Mapping of new skin tumor susceptibility loci by a phenotype-driven congenic approach

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Abstract. As cancer susceptibility varies among mouse strains, mouse models are powerful tools for the identification of genes responsible for cancer development. Several cancer susceptibility loci have been mapped by genetic analysis using cancer-resistant and cancer-susceptible mouse strains. However, only a few corresponding genes for these loci have been identified, because most of the cancer susceptibility loci are low-penetrance alleles. We reported previously that wild-derived PWK mice showed no tumor development on treatment with the two-stage skin carcinogenesis protocol [induced by 7.12-dimethylbenz(a) anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA)], and that this phenotype is dominant-resistant when crossed with the highly susceptible strain FVB. From the analysis of the F1 backcross generation between PWK and FVB, we have mapped the new significant locus *Skts-fp1* on chromosome 4. In the present study, congenic strains were generated with the PWK resistance allele in the FVB background using a phenotype-driven approach, and sought to narrow down the candidate loci and find the responsible gene(s). One of the resistant mice in the N_6 generation carried the remaining PWK allele on chromosomes 4, 7 and 11, and an association study using the progeny of this mouse suggested that the locus on chromosome 11 may affect the cancer susceptibility locus on chromosome 7. On the other hand, no skin tumor susceptibility locus was mapped on chromosome 11 as examined in N_2 progeny. These findings suggest that there is at least one tumor-resistance gene on chromosome 7, the function of which could be regulated by gene(s) located on chromosome 11.

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Introduction

Germline transmission of variant forms of tumor suppressor genes or oncogenes greatly increases the risk of developing certain types of cancer. These genetic variants associated with cancer predisposition have been identified through studies of familial cancers. However, the majority of cancers develop sporadically, and the epidemiological data suggest that cancer susceptibility in the general population is affected by multiple low-penetrant tumor susceptibility genes (1,2). Mouse cancer models are powerful tools for identification of these tumor susceptibility genes. As they have various advantages such as well-controlled genetic background and environment, mouse models have been utilized to analyze cancer susceptibility, and many genetic loci associated with cancer susceptibility have been mapped (3,4).

Susceptibility to the two-stage skin carcinogenesis protocol varies among mouse strains (5,6), and genetic approaches have been used to identify the specific loci related to the differences in tumor susceptibility among strains (3,4,7-11). For example, 15 skin tumor susceptibility loci, *Skts1-15* have been mapped by analysis of NIH and *Mus spretus* cross, in which *Mus spretus* showed the dominant resistance against NIH (12,13). *Psl1-4* were mapped by crossing of the resistant C57BL/6J (B6) strain and the susceptible DBA strain (14,15). *Skts-fp1-3* were found by crossing the resistant PWK strain and the susceptible FVB strain (8), and *Stmm1-3* were identified by crossing the resistant MSM/Ms strain and the susceptible FVB strain (11). Over the past 10 years, a number of responsible genes at each locus have been identified. *Skts13* and *Skts14* were identified as *Aurka* and *Tgfb1* genes, respectively (16,17). Recently, Okumura *et al* reported that the parathyroid hormone (Pth) gene is one of the genes responsible for *Stmm1* (18).

However, many of the responsible genes within the remaining loci have not yet been identified. As the contribution of each tumor susceptibility locus to the phenotype was relatively low and most of the loci were mapped to broad intervals, it is difficult to identify the precise locations of these tumor susceptibility loci. Derivation of congenic mice by multiple rounds of breeding to transfer the regions containing the variant from a resistant strain to a susceptible strain is a reliable approach for fine mapping of the responsible genes. However, the standard genotype-driven congenic approach,

in which mice that have inherited the resistance allele on susceptible loci are selected and bred with a susceptible strain to generate the next generation, may not be appropriate for precise mapping of low-penetrant genes. For example, we have shown that the contribution of the D4Mit26 locus to the *Skts-fp1* locus is approximately 15% of the PWK resistant phenotype, and therefore congenic mice carrying the PWK allele only at this locus in the FVB mouse background cannot be expected to show the resistant phenotype (8).

We used a phenotype-driven approach in the present study, in which mice were subjected to the two-stage skin carcinogenesis protocol in each generation. Mice showing the resistant phenotype were bred to generate progeny.

Materials and methods

Animals. The mice used in this study were bred in the specific pathogen-free (SPF) facility of the Department of Laboratory Animals at Roswell Park Cancer Institute (Buffalo, NY, USA) and were treated in accordance with Institutional Animal Care and Use Committee (IACUC) regulations. FVB/N mice were purchased from Taconic (Germantown, NY, USA). PWK/Rbrc inbred mice were obtained from RIKEN BioResource Center, (Tsukuba, Japan). (FVB/NxPWK/Rbrc) F1 males were backcrossed with FVB/N females to generate 198 F1 backcross progeny, abbreviated as FxFP. All were subjected to the two-stage skin carcinogenesis protocol, and then resistant FxFP mice were selected for further phenotype-driven backcrossing.

Skin carcinogenesis. 7,12-Dimethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma-Aldrich; Merck KGaA, (Darmstadt, Germany) for use as a carcinogen and a promoter, respectively. The back skin of each animal was carefully shaved with an electric shaver at 8-11 weeks old. Two days after shaving, 200 μl of DMBA (0.125 mg/ml solution in acetone) was applied to the back of each mouse. A total of 97.4 nmol of DMBA was applied to each mouse. One week after the treatment, mice were treated with 400 μl of TPA ($5x10^{-5}$ M solution in acetone), i.e., 32.4 nmol of TPA, twice a week for 20 weeks. Animals were assessed twice a week for the appearance of papilloma. The number of papillomas was counted every other week until 20 weeks after the initial DMBA treatment. The incidence of papilloma during the promotion phase and papilloma multiplicity 12 weeks after initiation were used for assessment of skin tumor susceptibility. Male mice of FxFP and congenic strains were subjected to the two-stages skin carcinogenesis protocol. After the final treatment with TPA, most of the animals were maintained for more than 10 months to obtain skin squamous cell carcinoma (SCC) tissues.

Genotyping. Genomic DNA was isolated from the tails of the mice using NaOH solution. Briefly, 2-3 mm sections of the tails were boiled in 300 μl of 50 mM NaOH for 30 min, and then 25 μl of 1 M Tris-HCl (ph 8.0) was added. Genomic DNA prepared from 199 FxFP backcross mice was genotyped based on 196 polymorphic microsatellite markers (MIT) spaced at approximately 8-cM intervals through chromosomes 1 to 19 by polymerase chain reaction (PCR). Information regarding the MIT markers used in this study is available upon request. PCR was carried out using a Biometra thermocyler (Analytik Jena AG, Jena, Germany), and PCR products were separated by electrophoresis through 4% Nusieve-GTG low-melting temperature agarose gels (FMC) in 0.5 x TBE buffer.

Analysis of allelic imbalances in skin tumor tissues. Genome DNA was prepared from skin tumor tissues developed in FxFP mice or from N_5-N_7 congenic mice sharing the PWK allele on chromosome 7. Genomic DNA extracted from the tails of F1 mice was utilized as a standard. PCR was performed to amplify the microsatellite markers on chromosome 7 and the PCR products were separated by electrophoresis as described above. The densities of the DNA bands for FVB and PWK alleles were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (19), and allelic imbalances were analyzed as described previously (20), with a modification. In the present study, differences of ≥25% in the band intensity ratios of the two alleles in tumor DNAs compared to F1 mouse tail DNA were considered to be allelic imbalances. Although previous report used ≥50% threshold, we realized that genome deletions or amplifications seemed to be too noncontiguous when we analyzed our data using ≥50% threshold. Therefore we used ≥25% threshold in the present study.

Statistical analysis. Interval mapping was performed using R/qtl software, an add-on package for the R statistical system, to map QTLs related to susceptibility for chemically induced skin tumors. One thousand permutations were performed to estimate the empirical threshold value for mapping. Statistical analyses were performed using one-way ANOVA and the Tukey's post hoc test using JMP software v11.2 (SAS Institute, Inc., Cary, NC, USA). Data are presented as means ± SD from at least three independent experiments. In all analyses, $P<0.05$ was considered to indicate a statistically significant difference.

Results

Linkages for papilloma multiplicity mapped to chromosome 3-7, 14, and 17 by analyzing FxFP male mice. Using the standard two-stage skin carcinogenesis protocol, 126 (63.6%) of 198 FxFP backcross males (N_2 progeny) developed at least one papilloma 12 weeks after the initial DMBA treatment. Average multiplicity was 5.08±0.55. Genome-wide linkage mapping using 196 polymorphic markers revealed a highly significant linkage ($P<0.01$) located on chromosome 4, significant linkages ($P<0.05$) on chromosomes 5 and 17, and suggestive linkages ($P<0.63$) on chromosomes 3, 6, 7, and 14 for papilloma multiplicity (Fig. 1A).

N_6 progeny of congenic strains obtained by phenotype-driven backcrossing. Forty-seven (23.7%) of 198 FxFP mice did not develop any skin tumors until at least until 1 year old. To generate a congenic strain with a skin tumor resistance phenotype, one of the completely resistant mice was picked and subjected to further phenotype-driven backcrossing. This mouse was crossed with FVB females. The resultant male offspring were processed for the two-stage carcinogenesis protocol, and individuals showing the resistant phenotype (12 weeks after the initial treatment) were again picked to

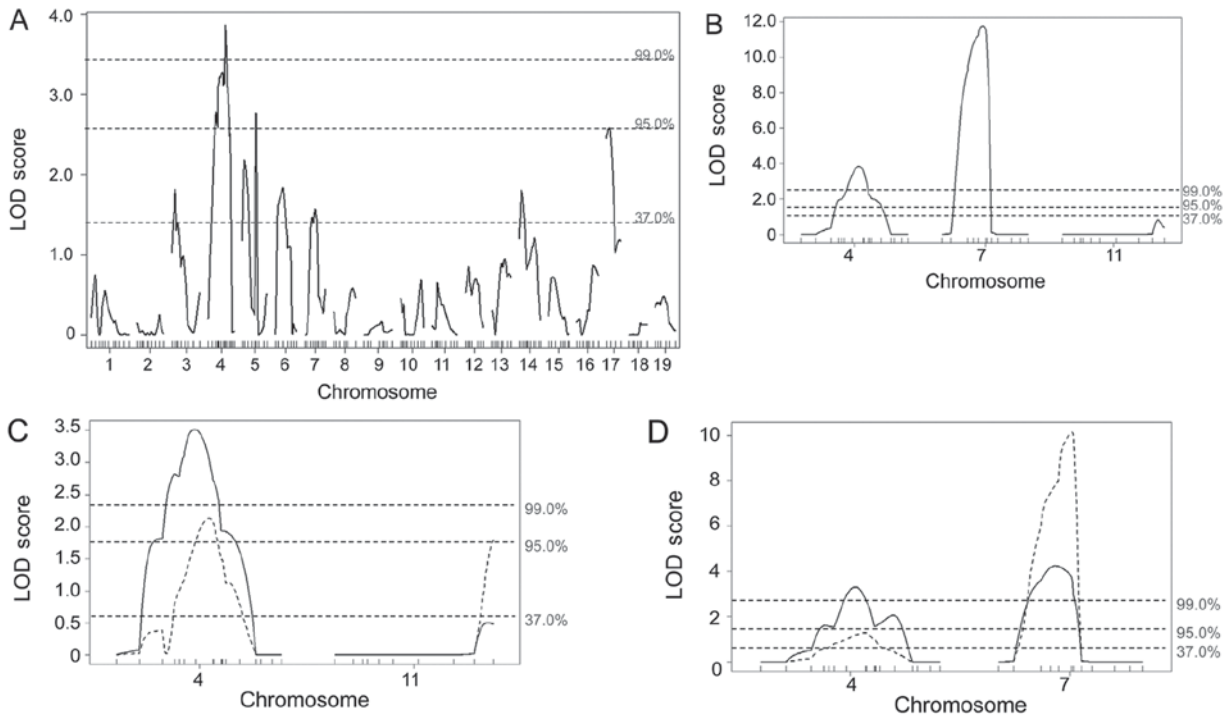

Figure 1. Interval mapping of skin tumor susceptibility loci. The number of papillomas 12 weeks after initiation was used as a trait. (A) Whole-genome scan of FxFP backcross, and (B) genome scan of chromosomes 4, 7, and 11 in N_7 congenic mice. N_7 congenic mice were divided into two groups according to the genotype at the (C) D7Mit31 locus or (D) D11Mit338 locus, and interval mapping was performed for each group separately. The solid lines indicate FVB homozygous at (C) D7Mit31 or at (D) D11Mit338. Dashed lines indicate heterozygous at (C) D7Mit31 or at (D) D11Mit338.

produce the next generation. This procedure was repeated to finally obtain N_6 progeny. The genotypes and phenotypes of each of eight N_6 progeny mice exhibiting the highest resistance or susceptibility are shown in Table I. Five of 69 males did not develop papillomas and all carried the PWK allele on chromosomes 4 and 7. In addition, they also harbored at least one PWK allele on chromosome 1, 3, 9, or 11 (Table I).

Linkage analysis of N_7 progeny exhibits significant linkage for papilloma multiplicity on chromosome 11 only in mice with the PWK allele on chromosome 7. To examine the possible effect of each PWK allele, N_7 congenic progeny were produced by crossing N_6-32 male, which retained the PWK allele on chromosomes 4, 7, and 11, with FVB females. Among 95 N_7 males, 88 (92.6%) developed papillomas 12 weeks after the initial DMBA treatment and the average multiplicity was 18.78±1.36. Linkage analysis of N_7 males indicated a highly significant linkage ($P<0.01$) for papilloma multiplicity mapped to chromosomes 4 and 7, whereas neither significant nor suggestive linkages were mapped to chromosome 11 (Fig. 1B). When the animals were grouped depending on the genotype at the D7Mit31 locus, a significant linkage was mapped to chromosome 11 only in N_7 progeny with the PWK allele at the D7Mit31 locus (Fig. 1C). Consistent with this, linkage on chromosome 7 was obviously higher in N_7 progeny with the PWK allele at the D11Mit338 locus than those homozygous for FVB at this locus (Fig. 1D).

Association studies confirmed the contribution of the remaining PWK allele to the resistant phenotype of the N_7 progeny. In association study, one-way ANOVA showed that at least one

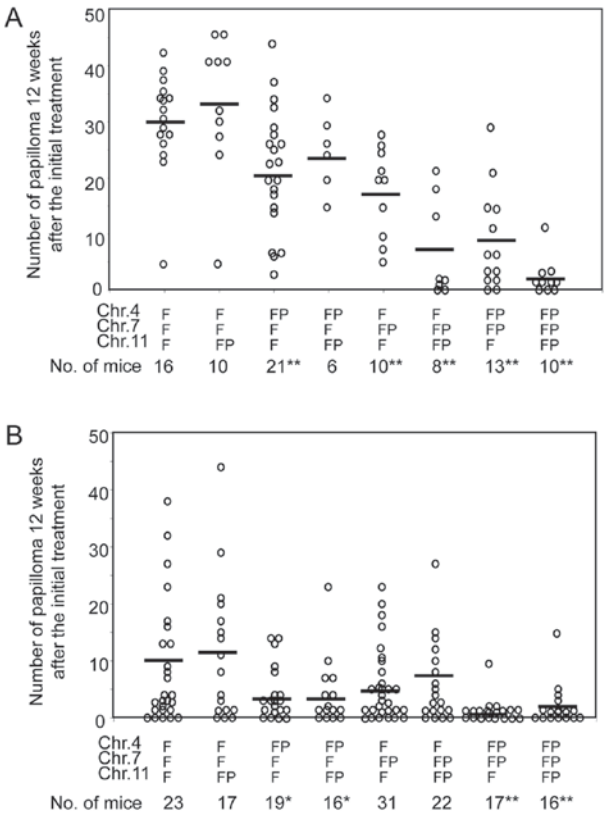

Figure 2. Association between genotypes of resistant loci and multiplicity of papilloma 12 weeks after the initial treatment. Means (lines) and distribution of individual mice (circles) are shown. F indicates FVB homozygous, FP indicates heterozygous. (A) Congenic mice in the N_7 generation derived from N_6-32. (B) FxFP backcross mouse. Asterisks next to the number of mice represent the significance of differences compared to mice with the FVB homozygous genotype at all three loci. $^{*}P<0.05$, $^{**}P<0.01$.

Table I. Genotype and phenotype of congenic mice in N6 generation.

	Chr	1					3			4													7							9					11		
	Mbp	129.3	149.1	167.2	182.9	196.2	7.5	26.8	30.4	33.9	53.5	74.4	81.7	88.0	94.7	102.8	109.4	117.2	120.7	123.8	137.4	26.5	31.1	58.6	94.6	116.0	119.0	122.7	37.2	46.5		58.1	115.3	108.2	115.2	
ID	Marker	D1Mit102	D1Mit102	D1Mit63	D1Mit151	D1Mit155	D3Mit164	D3Mit203	D3Mit222	D4Mit93	D4Mit111	D4Mit296	D4Mit289	D4Mit26	D4Mit9	D4Mit167	D4Mit146	D4Mit122	D4Mit76	D4Mit308	D4Mit54	D7Mit307	D7Mit246	D7Mit145	D7Mit31	D7Mit17	D7Mit370	D7Mit253	D9Mit2	D9Mit191	D10Mit198	D9Mit300	D9Mit214	D11Mit224	D11Mit338	No. of papilloma
N6-20			P	P	P						P	P	P	P	P	P	P	P							P	P	P		P	P	P					0
N6-22			P	P	P							P	P	P	P	P	P	P				P	P	P	P	P	P		P	P	P					0
N6-32												P	P	P	P	P	P	P	P	P				P	P	P	P							P	0
N6-40					P		P	P					P	P	P	P	P	P				P	P	P	P	P	P								0
N6-76											P	P	P	P	P	P	P	P	P	P				P	P	P	P		P	P	P	P				0
N6-24				P							P	P	P	P	P	P	P	P				P	P	P	P	P	P		P						P	1
N6-35														P	P	P	P	P	P	P				P	P	P	P								P	2
N6-38											P	P	P	P	P							P	P	P	P	P	P		P	P	P				P	2
N6-8							P	P			P	P	P	P	P	P	P	P																	P	30
N6-34													P	P	P	P	P	P	P	P										P	P				P	31
N6-39			P	P	P								P									P	P	P	P	P	P		P	P	P					33
N6-28																								P	P	P	P									34
N6-79					P						P	P	P	P	P	P	P	P	P	P					P	P	P									36
N6-7																												P	P	P					39
N6-16																												P	P	P					39
N6-29																								P	P	P	P								45

Blank, FVB homozygous; P, heterozygous.

of the remaining PWK alleles significantly reduced papilloma number in N_7 progeny ($P<0.0001$). The post hoc Tukey test to examine the effect of each remaining allele demonstrated that the mice carrying the PWK allele at D7Mit31 loci developed a significantly reduced number of papillomas compared to the mice without the PWK allele (Fig. 2A). Although it was not significant, the papilloma multiplicity of mice carrying the PWK allele at both D7Mit31 and D11Mit338 loci tended to be decreased compared to mice harboring the PWK allele only at the D7Mit31 locus. In addition, the number of papillomas in mice containing the PWK allele at the above-mentioned three loci also seemed to be lower than that of mice carrying the PWK allele at both D4Mit26 and D7Mit31 loci, but not at D11Mit338. In contrast, mice carrying the PWK allele only at D11Mit338 developed almost the same number of papilloma as mice without the PWK allele (Fig. 2A).

For FxFP mice, one-way ANOVA showed that at least one of the 3 PWK alleles significantly reduced papilloma number ($P<0.0005$), and Tukey test revealed that the presence of both PWK alleles at the D4Mit26 and D7Mit31 loci is needed to significantly reduce the number of papillomas as compared to mice with the FVB allele at the indicated three loci ($P<0.05$) (Fig. 2B). The presence of the PWK allele at the D11Mit338 locus had no detectable effect on papilloma multiplicity regardless of the genotype at the D7Mit31 locus (Fig. 2B). Consistent with these results, the degree of penetrance of the PWK allele in seven N_7 progeny that did not develop papilloma until at least 12 weeks after the initial DMBA treatment were 57.1, 100 and 71.4% for D4Mit26, D7Mit31 and D11Mit338, respectively. On the other hand, they were 60.3, 54.8 and 39.7% for D4Mit26, D7Mit31 and D11Mit338, respectively, in 73 of N_2 progeny that did not develop papillomas until at least 12 weeks after the initial treatment.

Allele imbalances in favor of FVB allele re more frequent compared with those in favor of PWK on chromosome 7 in tumor genome DNA obtained from congenic males. As the resistance allele-specific loss or susceptibility allele-specific amplification of chromosome 7 in the tumor genome DNA was frequently observed in NIH-spretus crosses (9,20), we sought to narrow down the putative susceptible loci on chromosome 7 utilizing skin tumors that developed in the congenic mice. Genomic DNA was purified from skin tumors that developed in N_5-N_7 congenic mice, which shared 40-122 Mb of the PWK allele, and allelic imbalances were analyzed using nine informative microsatellite markers (Fig. 3A). From the analysis of 27 skin tumor tissues, including nine papillomas and 18 skin cancers, all markers showed allelic imbalance, and the allele imbalances in favor of the FVB allele, i.e., loss of the PWK allele or amplification of the FVB allele, were observed more frequently than those in favor of the PWK allele.

Allele imbalance pattern at D7Mit31 is affected by the genotype at the D11Mit338 locus in tumor genome DNA obtained from congenic males. To examine whether the genotype of the D11Mit338 locus could affect the allelic imbalance, tumor tissues obtained from FVB mice homozygous and heterozygous at D11Mit338 were analyzed separately. From the analysis of 13 tumors, consisting of five papillomas and eight skin cancers, obtained from mice with FVB homozygous genotype in the D11Mit338 region, we found that the extent of allelic imbalance at D7Mit31 in favor of the FVB allele was

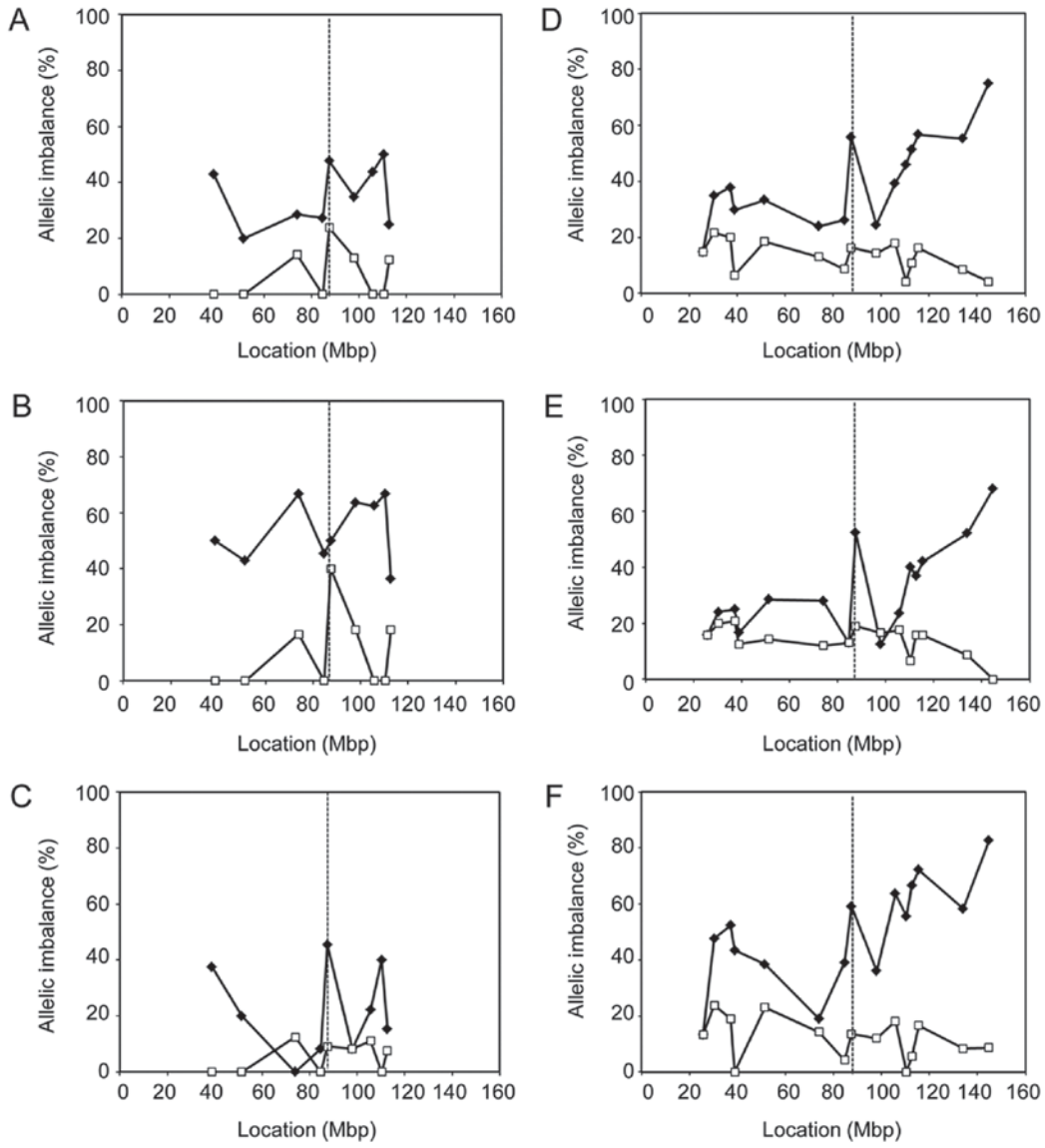

Figure 3. Analysis of allelic imbalance on chromosome 7. Frequencies of allelic imbalances in skin tumor tissues obtained from N_5-N_7 congenic mice (A-C) or from FxFP mice (D-F). The results obtained from (A) all of the N_5-N_7 congenic mice, (B) N_5-N_7 congenic mice with FVB homozygous genotype, or (C) with FP heterozygous genotype at the D11Mit338 locus, (D) all of the FxFP mice, (E) FxFP mice with FVB homozygous genotype, or (F) with FP heterozygous genotype at the D11Mit338 locus are shown. Filled squares and open squares indicate allelic imbalances in favor of the FVB allele and the PWK allele, respectively. Dashed lines indicate the position of D7Mit31.

similar to that of the PWK allele (Fig. 3B). On the other hand, analysis of 12 heterozygous tumors, consisting of eight skin cancers and four papillomas, indicated that 45 and 9.1% of tumors showed allelic imbalance at the D7Mit31 locus in favor of FVB and PWK alleles, respectively (Fig. 3C).

Differences in allele imbalance patterns at D7Mit31 are not observed in tumor genome DNA obtained from FxFP males with or without the PWK allele at D11Mit338. Finally, we examined 43 skin cancer tissues obtained from FxFP progeny carrying the PWK allele on chromosome 7. As shown in Fig. 3D, the allele imbalance in favor of the FVB allele was approximately four times higher than that in favor of the FVB allele through the entire chromosome 7. Cancer samples from D11Mit338 homozygous and heterozygous mice were analyzed separately, and no significant differences in patterns of allelic imbalance at the D7Mit31 locus were detected between the two groups (Fig. 3E and F).

Discussion

PWK is a dominant-resistant strain against two-stage skin carcinogenesis as shown by the crossing with the highly susceptible strain, FVB (8). As PWK is a wild-derived strain (5,6), we sought to identify the large genetic variation associated with skin tumor susceptibility between FVB and PWK, as seen between NIH and *Mus spretus* (12). Our previous study using FPxF backcrosses demonstrated the presence of only one significant linkage, *Skts-fp1*, and the suggestive linkage mapped to chromosome 4 and chromosomes 1/3/11/12/14, respectively (8). In the present study, we generated a congenic strain carrying the PWK resistance allele in the FVB strain background by utilizing a congenic-driven approach to narrow down the candidate loci and find corresponding genes. To obtain sufficient numbers of progeny in each generation, we performed FxFP crosses, in which FVB females were crossed

with FP males, rather than FPxF crosses. Our association study using N_7 progeny derived from N_6-32 mouse with PWK alleles on chromosomes 4, 7, and 11 indicated that both chromosomes 4 and 7 can significantly reduce the number of papillomas, whereas chromosome 11 required the PWK allele on chromosome 7 for the acquisition of this phenotype. These results indicated that there is at least one tumor resistance locus on chromosome 7, which may be regulated by certain genes(s) on chromosome 11.

In analysis of the FxFP backcross (N_2 generation), we did not observe a significant linkage on chromosome 11, and also a close association between papilloma multiplicity and chromosome 11 regardless of the genotype at D7Mit31. According to our previous results obtained from FPxF backcross, a suggestive linkage, *Skts-fp2,* was mapped to chromosome 11, and this linkage became evident in mice homozygous for the FVB allele at the *Skts-fp3* locus on chromosome 16 (8). Although *Skts-fp2* was present in the region between D11Mit155 (58.8 Mbp) and D11Mit178 (85.2 Mbp), the remaining PWK allele in the present congenic strain was present in the region between D11Mit338 (115.4 Mbp) and the telomere. These differences may have been due to putative effects of the other remaining resistance allele in the N_2 generation. As the N_2 generation theoretically retains around 50% of the PWK allele, the average papilloma number was estimated to be significantly lower than that in the N_7 generation. Thus, it is possible that the remaining resistance alleles of PWK made it difficult to detect the effect of each resistance allele in the N_2 generation.

It has been reported that loss of the allele inherited from a susceptible strain or amplification of the allele from a susceptible strain is frequently observed in tumors obtained from the progeny of F1 backcrosses between resistant and susceptible strains (9,20,21). Based on these observations, we sought to narrow down the tumor susceptibility loci by analyzing the allelic imbalances in skin tumors. These analyses indicated that the allele imbalances in favor of the FVB chromosomes are much more frequent than those in favor of PWK chromosomes in both of the congenic strains and FxFP mice. Using D7Mit31 as a marker, we detected one of the highly frequent allele-specific imbalances in both the congenic and N_2 progeny, indicating that at least one cancer susceptibility locus exists around this region. The allelic imbalance in favor of the FVB allele detected using this marker was observed more frequently than that in favor of the PWK allele in tumors carrying the heterozygous allele at D11Mit338 but not in tumors with the homozygous allele at D11Mit338 in congenic mice. On the other hand, we did not detect a clear difference in the allelic imbalance pattern at the D7Mit31 locus between D11Mit338 homozygous and heterozygous N_2 progeny. These results suggest that, around the D7Mit31 locus, there may be at least one tumor resistance gene, the function of which could be affected by the genotypes of certain genes located around D11Mit338. To adequately address this issue and identify the corresponding genes, further analyses, such as global gene expression analysis and allelic imbalance analysis with increased number of skin cancer tissues to narrow down the candidate loci are needed.

Although we did not analyze other N_6 congenic strains with the PWK allele on chromosome 1, 3, or 9 in the present study, it is likely that these mice will contribute to identification of novel gene(s) as well as alternative genetic interaction(s) affecting cancer susceptibility.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

KF and HN planned the experiments. KF performed the experiments. YI and MS performed statistical analysis of the data. KF wrote the paper. TO made substantial contribution to analysis and interpretation of data, revised the paper critically, and gave final approval of the paper to be published.

Ethics approval and consent to participate

All of the animal experiments in this study had ethics approval from the Institutional Animal Care and Use Committee (IACUC).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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