The *D4Mit12* locus on mouse chromosome 4 provides susceptibility to both γ-ray-induced and N-methyl-N-nitrosourea-induced thymic lymphomas

Hiroki Sato,[1,2] Yasushi Tamura,[1] Yukie Ochiai,[1] Yasumitsu Kodama,[1] Katsuyoshi Hatakeyama,[2] Ohtsura Niwa[3] and Ryo Kominami[1,4]

Departments of [1]Molecular Genetics and [2]Regenerative and Transplant Medicine, Graduate School of Medical and Dental Sciences, Niigata University, 1-757 Asahimachi, Niigata 951-8122; and [3]Radiation Biology Center, Kyoto University, Yoshida-Konoecho, Sakyou-ku, Kyoto 606-8315

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Low-penetrance genes control different susceptibilities to γ-ray-induced thymic lymphomas in mouse strains. Our previous genetic analyses with backcross mice between BALB/c and MSM strains and congenic lines localized one such gene near the *D4Mit12* locus on chromosome 4. N-Methyl-N-nitrosourea (MNU) is a guanine base-alkylating agent and differs from γ-radiation in its mechanism of mutagenic action. Accordingly, in this study, we examined whether or not the locus also provides susceptibility to MNU-induced thymic lymphomas using 84 offsprings derived from congenic mice for *D4Mit12*. Association analysis provided a suggestive linkage at *D4Mit12* (*P*=0.0075) and the linkage was sustained by the peak of likelihood ratio statistical values being at the same position as that for the γ-ray-induced lymphomas. The results strongly suggest that the BALB/c allele near *D4Mit12* is associated with susceptibility to lymphomas induced by two carcinogenic agents having different mechanisms of mutagenic action. (Cancer Sci 2003; 94: 668–671)

Genetic variation plays a key role in determining the range of individual susceptibility to cancers within human population, and there is increasing evidence that a high proportion of cancers arise in a susceptible subpopulation that carries low-penetrance genes.[1–5] The importance of susceptibility genes has been underlined, because the identification of such genes has possible public health implications, and can be achieved using information from the human genome project. Mouse thymic lymphomas are one of the classical models of radiation-induced and chemical carcinogen-induced malignancies,[6–8] and the model involves low-penetrance susceptibility genes in mouse strains. For instance, BALB/c (C) is susceptible to the development of γ-ray-induced thymic lymphomas[9,10] while MSM (M), an inbred strain derived from Japanese wild mice, *Mus musculus molossinus*, shows resistance.[11] Our previous analyses of backcross mice between sensitive and resistant strains and congenic lines localized one of the susceptibility genes near the *D4Mit12* locus on chromosome 4.[12] Mice with the C/M genotype at *D4Mit12* showed an incidence twice as high as that in mice with the M/M genotype. This indicated that the BALB/c allele near *D4Mit12* provides susceptibility in a dominant manner, although the nature of the susceptibility locus is not clear.

N-Methyl-N-nitrosourea (MNU) is an alkylating agent that can modify guanine bases in DNA and some of the modified guanines are changed to adenine after DNA replication. The mode of action differs from that of radiation; i.e., the major type of radiation-induced DNA change is large mutations, such as deletion and translocation.[13] It is of interest, therefore, to know whether or not the BALB/c allele near the *D4Mit12* locus provides susceptibility to MNU-induced thymic lymphomas as well. This may give a clue to the function of the susceptibility gene in lymphomagenesis. In this study we have investigated this issue using congenic mice for the *D4Mit12* locus.

Materials and Methods

Mice and lymphoma induction. A congenic mouse line for *D4Mit12* on chromosome 4 was obtained previously[12]; this line carries an MSM-chromosomal region spanning *D4Mit9* (39 cM) and *D4Mit203* (60 cM). The mice were mated with BALB/c (C) and heterozygotes carrying the same chromosomal region were crossed with MSM (M) mice. Progeny of the mice were hence of F_1 background and C/M heterozygous or M/M homozygous for the congenic region. They were given a single intraperitoneal dose, 75 mg/kg body weight, of MNU (Nacalai Co., Kyoto), when they were 6 weeks of age. Development of thymic lymphoma was diagnosed on the basis of inspection for labored breathing up to 250 days. Existence of tumors was confirmed upon autopsy of the mice. Sixty-eight of the 91 mice injected developed thymic lymphomas and seven developed other cancers such as skin tumors. The remaining 16 were free of lymphomas in the thymus, some of which were histologically examined.

Genetic analysis and statistics. Genotyping of mice and allelic loss analysis of lymphomas were carried out with microsatellite markers as described previously.[11,12] The γ-ray-induced lymphomas analyzed in this study were those previously obtained in our laboratory.[12] Analysis of *K-ras* mutations at codons 12 and 13 was carried out with SSCP and subsequent direct sequencing. Primers used were 5′-GCCTGCTGAAAATGACTGAG and 5′-TCAAAGCGCACGCAACTGTG. χ^2 and *P* values for association of markers with the development of MNU-induced thymic lymphoma were obtained by means of the χ^2 test and Mantel-Cox test with StatView-J 5.0 software on a Macintosh personal computer. QTLs were analyzed of the lymphoma incidence with the Map Manager QTXb14 software.[14] Evaluation of linkage followed the criteria of Lander and Kruglyak.[15] The statistical threshold of the congenic mouse data was corrected for multiple comparisons using the formula, $\mu(T)=[C+2\rho GT^2]\alpha(T)$: C (number of chromosomes)=1, ρ (to account for corrected results among linked loci: for backcross with 1 d.f.)=1, G (genome size in morgans)=0.25, T (for the χ^2 statistic with 1 d.f.)=3.84, and $\mu(T)$=0.05. Linkage was taken as suggestive in the congenic mice when *P* was less than 0.012 and as significant when *P* was less than 0.0060.

Results

Congenic mice used in this study was of BALB/c (C) background and harbored a chromosomal region of 25 cM between *D4Mit9* and *D4Mit203* on chromosome 4 that was derived from the MSM (M) chromosome.[12,16] The mice were mated with

[4]To whom correspondence should be addressed.
E-mail: rykomina@med.niigata-u.ac.jp

Table 1. The association of markers with the development of MNU-induced thymic lymphoma

Marker	(cM)	Lym(+)[1] C/M[2]	Lym(+) M/M	Lym(−) C/M	Lym(−) M/M	χ^2 value	*P* value
D4Mit9	(39.3)	28	40	5	11	0.535	0.47
D4Mit246	(45.9)	29	39	4	12	1.69	0.19
D4Mit331	(50.3)	30	38	4	12	1.97	0.16
D4Mit12	(54.6)	36	32	3	13	6.09	0.014
D4Mit338	(55.9)	36	32	3	13	6.09	0.014
D4Mit336	(56.6)	35	33	3	13	5.59	0.018
D4Mit203	(60.1)	33	35	3	13	4.69	0.030

The association of markers with the development of MNU-induced thymic lymphoma.
1) Lym(+), the number of lymphoma-bearing mice; Lym(−), the number of lymphoma-free mice.
2) C/M, heterozygous for BALB/c and MSM alleles; M/M, homozygous for MSM alleles.

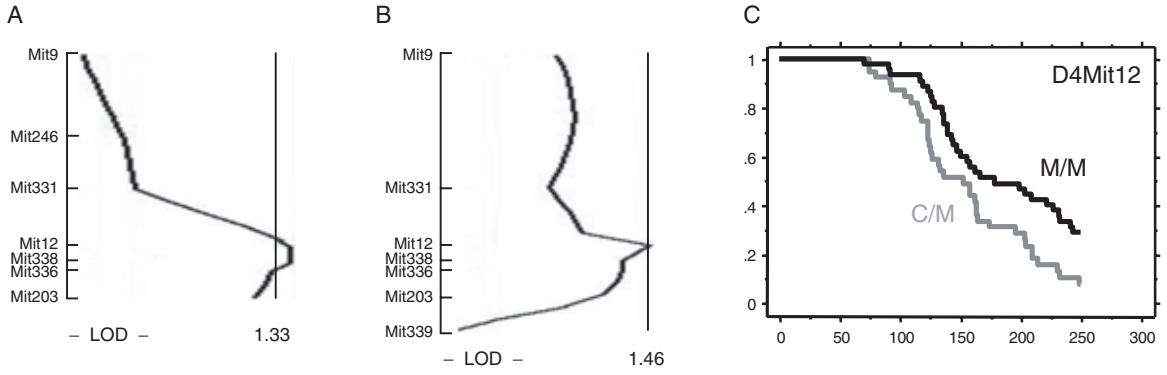

Fig. 1. Location of the susceptibility gene on chromosome 4. LOD scores on mouse chromosome 4 were calculated by using the Map Manager QTXb14 software[14] on a Macintosh personal computer. A, MNU-induced thymic lymphomas; B, γ-ray-induced thymic lymphomas.[12] A vertical line indicates the level giving statistical significance in this analysis. C, The cumulative frequency distributions of MNU-induced thymic lymphoma in mice that were produced by crossing the congenic mice for *D4Mit12* to MSM. C/M, heterozygotes at *D4Mit12*; M/M, homozygotes at *D4Mit12*.

BALB/c and the hybrids were then crossed with MSM. Ninety-one offsprings were intraperitoneally injected with MNU and followed up to 250 days after injection. Sixty-eight of them developed thymic lymphomas and 16 were free of lymphomas (Table 1). The remaining seven mice developing other cancers, such as skin tumors, were excluded from this study. Genotyping with seven markers (*D4Mit9*, *D4Mit246*, *D4Mit331*, *D4Mit12*, *D4Mit338*, *D4Mit336* and *D4Mit203*) revealed that 28 mice had received the BALB/c chromosome from the heterozygous mice, 42 had inherited the recombinant chromosome bearing the MSM genome, and 14 mice had received chromosomes that had undergone recombination in the heterozygous region. Table 1 summarizes the results of the χ^2 test for the association at each locus and Fig. 1A displays the distribution of likelihood ratio statistical values as LOD scores that was calculated using the Map Manager QTX software.[14] The highest value was at the *D4Mit12*/*D4Mit338* region. Fig. 1B shows a comparison of the likelihood ratio statistics in the association study of γ-ray-induced lymphomas that we previously performed.[12] This shows the same peak of association between them. Fig. 1C shows the cumulative lymphoma incidences of the homozygotes and the heterozygotes at the *D4Mit12* locus. The two groups showed a difference in the incidence and latency (P=0.0075 in Mantel-Cox test). These results strongly suggested that the BALB/c allele near the *D4Mit12* locus provides susceptibility to MNU-induced lymphomas, as found in γ-ray-induced lymphomas.

We previously showed that three loci, *Ikaros*, *Rit1/Bcl11b* and *D16Mit279* on chromosomes 11, 12 and 16, respectively, underwent allelic loss at high frequencies in lymphomas.[11, 17–20]

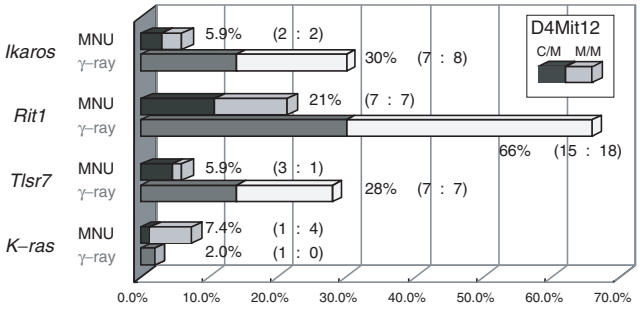

Fig. 2. Allelic loss frequencies of *Ikaros*, *Rit1* and *Tlsr7* on chromosomes 11, 12 and 16, respectively, and the frequency of *K-ras* mutations. Columns display the numbers of the allelic losses or *K-ras* mutations in MNU- and γ-ray-induced thymic lymphomas that were developed in mice of C/M and M/M genotypes at *D4Mit12*. Samples of the radiogenic lymphomas used were those previously obtained.[12] Among five *K-ras* mutations detected in MNU-induced thymic lymphomas, two were GGT to GAT change of codon 12, two were GGT to GTT change of codon 12, and the other was a GGC to GTC change of codon 13. One in a γ-ray-induced lymphomas was a GGC to GAC change of codon 13.

Thus, the allelic loss frequency of these loci was examined in the MNU and γ-ray-induced lymphomas. Fig. 2 summarizes the results. All three loci showed lower frequencies of loss in chemically induced lymphomas than in γ-ray-induced lymphomas, and no significant difference was observed between C/M heterozygous and M/M homozygous lymphomas of *D4Mit12*.

This suggested that the susceptibility-giving BALB/c allele in this region did not affect the recombination or deletion frequency.

K-ras is much more frequently mutated in mouse thymic lymphomas than the other *H-ras* and *N-ras* genes, and the activation is attributed mostly to single point mutations localized to codon 12 or codon 13.[21, 22] Therefore, mutations of codons 12 and 13 of the *K-ras* gene was examined by SSCP and subsequent sequence analysis. Missense mutations were identified in five of the MNU-induced lymphomas, one being in the C/M lymphomas and four in the M/M lymphomas (Fig. 2). Comparison with γ-ray-induced lymphomas showed a lower frequency, consistent with previous reports.[21, 22]

Discussion

In the present study, we examined whether or not the susceptibility locus near *D4Mit12* found in γ-ray-induced thymic lymphomas[12] also provides susceptibility to MNU-induced thymic lymphomas. When the association is tested independently of the linkage with γ-ray-induced thymic lymphomas, the evidence for the linkage (P=0.0075) is suggestive (much lower than the limit for a suggestive linkage, P=0.012), but not significant (P=0.0060) according to the criteria of Lander and Kruglyak.[15] However, the linkage is sustained by the finding that the peak position of the likelihood ratio statistics is the same as that in the case of γ-ray-induced lymphomas. Therefore, we conclude that the BALB/c allele near *D4Mit12* also provides susceptibility to MNU induction of thymic lymphomas in a dominant manner. This seems to be consistent with the recent report analyzing backcross mice between AKR and C57 mouse strains,[23] which assigned, although without statistical significance, MNU susceptibility to a region between *D4Mit306* and *D4Mit124*, 0.2 cM apart from the *D4Mit12* locus. The *D4Mit12* region is syntenic to human chromosome 1p34-36 and comprises several candidate susceptibility genes, such as *Lmyc1*, on the database. However, an attempt at identification is probably not yet justified, because the region is so large. Efforts should be made to generate congenic strains for lymphoma susceptibility testing to narrow down the candidate region.

The nature of the gene product encoded by the susceptibility allele remains to be identified. The product might function in recombination/repair processes and allelic difference of the function may affect the frequency of oncogene activation and inactivation of tumor suppressor genes required for tumor development. To address this issue, we examined allelic loss frequencies at the *Ikaros*, *Rit1/Bcl11b* and *Tlsr7* loci on chromosomes 11, 12 and 16, respectively. Allelic loss frequencies at the three loci were lower in MNU-induced lymphomas than in γ-ray-induced lymphomas, whereas the mutation frequency of *K-ras* showed an inverse relationship, consistent with their different modes of carcinogenic action.[13] However, no significant difference in the allelic loss frequency was observed between C/M heterozygous and M/M homozygous lymphomas of *D4Mit12* (Fig. 2). Further, the *K-ras* mutation frequency was higher in lymphomas of the resistance-giving M/M genotype, although the difference was not statistically significant. These results suggest that the susceptibility locus does not affect the recombination/repair process that increases mutation frequency.

In addition to such action at the cellular level, the susceptibility gene may operate at the tissue level. The importance of the tissue microenvironment in radiation carcinogenesis is well recognized.[24] Classical studies of radiation induction of mouse thymic lymphomas demonstrated that fractionated whole-body irradiation causes thymic atrophy and depletion of bone marrow cells, which lead to regeneration and differentiation arrest of thymocytes surviving the exposure.[6–8, 25–27] Interestingly, the lymphomagenesis is suppressed by bone marrow transplantation.[7, 8, 25–28] Potworowski *et al*.[29] reported that dendritic cells, but not thymocyte precursors, supplied by bone marrow to the thymus play a key role in the prevention of lymphoma development. On the other hand, involvement of the thymus and bone marrow in chemical carcinogen-induced lymphomagenesis is not well studied, despite the similarity of serological properties.[30] It is noteworthy, however, that the development of 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced thymic lymphomas was not suppressed by bone marrow transplantation.[31, 32]

Strain difference in radiation effects on the thymus and bone marrow was investigated using susceptible B10 and resistant C3H or STS strains.[33] A study with radiation bone marrow chimeras revealed that the difference between B10 and C3H mice in lymphoma susceptibility was due to that of bone marrow-derived cells, and the resistance of STS, but not C3H, was ascribed to an intrinsic property of thymocytes. It was also reported that the distribution of thymocyte subsets after irradiation was different between B10 and C3H within the thymus, as indicated by the accumulation of immature thymocytes in B10 mice.[34] Such a complex outcome may be due to the existence of different multiple genes controlling the susceptibility. It is less likely that the gene in the vicinity of *D4Mit12* affects the properties of bone marrow cells or dendritic cells for lymphoma prevention, since the *D4Mit12* locus influenced susceptibility to both radiation-induced and MNU-induced lymphomas.

Apoptosis is another process that regulates the net growth rate of thymocytes or pre-lymphoma cells, and hence the susceptibility gene product may confer a growth advantage by controlling apoptosis. Genetic dissection of the susceptibility to radiation-induced apoptosis of thymocytes was performed using recombinant congenic strains derived from BALB/c and STS strains. Three loci were mapped on chromosomes 3, 9 and 16, but not the *D4Mit12* locus on chromosome 4.[35, 36] Therefore, the susceptibility controlled by the gene near *D4Mit12* may not be involved in the different sensitivity to apoptosis of thymocytes after irradiation.

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