Quantitative Trait Loci Affecting 4-Nitroquinoline 1-oxide-induced Tongue Carcinogenesis in the Rat

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ABSTRACT

The incidence of tongue carcinomas (TCs) induced by oral administration of 4-nitroquinoline 1-oxide in rats is strain dependent. The inbred Dark-Agouti (DA) strain showed a much higher susceptibility to large mass-forming infiltrative TCs than did the Wistar-Furth (WF) strain. Our previous study (M. Kitano et al., Jpn. J. Cancer Res., 87: 1097—1101, 1996) on crosses between these two strains postulated a dominant susceptibility gene in DA and a dominant resistance gene in WF rats. The present study mapped these loci by analyzing the backcrosses to each parent with simple sequence repeat polymorphisms. Five quantitative parameters were analyzed: (a) the number of TCs > 5 mm in diameter; (b) the total number of TCs per rat; (c) the diameter of the largest TCs (DTCmax values); (d) the number of non-TC cancers per rat; and (e) the number of cancers of any site per rat. All of these parameters were closely correlated (P < 0.0001). DA rats had a semidominant gene (Stc1) favoring the development of 4-nitroquinoline 1-oxide-induced cancers on chromosome 19, closely linked to D19Mit9. Peak linkage was observed 4 cM distal from D19Mit9, with a logarithm of the odds (lod) score of 5.72 for the number of large TCs and 6.08 for the DTCmax. On the other hand, WF rats had a semidominant gene (Rtc1) mapped between D1Mit1 and D1Mit3, ~20 cM from D1Mit1, with a peak lod score of 3.30 for both the number of large TCs and the DTCmax. The main effect of Rtc1 seemed to be to reduce the size of the TCs. The action of these genes was dose dependent and cooperative. The final incidence of TC in DA, WF, F1, and backcross rats seemed to be explained by combinations of genotype at these two loci. Possible candidate genes for Stc1 and Rtc1 are discussed.

INTRODUCTION

Carcinogenesis is a multistep phenomenon modified by a number of host genetic and epigenetic factors. Such modifier effects of the host are poorly understood. To elucidate host genetic effects, the analysis of appropriate animal models is indispensable. Etiological factors of TC and other oral cancers are assumed to be mostly environmental (for example, smoking or alcohol drinking), but several epidemiological studies (1–3) and reports on familial clustering (4, 5) suggest that genetic factors contribute to susceptibility to these cancers. Such genetic susceptibility, if recognized, would be important in identifying risk groups and elucidating critical steps in carcinogenesis. Oral administration of the carcinogen 4NQO to rats induces TC and other oral cancers at a high incidence, thus providing an excellent model of these cancers (6). In a previous study (7), we found that susceptibility to the 4NQO-induced TCs was highly variable among inbred strains of rats. Of the seven strains, DA rats were the most susceptible, and WF rats were the most resistant. Based on the segregation of the incidence of 4NQO-induced TC#5 in reciprocal F1, F2, and backcrosses to either parent, we assume that DA rats have a semidominant susceptibility gene, Stc (susceptibility to TC), and that WF rats have a semidominant resistance gene, Rtc (resistance to TC). In this report, we attempted to identify and map these loci affecting chemically induced TC and other oral cancers by analyzing the crosses between DA and WF rats.

MATERIALS AND METHODS

Rats. Inbred DA (DA/Slc) rats were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). WF rats were originally obtained from Hiroshima University (Hiroshima, Japan) and maintained by brother-sister matings for over 46 generations in our laboratory. F1 hybrids and backcrosses to either DA or WF rats were produced by the appropriate matings. All rats were weaned 3 weeks after birth, individually numbered, and housed in plastic cages in an air-conditioned room at 22°C ± 2°C. They were fed commercial rat pellet CE-2 (Nippon Clea Co., Tokyo, Japan). No spontaneous tumor was evident by 6 months of age in intact rats of either strain.

Carcinogen Treatment. The carcinogen stock solution was prepared by dissolving 4NQO (Nakalai Tesque, Kyoto, Japan) in 5% ethanol at 200 mg/liter and stored frozen at −20°C until use. Starting at 6 weeks of age, all of the rats were allowed access to drinking water containing 0.001% 4NQO ad libitum from 5 p.m. to 9 a.m.; outside of this period, no water was given. The rats were inspected twice a day and weighed once a week. The rats were killed when they became moribund or on the 180th day of the experiment. A full autopsy and histopathological examination were carried out. The DTCmax and the number of cancers in the tongue and elsewhere were recorded individually.

Genetic Analysis. For linkage analysis, we used the simple sequence repeat (microsatellite) length polymorphism method, using genomic DNAs extracted from the kidney as templates. All primers for microsatellite analysis were purchased from Research Genetics, Inc. (Huntsville, AL). Methods for PCR and for the agarose electrophoresis of PCR products were described previously (8). The relative map positions of microsatellite loci were based on Jacob et al. (9). Of 432 microsatellite loci examined, 140 (32.4%) were polymorphic between DA and WF. To find the loci associated with either susceptibility or resistance to TC, a genome-wide screening with 60 polymorphic microsatellite loci, 2–4 loci/CHR, was carried out. The approximate coverage was ~63% of the entire rat genome, where one supposes that a marker locus detects linkage within a 15-cM chromosomal segment. Such preliminary screening was done with 32 backcross rats, 16 each with or without TC#5s. For CHRs containing loci with a χ2 value > 6.4 (P = 0.01), the genotypes of all backcross rats were determined for all available polymorphic loci. QTL analysis was performed, and the lod score was calculated using the Mapmaker/QTL computer package as described previously (10).

Statistical Analysis. Linkage was evaluated by the χ2 test. According to the criterias of Lander and Kruglyak (11), linkage was taken as suggestive (P < 3.4 × 10−2) or significant (P < 1 × 10−4). The size and number of tumors were evaluated by the unpaired Student’s t test. Correlations between the number of cancers and tumor diameter were evaluated by correlation analysis with StatView Ver. 4.0 software (Abacus Concepts, Inc., Berkeley, CA) on a Macintosh personal computer.
Table 1 4NQO-induced cancers of the tongue and other sites in DA, WF, and F1 rats

<table>
<thead>
<tr>
<th>Locus</th>
<th>DA rats (n = 47)</th>
<th>WF rats (n = 50)</th>
<th>DA × WF/F1 rats (n = 59)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of TCs5a</td>
<td>1.17 ± 0.64</td>
<td>0.04 ± 0.20b</td>
<td>0.61 ± 0.70a</td>
</tr>
<tr>
<td>No. of TCs overallb</td>
<td>1.49 ± 0.66</td>
<td>0.42 ± 0.50b</td>
<td>1.01 ± 0.58a</td>
</tr>
<tr>
<td>DTCmax (mm)</td>
<td>12.77 ± 4.49</td>
<td>1.18 ± 1.64b</td>
<td>6.15 ± 4.83b</td>
</tr>
<tr>
<td>No. of non-TC cancersb</td>
<td>1.83 ± 1.26</td>
<td>0.78 ± 1.16b</td>
<td>0.85 ± 1.39a</td>
</tr>
<tr>
<td>No. of cancers overallb</td>
<td>3.34 ± 1.58</td>
<td>1.24 ± 1.45b</td>
<td>1.86 ± 1.77a</td>
</tr>
</tbody>
</table>

a Number per rat.
b Significant to the values for DA rats (P < 1 × 10^-4).

c Significant to the values for DA rats (1 × 10^-4 > P > 1 × 10^-6).

RESULTS

4NQO-Induced TCs in DA, WF, and F1 Rats. The administration of 4NQO in drinking water to rats induced multiple squamous cell carcinomas in the mucosa of the tongue, hard palate, pharynx, larynx, gingiva, trachea, and esophagus. The tongue and hard palate were the sites most consistently involved, and the largest tumors were consistently observed in the tongue. Table 1 shows the number of TCs per rat, the overall number of TCs, the DTCmax, and the number of non-TC cancers, and the overall number of cancers in DA, WF, and (DA × WF)F1 rats. These parameters were found to correlate with each other (r > 0.5; P < 1 × 10^-4). Among them, the number of TCs and the DTCmax were significantly higher in DA rats than those in WF rats (P < 1 × 10^-6). Except as otherwise noted, the data were analyzed as quantitative parameters. Sex difference was not significant in any combinations (data not shown), so the data for both sexes were pooled. All of these parameters indicated that DA rats were more sensitive to 4NQO-induced cancers than were WF rats, and that their F1 hybrids were intermediate sensitivity to 4NQO-induced cancers.

(DA × WF)F1 × WF Backcross Rats. To identify and map the genes affecting tongue carcinogenesis, a linkage analysis of the backcross rats to each parent was performed using microsatellite marker loci as described in "Materials and Methods." The preliminary genome-wide scanning using 16 each of (DA × WF) × WF backcross rats with or without TC#5 revealed a significant linkage on CHR 19. At 6 marker loci on CHR 19, we determined the genotypes of all 137 (DA × WF)F1 × WF backcross rats, of which 34 (24.8%) developed TC#5. Table 2 summarizes the genotype of backcross rats with or without TC#5. The highest linkage was observed at D19Mit9; 29 of 34 rats with TC#5 and 42 of 103 rats without TC#5 had the DA-derived allele (χ^2 = 20.3; P = 6.7 × 10^-4). Heterozygotes at this locus were significantly more sensitive than WF homozygotes in all five parameters (Table 3). A Mapmaker/QTL-driven QTL analysis using the number of TC#5s showed that the highest lod score, 5.72, was observed 4 cm distal from D19Mit9, where the lod score for DTCmax was 6.08 (Fig. 1). This observation indicated the presence of a DA-derived dominant cancer susceptibility gene on CHR 19. We named this locus Stc1 (susceptibility to TC-1). At this locus, the numbers of TCs, non-TCs, and cancers overall were higher in DA rats than they were in WF rats (3.4 × 10^-3 > P > 1 × 10^-4). Therefore, Stc1 seemed to increase susceptibility not only to larger TCs, but to all cancers induced by 4NQO.

In addition, weak linkages were observed at two other loci, i.e., D3Mgh11 on CHR 3 (χ^2 = 6.87; P = 8.8 × 10^-3) and D14Mit6 on CHR 14 (χ^2 = 7.45; P = 6.2 × 10^-3), respectively. However, these linkages were not statistically significant. Genome-wide screenings with parameters such as TC and non-TC cancers did not reveal any other loci with significant linkage (data not shown).

(DA × WF)F1 × DA Backcross Rats. On the other hand, of 130 (DA × WF)F1 × DA backcross rats, 98 (75.4%) developed TC#5. The preliminary genome scanning indicated a linkage on CHR 1. Table 4 summarizes the genotype of the marker loci on CHR 1 in all (DA × WF)F1 × DA backcross rats with regard to the presence or absence of TC#5. A suggestive linkage was observed at D1Mit3 in which the χ^2 value was 11.47 (P = 7.1 × 10^-5). A QTL analysis with Table 4 Microsatellite analysis of (DA × WF)F1 × DA backcross rats with or without TC#5

<table>
<thead>
<tr>
<th>Locus</th>
<th>Rats with TC#5</th>
<th>Rats without TC#5</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1Mit5</td>
<td>23</td>
<td>11</td>
<td>1.1 ± 2.0</td>
</tr>
<tr>
<td>D1Mit6</td>
<td>29</td>
<td>5</td>
<td>3.7 ± 3.7</td>
</tr>
<tr>
<td>D1Mit9</td>
<td>28</td>
<td>8</td>
<td>1.8 ± 1.8</td>
</tr>
<tr>
<td>D1Mit9</td>
<td>28</td>
<td>6</td>
<td>1.3 ± 1.3</td>
</tr>
<tr>
<td>D1Mit9</td>
<td>27</td>
<td>7</td>
<td>1.4 ± 1.4</td>
</tr>
</tbody>
</table>

a Genotype of rats; D/W, heterozygote; W/W, homozygote of the WF allele.

Fig. 1. Genetic linkage map indicating the lod score for the number of TC#5s and tumor size on mouse CHR 19 using the Mapmaker/QTL program. Scale unit, 2.5 cM

Table 4 Microsatellite analysis of (DA × WF)F1 × DA backcross rats with or without TC#5

<table>
<thead>
<tr>
<th>Locus</th>
<th>Rats with TC#5</th>
<th>Rats without TC#5</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1Mit15</td>
<td>65</td>
<td>33</td>
<td>3.80 ± 1.37</td>
</tr>
<tr>
<td>D1Mit9</td>
<td>57</td>
<td>41</td>
<td>4.91 ± 3.77</td>
</tr>
<tr>
<td>D1Mit1</td>
<td>53</td>
<td>45</td>
<td>9.35 ± 2.77</td>
</tr>
<tr>
<td>D1Mit3</td>
<td>53</td>
<td>45</td>
<td>11.47 ± 7.10</td>
</tr>
<tr>
<td>D1Mit3</td>
<td>41</td>
<td>57</td>
<td>5.47 ± 3.77</td>
</tr>
<tr>
<td>D1Mit11</td>
<td>38</td>
<td>60</td>
<td>7.02 ± 8.10</td>
</tr>
<tr>
<td>D1Mit7</td>
<td>44</td>
<td>54</td>
<td>4.15 ± 4.20</td>
</tr>
</tbody>
</table>

a Genotype of rats; D/W, heterozygote; D/D, homozygote of the DA allele.
Genetic Susceptibility to 4NQO-Induced TC

The number of TC#5s as well as the DTC_max were significantly higher in the DA homozygotes than in the heterozygotes. The LOD scores for the numbers of TC, non-TC, and cancers overall at this locus were < 1.9 (P > 3.4 × 10^{-2}), so that the action of Rtc1 seemed primarily to reduce the size of TC, and the effect on the number of tumors induced by 4NQO was weaker.

Cooperative Effects of Host Genes. To explain the segregation of cancer susceptibility among crosses between DA and WF rats, we have assumed the existence of an independently segregating dominant susceptible gene of DA and a dominant resistance gene of WF rats (7).

To evaluate the possible interactions of these genes, genotypes at D19Mit9 as well as D1Mit1 and the parameters of 4NQO-induced carcinogenesis were compared (Table 6). Both Stc1 and Rtc1 affected carcinogenesis, either positively or negatively, in a dose-dependent manner. The highest incidence of TC#5, TC, and cancers overall was observed in (DA × WF)F1 × DA rats homozygous for the DA-derived allele at both loci, and the lowest incidence of TC#5, TC, and cancers overall was observed in (DA × WF)F1 × WF rats homozygous for the WF-derived allele at both loci. In both backcrosses, rats heterozygous at either locus showed an intermediate incidence of TCS, equivalent to the incidence observed in F1 hybrids. No such cooperative effect was evident in the incidence of non-TC cancers.

**DISCUSSION**

The induction of TCs in rats by 4NQO, a potent carcinogen, is under host genetic control. In the crosses between DA and WF rats, we identified and mapped two major independently segregating host loci positively or negatively influencing the cancer development. DA rats had a semidominant susceptible gene, Stc1, closely linked with D19Mit9 on chromosome 19, which was on the segment syntenic to human chromosome 11. The presence of two other dominant susceptibility loci on chromosomes 13 and 14 was suspected, but their linkages were not statistically significant. On the other hand, WF rats had a semidominant resistance gene, Rtc1, on a 46-cM segment between D1Mit1 and D1Mit3 on chromosome 1, which is syntenic to human chromosome 11. The action of these genes seemed dose dependent and cooperative. As seen in Tables 3 and 5, Stc1 has a stronger phenotypic effect than Rtc1. As predicted in our previous paper (7), F1 hybrids bearing both genes showed an intermediate incidence of TCs. Backcrosses to DA and to WF rats showed a reasonable segregation of cancer incidence, as expected from the combination of their genotypes. These findings provide powerful evidence that chemically induced tongue carcinogenesis is a multigenic event.

Many chemical carcinogens have been shown to require activation in vivo to achieve ultimate carcinogenic activity. 4NQO is one such carcinogen. It is enzymatically activated to yield 4HAQO. 4HAQO is further converted to aminoacyl-4HAQO-tRNA synthetase to form guanine or adenine adduct (12—14). If these adducts are not properly repaired, they can lead to DNA damage and mutations, thus promoting carcinogenesis.
modified bases are not appropriately repaired, erroneous genetic information is fixed to induce mutation or carcinogenesis. The processes of activation or inactivation of chemical carcinogens and the ability to recognize and repair DNA damage could be critical factors for determining susceptibility and resistance to carcinogenesis. To date, several enzymes have been identified that are involved in such processes: (a) P-450 (15, 16); (b) aryl hydrocarbon hydroxylase (17); (c) O⁶-methylguanine-DNA methyltransferase (18, 19); (d) seryl-RNA synthetase (20, 21); (e) GST (22, 23); and (f) DT-diaphorases (24). In the present study, we mapped a major susceptibility gene, Stc1, to rat CHR 19. As candidate genes involved in the metabolism of 4NQO in this region, we nominated DT-diaphorase, NADH-cytochrome b₅ reductase, carboxylesterase, and butyl-esterase. One of the major enzymes that convert 4NQO to the more active 4HAQO is DT-diaphorase (25, 26). This enzyme is distributed in most target tissues of 4NQO including the skin, lymph nodes, lungs, stomach, pancreas, and oral cavity (27, 28). Previous studies (29, 30) showed that the enzyme activity of DT-diaphorase is polymorphic among rat strains. The tongue mucosa of DA rats has a significantly higher activity than that of WF rats. Another candidate for Stc1 may be NADH-cytochrome b₅ reductase on rat CHR 19 (24). This enzyme is postulated to transfer NADH-saturated electrons to endogenous substrate-metabolizing pathways, thereby allowing NADPH to be preferentially used in the P-450-dependent reaction (31, 32). There is evidence that b₅ participates in electron transfer to P-450 (33). The first electron donated to reduce P-450 is most likely provided by P-450 reductase, whereas the second electron donated to reduce oxychtochrome P-450 may be from another substance, presumably b₅. Candidate genes carboxylesterase and butyl-esterase are also mapped on rat CHR 19. Aminoacyl-4HAQO is hydrolyzed by esterase to form 4HAQO adducts of guanine or adenine (34–36). These adducts show intense carcinogenic activity.

On the other hand, another dominant host gene yielding resistance to Tcs, Rtc1, was mapped to rat CHR 1. One of the candidate genes for Rtc1 may be GST-α, mapped at 1q43 (37, 38). GSTs belong to a family of isoenzymes that play an important role in the protection of cells from cytotoxic and carcinogenic agents. Normally GST-α activity is very low in the liver, but it is induced enormously in response to some carcinogens and is constitutively expressed in hyperplastic nodules as well as hepatocellular carcinomas (39). Our concurrent study (40) showed that GST-α is also a promising marker for tongue carcinogenesis in the rat model. All Tcs invariably expressed GST-α, whereas specimens from the normal control animals were negative. The 4NQO-induced dysplastic tongue mucosa of DA rats contained a significantly greater number of GST-α-positive foci than that of WF rats. This may somehow contribute to the difference in susceptibility to 4NQO between these two strains.

O⁶-Methylguanine-DNA methyltransferase is a key enzyme for DNA repair (18, 19); its gene is mapped on CHR 1. Higher expression of this gene in transgenic mice suppresses chemical carcinogenesis (41), whereas knockout mice of this gene are highly sensitive to chemically induced carcinogenesis (42). The location of this gene, however, is more distal than that predicted for Rtc1.

Rat TC induced by 4NQO provides an excellent model of TC in humans, at least a part of which is due to exposure to environmental carcinogens, such as viruses or chemicals contained in food, alcoholic beverages, and tobacco. After the submission of this paper, Harty et al. (43) reported a genetic correlation between the alcohol dehydrogenase 3 genotype and the risk of oral cancers in drinkers. To our knowledge, the present study is the first experimental work on genetic susceptibility to oral cancers. The identification of host genes determining susceptibility and resistance in the rat is a new approach toward the study of susceptibility to oral cancers, e.g., the search for human homologues of Stc1 and Rtc1.

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