Genetic Resistance to Urethan-induced Pulmonary Adenomas in SMXA Recombinant Inbred Mouse Strains

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ABSTRACT

Development of pulmonary adenomas (PAs) in mice is under the genetic control of multiple host genes. We have established a new set of SMXA recombinant inbred strains from PA-susceptible A/J and PA-resistant SM/J mice. The number of urethan-induced PAs was variable among substrains of the SMXA recombinant inbred strains, indicating the involvement of multiple genes. SMXA24 mice were highly resistant to PA, although they had susceptible alleles at all four known susceptibility genes, including kras2 and MHC. To identify the resistance gene in SMXA24, progeny of reciprocal F1 crosses and progeny of backcrosses to A/J were given urethan at 4 weeks of age and examined for induced PA at the age of 5 months. In reciprocal F1 cross progeny, the incidence of PA was very low, indicating that the resistance was a semidominant trait. Quantitative trait analysis of the backcross generation revealed significant linkages to loci on chromosome 12 (logarithm of odds score, 6.47) and chromosome 11 (logarithm of odds score, 4.35). To date, two PA resistance (PAR) genes, Par1 (located on chromosome 11) and Par2 (located on chromosome 18), have been reported. From the map position, one of the resistance genes on chromosome 11 was indistinguishable from Par1. However, another resistance gene on chromosome 12 was new, and we named this gene Par3. A likely candidate gene for Par3 is PKC@, which is expressed exclusively in skin and lung and is down-regulated in PA. Par1 and Par3 seemed to act synergistically.

INTRODUCTION

The genetics of lung tumor susceptibility have been extensively studied in the mouse. The new paradigm emerging from these studies is that lung cancers are multifactorial genetic diseases. Among laboratory strains of mice, A/J mice are the most susceptible to PA (1, 2). During a normal life span, these animals will spontaneously develop at least one PA. Also, this strain is highly susceptible to chemical induction of lung tumors and will acquire several tumors within a few months after exposure to carcinogens such as urethan. Genetic analysis of crosses between A/J mice and low PA strains revealed that at least four PAS genes determine development of urethan-induced PA in mice (3). Pas1 is a representative PAS gene that is closely linked with the kras2 locus, a proto-oncogene, on chromosome 6 (4, 5). Spontaneous and chemically induced PA in A/J mice show kras2 mutations at a high frequency (6). Molecular polymorphism of kras2 is associated with PAS in a number of mouse strains (7, 8). Mutation of kras2 has also been reported in human lung cancers (9). Furthermore, PAS can be partially explained by other PAS genes linked with HHC (Pas2; Refs. 10 and 11), D9Mit11 (Pas3; Ref. 12), and D19Mit16 (Pas4; Ref. 13). Recently, two dominant PAR genes have been described (14, 15). These observations indicate that pulmonary carcinogenesis is indeed a multifactorial genetic disease.

RI strains are powerful genetic tools with which to analyze multifactorial diseases. Recently, we established a new set of 26 RI strains of the mouse SMXA from A/J and SM/J and published a panel of strain distribution patterns of 200 polymorphic genetic markers (16). Unlike A/J, SM/J mice rarely develop spontaneous PA and are resistant to PA induction by carcinogens. Here, we first examined the incidence of PA in 20 SMXA RI strains. Of these strains, the SMXA24 attracted our attention because it was highly resistant to PA despite having A/J-derived alleles at all four PAS genes mentioned above. The progeny of reciprocal F1 crosses between A/J and SMXA24 were as resistant as SMXA24 mice. To identify dominant resistance genes in SMXA24, 188 progeny of backcrosses to A/J were given urethan and observed for PA development. Genetic analysis of the number of tumors in each animal as an index of PAS revealed two QTLs on chromosome 11 and chromosome 12. The resistant locus on chromosome 11 seemed to harbor Par1 (14), a known PAR gene, but the other locus on chromosome 12 seemed to be a novel gene, and so it was named Par3.

MATERIALS AND METHODS

Animals. The SMXA RI strain was established by Nishimura et al. (16). A/J, SM/J, and SMXA RI mice were bred and maintained as described previously (16). All in vivo experiments were performed at the Hamamatsu University School of Medicine (Hamamatsu, Japan). Reciprocal (SMXA24 × A/J)F1 hybrids and (SMXA24 × A/J)F1 × A/J as well as A/J × (SMXA24 × A/J)F1 backcross mice were produced. All animals were housed with four or five mice per cage, and cages were maintained at 22–25°C.

Carcinogen Treatment. All of the mice were injected i.p. with a single dose of 450 mg of urethan (ethyl carbamate; Katayama Chemical Industry Co., Nagoya, Japan) per kg of body weight, dissolved in 0.85% NaCl solution at 4 weeks of age. They were fed a standard pelleted diet and acidified tap water ad libitum. All mice were killed at 5 months of age.

Quantification of PA. At necropsy, all animals were examined for PA and other tumors or abnormalities. The lungs were fixed with Bouin’s solution overnight and transferred to 80% ethanol. The numbers of tumors on the lung surface were counted twice under a stereomicroscope, independently, by two examiners. Subsequently, regular histopathological examination was carried out to confirm histological types of tumors. All tumors were solid papillary adenomas.

Microsatellite Analysis. PCR primers for microsatellite loci were purchased from Research Genetics, Inc. (Huntsville, AL). Genomic DNA from kidney was used for PCR. Agarose gel electrophoresis of PCR products was performed as described previously (17). Map positions of microsatellite loci were based on the Chromosome Committee Report of 1996 (18, 19).

Genotypes of PAS and PAR genes shown in Table 1 were deduced from their map positions by referring to the strain distribution pattern of the SMXA RI strain (3).

Statistical Analysis. In the preliminary genome-wide screen, the association of animals with high or low numbers of PAs with alleles of the microsatellite loci was evaluated by χ² test. When a P < 0.01 was obtained at any locus, all of the backcross individuals were genotyped for available polymorphic loci on the respective chromosome. Linkage analysis was performed with MAP MANAGER, version 3.0, software as described previously (20). Interval mapping analysis for QTL detection was performed using the MAPMAKER/ QTL program.
RESULTS

Induction of Lung Tumor in SMXA RI Strains. Development of lung tumors was observed at 5 months of age in 20 SMXA RI strains and their parental strains, which had been injected with urethan at 4 weeks of age (Table 1). All strains were arranged by descending order of the number of tumors per mouse. Also shown are alleles of known PAS loci, the number of PAs was significantly higher in strains with higher than that in the susceptible parental NJ strain, whereas other PAS and PAR loci of all strains as estimated from their map positions were not detected in untreated mice from the backcross, F1, or parental strains (data not shown). There was no significant difference between male and female mice (data not shown). PAS Genes in SMXA RI Strains. To identify and map putative resistance genes, we observed urethan-induced PA in crosses between AJJ and SMXA24. Fig. 1 shows the distribution of urethan-induced PAs in 22 AJJ, 15 SM/J, 30 SMXA24 RI, 42 reciprocal F1 hybrid, and 188 reciprocal A/J backcross mice. AJJ mice developed 6.7 ± 4.6 PAs per mouse, and SMXA24 developed 0.4 ± 0.9 PAs per animal. The reciprocal (AJJ × SMXA24)F1 hybrids had an average of 1.2 ± 1.0 tumors, and (SMXA24 × A/J)F1 had an average of 1.4 ± 1.4. This observation suggested that the SMXA24 strain carries an autosomal semidominant genetic trait capable of suppressing PA. In the backcross to AJJ, the tumor numbers in individual mice showed a wide variety, ranging from 0 to 18, with an average of 4.5 ± 3.0. The percentage of mice with no lung tumors was 7%, although 48% of the mice had less than five tumors. This suggested that SMXA24-developed alleles at one or more loci strongly reduced PA. Lung tumors were not detected in untreated mice from the backcross, F1, or parental strains (data not shown). There was no significant difference between male and female mice (data not shown).

PA Genes in SMXA24 Mice. To identify and map PAR genes, we performed microsatellite analysis in backcross mice in two steps.
In the first step, a genome-wide screen for linkage was conducted with 20 randomly selected backcross mice with no or few tumors (<2 tumors per mouse) and 20 mice with >10 tumors per mouse. We selected 75 microsatellite loci polymorphic between A/J and SMXA24 mice (16), 3 or 4 for each chromosome, distributed 15–30 cM apart. Assuming that each marker detects linkage in 15-cM segments on both sides, the number of marker loci therein would be theoretically sufficient to cover the whole mouse genome. Significant linkage was found at D11Mit7O on chromosome 11 (χ² = 6.46; P < 0.01) and at D12Mit5 on chromosome 12 (χ² = 14.5; P < 0.001; Table 2). This indicated that there are two lung tumor suppressor genes derived from SMXA24 RI strain. Recently, Obata et al. (15) reported a dominant resistance gene Par2 on chromosome 18 in a cross between A/J and BALB/c. However, in our crosses, no linkage was found on chromosome 18 because the entire chromosome 18 was derived from A/J in the SMXA24 RI strain (16).

QTL Analysis. We next examined whether these resistance genes are QTLs that control the number of PAs. For this purpose, all 188 backcross mice were genotyped at four loci on chromosome 11 (D11Mit61, D11Mit14, D11Mit70, and D11Mit1) and at six loci on chromosome 12 (D12Mit37, D12Mit36, D12Mit52, D12Mit5, D12Mit6, and D12Mit17). The data from (SMXA24 × A/J)F₁ × A/J and A/J × (SMXA24 × A/J)F₁ backcross mice were pooled because both showed similar distributions of tumor multiplicity. As shown in Fig. 2A, a QTL with peak LOD score of 6.47 was found on chromosome 12, close to D12Mit5. This locus explained a

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**Table 2 Mapping of PAR genes**

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Locus</th>
<th>Map position (cM)</th>
<th>Low-tumor animals</th>
<th>High-tumor animals</th>
<th>χ²</th>
</tr>
</thead>
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<tr>
<td>11</td>
<td>D11Mit14</td>
<td>37</td>
<td>11</td>
<td>9</td>
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<td>15</td>
<td>5</td>
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<tr>
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<td>5</td>
<td>3.75</td>
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<td>7</td>
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</tr>
<tr>
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<td>7</td>
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</tr>
<tr>
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<td>14</td>
<td>6</td>
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<td>16</td>
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<td>D12Mit17</td>
<td>55</td>
<td>14</td>
<td>7</td>
<td>13.0.11</td>
</tr>
</tbody>
</table>

*a* Map positions are from Chow et al. (18), and Eustachio and Riblet (19).

*b* Low-tumor (0 or 1) or high-tumor (>10) animals were genotyped.
DEMITL4 and DEMITL70 (Fig. 2B). From its map position, this locus seemed to be identical with Par1, reported by Manenti et al. (14). Variance explained by Par1 was 10.2%.

We also investigated the possible interrelationship between Par3 and Par1. Mean tumor multiplicities for each of the four possible combinations of genotypes of Par3 and Par1 were compared by random permutation, with the data from all 188 backcross mice. Mice homozygous for A/A alleles at Par3 and Par1 had an average of 6.7 ± 3.0 tumors per animal, which was more than those with heterozygous A/S genotype at either of Par3 (4.7 ± 2.8) or Par1 (4.2 ± 2.7) and a heterozygous A/S genotype at both loci (3.0 ± 2.3; P = 10^-8). The P for the significance for Par1 was 0.0012, whereas that for Par3 was 0.00015. This result demonstrated that the PA-suppressive effect of Par3 was stronger than that of Par1. The overall percentage deviance explained by the combination of these two loci was 21.6% (LOD score, 9.88).

To evaluate the combined effect of the three resistance genes, we examined the genotypes of all SMXA RI strains at the Par1, Par2, and Par3 loci (Table 1). Homozygosity for the AJ-derived allele at all three loci seemed to be strongly associated with lung tumorigenesis and vice versa. However, it is also clear that the three resistance loci genotype cannot fully explain the suppression seen in these RI strains and backcross progeny. For example, SMXA8 had resistance alleles at all three PAR loci, but it showed relatively high tumor multiplicity. This may suggest possible presence of a susceptibility gene other than the known PAS genes that escapes the effect of these three PAR genes.

Further backcross study among SMXA RI strains, guided by the data in Table 1, will offer a rational approach to dissect the genes involved in such a multigene system, step by step.

**DISCUSSION**

RI strains provide excellent model systems for the analysis of multifactorial diseases, as they allow the investigation of the effects of genes in a genetically defined background. This study took advantage of the fact that the genotype of known relevant loci of RI strains can be estimated from their map positions. Another possible advantage of RI strains is that one can dissect the effect of susceptible and resistance genes localized in proximity on the same chromosome, if RI substrains showing recombination between these loci are available. Newly discovered genes using RI strains could be partly due to such segregation. Without such a tool, a huge number of backcross or intercross mice would be required just to recognize the presence of these loci.

Here, we demonstrated two dominant resistance genes in one SMXA RI strain homozygous for AJ alleles at four known PAS genes. These genes were QTLs, synergistically suppressing the number of PAs despite the presence of susceptible alleles on the four PAS genes. Here, we observed tumor multiplicity at 5 months of age, according to standard protocol for urethan-induced PA. Therefore, the possibility is not excluded that the action of these genes may affect the onset of tumor formation rather than the number of targets.

Development of mouse PAs has been shown to be determined by multiple host genetic factors. In addition to four PAS genes, several resistance genes have recently been identified. One of these, Par1, was found in a cross between AJ and Mus spretus, but it seems to be identical with the locus mapped to chromosome 11 in this study. Par1 strongly reduces the expressivity of the Pas1 allele but does not affect lung tumor susceptibility in mice without the susceptible Pas1 allele (14). Therefore, Par1 is epistatic to Pas1, as confirmed in this study. From its map position, the Retinoic acid receptor α gene has been mentioned as a candidate gene for Par1. Retinoic acid receptors are nuclear transcription factors that play a key role in retinoid physiology. A number of epidemiological examinations of experimental evidence have shown that synthetic retinoids can modulate epithelial cell differentiation (21) and prevent or revert carcinogenesis (22) including lung cancer in both experimental animals and in humans (23). More recent study showed, however, Retinoic acid receptor α seems irrelevant to Par1.4

The second resistance gene, Par2 (15), was mapped to chromosome 18 in a cross between AJ and BALB/c. Par2 is, however, irrelevant in the cross between AJ and SMXA24 because the entire chromosome 18 of SMXA24 was derived from AJ.

Here, we identified a novel resistance gene, Par3, on chromosome 12, and its SM1J-derived allele effectively reduced the number of PA. Par3 was mapped approximately 40 cM from the centromere of chromosome 12, for which fos, Tgfb-3, and nPKCη (encoding Ca2+—independent protein kinase η) are attractive candidate genes. Several investigators have shown that c-Fos is expressed at a significantly higher frequency in lung tumors (24). Tgfb-3 is a subfamily of transforming growth factor-β that modulates cellular growth and differentiation (25). Ca2+—independent protein kinase η is a member of the PKC family, originally discovered as a Mr 70,000 mouse nuclear protein, expressed exclusively in skin and lung (26). It has been reported that the level of nPKCη-related transcript is 5–10-fold lower in PA than in normal control lungs (27). Several studies showed that low levels of mouse lung nPKCη are correlated with increased growth rate and neoplasia (28). Most tumor promoters affect the phosphorylation state of proteins, either by chronically down-regulating PKC (29) or by stimulating phosphoprotein phosphatases (30). Strains of mice sensitive to tumor promotion by BHT respond to chronic BHT treatment by a sustained loss of PKC, whereas resistant strains do not (31). Furthermore, following chronic BHT treatment, the Ca2+-dependent protease responsible for initiating PKC down-regulation is elevated in Clara cells of PA-sensitive strains but not in those of resistant strains (32). These observations support the possible involvement of the nPKCη gene in lung tumorigenesis. Examination of synteny conservation between the mouse chromosome 12 regions containing Par3 and human chromosomes revealed that the murine Par3 region corresponds to human 14q11–24, the region showing frequent loss of heterozygosity (33) and translocation in a variety of human tumor types (34), including lung tumor (35).

After this manuscript was completed, Fijne et al. (36) reported the identification of four QTLs affecting pulmonary tumors using recombinant congenic strains Ocb/Dem and by applying multiple QTL marker mapping (36). These genes are designated susceptibility to lung cancer (Sluc) genes; specifically, they are Sluc1 (located on chromosome 19), Sluc2 (located on chromosome 2), Sluc3 (located on chromosome 6), and Sluc4 (located on chromosome 11). From the map data, these four genes are claimed to be distinct from the known PAS genes. One of reasons for the detection of a number of unique PAS genes is the contribution of 020 strain, a mouse strain of European origin that is genetically remote from most laboratory strains commonly used in the United States. The approach of Fijne et al. (36) is also highly effective in identifying multiple QTLs involved in lung tumorigenesis and in analyzing the interactions among them.

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