

Five Quantitative Trait Loci Affecting 4-Nitroquinoline 1-Oxide-induced Tongue Cancer in the Rat

Jun-ichi Tanuma,^{1,4} Kei Fujii,¹ Masato Hirano,¹ Hiroaki Matsuuchi,¹ Hayase Shisa,² Hiroshi Hiai³ and Motoo Kitano¹

¹Department of Oral Pathology, Kagoshima University Dental School, 8-35-1 Sakuragaoka, Kagoshima 890-8544, ²Laboratory of Basic Cancer Study, Saitama Cancer Center Research Institute, 818 Komuro, Ina-machi, Saitama 362-0800 and ³Department of Pathology and Biology of Diseases, Kyoto University Graduate School of Medicine, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501

In our previous study, Dark-Agouti (DA) rats were found to be highly susceptible to 4-nitroquinoline 1-oxide (4NQO)-induced tongue carcinoma (TC), whereas Wistar/Furth (WF) rats were barely susceptible. Interval mapping analysis of reciprocal backcross rats showed two quantitative trait loci (QTL) on rat chromosomes (RNO) 1 and 19. In the present study, a composite interval mapping analysis was applied to 4NQO-induced TC in 130 (DA×WF) F2 rats, demonstrating five independent QTL, *Tongue squamous cell carcinoma 1–5 (Tsccl–5)*, responsible for phenotypic differences in the size and number of TCs in the two strains. Two of these QTL were mapped on RNO1, and the others were mapped on RNO4, 14, and 19. The DA allele at these loci consistently yielded semidominant susceptibility to TC. Out of the five loci detected in this F2 generation, *Tsccl* 1 and 2 were identical to *Stc1* and *Rtc1* described in our previous study, but the other three were novel. We propose a new nomenclature consistent with their function. Genome-wide screening of the F2 progeny also suggested the presence of three additional QTL on RNO5, 6, and 10. The possible roles of these loci in tongue carcinogenesis are discussed.

Key words: Genetic susceptibility — QTL — Tongue cancer — Rat — 4-Nitroquinoline 1-oxide

Tongue carcinoma (TC) is one of the most frequent malignancies in the head and neck region. An increase in TC has been reported in the Western world over the past ten years. TC has a poor prognosis because therapeutic strategies provide limited effects.^{1–4} For effective prevention of TC, it would be necessary to identify the genetically predisposed risk group. However, virtually nothing is known about the genetic predisposition to TC.

Recently, we found that the Dark-Agouti (DA) strain of rats had an extremely high susceptibility to 4-nitroquinoline 1-oxide (4NQO)-induced squamous cell carcinoma of the tongue. Virtually 100% of DA rats given drinking water containing 0.001% 4NQO developed one or more large TC within 180 days of administration, whereas the Wistar/Furth (WF) strain of rats was much less susceptible.^{5–8}

In the previous study with reciprocal backcrosses between DA and WF rats,^{7,8} DA rats were shown to have a semidominant susceptibility locus, *Stc1 (Susceptibility to TC-1)* on rat chromosome (RNO) 19, and WF rats, another semidominant resistance gene *Rtc1 (Resistance to TC-1)* on RNO1.

In this study, to scrutinize further the genetic predisposition to TC in the rat, we studied F2 progeny between DA and WF strains by means of a composite interval mapping analysis. After several statistical trials, we employed a quantitative parameter for tumor number, TC >3 mm in diameter, rather than TC >5 mm. In addition to two quantitative trait loci (QTL) described in the previous study, three novel QTL have been found to affect TC susceptibility. These five QTL explain almost all phenotypic variations between the two strains.

MATERIALS AND METHODS

Animals and phenotype The origin of DA rats is related to Copenhagen rats, although there is no detailed information.^{9,10} They were established as an inbred strain in Columbia University. They were first introduced to Japan from the National University of Australia to Kumamoto University in 1973, and transferred to Shizuoka Laboratory Animal Center (Hamamatsu) in 1983. We purchased DA rats from the latter. WF rats were originally obtained from Hiroshima University (Hiroshima). They were maintained by brother-sister mating for more than 50 generations in our laboratory. A total of 130 F2 rats (62 males and 68 females) were obtained by mating (DA×WF) F1 hybrids. All rats were weaned three weeks after birth, individually numbered, and housed in plastic cages in an air-conditioned room at 22°C. They were fed on commercial rat pellet CE2 (Nippon Clea Co., Tokyo).

⁴ To whom correspondence should be addressed.

E-mail: tanuma@dentb.hal.kagoshima-u.ac.jp

Abbreviations: TC, tongue carcinoma; 4NQO, 4-nitroquinoline 1-oxide; DA, Dark-Agouti; WF, Wistar/Furth; RNO, rat chromosome; QTL, quantitative trait loci; TC#3, number of TCs >3 mm in diameter; DTC_{max}, diameter of the largest TC; Lod, logarithm of odds.

No spontaneous tumors were observed at up to six months of age in rats of either parental strain.

Carcinogen treatment The stock solution of 4NQO (Nacalai Tesque, Kyoto) had been prepared by dissolving it in 5% ethanol at 200 mg/liter and was stored at 4°C until use. Starting at six weeks of age, all 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. A full autopsy and histological examination were carried out. The diameters of all TCs of each rat were measured in millimeters using calipers or a ruler and the number of all TCs and cancers other than tongue were recorded individually.

Genetic analysis For linkage analysis, we used microsatellite (simple sequence repeat) analysis, with 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.¹¹ The relative map positions of microsatellite loci were based on Jacob *et al.*¹² and Watanabe *et al.*¹³ Of 873 microsatellite loci examined, 267 (30.5%) were polymorphic between DA and WF. The approximate coverage was ~92% of the entire rat genome, supposing that a marker locus detects linkage within a 15 cM chromosomal segment. Interval mapping and calculation of the logarithm of odds (Lod) scores were carried out with Mapmaker/QTL software.¹⁴ For RNO1, composite interval mapping was applied to confirm the presence of two independent peaks of linkage with Cartographer QTL software, version 1.13.¹⁵ In this study, we used two quantita-

tive parameters: (a) TC#3: the number of the TCs >3 mm in diameter, and (b) DTC_{max}: the size of the diameter of the largest TC. TC#3 was employed because the smallest diameter of the TCs among the 4NQO-treated DA rats was 3 mm.⁸

Statistical analysis Linkage was evaluated by the χ^2 test. According to the criteria of Lander and Kruglyak,¹⁶ a Lod score of 4.3, corresponding to a P value of 5×10^{-5} , is a threshold value for significance in an F2 intercross. Correlations between the number of cancers and tumor diameters were evaluated by correlation analysis with StatView-J Ver4.11, software (Abacus Concepts, Inc., Berkeley, CA) on a Macintosh personal computer.

RESULTS

Tongue carcinogenesis induced by 4NQO Fig. 1 shows the distribution of phenotypic parameters in tongue carcinogenesis in parental strains, F1 and F2. Fig. 1A plots the number of rats vs. the number of TC#3 (TC with >3 mm diameter), and Fig. 1B, the number of rats vs. the tumor diameter. The distribution in parental strains was evidently different, and that in F1 and F2 showed similar intermediate patterns for both parameters. In this work, we selected TC#3 rather than TC#5 because the former more clearly distinguished the distribution of tumor numbers between parental strains (data not shown). TC#3 and DTC_{max} in the F2 intercross were highly correlated ($r=0.803$).

Genetic analysis A preliminary genome-wide scan with 30 out of the 130 experimental F2 rats showing either high or low extreme values of both TC#3 and DTC_{max} revealed significant linkages on RNO19, 1, 4, and 14. On RNO19,

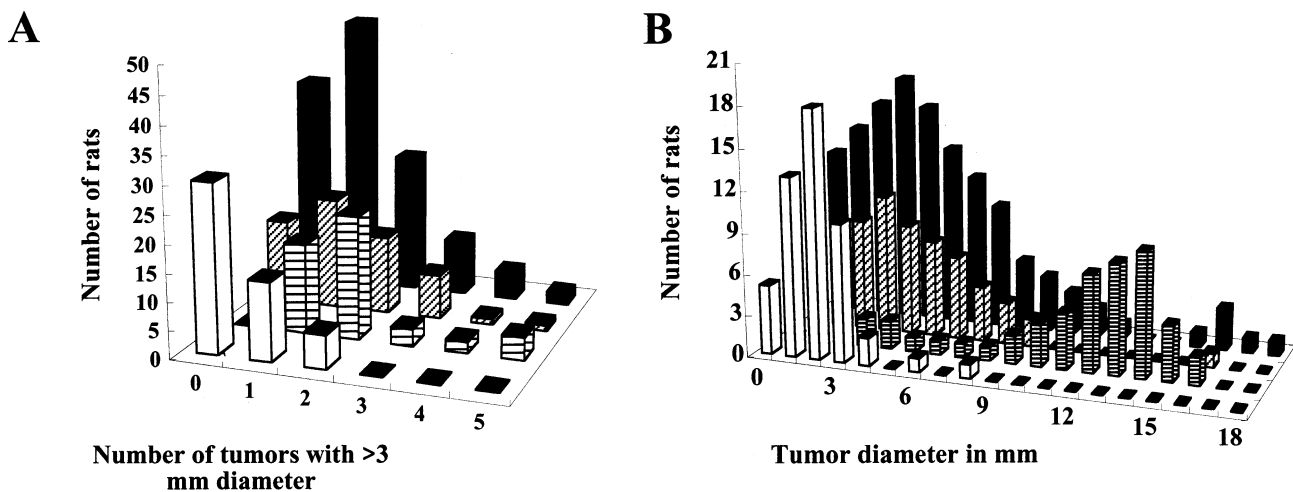


Fig. 1. (A) Distribution of the number of tumors with >3 mm diameter (TC#3) and (B) diameter of the largest TC (DTC_{max}) in 47 DA, 50 WF, 59 (F344×DA) F1 and 130 F2 rats. DA (striped bar), WF (open bar), F1 (hatched bar), and F2 (closed bar).

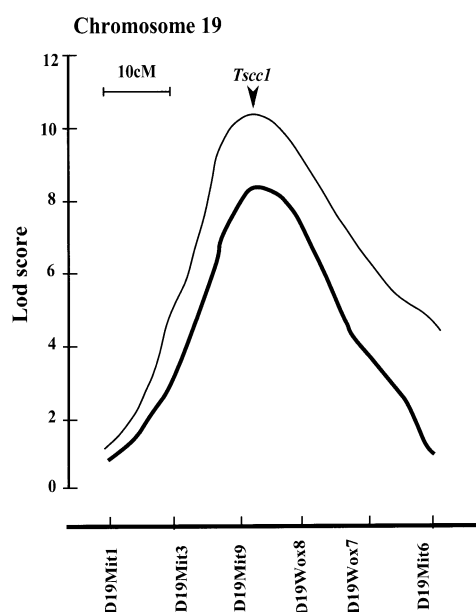


Fig. 2. A composite interval mapping of *Tsccl* on RNO19 based on 130 F2 rats (62 males and 68 females) treated with 4NQO. Lod score plots for TC#3 and DTC_{max} are shown. The bar indicates 10 cM distance. TC#3 (thin solid line) and DTC_{max} (thick solid line).

subsequent genotyping of all 130 F2 rats and interval mapping with Mapmaker/QTL using TC#3 as a quantitative parameter revealed a sharp peak with a Lod score of 10.04 for TC#3, at 4 cM distal from *D19Mit9* (Fig. 2). At the same map location, we observed a peak linkage for DTC_{max} with a Lod score of 8.25 (Fig. 1). As shown in Table I, the mode of inheritance was semidominant: the DA allele yielded a higher susceptibility than the WF allele, and heterozygotes had intermediate phenotypic values between homozygotes for the DA and WF alleles. The map position of this peak is identical to that of *Stc1* (*Susceptibility to tongue cancer-1*) reported previously, now renamed as *Tsccl*.

The genome-wide scanning of the F2 population also detected two linkage peaks on the distal segment of RNO1 (Fig. 3). To confirm that the two peaks are independent rather than a random fluctuation of phenotypes, a composite interval mapping was carried out with Cartographer software¹⁵⁾ as seen in Fig. 3. One of the peaks with Lod scores of 6.85 for TC#3 and 6.79 for DTC_{max} was observed 4 cM distal from *D1Rat320* (Fig. 3). This was named *Tsccl2*. Another peak affecting TC#3 with a Lod score of 4.93 and DTC_{max} with a Lod score of 3.66 was found 2 cM distal from *D1Mit5* (Fig. 3). The latter was

Table I. QTLs Affecting Susceptibility to Tongue Cancers Induced by 4NQO

Locus	Chr. ^{a)}	Phenotype-genotype correlation at each locus (Average±standard deviation) (No. of rats)							
		TC#3				DTC _{max}			
		Lod score	DD ^{b)}	DW ^{c)}	WW ^{d)}	Lod score	DD ^{b)}	DW ^{c)}	WW ^{d)}
<i>Tsccl</i>	19	10.04	3.23±1.28 (34)	1.79±0.96 ^{f)} (65)	0.36±0.84 ^{g)} (31)	8.25	9.86±4.95 (34)	5.45±3.68 ^{e)} (65)	2.13±2.98 ^{g)} (31)
<i>Tsccl2</i>	1	6.85	2.12±1.05 (28)	0.80±0.85 ^{g)} (67)	0.30±0.74 ^{g)} (35)	6.79	9.42±4.46 (28)	4.02±4.52 ^{f)} (67)	1.81±2.63 ^{g)} (35)
<i>Tsccl3</i>	1	4.93	1.34±1.22 (28)	0.57±0.83 ^{g)} (68)	0.35±0.56 ^{g)} (34)	3.66	9.68±4.39 (28)	4.46±4.33 ^{f)} (68)	2.05±2.78 ^{g)} (34)
<i>Tsccl4</i>	4	6.88	1.43±1.37 (32)	0.55±0.98 ^{g)} (60)	0.37±0.91 ^{g)} (38)	3.13	9.13±4.81 (32)	4.38±3.84 ^{f)} (60)	1.91±4.02 ^{g)} (38)
<i>Tsccl5</i>	14	7.29	2.83±1.17 (31)	1.35±0.63 ^{f)} (67)	0.32±0.83 ^{g)} (32)	3.10	9.25±4.72 (31)	5.76±4.01 ^{e)} (67)	2.30±3.12 ^{g)} (32)
<i>D5Mgh4</i>	5	3.47	1.82±2.01 (32)	0.79±1.03 ^{f)} (65)	0.23±0.87 ^{g)} (33)	2.83	8.98±3.95 (32)	5.82±3.78 ^{e)} (65)	2.01±3.65 ^{g)} (33)
<i>D6Rat55</i>	6	3.29	1.76±2.42 (29)	0.72±2.94 ^{f)} (66)	0.24±0.73 ^{g)} (35)	2.75	8.56±4.07 (29)	5.71±3.15 ^{e)} (66)	2.11±2.91 ^{g)} (35)
<i>D10Mit8</i>	10	3.12	1.84±2.73 (35)	0.71±0.96 ^{f)} (64)	0.27±0.38 ^{g)} (31)	2.13	9.07±3.09 (35)	5.68±2.79 ^{e)} (64)	2.06±2.46 ^{g)} (31)

a) Chromosome.
 b) Homozygote of the DA allele.
 c) Heterozygote.
 d) Homozygote of the WF allele.
 e) Significant difference from the values for homozygote of the DA allele ($5 \times 10^{-2} > P > 1 \times 10^{-3}$).
 f) Significant difference from the values for homozygote of the DA allele ($1 \times 10^{-3} > P > 1 \times 10^{-4}$).
 g) Significant difference from the values for homozygote of the DA allele ($P < 1 \times 10^{-4}$).

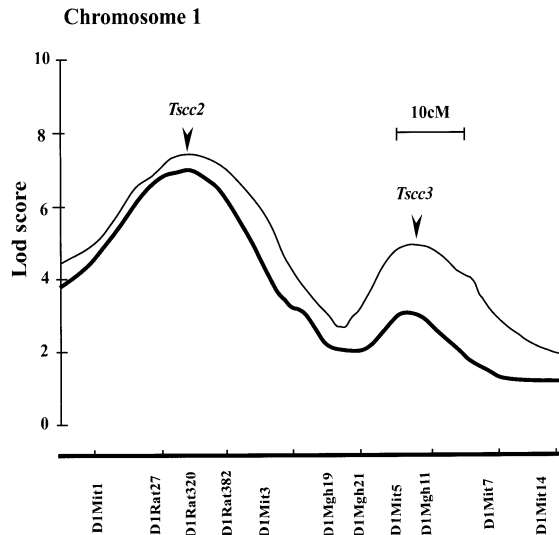


Fig. 3. *Tsc2* and *Tsc3* on RNO1. TC#3 (thin solid line) and DTC_{max} (thick solid line).

named *Tsc3*. The modes of inheritance of *Tsc2* and 3 were semidominant.

On RNO4, the fourth peak with a Lod score of 6.88 for TC#3 was observed 4 cM distal from *D4Mgh10* (Fig. 4). This locus was named *Tsc4*, which exhibited a stronger phenotypic effect on the number of TCs than on their size, since the Lod score for DTC_{max} was 3.13, which was below the level of significance.

The fifth peak with a Lod score of 7.29 was observed for TC#3 2 cM distal from *D14Wox4* on RNO14 (Fig. 5). This locus was named *Tsc5*. The DA allele of *Tsc5* favored an increase in the number of TCs. The Lod score for DTC_{max} was considerably lower than the level of significance (Fig. 5).

The phenotype-genotype correlation of F2 rats is summarized in Table I. TC#3 and DTC_{max} in F2 rats were found to correlate with each other ($r > 0.5$). At all five QTL, the phenotypic values for these parameters were consistently higher in the DA allele homozygote than in the WF allele homozygote ($P < 1 \times 10^{-4}$) and intermediate in the heterozygote.

In addition, weak linkage peaks were observed on three other chromosomes, i.e., on RNO5 (Lod score 3.47 at *D5Mgh4*), RNO6 (Lod score 3.29 at *D6Rat55*), and RNO10 (Lod score 3.12 at *D10Mit8*), respectively. Lod values at these loci were < 4.3 and > 2.8 ; therefore, their presence was only suggestive.

DISCUSSION

Previously, by analyzing reciprocal backcrosses between DA and WF strain rats, we defined two semidom-

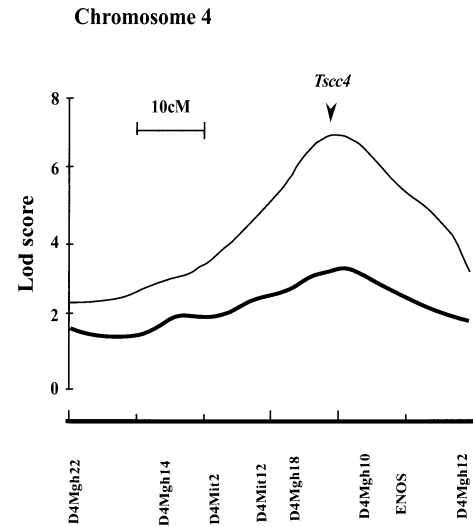


Fig. 4. *Tsc4* on RNO4. TC#3 (thin solid line) and DTC_{max} (thick solid line).

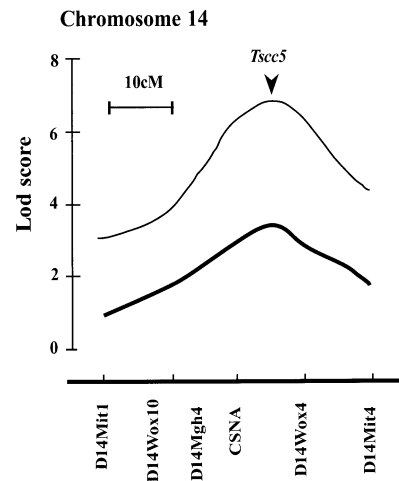


Fig. 5. *Tsc5* on RNO14. TC#3 (thin solid line) and DTC_{max} (thick solid line).

inant QTL, *Rtc1* and *Stc1*.^{7,8} The genetic predisposition to TC, however, is more complicated than we had initially assumed. The present analysis of the F2 generation with two quantitative parameters for size (DTC_{max}) and number (TC#3) detected these two loci and three additional QTL. Since the DA allele at all of these loci yields semidominant susceptibility to TC, we propose renaming these loci as *Tongue squamous cell carcinoma 1–5* (*Tsc1–5*) for consistency. In DA rats, therefore, these multiple QTL funnel to dictate high susceptibility to TC.

In the previous study, we used TC#5, the number of TCs more than 5 mm in diameter, as a quantitative param-

eter for QTL analysis. The analysis of the current F2 progeny with TC#5 displayed significant peaks for *Tscc1* and 2 but statistically insignificant peaks for *Tscc3*, 4, and 5. After several preliminary attempts, we selected TC#3, the number of TCs more than 3 mm in diameter, as a quantitative parameter because it distinguished the distribution of the number of tumors in both parental strains more clearly. With this parameter, three novel QTL *Tscc3*, 4, and 5 were significant according to the criteria of Lander and Kruglyak for F2 (Lod score >4.3).¹⁶⁾

Tscc1, formerly *Stc1*, was mapped on RNO19 and had the biggest phenotypic effect on TC resistance. In the light of the more precise map position of *Tscc1* determined in this study, one of the candidate genes for *Tscc1* is *NQO1* (the gene for quinone oxidoreductase, synonymous with DT-diaphorase or NADH-cytochrome *b₅* reductase).^{17,18)} Quinone oxidoreductase is one of the major enzymes that converts 4NQO to the more active metabolite, 4-hydroxy-aminoquinoline 1-oxide (4HAQO).^{19,20)} Our preliminary study showed that the enzyme activity is higher in DA than in WF rats. *Junb*,^{21,22)} a protooncogene, is another potential candidate for *Tscc1* mapped on the homologous segment on human chromosome 19 and mouse chromosome 8. The expression of immediate-early response genes of the *jun/fos* leucine zipper family could be critical in cell proliferation, differentiation, and apoptosis.

There were two QTL affecting TC on RNO1, *Tscc2*, and *Tscc3*. *Tscc2* (formerly *Rtc1*) was 4 cM distal from *D1Rat320*. One of the candidate genes for *Tscc2* is *Cyp2b1*, a structural gene for cytochrome P450, a phenobarbital-inducible enzyme of the superfamily of membrane-bound enzymes that function as terminal monooxygenases in the metabolism of a broad variety of endogenous and exogenous compounds.^{23,24)} The quinoline and 4NQO are hepatocarcinogens in rats and mice²⁵⁾ and well-known mutagens in bacteria²⁶⁾ after incubation with rat liver microsomes, of which the membrane is likely to be closely associated with the cytochrome P450 gene families.²⁷⁾ Another candidate gene for *Tscc2* is the *p57* protein-gene.²⁸⁾ The control of the cell cycle and suppression of cell transformation by the *p57* protein require both cyclin-dependent kinase and proliferating cell nuclear antigen inhibitory activity. Disruption of either or both functions may lead to uncontrolled cell growth.²⁹⁾

At the map position of *Tscc3*, there are *Ha-ras*,^{30,31)} *CyclinD1*,^{32,33)} and *Gstp*.³⁴⁾ The *Ha-ras* gene has been implicated in cancer predisposition in the human, mouse, and rat. Loss of the wild-type *Ha-ras* allele is considered to result in an unopposed mutant activated by the *p21* protein that could potentially lead to gene amplification. In our parallel study with 4NQO-induced TC in F1 rats, frequent loss of the WF allele and point mutations in the remaining allele at Ha-Ras were observed (Tanuma *et al.*, to be published elsewhere). The human oral cancers as

well as 4NQO-induced tumors with amplification in the *Ha-ras* region show an increased *CyclinD1* expression, which dramatically shortens the G1/S transition, presumably preventing apoptosis and enabling the cells with DNA damage to enter the S phase more rapidly.³⁵⁾ Our previous study showed that *Gstp* is a promising marker for tongue carcinogenesis in the rat model.³⁶⁾ All TCs invariably express *Gstp*, whereas specimens from the normal control animals are negative.

The candidate genes for *Tscc4* mapped on RNO4 are *Tgf α* (transforming growth factor- α)³⁷⁾ and *p27*.³⁸⁾ *TGF- β* exerts anti-mitogenic effects through a kinase-inhibitory protein (*p27*) that can inhibit both *cyclin D-Cdk4* and *cyclin E-Cdk2*. This protein may be the primary regulator of *Cdk* activity when cells enter and leave a quiescent state. The autocrine production of *Tgf α* and *TGF- β* is a marker of tumor progression in the rat 4NQO model of oral carcinogenesis.

As candidate genes for *Tscc5* on RNO14, we can mention the epidermal growth factor receptor gene (*Egfr*)³⁹⁾ and the gene of tumor protein *Tp53*-like 2 (*Tp53l2*).⁴⁰⁾ Epidermal growth factor is a potent mitogen of normal epithelial cells and mediates its signal through binding to the cell surface receptor. Polymorphism in *Egfr* can be a constitutional factor that determines susceptibility to cancer development. The *Tp53l2* belongs to the *Tp53*-superfamily and codes a tumor protein, a *Tp53*-like 2 protein, which acts as a *Tp53* protein in regulation of the cell cycle and cellular apoptosis.

Genome-wide screening followed by a composite interval mapping analysis revealed five QTL affecting 4NQO-induced TCs in the rat. The size of DTC_{max} and the number of TC#3 are apparently correlated. However, the degree of linkage to each phenotype is variable by locus. The number of TCs is significantly linked to all five QTL, but the size of the largest tumor is linked only to *Tscc1* and 2. Linkages of *Tscc3* and 4 to DTC_{max} are only suggested, and there is no linkage to *Tscc5*. Therefore, the size of TCs and their number were correlated to each other, but the contribution of each locus to the phenotypes was different.

Genetic predisposition to cancer is a complicated multigenic trait. Chemically induced tongue cancer in rats is one of the best models for quantitative genetic analysis. In rodents, a number of resistance or susceptibility loci for liver cancers,^{41,42)} as well as pulmonary cancers, have been identified.^{43,44)} These provide not only important clues for understanding the polygenetic nature of cancer development, each step of which is controlled by multiple host genes, but also clues to genetic steps possibly shared with human cancers.⁴⁵⁾ We now understand that chemical activation of 4NQO and cell growth regulation are critical steps in determining genetic susceptibility. Further characterization of candidate genes is required, since they might

have relevance to chemoprevention and lead to novel therapeutic approaches to human oral cancers.

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