Genetic and epigenetic control of 4NQO-induced tongue carcinogenesis in the rats

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Abstract

Oral administration of 4-nitroquinoline 1-oxide (4NQO) can induce a high incidence of tongue cancers (TCs) in rats; inbred Dark-Agouti (DA) strain rats are highly susceptible, whereas Wistar/Furth (WF) rats are resistant. Our earlier studies confirmed that susceptibility depended on the respective genetic background, and 5 quantitative trait loci (QTLs), *Tongue squamous cell carcinoma 1-5 (Tscc1-5)*, were identified as influencing susceptibility/resistance to 4NQO-induced TCs; however, the gene products encoded by the Tscc loci, and the molecular mechanisms responsible for their allele-specific cancer-modifier activities, remain unknown. To test whether *Tscc* genes display either oncogene or allele-specific tumour suppressor-like activity in TCs, we examined 4NQO-induced TCs in (DA x WF) F1 rats for loss of heterozygosity (LOH) at the loci known to be involved in cancer to characterize genetic alterations in TCs and their possible relationship with the *Tscc* loci. We examined 40 tongue tumours $\geq$ 5 mm in diameter from (DA x WF) F1 rats for LOH at the *Tscc* loci, revealing a high frequency of LOH in chromosomal regions where the *Tscc 2, 3* and *4* loci map. In most LOH, the allele of the resistant WF strain was lost, suggesting that these loci encode tumour suppressor genes. LOH was frequently found on rat chromosomes 5 (RNO5) and 6 (RNO6). In some advanced TCs, 5' CpG island methylation and mutation of $p15^{INK4B}$ and $p16^{INK4A}$ genes were also detected. The accumulation of LOH, mutation and methylation in tumour suppressor genes in larger tumours suggests that they may play a role in TC progression. This latest work represents an important step toward the identification of clinically significant biomarkers for tongue carcinogenesis.

Key words: 4NQO, Tongue Cancer, QTL, LOH, Methylation

Introduction

Carcinogenesis is a multistep phenomenon modified by a number of host genetic and epigenetic factors, though such modifier effects of the host are still poorly understood. Etiological factors of TCs and other oral cancers are assumed to be mostly environmental, such as smoking, alcohol intake or viral infection, but several epidemiological studies and reports on familial clustering suggest that genetic and epigenetic factors also contribute to susceptibility to these cancers.[1, 2, 3] Such genetic susceptibility, if recognized, would be important in identifying risk groups and elucidating critical steps in carcinogenesis.[4, 5]

To elucidate host genetic and epigenetic effects, analyses of appropriate animal models are very important; indeed, the rat model of TC induced by 4NQO is one of the best. The induction of TC in rats by 4NQO, a potent carcinogen, is under genetic and epigenetic control. We have reported that the DA strain of rats is highly susceptible to 4NQO-induced TC, but that the WF strain is highly resistant.[6, 7] Our studies in F2 rats using interval mapping

methods have shown that susceptibility to 4NQO-induced rat tongue carcinogenesis is determined by five QTLs of a significant level, *Tscc1-5* and, in addition, four QTLs of suggestive level located on rat chromosomes 5, 6, 10 and 17.[8, 9, 10] To test whether an individual locus has an allele-specific tumour suppressor-like activity on TC, 4NQO-induced TCs in (DA x WF) F1 hybrid rats were examined for LOH, methylation and mutation at each chromosomal region containing these QTLs.[11, 12, 13] This report is based on our studies in respect to chemical carcinogenesis in the rat tongue.

Animals and Carcinogen

DA rats were purchased from the Shizuoka Laboratory Animal Centre (Hamamatsu, Japan). WF rats were originally obtained from Hiroshima University (Hiroshima, Japan), and have been maintained by brother-sister mating for over 90 generations in our laboratory. Reciprocal mating between DA and WF rats derived a total of 100 F1 rats (50 females and 50 males). In addition, a total of 130 F2 rats (68 females and 62 males) were obtained by mating (DA x WF) F1 hybrids. A stock solution of 4NQO (Nacalai Tesque, Kyoto, Japan) was prepared at 200 mg/litre in a 5% ethanol solution and stored at 4℃ until use. From 6 weeks of age, all rats were allowed access to drinking water containing 0.001% 4NQO *ad libitum* from 5 pm to 9 am. Rats were sacrificed when they became moribund or on the 180th day of the experiment. A full autopsy with histopathological examination of various organs, including the tongue, maxilla and mandible was carried out on each animal, and number and size of carcinomas of the tongue and those of the oral cavity other than the tongue were recorded.

QTLs in the F2 progeny (Table 1 & Figure 1)

QTL analysis in F2 rats was carried out as described previously.[10, 14] We used DNA of 130 F2 rats. Primers for microsatellite analyses were purchased from the Invitrogen Corporation (Carlsbad, CA, USA). The relative map positions of the microsatellite loci were based on Jacob,[15] Watanabe,[16] and the RAT MAP (http://ratmap.gen.gu.se/). For mapping, Mapmaker/EXP version 3.0b was used to create genetic maps for the datasets, and calculations of the logarithm of the odds (Lod) scores was carried out with

Table 1. QTL affecting susceptibility to 4NQO-induced tongue carcinoma

Locus	RNO[a]	Linkage indexes and %variance explained at each locus					
		Number of TC			Size of the largest TC		
		p	Lod Score	%[b]	p	Lod Score	%[b]
Tscc1	**19**	**4.82×10^{-10}**	**10.04**	**20.9**	**1.48×10^{-9}**	**8.25**	**17.9**
Tscc2	**1**	**5.07×10^{-8}**	**6.85**	**13.8**	**4.76×10^{-8}**	**6.79**	**12.9**
Tscc3	**1**	**2.48×10^{-7}**	**4.93**	**10.7**	**5.26×10^{-6}**	**3.66**	**9.21**
Tscc4	**4**	**3.34×10^{-8}**	**6.88**	**14.4**	**8.72×10^{-4}**	**3.13**	**7.01**
Tscc5	**14**	**7.48×10^{-9}**	**7.29**	**15.3**	**1.92×10^{-3}**	**3.10**	**6.81**
D5Mgh4 [c]	**5**	**1.36×10^{-4}**	**3.47**	**7.92**	**3.25×10^{-3}**	**2.83**	**5.92**
D6Rat55 [c]	**6**	**2.23×10^{-4}**	**3.29**	**7.32**	**5.44×10^{-4}**	**2.75**	**5.78**
D10Mit5 [c]	**10**	**1.45×10^{-3}**	**3.12**	**6.91**	**9.47×10^{-3}**	**2.13**	**5.49**
D17Wox22 [c]	**17**	**1.98×10^{-3}**	**3.01**	**5.41**	**1.47×10^{-2}**	**2.03**	**4.49**

[a] Rat Chromosome

[b] %Variance of phenotype explained

[c] The marker locus closest to the suggested QTL peak.

Mapmaker/QTL software.[17, 18]

Whole-genome scanning of 130 extreme-phenotype F2 rats with 267 markers showed five significant linkages to susceptibility to 4NQO-induced tongue carcinomas. We found a significant linkage of a RNO19 region with both TC#3 (TC with ≥ 3 mm diameter) and DTCmax (maximum diameter of the largest TC in mm) at the point 4cM distal from *D19Mit9* (Fig.1) (A). This confirmed linkages, between TC#3 and *D19Mit9* and also between DTCmax and *D19Mit9*, supported by χ^2 values of 46.18 and 37.95

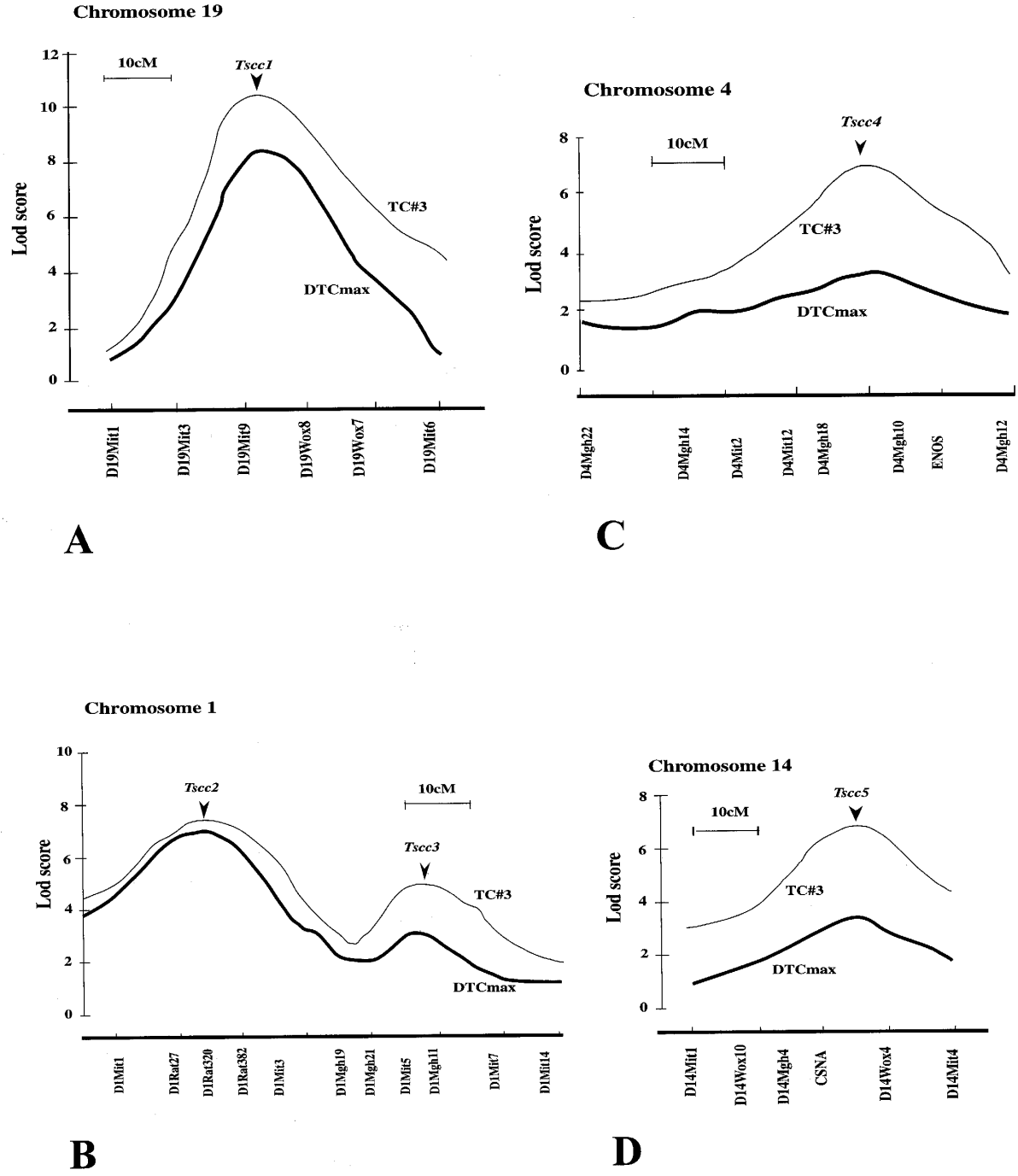

Fig. 1. A composite interval mapping of *Tscc1* on RNO 19 based on 130 F2 rats (62 male and 68 female) treated with 4NQO (A). Lod score plots for TC#3 and DTCmax are shown. The bar indicates 10 cM distance. TC#3 (Thin solid line) and DTCmax (Thick solid line). *Tscc2* and *Tscc3* on RNO1 (B). *Tscc4* on RNO4 (C). *Tscc5* on RNO14 (D). Reproduced from Tanuma, J., et al. with copyright permission of the publisher (JJCR 92, 610-616, 2001).

and p values of 4.82×10^{-10} and 1.48×10^{-9} (Lod scores for 10.04 and 8.25), respectively. The major locus was *Tscc1*, explaining the 20.9% variance in phenotype DTCmax and 17.9% in TC#3.

Another significant linkage with the distal region of RNO1 was observed 4cM distal from *D1Rat320*, with both the number of TC#3 and the size of DTCmax, and was supported by χ^2 values of 31.5 and 31.23, p values of 5.02×10^{-8} and 4.76×10^{-8} (Lod score of 6.85 and 6.79), respectively (Fig.1) (B).

The association of the same chromosome with TC#3 showed a significant linkage for the marker locus *D1Mit5*, besides *Tscc2*. To confirm that the two linkages are independent rather than a random fluctuation of phenotypes, composite interval mapping was carried out with the Cartographer software. We named this locus *Tscc3*, and the mapping was supported by an χ^2 value of 22.68, a p value of 2.48×10^{-7} (lod score of 4.93) for TC#3, 2cM distal from *D1Mit5*. This QTL, *Tscc3*, showed considerably weaker effects than *Tscc2*, because the linkage was smaller than that of *Tscc2* when found with DTCmax (lod score of 3.66) (Fig.1) (B).

On RNO4, we mapped another locus linked to TC#3. The mapping was supported by an χ^2 value of 31.65 and a p value of 3.34×10^{-8} (Lod score of 6.88) for TC#3, 4cM distal from *D4Mgh10* (Fig.1) (C). We named this locus *Tscc4* and the gene showed a stronger effect for TC#3 than for DTCmax; because the significant linkage was much smaller than found with DTCmax (Lod score of 3.13).

On RNO14, we mapped another locus linked to the number of TC#3. The DA allele is associated with increased phenotype expression, and the mapping was supported by an χ^2 value of 33.53 and a p value of 7.48×10^{-9} for TC#3 (Lod score of 7.29) in this locus 2cM distal from D14Wox4 (Fig.1) (D). We named this locus *Tscc5*; and it specifically affected TC#3, because the significant linkage was not much higher than found with DTCmax (Lod score of 3.10).

In addition, weak linkages for values of either TC#3 or DTCmax were observed at three loci, i. e., *D5Mgh4* on RNO5, *D6Rat45* on RNO6, *D10Mit8* on RNO10, and *D17Wox22* on RNO17. However, these linkages were not statistically significant (all of the Lod scores were of less than 4.3), but were suggestive. Tentatively, we have called them suggestive QTLs.

LOH analysis for F1 rats at QTLs

In 40 of 100 experimental F1 rats, at least one TC with a diameter of $\geq$ 5 mm was found at necropsy (Fig. 2). LOH analysis in F1 rats was carried out as described previously.[11, 12, 13] Data was collected automatically and analysed using the GeneScan software (Applied Biosystems). A Genotyper (Applied Biosystems) was used for allele scoring and assessment of LOH. In the case of constitutional heterozygotes, two alleles were detected in normal tissue, and if one was absent in the tumour, the result was classed as LOH. When a tumour showed an allelic imbalance rather than 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.

In all the *Tscc* loci including the suggestive QTLs, DA alleles lead to susceptibility to TC development, while WF alleles lead to resistance. To determine whether the resistant WF alleles of these loci are lost by hemizygous deletion, as are tumour suppressor genes, we first examined LOH in the TCs of the 40 (DA x WF) F1 rats with tumours $\geq$ 5 mm in diameter. LOH was frequently found on RNO1, 4, 5, and 6 and less frequently on RNO10, 14, 17 and 19. Among *Tscc* loci, *Tscc2* and *3* (on RNO1) and *Tscc4* (on RNO4) were within the segment frequently involved in LOH. *Tscc1* (RNO19) and *Tscc5* (RNO14) were not involved in LOH. In *Tscc2*, *Tscc3* and *Tscc4* in regions, the WF allele was preferentially lost. LOH frequency at *Tscc 2, 3*, and *4* increased in parallel with the size of the TC, raising the possibility that the functions of *Tscc 2*, *3* and *4* are to suppress tumour progression. Other hot-spots showing $\geq$ 30% frequency were on RNO5 and 6 bearing $p15^{INK4}$, $p16^{INK4A}$ and *Msh2*, respectively.

DNA sequencing

Samples were loaded into an ABI 310 Genetic Analyzer, electrophoresed in the analyser's capillaries through Performance Optimised Polymer 6 (ABI) at 15 kV for 25 min, and data analysed using the ABI 310 Sequence Analysis software.[11, 13]

Tumours and normal tissue in 40 F1 rats were screened for *Ha-ras* gene exon 1 and 2 mutations. DNA sequence analysis revealed a G→A transition at codon 12 in the *Ha-ras* exon 1 coding sequence, resulting in a change from Gly to Glu (shown by the symbol *a* in Fig.2b).

Another C→G mutation was found at codon 61 in the exon 2 coding sequence, resulting in a Gly rather than a Gln residue (*b* in Fig.2b). The incidence of *Ha-ras* mutations was 70% (28/40 tumours) for codon 12 and 25% (10/40 tumours) for codon 61. The presence of *Ha-ras* mutations in almost all tumours points to a role for this gene in TC pathogenesis.[11]

Analysis of $p15^{INK4B}$ revealed two mutations, one at codon 9, GGC (Gly) to GAC (Asp) in exon 1 (*c* in Fig.2b), and one at codon 87, ACG (Thr) to ACA (Thr) in exon 2 (*d* in Fig.2b). In $p16^{INK4A}$, one of the mutations was found at codon 9, GCC (Ala) to GC (one base deletion with frameshift) in exon 1 (*e* in Fig.2b), and the other at codon 16, CGT (Arg) to CAT (His) in exon 1 (*f* in Fig.2b). The two missense mutations were accompanied by LOH, possibility leading to the loss of oncosuppression.[13]

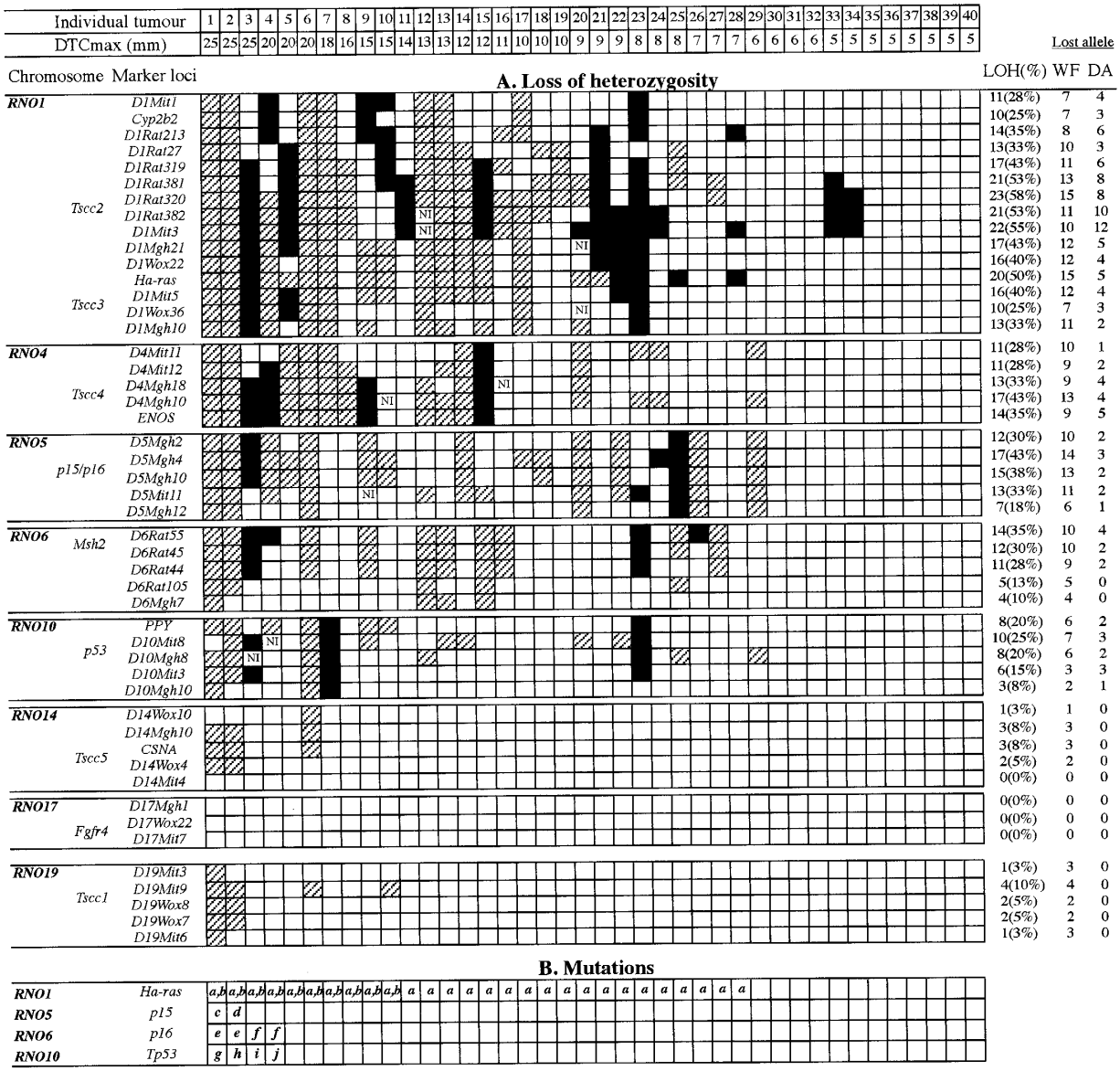

Fig. 2. Genetic alterations in individual TCs arranged in descending order of tumour size. DTCmax, maximum diameter of the largest TC in mm.

a. LOH. W, loss of the WF allele; D, loss of the DA allele; empty box, no LOH; NI, not informative.

b. Mutations. *Ha-ras*; mutation at (a) codon 12 or (b) codon 61. *p15*: mutation at (c) codon 9 in exon 1 and (d) codon 87 in exon 2. *p16*: mutation at (e) codon 9 in exon 1 and (f) codon 16 in exon 1. *Tp53*: mutation at (g) codon 174 in exon 5, (h) codon 211 in exon 6, (i) codon 247 in exon 6, and (j) codon 256 in exon 6.

A low frequency of LOH was observed at *D10Mit8* (25%) on RNO10, close to the *Tp53* location. Analysis of individual tumours for mutation of *Tp53* revealed only four large tumours ≥ 20 mm in diameter with a mobility shift, the 1st in exon 5, due to a transitional mutation at codon 174, TGC (Cys) to TAC (Tyr) (*g* in Fig.2b), the 2nd in exon 6 at codon 211, CGG (Arg) to CTG (Leu) (*h* in Fig. 2b), the 3rd at codon 247, CGG (Arg) to CGT (Arg) (*i* in Fig. 2b), and the 4th at codon 256, GAA (Glu) to CAA (Gln) (*j* in Fig.2b). These results indicate that mutations in *Tp53* are rare in TCs occurring only in the largest tumours. Furthermore, no mutations in *Msh2* and *Fgfr4* were found in any of the 40 TCs (data not shown).

Methylation analysis

DNA methylation of the $p15^{INK4B}$ and $p16^{INK4A}$ genes was examined by amethylation-specific polymerase chain reaction (MS-PCR), as previously reported by Herman et al.[19] Briefly, 3μg genomic DNA was treated with 3 M sodium bisulfite and 10 mM hydroquinone, purified using the Wizard DNA purification system (Promega, Madison, WI) and desulfonated with 0.3 M NaOH. PCR amplification was performed with approximately 200 ng of treated DNA as the template. Primers for $p15^{INK4B}$ and $p16^{INK4A}$ genes were synthesized as previously reported by Swafford[20] and Abe et al.[21] Two sets of primers were designed, one specific for DNA methylated at CpG sites and another specific for fully unmethylated DNA.

We observed that $p16^{INK4A}$ inactivation was mostly associated with aberrant methylation in the 5'CpG island of its promoter region, whereas $p15^{INK4B}$ was rarely affected. Methylation was observed in 2/40 (5%) for the $p15^{INK4B}$ gene and 8/40 (20%) for the $p16^{INK4A}$ gene, shown in Fig.3.

mRNA expression of the $p15^{INK4B}$ and $p16^{INK4A}$ genes

Expressions of the $p15^{INK4B}$ and $p16^{INK4A}$ genes were analyzed in normal and the 40 TC samples by quantitative real-time RT-PCR.[13] $p15^{INK4B}$ and $p16^{INK4A}$ gene expressions were associated with DTCmax and TC#5, as shown in Table 1. $p15^{INK4B}$ and $p16^{INK4A}$ genes mRNA expression levels are shown as the ratio of $p15^{INK4B}$ and $p16^{INK4A}$ / β-actin in Fig 4.

Candidate genes for QTLs

Tscc1: We considered that the strongest linkage of *Tscc1* was to the locus of the quinone oxidoreductase gene, *NQO1 (Dia4)*, which is the gene of DT-diaphorase or NADH-cytochrome b5 reductase) on RNO19.[22, 23] Quinone oxidoreductase is one of the major enzymes that convert 4NQO to the more active metabolite, 4-hydroxyaminoquinoline 1-oxide (4HAQO). Recently, we clarified that the locus of *NQO1* is located between *D19Mit9* and *D19wox8*, as is the locus of *Tscc1* in rat.[24, 25] *JunB* a proto-oncogene, is another potential candidate gene for *Tscc1*,[26, 27] and may act to either promote or inhibit carcinogenesis, playing a role in critical cell functions such as proliferation, differentiation

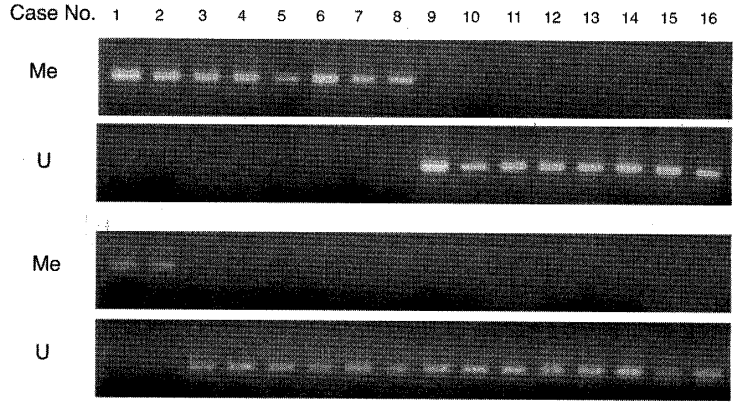

Fig. 3. Methylation-specific PCR of the 5'CpG islands of $p15^{INK4B}$ and $p16^{INK4A}$ in 4NQO-induced TC. Methylation-specific PCR was carried out according to the method of Herman[19] with minor modifications. Primers for the $p16^{INK4A}$ and $p15^{INK4B}$ genes were synthesized as reported by Swafford et al.[20] and Abe et al.[21], respectively. Me, methylated; U, unmethylated. Case numbers correspond with those in Table 1. Reproduced from Ogawa, K., et al. with copyright permission of the publisher (Oral Oncology 2006, in press).

and apoptosis.

Tscc2: One of the candidate genes for *Tscc2* may be at the *cyp2a* locus located at 19q13.2; this is a structural gene for the cytochrome P450 enzyme, phenobarbital inducible, constituting a superfamily of membrane-bound enzymes that function as terminal mono-oxygenizes in the metabolism of a broad variety of endogenous and exogenous compounds including chemical carcinogens.[28, 29] When searching for LOH at *Tscc* loci in 4NQO-induced TCs in (DA x WF) F1 rats, we found that the chromosomal region of the *Tscc2* was a frequent target of LOH; the frequency increasing as the size of the TCs increased. The resistant WF allele was selectively lost, which suggests that *Tscc2* might encode a tumour suppressor gene.

Tscc3: From its map position, we can assume several candidate genes for *Tscc3* from its map position, including *Ha-ras*[13, 30, 31] and *Gstp*.[32, 33] *Ha-ras* genes have previously been implicated in cancer predisposition in humans, mice, and rats. Loss of the wild-type *Ha-ras* allele may cause an unopposed mutant activated by p21 protein that could potentially lead to gene amplification. *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 >7 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.

A earlier study of ours showed *Gstp* to be a promising marker for 4NQO-induced tongue carcinogenesis in rat. All TCs invariably expressed *Gstp*, whereas tongue tissues from normal control animals were negative. This may contribute in some way to the difference in susceptibility to 4NQO between the DA and WF strains.

Tscc4: One of the candidate genes for *Tscc4* is *Tgf α* , located on RNO4 and encoding the transforming growth fac tor- α (TGF α).[34, 35] Other candidate genes for *Tscc4*

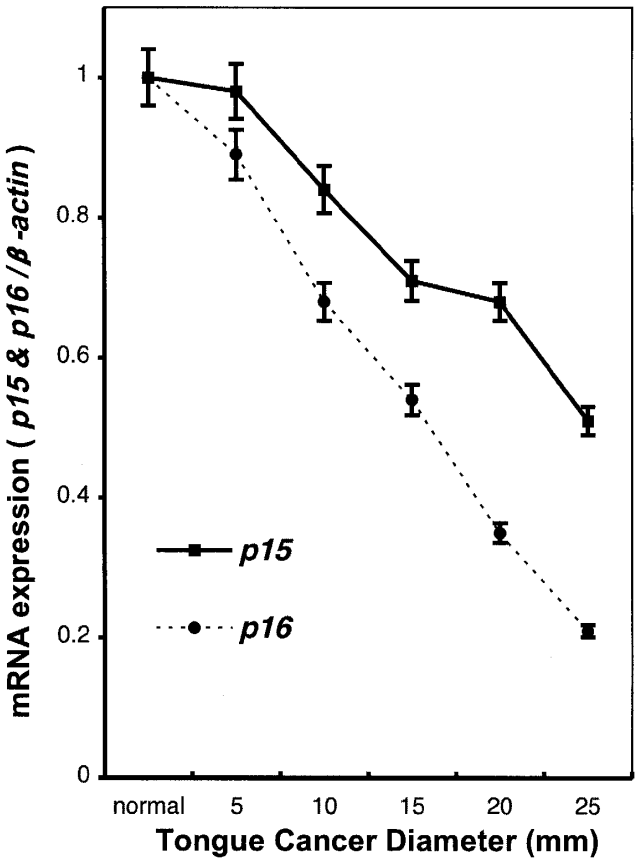

Fig. 4. Expression levels of mRNA of the $p15^{INK4B}$ and $p16^{INK4A}$ genes. The level is shown as the ratio of $p15^{INK4B}$ and $p16^{INK4A}$ genes / b-actin. $p15^{INK4B}$ and $p16^{INK4A}$ gene expressions in 40 TCs. Reproduced from Ogawa, K., et al. with copyright permission of the publisher (Oral Oncology 2006, in press).

mapped on RNO4 may be *Ki-ras*[36, 37] and *Pthlh*.[38, 39] *Pthlh* encodes the parathyroid hormone-related protein (PTHrP), which is regarded as a predominant cause of hypercalcemia of malignancies observed in several types of human cancers. Manenti et al.[40] 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 survery on *Pthlh*, in respect to 4NQO-induced rat tongue carcinogenesis, and some important and interesting results will hopefully be presented in the future (Tanuma, unpublished data).

Tscc5: For *Tscc5*, *Egfr* and *Tp53l2* can be mentioned as possible candidate genes. Some important and novel aspects of the biological behaviours of oral cancers should be brought by further surveys regarding these genes.

Suggestive-QTL regions: Finally, the candidate genes for RNO5, RNO6, RNO10 and RNO17 may be mutations in tumours - the suppressor genes *p15*, *p16*, *p53* and *Fgfr4* were only observed in a few large TCs in our model.

Their contribution to the tumour progression in TCs was unclear but they appeared to be somehow involved at an enlarged stage, possibly by genetic instability. The lower frequency of genetic alterations in *p53* in this model contrasts with other reports of high levels of *p53* expression and its frequent mutation in human TCs, and of LOH in oral cancers.

Conclusive remarks

We have identified five susceptibility/resistance genes for 4NQO-induced rat tongue carcinogenesis, *Tscc1, Tscc2, Tscc3, Tscc4* and *Tscc5* on RNO19, 1, 4, and 14. The mapping of the genetic loci conferring susceptibility/resistance to TC opens up important avenues for future studies. A high incidence of LOH was observed at the chromosomal sites where genes involved in susceptibility/resistance to tongue carcinogenesis are located, and indicates that putative oncosuppressor genes also play important roles in rat tongue carcinogenesis. This article presents our extensive genetic and epigenetic, including assayed on cDNA Microarrays and PowerBlot analysis of rat 4NQO-induced TCs, and a discussion of possible candidate genes with special reference to TCs.

In conclusion, genetic and epigenetic studies of susceptibility/resistance to chemical carcinogenesis have revealed polygenic control to include several QTLs, each locus providing only a small phenotypic effect. Identification of involved genes and their functions should lead to a better fundamental understanding of the fundamentals of carcinogenesis and cancer prediction in humans, and will aid in devising novel therapeutic strategies.

Acknowledgments

The author thanks Dr. Hiai, H., (Shiga Medical Centre for Adults), Dr. Shisa, H., (Saitama Cancer Centre) and Emeritus Professor Kitano, M., for critical reading of the manuscript, and also express thanks to Professor Semba, I., at Department of Oral Pathology, Kagoshima University Graduate School of Medical and Dental Sciences and staff members for their various contributions. This research was supported by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan (No. 13-2), and Grants-in-Aid from the Japanese Ministry of Education, Science, Sports and Culture of Japan (No. 11470399, 12470399).

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