (Fig. 2B). The expression was detected in 14 of the 20 available thymic lymphomas, consistent with the frequency (78%) of the wild-type allele retained. Comparison of this loss frequency (22%) to 54% (14/

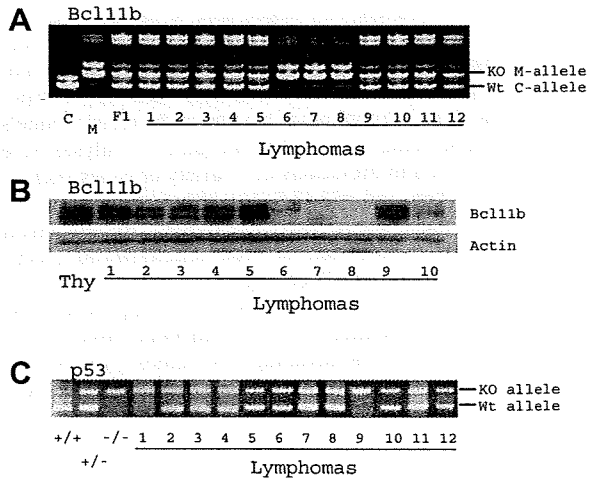

Fig. 2. Detection of the functional alleles of *Bcl11b* and *p53* in γ-irradiated *Bcl11b*$^{+/-}$*p53*$^{+/-}$ mice. (A) Allelic loss analysis of *Bcl11b* locus using *Mit181* marker in the vicinity of *Bcl11b*. Wild-type and KO alleles are indicated at the right. (B) Western blot analysis of thymus (lane Thy) and thymic lymphomas (lanes 1–10) using anti-Bcl11b antibodies. Anti-β-actin antibodies were used as a control. (C) Allelic loss analysis of *p53* locus. Wild-type and KO alleles are indicated at the right.

Table 2
Frequencies of loss of wild-type alleles and allelic losses in thymic lymphomas developed in irradiated and unirradiated mice of *Bcl11b*$^{+/-}$*p53*$^{+/-}$ genotype

Irradiation	Bcl11b wt-allele loss	p53 wt-allele loss	Pten LOH	Ikaros LOH
(+)	22% (6/27)	19% (5/27)	22% (6/27)	33% (9/27)
(−)	14%[a] (2/14)	29% (4/14)	29% (4/14)	43% (6/14)

[a] Data of *Bcl11b* in unirradiated mice are from Ref. 15.

26) in irradiated *Bcl11b*$^{+/-}$ mouse lymphomas [26] showed a significant decrease in the frequency ($P = 0.024$). A similar analysis was performed for *p53* (Fig. 2C). Loss of the wild-type allele was observed in five (19%) of the 27 lymphomas but in three of the five *p53*$^{+/-}$ mouse lymphomas. The frequency of 19% was also significantly lower than 137/160 (85.6%) in irradiated *p53*$^{+/-}$ mouse lymphomas [6] ($P < 0.0001$). Intriguingly, however, such low frequencies were seen in spontaneously developed thymic lymphomas in mice of the same genotype [15]. Table 2 summarizes the comparison of loss of wild-type alleles between irradiated and unirradiated DH mice (see discussion). Those results indicated that most lymphomas in irradiated DH mice retained the wild-type *Bcl11b* and *p53* alleles.

We next examined allelic losses at the *Pten* and *Ikaros* loci in the 27 thymic lymphomas. Those analyses were also done using PCR primers that were able to detect allelic differences between BALB/c and MSM chromosomes (data not shown). Allelic loss was detected in six (22%) for *Pten* and in nine (33%) for *Ikaros* (Table 2). For comparison, we examined the 14 thymic lymphomas that spontaneously developed in DH mice, which were previously obtained [15]. Allelic loss was detected in four (29%) for *Pten* and in six (43%) for *Ikaros* (Table 2), indicating no particular difference between the irradiated and spontaneous DH mouse lymphomas.

Discussion

Bcl11b is a haploinsufficient tumor suppressor gene and the human *BCL11B* locus is involved in recurrent chromosomal rearrangements in leukemias mostly of T-cell origin [28–30]. This study examined whether or not the *Bcl11b* heterozygous genotype provides a preference for T-cell lymphoma development, using (BALB/c x MSM) F_1 mice of *p53*$^{+/-}$ genetic background. The use of *p53*$^{+/-}$ mice was based on the development of a broad spectrum of tumors [25]. Indeed, this study demonstrated that most of the tumors developed in irradiated *p53*$^{+/-}$ mice were skin tumors, and only 5 (36%) of the 14 tumors were thymic lymphomas (Table 1). In contrast, the majority of tumors developed in irradiated DH mice were thymic lymphomas, the incidence being 27 (79%) of the 34 tumors developed. This indicates that the presence of a knockout allele of *Bcl11b* leading to Bcl11b impairment gives a preference for the development of thymic lymphomas.

This study also examined the frequency of loss of the wild-type alleles of *Bcl11b* and *p53* in the irradiated DH mouse lymphomas. Unexpectedly, most of the thymic lymphomas retained the wild-type alleles, the loss frequency being 22% for *Bcl11b* and 19% for *p53*. The frequency of *Bcl11b* was lower than 54% in irradiated *Bcl11b*$^{+/-}$ mice and that of *p53* was also lower than 86% in irradiated *p53*$^{+/-}$ mice. On the other hand, no marked difference was observed in the loss frequencies when compared to the spontaneously developed thymic lymphomas in DH mice. The loss frequency in the spontaneous lymphomas was 14% (2/14) for *Bcl11b* [15] and 29% (4/14) for *p53* (data not shown). Also, no particular difference was seen in allelic loss at *Pten* or *Ikaros* locus between those irradiated and unirradiated DH mouse lymphomas (Table 2). Of note is a prominent difference in the latency, the mean latency being 159 days in irradiated DH mice whereas 203 days in unirradiated DH mice ($P = 0.0079$). This suggests that irradiation enhanced the lymphoma development but did not markedly increase the frequency neither in loss of the wild-type alleles nor in allelic loss at the *Pten* or *Ikaros* loci.

It is well accepted that the induction of allelic losses at tumor suppressor loci is a general phenomenon in the initiation of radiation carcinogenesis [1]. Allelic loss at the *p53* locus, for instance, has been reported in γ-ray induced thymic lymphomas in *p53* wild-type mice. In chemical carcinogen-induced thymic lymphomas, however, such allelic losses are not prevalent and instead point-mutations are seen at high frequencies [31,32]. Hence, the mechanism of gene inactivation significantly differs between γ-ray- and chemical carcinogen-induced thymic lymphomas. *p53*$^{+/-}$ heterozygous mice are more susceptible to radiation-induced tumors and most of those tumors lose the wild-type allele of *p53* [6,25]. These imply that radiation contributes to carcinogenesis through induction of allelic losses resulting from DSBs and subsequent DNA recombination. However, the results of high retention of the wild-type alleles described above seem to disagree with this implication. It is probably related with DH mice used in this study. Unlike *Bcl11b*$^{+/-}$ or *p53*$^{+/-}$ singly heterozygous mice, DH mice spontaneously develop thymic lymphomas at a high frequency and hence the DH genotype may have a propensity to initiate the development of thymic lymphomas. Such synergistic effect has been reported in various genes such as *Pten* and *p53* [13,33,34], although it remains open what mechanism governs the synergistic cooperativity. Interestingly, Mao et al. [13] analyzed thymic lymphomas in irradiated *Pten*$^{+/-}$*p53*$^{+/-}$ doubly heterozygous mice and reported loss of the remaining *Pten* wild-type allele in majority of the thymic lymphomas. The reason for this difference between *Bcl11b* and *Pten* is not clear but it may be due to difference in the consequence of their inactivation, the promotion of apoptosis for the former and the inhibition of apoptosis for the latter [18–20,34]. Alternatively, it may be ascribed to different chromosomal location as the authors interpreted that loss of the chromosome 19 harboring *Pten* is driven by loss of additional tumor suppressor genes on this chromosome but not by requirement for complete loss of *Pten*. Since it is unlikely that the DH genotype can suppress radiation-induced DSBs and recombination, the cells losing the wild-type allele probably are generated in irradiated thymus but those cells acquire a relatively weak selective advantage in DH mice [11,12]. In cells of the DH genotype, therefore, the combination of their heterozygous states could contribute to lymphomagenesis without further loss of the wild-type *Bcl11b* or *p53* allele.

Of importance in this paper is the demonstration of high retentions of the wild-type alleles even in radiation-induced tumors. This suggests that the main contribution of irradiation may not be for the tumor initiation by inducing DSBs. Rather, irradiation contributes to the promotion of thymic lymphoma development, possibly by changing the normal thymic microenvironment into impaired one that founds pre-lymphoma cells [3]. This provides an implication that the induction of allelic losses by γ-irradiation less contributes to lymphomagenesis than previously predicted [1].

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Mtf-1 lymphoma-susceptibility locus affects retention of large thymocytes with high ROS levels in mice after γ-irradiation

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Abstract

Mouse strains exhibit different susceptibilities to γ-ray-induced thymic lymphomas. Our previous study identified *Mtf-1* (metal responsive transcription factor-1) as a candidate susceptibility gene, which is involved in the radiation-induced signaling pathway that regulates the cellular reactive oxygen species (ROS). To reveal the mechanism for the increased susceptibility conferred by *Mtf-1* locus, we examined early effects of γ-ray on ROS levels *in vivo* and its difference between *Mtf-1* susceptible and resistant congenic mice. Here, we show the detection of clonally growing thymocytes at 4 weeks after irradiation, indicating the start of clonal expansion at a very early stage. We also show that large thymocytes with higher ROS levels and a proliferation capacity were more numerous in the *Mtf-1* susceptible mice than the resistant mice when examined at 7 days after irradiation, although such tendency was not found in mice lacking one allele of *Bcl11b* tumor suppressor gene. This high retention of the large thymocytes, at a high risk for ROS-induced mutation, is a compensatory proliferation and regeneration response to depletion of the thymocytes after irradiation and the response is likely to augment the development of prelymphoma cells leading to thymic lymphomas.

Keywords: Lymphoma susceptibility; Clonal proliferation; ROS; γ-Irradiation; *Mtf-1*

Fractionated whole-body γ-irradiation (4 × 1.75 Gy at weekly intervals) to mice renders the thymus atrophic by inducing thymocyte apoptosis, and atrophic thymuses develop thymic lymphomas, though the incidence varies among mouse strains [1–3]. Prevention of the lymphoma development was demonstrated by transfer of unirradiated bone marrow cells to irradiated mice, which was presumed to supplement intrathymic pre-T cells [4], within 1 week after the last irradiation but not 1 month or later [5,6]. On the other hand, impaired thymocytes at 2 weeks after irradiation, when transferred to thymus of unirradiated mice, were able to develop into lymphomas [7]. These data suggest the generation of 'prelymphoma' cells in the atrophic thymus at a very early stage.

BALB/c and C57BL/6(B6) mouse strains are highly susceptible to radiogenic thymic lymphomas whereas MSM and C3H strains are resistant [8–10]. Our previous genetic study using BALB/c and MSM strains identified *Mtf-1* (metal responsive transcription factor-1) [11,12] as a candidate susceptibility gene to γ-ray-induced thymic lymphomas [13]. The two different *Mtf-1* alleles, BALB/c encoding the serine-type MTF-1 and MSM encoding the proline-type, exhibited distinct transcriptional activation and responses to ionizing radiation (IR).

Exposure of cells to IR leads to production of reactive oxygen species (ROS) in irradiated cells and their progeny which are thought to be the main cause for the delayed genomic instability [14–16]. In response to physiological

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growth stimulation, the level of ROS also increases and the increase is sufficient for significantly damaging DNA [17–19]. The elevation of ROS activates p53 signaling pathway which in turn controls the ROS level, proliferation, and apoptosis [20,21]. MTF-1, activated by heavy metals such as zinc, is also included in the radiation-induced signaling pathway that responds to and regulates the cellular ROS level [22]. We reasoned that ROS in thymocytes after IR may differ in amount between congenic mice of the different *Mtf-1* genotype. Since ROS is a well-known mutagen contributing generation of more advanced precancerous cells, the number of prelymphoma cells generated may differ between them. In this paper, we examine clonal growth and the ROS levels in thymocytes between susceptible and resistant congenic lines after IR.

Materials and methods

Mice and irradiation. The congenic mouse strain (line-5) of BALB/c background used in this study carried an MSM-derived chromosomal region spanning an approximately 4 Mb interval between *Nds2* and *D4Mit336* on chromosome 4. The mice were obtained by mating of the previously used congenic strain (line-3) [13] with BALB/c after genotyping with nine microsatellite markers within this region. BALB/c mice were purchased from CLEA Japan Inc. (Tokyo, Japan). Mice used in this study were maintained under specific pathogen-free conditions in the animal colony of the Niigata University. Genotyping was carried out with PCR as described previously [13]. Mice were given a whole-body dose of 3 Gy at a dose rate of 1 Gy/min from a broad-beam cesium-137 source when they were at age of 8 weeks. The Ethics Committee for Animal Experimentation of Niigata University approved all experimental procedures involving the mice.

Flow-cytometric analysis and cell sorting. Progeny obtained by mating between the heterozygous congenic mice were irradiated and thymus was isolated at 3, 5, 7, and 14 days after irradiation. Flow-cytometric analysis was performed as previously described [23,24]. In brief, single cell suspensions of thymocytes were prepared from thymus and, $1–2 \times 10^6$ cells were incubated with monoclonal antibodies (mAbs) in phosphate-buffered saline containing 2% fetal calf serum and 0.2% NaN_3 for 20 min at 4 °C. The following monoclonal antibodies were purchased from eBioscience: anti-CD4-FITC or -APC (RM4-5) and anti-CD8-APC (53-6.7). To prevent nonspecific binding of mAbs, we added CD16/32 (93; eBioscience) before staining with labeled mAbs. Dead cells and debris were excluded from the analysis by appropriate gating of FSC and SSC.

ROS levels were determined by incubating the thymocytes with 10 μg/ml dichlorodihydrofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR) for 15 min at 37 °C in the dark. Cells were then placed on ice and kept in the dark until analysis, which was carried out within 30 min. The percent of dead cells was determined by the uptake of propidium iodide (PI) (10 μg/ml) and excluded from analysis. A minimum of 10,000 cells for each sample were analyzed by a FACScan (Becton–Dickinson) flow cytometer. Data were analyzed using the Flow-Jo software (Tree-Star, Inc.).

For BrdU incorporation experiments, we injected mice at 5 days after γ-irradiation at 1.5 Gy intraperitoneally with 100 μl of BrdU solution (10 mg/ml) and thymus was isolated 1 h after. Thymocytes were prepared from the thymus and analyzed with the use of the BD Pharmingen BrdU Flow Kit according to manufacturer's instruction. In brief, cells were suspended at a concentration of $1–2 \times 10^6$ cells/ml, fixed, permeabilized, and incubated with a murine anti-BrdU antibody for 60 min on ice. After washing, cells were incubated with FITC-conjugated goat anti-mouse antibodies for 30 min on ice, washed, and resuspended in PBS containing 20 μl of the 7AAD solution. Cells were resuspended in staining buffer and analyzed by FACScan. As for separation of large and small thymocytes, sorting was carried out on a FACSAria (BD Biosciences).

Nested PCR assay and estimation of deletion frequency. Genomic DNA was isolated from the thymus of mice at 28 days after irradiation. The nested PCR assay for *Bcl11b*/*Rit1* internal deletions was performed as described previously [25]. First PCR was done with outer primers F1 and R1 for 30 cycles, and the second PCR was done for another 30 cycles with inner primers F1-2 and R1-2. Reaction mixtures were then analyzed by electrophoresis on agarose gels. Sequence analysis was performed to confirm that DNA in the band consisted of *Bcl11b*/*Rit1* recombinant molecules.

Western blotting. The large and small thymocytes were separated by flow cytometry and analyzed as described previously [23]. Anti-p53 (#9282) was purchased from Cell Signaling Technology.

Results

Clonally growing thymocytes in mice after irradiation

Fig. 1A shows the number of thymocytes in BALB/c mice at various days after irradiation. The number on average at 5 days after was less than 10% of that in unirradiated mice, and then increased greatly in approximately a half of thymuses at 7 days after. The numbers at 10 and 28 days post-irradiation were similar but generally less than those in unirradiated mice. The proliferation of thymocytes seen from 5 to 10 days after is probably a compensatory reaction of thymocytes to depletion of the cells after irradiation.

Our previous study showed that there are a considerable number (10^3–10^4) of thymocytes in unirradiated thymus with intragenic deletions of *Bcl11b*/*Rit1*, a tumor suppressor gene for thymic lymphomas [23,25,26], because these deletions are frequently generated by aberrant V(D)J recombination during the thymocyte development [25]. Hence, if a thymocyte with the mutation preferentially proliferates in atrophic thymus following irradiation, the clonal growth should be detectable by examining *Bcl11b* intragenic deletions with nested PCR. DNA was extracted from thymus in mice at 28 days after irradiation and examined with nested PCR (Fig. 1B). The bands of *Bcl11b* intragenic deletions were detected in approximately a half of thymuses under these PCR condition (see Materials and methods). Although the intensities varied, the sizes of the bands were similar to that of the lymphoma DNA diluted 10^2- to 10^3-fold with brain DNA. Assuming that the thymus contained 3×10^7 cells, the number of thymocytes with *Bcl11b* mutations was estimated from the band intensities as approximately 10^5 in a thymus. The result indicates the presence of clones in some of thymuses at 28 days after irradiation, which suggests that the clonal growth of possible prelymphoma cells starts at a very early stage during the lymphoma development.

Detection of ROS in thymocytes in vivo

Radiation has been shown to increase ROS levels in *in vitro* irradiated cells and their progeny [14,15,27]. However, a few studies investigate the *in vivo* effect of IR on the ROS

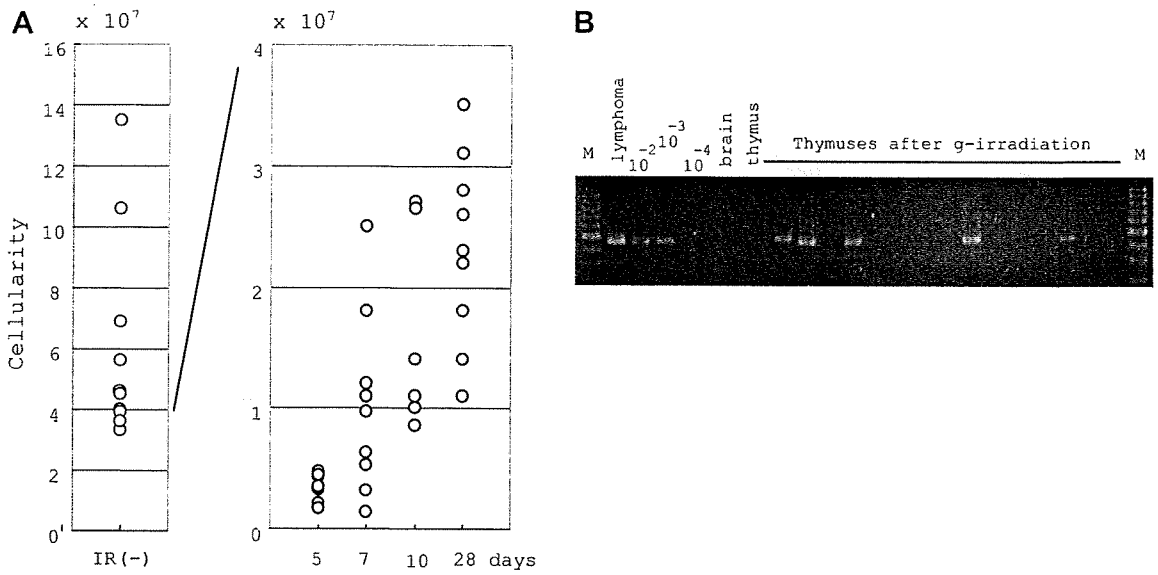

Fig. 1. (A) Cellularity of thymocytes at various times after 3 Gy γ-irradiation. (B) Existence of clonally growing thymocytes in thymuses at 28 days after irradiation. Nested PCR of *Bcl11b* intragenic deletions was performed and the PCR products were compared to the deletion-positive lymphoma DNA which had been 10^2-, 10^3-, and 10^4-fold diluted with brain DNA.

levels. We examined such effects on thymocytes that were isolated from BALB/c mice at 5, 7, 10, and 28 days after the irradiation. Thymocytes were analyzed with flow cytometry after incubation with DCFH-DA, which is a membrane-permeable fluorescent dye and the oxidization of DCFH-DA to DCF by ROS results in increased fluorescence of the dye.

Fig. 2A shows examples of the forward scatter and side scatter analysis of unirradiated thymus and thymus at 7 days after 3 Gy irradiation. The proportion of dead cells and debris increased after irradiation. The dead cells and debris-excluded fraction of thymocytes showed significant increase in DCF fluorescence after IR and the increase was higher at 5 days than at 7 days after (upper panels in Fig. 2B). These results suggested an increase in ROS in thymocytes of irradiated mice. The ROS levels of thymocytes at 10 and 28 days after irradiation were similar to those of unirradiated thymocytes (data not shown). In parallel with the increase in ROS, however, the proportion of large cell-sized thymocytes increased in the irradiated samples (lower panels in Fig. 2B). Therefore, the ROS level was separately examined in the large cell-sized and small cell-sized fractions of thymocytes. The large thymocytes were found to have higher levels of ROS than the small thymocytes did (Fig. 2C). Of note is that although the proportion of large thymocytes was very low, the level of ROS in the large thymocytes from unirradiated thymus was as high as that from irradiated thymus. This indicated that the high ROS was the feature of large thymocytes and not due to irradiation.

Properties of small and large thymocytes were investigated by flow-cytometric analysis of cell surface markers. A lower proportion of $CD4^+CD8^+$ double positive cells was found in large thymocytes than in small thymocytes, suggesting that large thymocytes comprised more immature cells than small thymocytes (Fig. 2D). This characteristic of large thymocytes was independent of irradiation status. However, irradiation increased the proportion of immature subsets of thymocytes ($CD4^-CD8^-$ and $CD4^-CD8^+$ cells) in both small and large thymocytes. We next examined BrdU uptake in thymocytes to test the capability for those small and large thymocytes to proliferate (Fig. 2E). Only large thymocytes incorporated BrdU, irrespective of irradiation (data not shown), indicating that the large cells comprise of cycling cells. A high ROS level has been shown to activate p53 tumor suppressor. Hence, the activation was examined by Western blotting. A higher expression of p53 was found in the large thymocytes relative to the small thymocytes (Fig. 2F). Taken together, these results suggest that irradiation increases the number of the immature large thymocytes with high ROS levels and a proliferation capacity. Since ROS can cause DNA damage, the large thymocytes are the likely target for precancerous conversion with clonal expansion.

Higher retention of large thymocytes in susceptible strain than resistant strain

Mtf-1 congenic heterozygous mice of BALB/c(C) background carrying an MSM(M)-derived 4 Mb interval (Fig. 3A) were mated with each other, and progeny were used for flow-cytometric analysis at 7 days after 3 Gy irradiation. We always used unirradiated thymocytes as a control, setting region gates for large thymocytes to range from 15% to 18%. Under the same gate condition, the proportion of large thymocytes was shown to range from 15%

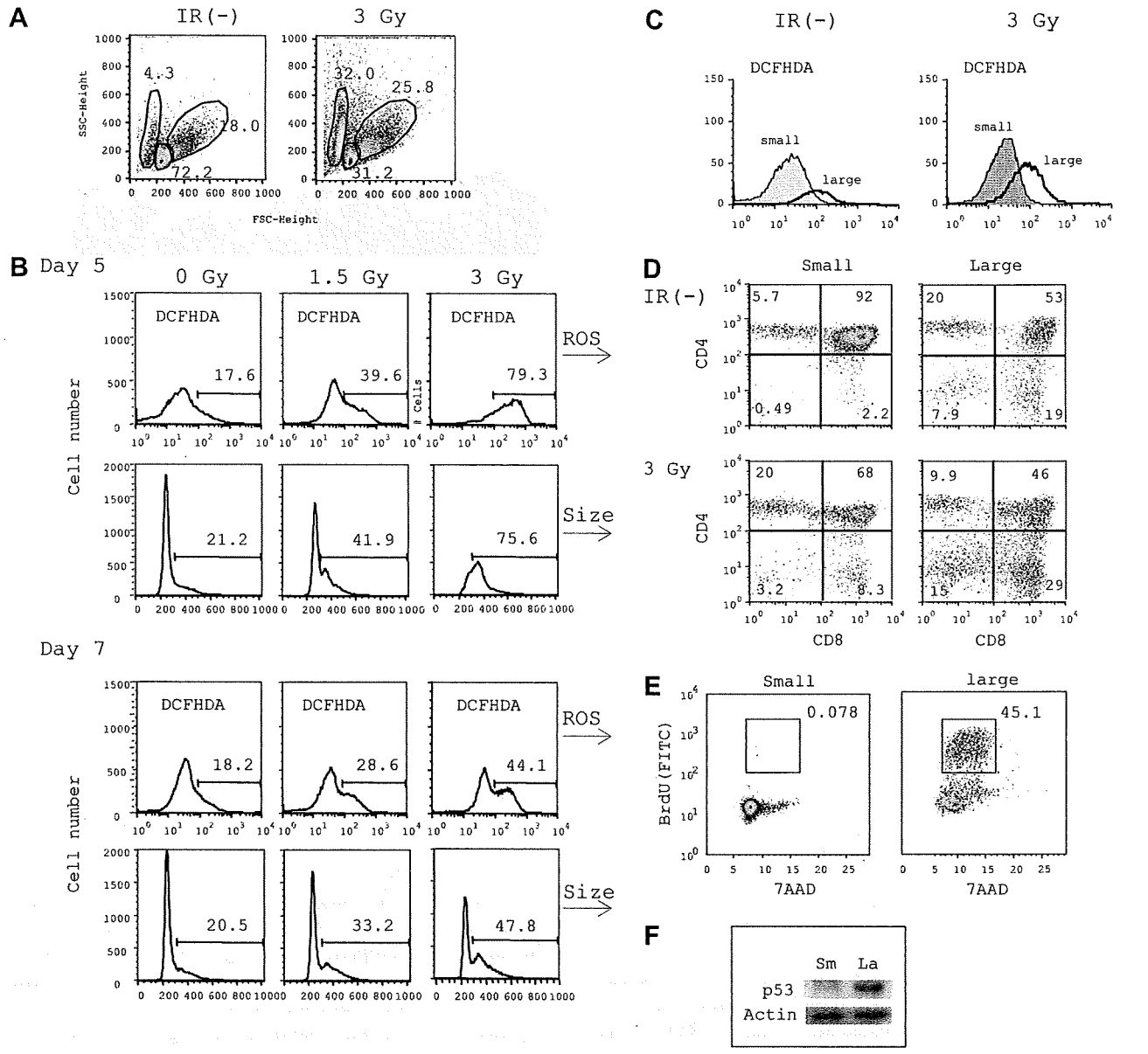

Fig. 2. IR increases the number of large thymocytes with high levels of ROS. (A) Cell size of thymocytes by forward scatter (FSC) versus side scatter (SSC) was analyzed by flow cytometry in unirradiated BALB/c mice and mice at 7 days after irradiation. The percentages of cells are shown in corresponding areas. (B) ROS levels (upper) and the cell size (lower) of thymocytes at 5 and 7 days after irradiation. The horizontal bars indicate percentages of cells in corresponding areas. (C) ROS levels of small and large thymocytes at 7 days after irradiation. (D) Surface expression of CD4 and CD8 on thymocytes was analyzed by flow cytometry in small and large thymocytes at 7 days after irradiation. (E) The measurement of cells incorporated BrdU and total DNA content in small and large thymocytes. The region gate in FACS analysis of large thymocytes indicated that 45.1% of the cells actively incorporated BrdU whereas that of small thymocytes showed that only 0.078% of the cells actively incorporated BrdU. (F) Western blotting indicated an increase of p53 in large thymocytes relative to small thymocytes.

to 60% in irradiated mice. Thymuses were analyzed for the proportion of large thymocytes in mice of the three different *Mtf-1* genotypes (Fig. 3B). We then divided thymuses into two groups, one harboring large thymocytes above the level of 20% and the other harboring large thymocytes below 20%. The former retains radiation influences and can be regarded as a high-risk group with clonally expanding precancerous cells. The latter, on the other hand, seems to be the thymuses well recovered from radiation influences.

Our previous results showed that mice of *Mtf-1* C/C and C/M genotypes are susceptible to γ-ray-induced thymic lymphomas and those of *Mtf-1* M/M genotype resistant [9,13]. A total of 18 mice of C/C and C/M genotype belonged to the former with more than 20% large thymocytes and only four belonged to the latter with less than

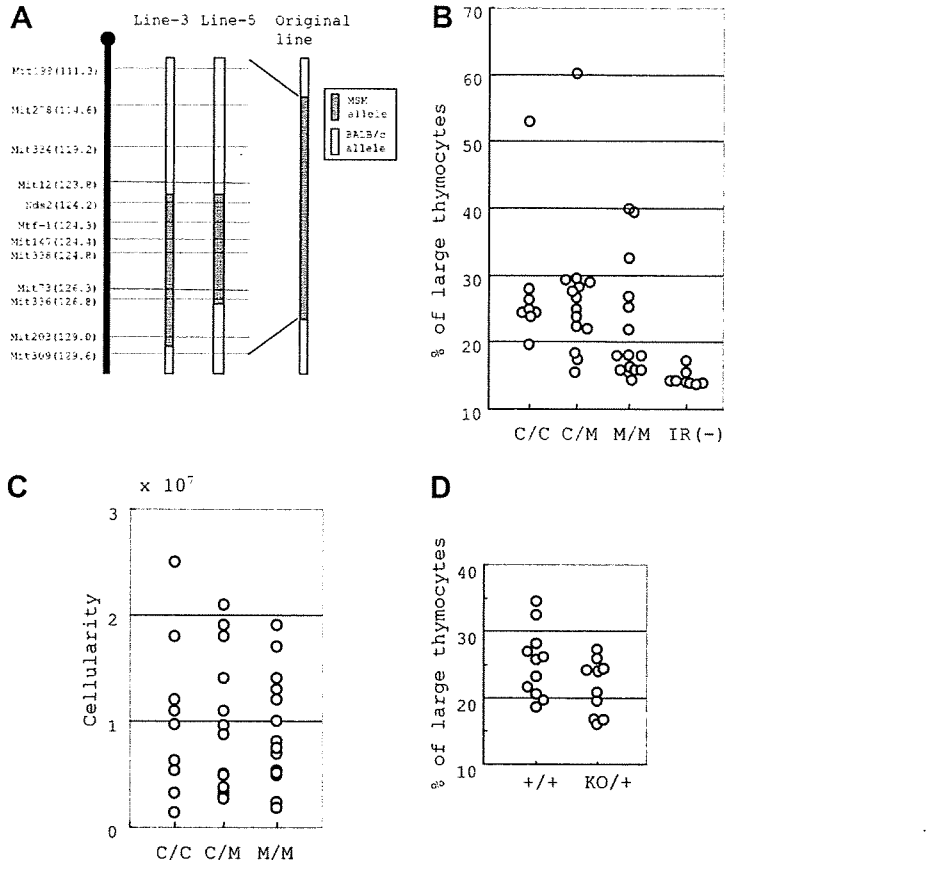

Fig. 3. Higher retention of large thymocytes in susceptible congenic mice. (A) Genetic constitution is displayed of congenic mouse strains (line-3 and line-5) of BALB/c background. The line-5 is a subcongenic line derived from the line-3 mice. Vertical bars represent a chromosomal region with map positions (in Mb from the centromere) and shadowed portions carry an MSM-derived region. (B) Distribution of thymuses with different percents of large thymocytes was analyzed in mice of the three different *Mtf-1* genotypes: C/C, C/M, and M/M genotypes. (C) Cellularity of thymocytes was analyzed in mice of the C/M and M/M *Mtf-1* genotypes at 7 days after γ-irradiation. (D) Distributions of thymuses with different percents of large thymocytes were analyzed in mice of $Bcl11b^{+/+}$ and $Bcl11b^{KO/+}$.

20%. In contrast, six mice of M/M genotype were belonged to the former and nine to the latter. This difference between susceptible mice and resistant mice is statistically significant (χ^2 test, $P = 0.0089$), indicating the tendency of susceptible strains retaining large thymocytes more than the resistant strain. We noted that the four thymuses possessing more than 40% of large thymocytes were very atrophic (see the open circles in Fig. 3B). On the other hand, no significant difference in the cellularity was observed between mice of *Mtf-1* C/C and C/M genotypes and mice of M/M genotype (Fig. 3C). Analysis of thymocytes with different ROS levels showed a similar pattern of different distributions (data not shown).

Bcl11b-knockout heterozygous mice are more susceptible to γ-ray-induced thymic lymphomas than *Bcl11b* wild-type mice (Kamimura et al., accompanying paper). Thus, analyses of large thymocytes were carried out on *Bcl11b* wild-type and heterozygous mice (Fig. 3D). The proportion of large thymocytes ranged from 16% to 40% in irradiated mice and no significant difference was observed between *Bcl11b* wild-type and knockout heterozygous mice. These results suggest that *Mtf-1* susceptibility locus and *Bcl11b* tumor suppressor gene contribute to the development of radiogenic thymic lymphomas by distinct mechanisms.

Discussion

This paper examines clonal growth of thymocytes after irradiation, early effects of IR on cell behavior *in vivo* and its difference between *Mtf-1* susceptible and resistant congenic mice for γ-ray-induced thymic lymphomas, to reveal the basis for the increased susceptibility given by *Mtf-1* locus. Here, we show thymocytes clonally growing at 28 days after irradiation, indicating the start of clonal expansion at a very early stage. We also show that susceptible mice tended to have more of proliferating immature large thymocytes with higher ROS levels than resistant mice when examined at 7 days after irradiation. This increase of large thymocytes may be a compensatory reaction of thymocytes to depletion of the cells after irradiation. Of particular importance is that the mode of the

compensatory reaction differed between susceptible and resistant mice. The high proportion of the large thymocytes is likely to contribute to lymphoma susceptibility because they are vulnerable to ROS-induced mutation. The importance of the cell death and complementary proliferation for carcinogenesis is also provided by the study on hepatocellular carcinogenesis [28].

Genetic factors underlying cancer susceptibility are best treated as quantitative trait loci [29,30] and in most cases the susceptibility does not operate in the gain-or-loss mechanism which is for the oncogenes and tumor suppressor genes conferring cancerous phenotype of the cell [31,32]. An individual mouse with a predisposing genotype may not develop cancer, while mice with the predisposing genotype will on average exhibit a higher incidence of cancer over those without. Indeed, *Mtf-1* susceptible mice developed thymic lymphomas at the incidence of approximately 70% whereas resistant mice exhibited that of 40% [9,13]. Susceptibility genes may predispose by affecting the level of genomic insult to the cancer target cells (that is, the initiation frequency), the promotion of tumor cell proliferation and growth, and the number of the target cells. This study showed that the *Mtf-1* susceptible gene contributes to the persistence of large thymocytes after irradiation but not to the total number of thymocytes. These suggest that the *Mtf-1* susceptibility affects the number of the large thymocytes, the candidate target cells for malignant conversion. This may be supported by the fact that the *Mtf-1* susceptibility gave the higher incidence of thymic lymphomas but did not affect the latency [9,13]. In contrast, KO heterozygosity of the *p53* and *Bcl11b* genes gave both the higher incidence and the shorter latency probably due to the pre-existence of one hit of mutation ([33]; Kamimura et al., unpublished paper).

In the present study, differences were found in radioresponses of thymocytes in the congenic mice of BALB/c background carrying an MSM-derived 4 Mb region between *Nds2* and *D4Mit336*. Therefore, the difference can be ascribed to a gene or genes in this region and the *Mtf-1* gene in this interval is obviously a strong candidate for the susceptibility gene. At present, the mechanism of *Mtf-1* alleles for affecting the retention of large thymocytes is unclear. In addition, there are many genes within this interval other than *Mtf-1*. Therefore, the observed phenotypic difference may not solely be attributable to the *Mtf-1* polymorphism. The relationship between *Mtf-1* and the susceptibility remains to be further investigated.

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Polymorphisms of DNA damage response genes in radiation-related and sporadic papillary thyroid carcinoma

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Abstract

Papillary thyroid carcinoma (PTC) etiologically occurs as a radiation-induced or sporadic malignancy. Genetic factors contributing to the susceptibility to either form remain unknown. In this retrospective case–control study, we evaluated possible associations between single-nucleotide polymorphisms (SNPs) in the candidate DNA damage response genes (*ATM*, *XRCC1*, *TP53*, *XRCC3*, *MTF1*) and risk of radiation-induced and sporadic PTC. A total of 255 PTC cases (123 Chernobyl radiation-induced and 132 sporadic, all in Caucasians) and 596 healthy controls (198 residents of Chernobyl areas and 398 subjects without history of radiation exposure, all Caucasians) were genotyped. The risk of PTC and SNPs interactions with radiation exposure were assessed by logistic regressions. The *ATM* G5557A and *XRCC1* Arg399Gln polymorphisms, regardless of radiation exposure, associated with a decreased risk of PTC according to the multiplicative and dominant models of inheritance (odds ratio (OR) = 0.69, 95% confidence interval (CI) 0.45–0.86 and OR = 0.70, 95% CI 0.59–0.93 respectively). The *ATM* IVS22-77 T > C and *TP53* Arg72Pro SNPs interacted with radiation (P = 0.04 and P = 0.01 respectively). *ATM* IVS22-77 associated with the increased risk of sporadic PTC (OR = 1.84, 95% CI 1.10–3.24) whereas *TP53* Arg72Pro correlated with the higher risk of radiogenic PTC (OR = 1.80, 95% CI 1.06–2.36). In the analyses of *ATM*/*TP53* (rs1801516/rs664677/rs609429/rs1042522) combinations, the GG/TC/CG/GC genotype strongly associated with radiation-induced PTC (OR = 2.10, 95% CI 1.17–3.78). The GG/CC/GG/GG genotype displayed a significantly increased risk for sporadic PTC (OR = 3.32, 95% CI 1.57–6.99). The results indicate that polymorphisms of DNA damage response genes may be potential risk modifiers of ionizing radiation-induced or sporadic PTCs.

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Introduction

Thyroid cancer accounts for more than 90% of all endocrine malignancies. The incidence of thyroid cancer in the world is increasing during the past three decades, mainly due to the papillary thyroid carcinoma (PTC) which is the predominant type of malignant thyroid tumors (Davies & Welch 2006).

Most thyroid cancer patients do not have the history of radiation exposure, yet ionizing radiation (IR) is a recognized etiological factor of the disease. An increased risk of thyroid cancer has been documented after external irradiation (Ron *et al.* 1995) and after environmental exposure to ^{131}I, such as after the Chernobyl fallouts in Belarus, Ukraine, and Russia (Bennett *et al.* 2006).

Although radiation thyroid doses in Chernobyl PTC cases are generally greater than in controls in epidemiological studies (Cardis *et al.* 2005, Jacob *et al.* 2006, Likhtarev *et al.* 2006), thus confirming radiation to be a risk factor for thyroid cancer, those in

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controls are non-zero. Furthermore, there were some 14 million residents in the contaminated territories at the time of exposure (Bennett *et al.* 2006). Conceivably, at least some of them might have accumulated thyroid doses comparable with doses in diseased individuals. However, thyroid cancer developed only in a small fraction of irradiated population.

Among the variety of DNA damage types induced by radiation, double-strand DNA breaks are considered to be the most significant for chromosomal aberrations, mutagenesis, genetic instability, and carcinogenesis (Khanna & Jackson 2001). PTC is one of the rare human cancers of epithelial origin in whose oncogenesis gene rearrangements play a noticeable role. Several variants of rearrangements are described in PTC, with RET/PTC occurring most frequently (Nikiforov *et al.* 1997, Rabes *et al.* 2000).

While in the exposed individuals DNA damage could be attributed to IR, the origination of genetic alterations in sporadic cancers remains obscure. Nevertheless, the spectrum of oncogenic changes in radiation-related and sporadic PTCs is largely common. Such similarities imply the resemblance of molecular reactions on DNA damage in exposed and non-exposed thyrocytes. These reactions involve first of all DNA damage response factors, including DNA repair and checkpoint complexes.

The vast majority of Chernobyl thyroid malignancies were PTCs which displayed wide variations in clinical course, from highly aggressive tumors developing after the shorter latency to more indolent carcinomas with the longer latent period (Williams 2006). The randomness and multiplicity of forms of genetic alterations caused by IR can only partly explain these differences in the individual reactions on exposure as well as why cancer develops only in some of the exposed individuals.

It is attractive to hypothesize that inherited variability in the genes directly or indirectly involved in the maintenance of genome stability in response to environmental carcinogens such as IR or chemicals that may play a role in susceptibility for radiation-related or sporadic PTC or may be a marker of it. In this work, we tested the relation of genetic variants of some of such genes, namely *ATM*, *TP53*, *XRCC1*, *XRCC3*, and *MTF1* to PTC of different etiology.

The ataxia-telangiectasia mutated (*ATM*) gene plays a key role in the sensing and repair of DNA double-strand breaks. Activation of the ATM protein kinase by IR results in the subsequent initiation of several molecular pathways of DNA damage repair (Shiloh 2003). One of the ATM targets is the p53 pathway. Overexpression of *TP53* arrests the cell cycle and affects DNA repair and apoptosis.

The *ATM* and *TP53* genes play a significant role especially in the tumors that are induced by IR. A number of single-nucleotide polymorphisms (SNPs) in the *ATM* and *TP53* genes studied in populations of different ethnicities have been reported to associate with the risk of different radiogenic tumors (Hu *et al.* 2002, Angele *et al.* 2003, Thorstenson *et al.* 2003, Malmer *et al.* 2007). By contrast, studies of post-Chernobyl pediatric thyroid cancers demonstrated a low mutation and polymorphism rate in the *TP53* gene (Nikiforov *et al.* 1996, Hillebrandt *et al.* 1997). It, however, should be mentioned that after exposure to radiation p53 facilitates DNA repair in normal thyrocytes *in vitro* (Yang *et al.* 1997).

The base excision repair (BER) and homologous recombination repair (HRR) pathways are particularly important for genomic integrity restoration (Hoeijmakers 2001). The product of the X-ray repair cross complementing 1 (*XRCC1*) gene acts as a scaffold and a modulator of different enzymes involved in BER. The *XRCC1* Arg399Gln and Arg280His variants have been extensively investigated for their function and association with cancer risk; however, the results remain contradictory rather than conclusive (Hu *et al.* 2005). The *XRCC3* gene is a member of the *Rad51* DNA-repair gene family. Its product is a factor of the HRR. The *XRCC3* Thr241Met polymorphism has been controversially associated with different human malignancies (Han *et al.* 2006). Sturgis *et al.* (2005) reported 241Met allele association with the risk of differentiated thyroid cancer.

The metal-responsive transcription factor 1 (*MTF1*) gene has been implicated in tumor initiation and progression to malignant growth. MTF1 protein interacts with metallothioneins that are able to suppress cellular stresses generated by IR and other agents (Tamura *et al.* 2005). Polymorphism in murine *Mtf1* gene has been found to associate with the susceptibility to experimental γ-ray-induced thymic lymphomas. This observation points at possible involvement of human *MTF1* polymorphisms in the modulation of radiation-induced malignancies (Tamura *et al.* 2005).

To date no polymorphisms of the *ATM*, *XRCC1*, and *MTF1* genes have been studied neither in human sporadic or radiation-induced PTCs. Data on the *TP53* and *XRCC3* polymorphisms associations are quite limited (Hillebrandt *et al.* 1997, Boltze *et al.* 2002, Granja *et al.* 2004, Sturgis *et al.* 2005, Rogounovitch *et al.* 2006). Therefore, in this study, we addressed the relation of SNPs in aforementioned DNA damage response genes to the risk of PTCs of different etiology.

Materials and methods

Study population

A total of 255 histologically verified PTC cases and 596 healthy controls, all Caucasians, were included in the study. Among the patients, 123 individuals with PTC (24 males and 99 females) lived in the areas of the Russian Federation (38 patients) and Belarus (85 patients) contaminated with radionuclides from Chernobyl fallouts. At the time of the Chernobyl accident, these subjects were younger than 18 years old (mean age at exposure ± S.D., 9.8 ± 5.1 years old; 1–18 years old, range). The mean age at diagnosis was 24.4 ± 4.9 years old, range 19–37 years old (IR-induced PTCs). Information about individual radiation thyroid doses was available for PTC cases from Russia as reconstructed in previous studies (Davis *et al.* 2004, Stepanenko *et al.* 2004). The doses varied from 43 to 2640 mGy. Radiation thyroid doses for PTC patients and controls from Belarus evaluated in dosimetric investigations at the places of residence ranged 21–1500 mGy (Bouville *et al.* 2007). Among the controls, 198 individuals (65 males and 133 females, mean age at sampling 22.2 ± 3.2 years old; 19–35 years old, range) were residents of the Chernobyl areas (60 from the Russian Federation and 138 from Belarus). The averaged thyroid radiation dose in the exposed control subjects from Russia is 41 mGy (Bouville *et al.* 2007). All exposed control individuals were aged < 18 years at the time of the accident (mean age at exposure 1.8 ± 3.2 years old; 1–16 years old, range) (IR-exposed controls). IR-exposed controls and patients with IR-induced PTCs not were individually matched; however, they were residents of the same settlements. This, given the uncertainty with individual radiation thyroid doses, was supposed to partly reduce exposure bias. Age of IR-exposed control subjects was set to be ±3 years of that of IR-induced PTC individuals.

One hundred and thirty-two PTC cases (21 males and 111 females, mean age at diagnosis 47.8 ± 11.4 years old; 19–76 years old, range) were adults without history of radiation exposure (sporadic PTCs). The remaining 398 control participants (180 males and 218 females, mean age at sampling 45.0 ± 10.3 years old; 16–65 years old, range) had no previous history of radiation exposure (non-exposed controls); their age was also set to be ±3 years of that of patients with sporadic PTC. Both sporadic PTCs and non-exposed controls originated from the European part of Russia not contaminated by the Chernobyl fallouts.

Thyroid tissues and/or blood samples were collected from patients during surgery or further follow-up. Blood samples and information from the controls were obtained during a routine health examination or complex screening for thyroid diseases.

Written informed consent was obtained from all participants. Protocols of the present study were approved by the Committee for Ethical Issues of Human Genome Analysis of Nagasaki University.

SNP selection

The candidate SNPs (Table 1) were selected based on their reported functional role (if available), associations with radiosensitivity or (thyroid) cancer risk. Accordingly, we did not search for tag SNPs or account for the genetic variability in the regions of SNP location. All SNPs are listed in a public database, dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), with validated status in ethnically diverse populations. To ensure sufficient power for calculations, only SNPs with minor allele frequency (MAF) of >1% were included.

SNP genotyping

DNA was extracted from normal thyroid tissues using proteinase K/phenol–chloroform method or from the whole blood lymphocytes with Puregene DNA Purification Kit (Gentra Systems, Inc., Minneapolis, PA, USA). All specimens were genotyped using various techniques (Table 1). Primers and probes (Table 2) were designed with Primer Express Version 1.0 (Applied Biosystems, Foster City, CA, USA) software.

Briefly, 25 μl PCR mixtures generally contained 50 ng DNA, 1.5 mM $MgCl_2$, 200 μM each dNTP, optimized concentrations of corresponding primers and 0.625 U AmpliTaq Gold (Applied Biosystems). All restriction endonucleases for PCR/RFLP were from New England BioLabs (Ipswich, MA, USA). TaqMan allelic discrimination assay for *TP53* variants was done essentially as described previously (Rogounovitch *et al.* 2006). Melting curve T_m-shift assay for *MTF1* genotyping was designed according to the described technology (Wang *et al.* 2005) and done in a Thermal Cycler Dice Real Time System TP800 (TaKaRa, Ohtsu, Japan). Technical details are available from the authors upon request.

For every SNP, some 20–30 randomly chosen DNA samples, unless otherwise specified, were also analyzed by direct sequencing with a Big Dye Terminator sequencing kit v 3.1 (Applied Biosystems) in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). A complete concordance between different techniques was observed.