Genetic Predisposition to 4NQO-Induced Tongue Carcinogenesis in the Rat

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Key Words

4-Nitroquinoline 1-oxide · Tongue cancer · Rat · Genetic susceptibility · Quantitative trait loci · Loss of heterozygosity

Abstract

Objective: This study aims to elucidate the genetic basis of predisposition to 4-nitroquinoline 1-oxide (4NQO)-induced tongue cancers (TCs). **Materials and Methods:** We have reported that inbred Dark-Agouti (DA) strain rats were highly susceptible to 4NQO-induced TCs, whereas Wistar/Furth (WF) rats were resistant to tongue squamous cell carcinomas induced by oral administration of 4NQO. Using size and number of the tumours as quantitative parameters, responsible host loci were analysed by an interval mapping of F₂ intercross of DA and WF given carcinogenic regimen. Also, loss of heterozygosity (LOH) at these loci was analysed in tongue cancers in (DA × WF) F₁. **Results:** We identified and mapped 5 significant quantitative trait loci (QTL), the *Tongue squamous cell carcinoma 1–5* (*Tscc1–5*), and several other suggestive QTL that determine susceptibility to 4NQO-induced TC. Study of TCs induced in (DA × WF) F₁ rats revealed a high frequency of LOH in the chromosomal regions of *Tscc2*, 3, and 4 and also of suggestive QTL on chromosomes 5 and 6. The fact that LOH was found only

in larger TCs indicates that LOH occurred in the process of tumour progression. In most LOH, the allele of the resistant WF strain was lost, suggesting that these loci may encode tumour suppressor genes. In larger TCs, in addition to LOH, point mutations and the methylation of possible candidate genes were accumulated. **Conclusion:** These observations indicate that the 4NQO-induced TC in the rat is a multifactorial disease of a polygenic trait. This model will be useful to understand the complicated genetic basis of predisposition to oral cancers.

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Introduction

Oral cavity cancers, including tongue cancer (TC), are ranked sixth as a cause of death due to malignancy, and their incidence is increasing [1–5]. It is important to identify the genetically predisposed group that is at risk for oral cavity cancers, and to determine molecular targets for effective treatment. We have established a rat model to dissect the genetic predisposition to TCs induced by a chemical carcinogen, 4-nitroquinoline 1-oxide (4NQO). The model is based on our observation that Dark-Agouti (DA) rats are extremely susceptible to TC by oral administration of 4NQO, while Wistar/Furth (WF) rats are resistant [6–9].

To elucidate the genetic predisposition to TC, (DA × WF) F₂ rats were given 4NQO, and interval mapping of the genetic loci affecting the TC phenotype was carried out to find multiple quantitative trait loci (QTL). To test whether an individual locus has an allele-specific tumour suppressor-like activity against TC, 4NQO-induced TCs in (DA × WF) F₁ hybrid rats were examined for loss of heterozygosity (LOH) at each chromosomal region containing these QTL. This report reviews our extensive genetic analysis of rat 4NQO-induced TCs and discusses possible candidate genes with special reference to genetic alterations in TCs and the metabolic activation of the carcinogen [6–10].

Materials and Methods

Animals

Inbred DA rats were established at Columbia University (New York, N.Y., USA) from a progenitor related to Copenhagen rats, and were introduced to Kumamoto University (Kumamoto, Japan) in 1973 from the Australian National University (Canberra, Australia). At present, they are commercially available from the Shizuoka Laboratory Animal Centre (SLC, Hamamatsu, Japan). The DA rats used in this study were purchased from SLC. WF rats were originally obtained from Hiroshima University (Hiroshima, Japan) and have been maintained by brother-sister mating for over 90 generations at Kagoshima University (Kagoshima, Japan).

For genetic analysis, reciprocal F₁ and F₂ intercrosses between DA and WF rats were prepared. All rats were individually numbered and housed in plastic cages in a room air-conditioned to 22 ± 2°C and fed commercial rat pellets CE-2 (Nippon Clea Co., Tokyo, Japan). No spontaneous tumours were observed at 6 months of age in rats of either parental strain.

Induction of Tongue Cancers

Starting at 6 weeks of age, rats in the experimental group were given drinking water containing 0.001% 4NQO ad libitum from 5 p.m. to 9 a.m., but no water was given at other times. They were sacrificed when they became moribund or on the 180th experimental day if still alive. A full autopsy with histopathological examination was carried out. The number of TCs with diameters >3 mm (TC#3), and the diameter of the largest TC were selected as parameters for QTL analysis.

Linkage Analysis

Linkage analysis was done by interval mapping with a PCR-based method of microsatellite analysis [11]. Of 873 microsatellite loci examined, 267 (30.5%) were polymorphic between DA and WF. The approximate coverage was ~92% of the entire rat genome when a marker locus was assumed to detect linkage within a 15-cM chromosomal segment. QTL analysis and lod score calculations were carried out using a Mapmaker/QTL computer package as described previously [8]. For RNO1, composite interval mapping was used to confirm the presence of two independent peaks of linkage with Cartographer QTL software, version 1.13 [12].

According to the criteria of Lander and Kruglyak [13], linkages in the F₂ intercross were taken as significant when the *p* value was <1 × 10⁻⁴ (lod score >4.3), and as suggestive if 1 × 10⁻⁴ < *p* < 3.4 × 10⁻³ (lod score <4.3 and >2.8). Correlations between the number of TCs and tumour diameter were evaluated by correlation analysis with StatView, version 4.02, software (Abacus Concepts, Inc., Berkeley, Calif., USA). The relative map positions of microsatellite loci were based on the findings of Jacob et al. [14] and Watanabe et al. [15].

LOH Analysis

Of 88 (DA × WF) F₁ rats given 4NQO as described above, 40 developed at least one TC with a diameter >3 mm. Paired samples of the largest TC and the kidney or tail from each of a total of 51 tumour-bearing F₁ rats were obtained and stored at -80°C. Tumour tissues were carefully enucleated to avoid normal tissue contamination under a dissecting microscope. LOH was detected using PCR-based microsatellite analysis with fluorescence-tagged primers (Research Genetics, Inc., Huntsville, Ala., USA). One microlitre of PCR product was added to 10 µl formamide and 0.5 µl of TAMRA 500 size standard (Applied Biosystems, Foster, Calif., USA), and the mixture was applied on a 4% polyacrylamide 6 M urea gel. PCR conditions for each marker locus were as determined in a preliminary study [10]. The gel was scanned using an ABI Prism 310 Genetic Analyser (Applied Biosystems), and data were collected automatically and analysed using GeneScan software (Applied Biosystems). Finally, a Genotyper (Applied Biosystems) was used for allele scoring and assessment of LOH. In constitutional heterozygotes, two alleles are detected in normal tissue; if one is absent in a tumour, the result is LOH. When tumours showed allelic imbalance rather than the complete loss of one allele, the ratio of the tumour signal to that of the normal signal (T1/T2 over N1/N2) was calculated. Ratios of <0.67 or >1.35 were considered indicative of LOH for that locus.

Results

Genetic Susceptibility of DA Rats to 4NQO-Induced TC

4NQO is a potent pleiotropic carcinogen for various organs in many animal species. Oral administration to rats of 4NQO dissolved in drinking water induced mass-forming squamous cell carcinomas of the tongue (fig. 1) and the oral cavity. Among inbred rat strains of DA, Long-Evans/stm, Sprague-Dawley, ACI/Ms, Fischer 344, Donryu, and WF, there were remarkable differences in susceptibility to 4NQO-induced TCs (table 1). DA rats developed the highest number of TCs with larger sizes in shorter latent periods, whereas WF rats developed fewer and smaller TCs than any other strains. Other strains showed susceptibility intermediate between these two strains. From this observation, we concluded that the set of DA and WF rats would be an excellent model to study genetic factors in tongue carcinogenesis.

Table 1. Difference in susceptibility of 4NQO-induced TC among rat strains^a

Strain	Rats	Rats with TC#5 (incidence, %)	DTC _{max} , mm (average ± SD)	Survival time, days (average ± SD)
Dark-Agouti	47	44 (94%)	12.77 ± 4.49	171.1 ± 29.6
Long-Evance/Stm	42	25 (60%)	8.08 ± 3.67	182.0 ± 38.8
Sprague-Dawley	48	33 (69%)	9.03 ± 4.32	186.2 ± 31.2
ACI/Ms	41	33 (81%)	10.07 ± 5.39	189.4 ± 22.5
Fischer 344	48	40 (83%)	10.92 ± 3.83	199.5 ± 30.1
Donryu	46	35 (76%)	9.67 ± 2.54	205.4 ± 31.0
Wistar/Furth	50	2 (4%)	1.18 ± 1.64	238.2 ± 23.6

TC#5 = Number of TCs ≥ 5 mm in diameter; DTC_{max} = diameter of the largest TC in millimetres.

^a Cited from Kitano et al. [7] with copyright permission of the publisher.

Fig. 1. 4NQO-induced tongue carcinomas in an F₂ rat. **A** Large mass forming cancer. **B** No macroscopic tumour in this individual.

QTL Determining Host Susceptibility to TCs

A preliminary whole-genome scanning of 130 extreme-phenotype F₂ rats with 267 markers showed five significant linkages for susceptibility to 4NQO-induced TCs (table 2). The highest linkage peak by the number and size of TC was found on rat chromosome (RNO) 19, at 4 cM

distal from *D19Mit9*. The p values at the peak were 4.82×10^{-10} for number and 1.48×10^{-9} for size of TCs (lod scores, 10.04 and 8.25, respectively, fig. 2). We named this QTL *Tongue squamous cell carcinoma-1* (*Tscc1*). The function of *Tscc1* was semi-dominant because the number and size of TCs in heterozygous F₂ rats were intermediate between the high values in the DA-allele homozygotes and the low ones in the WF-allele homozygotes. *Tscc1* explains the 20.9% variance of phenotype for number and 17.9% for size.

A significant QTL peak was observed in the distal region of RNO1, 4 cM distal from *D1Rat320*, in both number and size (fig. 3). At this peak, the p values for TC number and size were 5.02×10^{-8} and 4.76×10^{-8} (lod score, 6.85 and 6.79), respectively. This locus was named *Tscc2*.

On RNO1, besides *Tscc2*, there was a second peak of QTL (*Tscc3*) for the number of TC at 2 cM distal from *D1Mit5*. To confirm that the two QTL are independent rather than a random fluctuation of phenotypes, composite interval mapping was carried out with the Cartographer software. It was concluded that this *Tscc3* locus was independent from *Tscc2*. At *Tscc3*, the p value for the number of TCs was 2.48×10^{-7} (lod score, 4.93). The semi-dominant phenotypic effect of *Tscc3* was considerably weaker than that of *Tscc2*, and its effect on TC size was not significant.

On RNO4, we mapped another locus (*Tscc4*) linked to the number of TCs. Again, the DA alleles were associated with higher TC phenotype values. At 4 cM distal from *D4Mgh10*, the p value for the number of TCs was 3.34×10^{-8} (lod score, 6.88) (fig. 4). The phenotypic effect of *Tscc4* on the size of TCs was below a significance level (lod score, 3.13).

Table 2. QTL affecting susceptibility to 4NQO-induced TC

Locus	RNO	Linkage indexes and % variance explained at each locus					
		TCs			size of the largest TC		
		p	lod score	% ^a	p	lod score	% ^a
<i>Tscc1</i>	19	4.82×10^{-10}	10.04	20.9	1.48×10^{-9}	8.25	17.9
<i>Tscc2</i>	1	5.07×10^{-8}	6.85	13.8	4.76×10^{-8}	6.79	12.9
<i>Tscc3</i>	1	2.48×10^{-7}	4.93	10.7	5.26×10^{-6}	3.66	9.21
<i>Tscc4</i>	14	3.34×10^{-8}	6.88	14.4	8.72×10^{-4}	3.13	7.01
<i>Tscc5</i>	14	7.48×10^{-9}	7.29	15.3	1.92×10^{-3}	3.10	6.81
<i>D5Mgh4</i> ^b	5	1.36×10^{-4}	3.47	7.92	3.25×10^{-3}	2.83	5.92
<i>D6Rat55</i> ^b	6	2.23×10^{-4}	3.29	7.32	5.44×10^{-4}	2.75	5.78
<i>D10Mit5</i> ^b	10	1.45×10^{-3}	3.12	6.91	9.47×10^{-3}	2.13	5.49

^a % variance of phenotype explained.
^b The marker locus closest to the suggested QTL peak.

Fig. 2. Rat chromosome 19 (RNO19) bearing *Tscc1* and syntenic chromosomal regions of mouse (MMU) and human (HSA). Bar represents the *Tscc1* region of lod score >4.3.

On RNO14, we mapped a fifth QTL (*Tscc5*) linked to the number of TCs. The DA allele is associated with an increased phenotype value. At 2 cM distal from *D14Wox4*, the p value for TC#3 was 7.48×10^{-9} (lod score, 7.29) (fig. 5). *Tscc5* mainly affected the number of TCs in a semi-dominant trait, while the linkage for TC size of was just suggestive (lod score, 3.10).

In addition to the 5 QTL noted above, a suggestive linkage for phenotypic values either by number or size was observed at four loci: *D5Mgh4* on RNO5, *D6Rat45*

on RNO6, *D10Mit8* on RNO10, and *D17Wox22* on RNO17. Tentatively, they were identified as suggestive QTL. Among them, special attention should be given to the QTL on RNO5, as frequent loss of the WF chromosomal segment of RNO5 was found where this QTL and the tumour suppressor genes *p15* and *p16* were mapped. In larger TCs, point mutations and methylation of *p15* and *p16* also occurred [16]. Polymorphism in the germline as well as somatic changes may contribute to the progression of TCs. Unlike human TCs [16], genetic changes

Fig. 3. RNO1 bearing *Tscc2* and *Tscc3* and syntenic chromosomal regions of mouse (MMU) and human (HSA). Bars represent *Tscc2* and *Tscc3* regions of lod score >4.3.

Fig. 4. RNO4 bearing *Tscc4* and syntenic chromosomal regions of mouse (MMU) and human (HSA). Bar represents *Tscc4* regions of lod score >4.3.

Fig. 5. RNO14 bearing *Tsc5* and syntenic chromosomal regions of mouse (MMU) and human (HSA). Bar represents *Tsc5* regions of lod score >4.3.

Fig. 6. Distribution of QTL affecting the size of TCs and percent frequency of LOH at each QTL.

in *p53* were not frequent in the 4NQO-induced rat TCs except in some large TCs.

Survey of LOH in TCs Induced in *F*₁ Rat

To determine whether the resistant WF alleles of these loci are lost by hemizygous deletion, as seen in tumour suppressor genes, we first examined LOH in the TCs

of the 40 reciprocal (DA × WF) *F*₁ rats with tumours ≥ 5 mm in diameter. LOH was frequently found on RNO1, 4, 5, and 6 and less frequently on RNO10, 14, and 19 (fig. 6). Among *Tsc* loci, *Tsc2*, *Tsc3* (on RNO1) and *Tsc4* (on RNO4) were within the segments frequently involved in LOH. In contrast, *Tsc1* (RNO19) and *Tsc5* (RNO14) were not involved in LOH. In *Tsc2*, *Tsc3*, and

Fig. 7. LOH at each *Tsc2* locus and size of TCs.

Tsc24, the WF allele was preferentially lost and LOH frequency increased in parallel with the size of the TC, raising the possibility that the functions of these QTL are to suppress tumour progression [16]. Other hot spots showing >30% frequency were on RNO5 and 6, bearing *p15^{INK4B}/p16^{INK4A}* and *Msh2*, respectively. We also examined TCs in reciprocal F₁ rats; however, no imprinting was observed at any of these loci.

Discussion

The plethora of host genetic control of 4NQO-induced tongue cancers in rats is not exceptional for the model introduced here. Similar complexity has been observed in chemically induced lung adenomas [17–19], liver cancers [20, 21], and skin cancers [22, 23]. The molecular bases of QTL found in this study have not been elucidated, but candidate genes for them include genes encoding enzymes metabolising the carcinogen, growth factors, and their receptor, oncogene, and onco-suppressor genes. It is also possible that some of them are responsible for immune responses to developing TCs. At present, it would be premature to assign them to each QTL, but the following discussion serves as a basis for further investigation.

One of important candidate genes for *Tsc21* was *NQO1* (*Dia4*), the gene encoding quinone oxidoreductase (DT-

diaphorase or NADH-cytochrome b₅ reductase) [24, 25]. A precisely mapped location of *NQO1* in the rat has not been reported, but it is in the syntenic regions of the human chromosome 16q22.1 and the mouse chromosome 8 [26–28]. Quinone oxidoreductase is one of the major enzymes that convert 4NQO to the more active metabolite, 4-hydroxyaminoquinoline 1-oxide [25, 27]. Recently, we clarified that the locus of *NQO1* is co-mapped with *Tsc21* between *D19Mit9* and *D19wox8* [29]. The polymorphism of *NQO1* may well modulate the enzyme activity to activate 4NQO and, thus, modify the susceptibility to TCs. Actually DA rats show a very high level of *NQO1* mRNA as well as enzymal activity of quinone oxidoreductase [30].

An important candidate for *Tsc22* is *Cyp2a*, located at 19q13.2, a structural gene for one of the cytochrome P-450 enzymes. The P-450 enzymes constitute a superfamily of membrane-bound enzymes that function as terminal mono-oxygenase in the metabolism of a broad variety of endogenous and exogenous compounds, including chemical carcinogens [31, 32]. Another candidate gene for *Tsc22* may be *p57* of the p57Kip2 protein [33], which is one of the cyclin-dependent kinase (Cdk) inhibitors [34]. When searching for LOH at *Tsc2* loci in 4NQO-induced TC in (DA × WF) F₁ rats, we found that the chromosomal region of the *Tsc22* was a frequent target of LOH; the frequency of which increased as the size of the TCs increased. The resistant WF allele was selectively lost, which suggests that *Tsc22* might encode a tumour suppressor gene.

We can assume several candidate genes for *Tsc23* from its map position, including *Ha-ras* [35, 36], *CyclinD1* [37], *IGF2* [38], and *Gstp* [39, 40]. *Ha-ras* genes have previously been implicated in cancer predisposition in humans, mice, and rats [35]. Loss of the wild-type *Ha-ras* allele may cause an unopposed mutant activated by p21 protein that could potentially lead to gene amplification [36]. *Ha-ras* point mutations at codon 12 or 61 were detected in 28 of the 40 TCs, among which LOH of wild-type *Ha-ras* was observed in 20 of the TCs >8 mm in diameter. The high incidence of *Ha-ras* mutations raises the possibility that they represent an early event in TC development. In our parallel study with 4NQO-induced TCs in F₁ rats, frequent loss of the WF allele and point mutations in the remaining DA allele at the *Ha-ras* gene were observed [16].

An earlier study of ours showed *Gstp* to be a promising marker for 4NQO-induced tongue carcinogenesis in the rat [39]. All TCs invariably expressed *Gstp*, whereas tongue tissues from normal control animals were nega-

tive. This may contribute somehow to the difference in susceptibility to 4NQO between the DA and WF strains [39, 40].

One of the candidate genes for *Tscc4* is *Tgfa*, located on RNO4 and encoding the transforming growth factor- α [41, 42]. Another candidate gene is p27Kip1, a 27-kDa Cdk inhibitor [43]. Moreover, other candidate genes for *Tscc4* mapped on RNO4 may be *Ki-ras* [44, 45], *Ret* [46], *Raf-1* [47], and *Pthlh*. *Pthlh* encodes the parathyroid hormone-related protein, which is regarded as a predominant cause of hypercalcaemia of malignancies observed in several types of human cancers [48, 49]. Manenti et al. [50] reported that an amino acid polymorphism of *Pthlh* showed cancer modifier effects in a human squamous cell carcinoma cell line. We also are now engaged in an exhaustive molecular survey on *Pthlh*, in respect to 4NQO-induced rat tongue carcinogenesis, and some important and interesting results will hopefully be presentable in the future [Tanuma, unpublished data]. For *Tscc5*, *Egfr* [51] and *Tp53l2* [52] can be mentioned as possible candidate genes.

Carcinogenesis is a multifactorial disease. Numerous genes in the complicated biochemical steps involved in carcinogenesis may be genetically polymorphic. A sum of

such a polymorphic effect is the visible difference between strains. Although it is not straightforward to compare the results in rats with those in humans, it is possible that some of the steps in carcinogenesis are shared across species. We believe that the comparative approach is still an effective means of understanding the genetic basis of differences in cancer susceptibility among individuals.

Conclusion

The results of this study indicate that the 4NQO-induced TC in the rat is a multifactorial disease of a polygenic trait. This model will be useful to understand the complicated genetic basis of predisposition to oral cancers.

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