Generation of Large Homozygous Chromosomal Segments by Mitotic Recombination during Lymphomagenesis in F$_1$ Hybrid Mice

DOO-PYO HONG$^{1,2}$, KIHEI KUBO$^2$, NAOMI TSUGAWA$^2$, NOBUKO MORI$^1$, SEIICHI UMESAKO$^{1,2}$, CHANG-WOO SONG$^3$ and MASAAKI OKUMOTO$^{1,2}$*

INTRODUCTION

Because LOH has often been observed in the chromosomal regions where tumor-suppressor genes reside$^{1,2}$, allelotype analyses of tumors have been performed to identify the tumor-suppressor genes responsible for tumorigenesis. One allele of the recessive tumor suppressor genes is often inactivated by methylation, or small mutations such as base substitutions or microdeletions. The function of the other allele has been thought to be subsequently lost by chromosomal rearrangements, e.g. deletion, mitotic recombination, nondisjunction with or without reduplication and gene conversion, which can be detected by LOH. Therefore, LOH studies have been performed to detect the secondary event, that is, the loss of remaining alleles of recessive tumor-suppressor genes in many tumors. The use of F$_1$ hybrid mice in LOH analysis has several advantages, i.e. many strains have been established and well characterized genetically, thousands of polymorphic markers are available$^3$, all tumors are informative at the polymorphic markers, and an adequate number of tumors can be provided. Previously, we and others have analyzed radiation-induced lymphomas in mice, and found several regions with highly frequent LOH$^{4-11}$. However, the chromosomal mechanisms causing the loss of function of the remaining allele of recessive tumor-suppressor genes as a secondary event

Lymphoma / Loss of heterozygosity (LOH) / Mitotic recombination / Tumor-suppressor gene / Mouse

The loss of heterozygosity (LOH) has been reported in numerous neoplasms in both human and animals, and has often been observed in chromosomal regions, which contain tumor-suppressor genes. We previously found frequent LOH on chromosomes 4, 12 and 19 in radiation-induced lymphomas from (BALB/cHeA x STS/A)F$_1$ hybrid mice by allelotype analysis at polymorphic microsatellite loci. In this study, to elucidate the nature of allelic losses, we refined the loss regions on chromosomes 4, 12 and 19 of the tumors from the F$_1$ mice and then analyzed them cytogenetically. The results represent evidence of a wide range of allelic losses owing to mitotic recombination on chromosomes 4 and 19 in the tumors, possibly reflecting functional losses of putative tumor-suppressor genes. It is suggested that the generation of these large homozygous chromosomal segments probably containing the affected genes is one of the genetic alterations responsible for tumorigenesis.
are unknown. In this study, we analyzed radiation-induced lymphomas in (BALB/cHeA x STS/A)F1 hybrid mice for LOH at polymorphic microsatellite loci and for cytogenetic changes.

**MATERIALS AND METHODS**

**Mice**

The origins of BALB/cHeA and STS/A mice and the conditions for breeding were described previously.

**Lymphoma induction**

Mice were exposed four times to X-rays at 1.7 Gy (0.5 Gy / min) at weekly intervals starting at 4 weeks of age, and the moribund mice were examined as previously described.

**DNA isolation and allelotype analysis**

DNA isolation, PCR of microsatellite markers, electrophoresis of PCR products and assessment of allelic losses were performed according to procedures reported previously. Oligonucleotide primers corresponding to microsatellite loci were purchased from Research Genetics, Inc. (Huntsville, AL). Chromosomal locations of the microsatellite markers and several loci were based on the 2000 Chromosome Committee Reports in the Mouse Genome Informatics (Jackson Laboratory, Bar Harbor, ME).

**Cytogenetic analysis**

Moribund animals were sacrificed by cervical dislocation, lymphomas were removed, and the lymphoma cells were suspended in phosphate-buffered saline containing 0.075 µg/ml of Colcemid (Ciba). After centrifugation at 1,000 rpm for 5 min, cells were resuspended in a hypotonic solution (0.075 M KCl) and incubated at room temperature for 15 min. Cells were fixed three times with a methanol: acetic acid (3:1) solution, then dripped onto slide glasses, and dried at 37°C for 24 hr. The specimens were stained with 0.5 µg/ml of Hoechst Dye 33258 (Wako Pure Chemical Industries, Ltd.) at room temperature for 10 min, and then stained with 50 µg/ml of quinacrine mustard (Wako Pure Chemical Industries, Ltd.) in MacIlvaine buffer, pH 6.8 (0.06 M citric acid, 0.09 M disodium hydrogen phosphate) for 10 min. The slides were observed under a fluorescence microscope with a BV filter, and photographed using Kodak plus-Xpan film.

**RESULTS**

**Allelotype analysis of lymphomas**

We precisely analyzed 43 radiation-induced lymphomas in (BALB/cHeA x STS/A)F1 hybrid mice for LOH at more polymorphic microsatellite loci than those which we reported previously. The patterns of LOH on chromosomes 4, 12 and 19 are shown in Fig. 1A, 1B and 1C. Although allelic losses were observed at D4Mit149 (0 cM from the centromere: 0 cM) on
Fig. 1. LOH patterns on chromosomes 4, 12, and 19 in 43 radiation-induced lymphomas of (BALB/cHeA x STS/A)F_1 mice. Only the tumors which indicate LOH on chromosomes 4, 12 and 19 are shown in A, B and C, respectively. The order and map positions of the microsatellite loci indicated at the top are according to the 2000 chromosome committee reports in the Mouse Genome Informatics (Jackson Laboratory, Bar Harbor, ME). The map positions of the loci are indicated by distances (cM) from the centromere. Tumor numbers are shown to the left. Black and stippled boxes represent loss of alleles derived from STS/A and BALB/cHeA, respectively, and white boxes represent retention of both alleles. A, chromosome 4; B, chromosome 12; C, chromosome 19.
chromosome 4 in only 2 out of 43 lymphomas, 7 lymphomas showed LOH at D4Mit19 (5 cM) (Fig. 1A). At D4Mit17 (31.4 cM), 14 of 43 lymphomas demonstrated LOH, and the regions lost continued to the telomere through the loci of D4Mit7 (35.5 cM), D4Mit9 (44.5 cM), D4Mit31 (51.3 cM), D4Nds2 (55.6 cM), D4Mit11 (57.4 cM), D4Mit13 (71 cM), and D4Mit42 (81 cM). Only two tumors showed LOH at all of the loci examined on chromosome 4. The lengths of the loss regions on chromosome 12 were considerably restricted as compared with those on chromosomes 4 and 19 (Fig. 1A, B and C). On chromosome 12, the peak of the incidence of LOH appeared at D12Mit17 (55 cM) (Fig. 1B). No entire loss of chromosome 12 was observed. Only 4 tumors indicated LOH at D19Mit32 (0 cM) on chromosome 19 (Fig. 1C). Approximately 50% (22 of 43) of lymphomas, however, showed LOH at D19Mit56 (5 cM) near the centromere. Thus, 18 of the 22 lymphomas retained heterozygosity within a small 5 cM interval between D19Mit32 and D19Mit56. Nineteen of the 22 lymphomas which showed LOH at D19Mit56 exhibited allelic loss through D19Mit61 (9 cM), D19Mit41 (16 cM), D19Mit12 (35 cM), D19Mit17 (46 cM), D19Mit25 (50 cM) and D19Mit104 (53 cM), until D19Mit6 (55 cM) near the telomere.

**Cytogenetic analysis**

Six lymphomas with wide ranges of LOH on chromosome 4 and LOH on chromosome 12 or 19

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**Fig. 2.** Representative cytogenetic features of lymphomas T5 (A) and T38 (B). A, The number of chromosomes is 40. Translocation between chromosome 7A and chromosome 9F is shown. B, The number of chromosomes is 42. Chromosome 14 shows trisomy. Translocations between chromosomes 6F and 12F, between chromosomes 8E and 15B, and between chromosome 13A and an unknown chromosome are shown. In T5 and T38, the loss of a whole chromosome or a large deletion of chromosome 4 or 19 is not observed.
were analyzed cytogenetically. Representative karyotypes of lymphomas no. 5 (Tumor 5; T5) and no. 38 (T38) are shown in Figs. 2 A and B, respectively. In LOH studies, T5 and lymphoma no. 18 (T18) showed probably an entire loss of chromosome 4; lymphomas no. 11 (T11), 35 (T35) and T38 showed a loss of 76 cM or more, ranging from \(D4\text{Mit}19\) (5 cM) to \(D4\text{Mit}42\) (81 cM); lymphoma no. 12 (T12) showed an approximately 50 cM LOH, ranging from \(D4\text{Mit}17\) (31.4 cM) to \(D4\text{Mit}42\) (81 cM). Further-
more, T5 had more than a 54 cM LOH ranging from \(D19\text{Mit}43\) (0.5 cM) to \(D19\text{Mit}6\) (55 cM) on chromosome 19; T12 had more than an approximately 50 cM LOH from \(D19\text{Mit}56\) (5 cM) to \(D19\text{Mit}6\) (55 cM); and T18 had an about 20 cM LOH from \(D19\text{Mit}12\) (35 cM) to \(D19\text{Mit}6\) (55 cM) in T35 also had a somewhat wide range (24 cM or more) of LOH on chromosome 12. However, none of these lymphomas showed any obvious abnormalities on chromosomes 4, 12 or 19, except for translocation between 6F and 12F in T38 (Table 1, Fig. 2 A and B). The regions on chromosomes 4 and 19 with wide ranges of LOH did not appear to have chromosomal aberrations, such as large deletions. It appears that a set of alleles are retained in these large loss regions.

### DISCUSSION

To examine the mechanism of genomic alterations in lymphoma development, precise LOH and cytogenetic analyses have been performed. LOH has been known to reflect the occurrences of some chromosomal rearrangements, such as deletions, mitotic

### Table 1. Comparison of LOH regions of lymphomas with their cytogenetic features

| Lymphomas | Range of LOH | Length of LOH | Basic karyotypes |
|-----------|--------------|---------------|-----------------|
| T5        | \(D4\text{Mit}149\) (0 cM) – \(D4\text{Mit}42\) (81 cM) | 81 cM | \(2n=40^b\), XX |
|           | \(D12\text{Mit}28\) (52 cM) | 54.5 cM | \(t (7A; 9F)\) |
| T11       | \(D4\text{Mit}19\) (5 cM) – \(D4\text{Mit}42\) (81 cM) | 76 cM | \(2n=41, XX, +15\) |
| T12       | \(D4\text{Mit}17\) (31.4 cM) – \(D4\text{Mit}42\) (81 cM) | 49.6 cM | \(2n=41, XX, marker\) |
| T18       | \(D4\text{Mit}149\) (0 cM) – \(D4\text{Mit}42\) (81 cM) | 81 cM | \(2n=40b, XX\) |
| T35       | \(D4\text{Mit}19\) (5 cM) – \(D4\text{Mit}42\) (81 cM) | 76 cM | \(2n=41, XY, +16\) |
| T38       | \(D4\text{Mit}19\) (5 cM) – \(D4\text{Mit}42\) (81 cM) | 76 cM | \(2n=42, XY, +14\) |

\(a\) Numbers in parentheses show distance in centimorgans from the centromere.

\(b\) Number of chromosome (2n) is shown by mode of the number.
recombinations, nondisjunctions with or without replication and gene conversions. In the present study on radiation lymphomagenesis in mice, a substantial number of tumors revealed a wide range of LOH on chromosome 4 and/or 19. T5, T11, T12, T18, T35 and T38 had a wide range (at least 50 cM or more) of LOH on chromosome 4. T5, T12 and T18 also had loss regions encompassing 20 cM or more of LOH on chromosome 19. However, none of these lymphomas showed any obvious cytogenetical abnormalities on chromosomes 4 and 19 (Table 1, Fig. 2 A and B). It is unlikely that such a wide range of LOH results from gene conversion. These results suggest that mitotic recombination or nondisjunction occurred on chromosomes 4 and 19 of these lymphomas. Chromosome 4 is reported to contain putative tumor-suppressor genes, such as p16\textsuperscript{Ink4a}, p15\textsuperscript{Ink4b}, p19\textsuperscript{Arf}, and p73\textsuperscript{13}, and chromosome 19 to contain Pten, multiple endocrine neoplasia type 1 (\textit{Men1}\textsuperscript{14}) and Mxi-1\textsuperscript{15}). On the other hand, although these lymphomas also did not show any obvious abnormalities on chromosome 12, except for translocation between 6F and 12F in T38 (Table 1, Fig. 2 A and B), the types of chromosomal rearrangements of chromosome 12 are unclear because of the short loss regions.

Although approximately 50\% (22 of 43) of the lymphomas showed LOH at \textit{D19Mit56} (5 cM), 18 of the 22 lymphomas retained heterozygosity at \textit{D19Mit32} (0 cM). Retention of the heterozygosity in these tumors excludes the possibility of nondisjunction. On chromosome 4, 12 of 14 lymphomas also retained heterozygosity at \textit{D4Mit149} (0 cM). These large homozygous regions on partial chromosomes are most likely generated through chromosomal mechanisms, such as mitotic recombination, which may be enhanced by irradiation. In some rare cases displaying LOH on entire chromosomes, the loss of one entire chromosome by nondisjunction, and subsequent repulsion of the remaining homologue might occur. Some recessive mutations or inactivated alleles at certain tumor-suppressor genes might result in a loss of function by the formation of homozygous regions through various chromosomal mechanisms, such as mitotic recombination and nondisjunction. Cells harboring homozygous recessive mutations may become malignant. These homozygous disfunctions in specific tumor suppressor genes might be important genetic alterations in tumorigenesis.

The STS/A mouse is extremely resistant to radiation lymphomagenesis\textsuperscript{16). We previously found STS/A-specific preferential allelic loss on chromosome 4 where the lymphoma resistance locus has been suggested to exist by the analysis of CXS recombinant inbred strains\textsuperscript{5,12). The preferential loss of STS/A-derived alleles in a large region from \textit{D4Mit17} (31.4cM) to \textit{D4Mit42} (81cM) was also shown in this study (Fig. 1A). A susceptibility locus for radiation lymphomagenesis was recently reported using the same BALB/cHeA and STS/A mice\textsuperscript{17). An analysis of the underlying gene for susceptibility to ionizing radiation and chromosomal instability is relevant for radiation oncology\textsuperscript{18).}

Allelic losses on chromosome 4 were detected in 29 of 61 (48\%) lung adenocarcinomas, and in most of these cases the losses appeared to occur by nondisjunction\textsuperscript{19). Hegi et al. reported that nondisjunction appeared to be the most common mechanism of chromosomal alteration because all 9 markers examined were lost in 24 of 26 methylene chloride-induced lung adenocarcinomas with LOH on chromosome 4 in (C57BL/6J X C3H/HeJ) F\textsubscript{1} mice\textsuperscript{20). A high frequency of loss of \textit{p53} has been found in benzene-induced thymic lymphomas in \textit{p53}\textsuperscript{+/-} mice, likely mediated by aberrant chromosomal recombination\textsuperscript{21). Mitotic recombination has been suggested as a common mechanism for the LOH in neurofibromas bearing both \textit{NF1} mutated alleles (\textit{NF1}\textsuperscript{-/-})\textsuperscript{22). In normal human T lymphocytes, mitotic recombination and, to a much lesser extent, deletion may be the primary mechanism for the high frequency of in vivo LOH, suggesting a significant role for LOH by mitotic recombination in tumor development\textsuperscript{23). Phenotypic reversions were observed at the \textit{W/Kit} locus in normal tissue of mice as a result of mitotic recombination occurring spontaneously\textsuperscript{24). More than 90\% of the \textit{γ}-ray-induced human lymphoblastoid cells, WR10, showed allelic loss, but approximately half of them retained two copies of the mutated allele at \textit{aprt} locus\textsuperscript{25). It is likely that ionizing
radiation enhances the rate of spontaneous mitotic recombination in the lymphomagenesis, resulting in the generation of frequent large homozygous regions. Therefore, this mitotic recombination might be an important genetic event responsible for tumorigenesis induced by ionizing radiation.

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