A novel rasH2 mouse carcinogenesis model that is highly susceptible to 4-NQO-induced tongue and esophageal carcinogenesis is useful for preclinical chemoprevention studies

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We investigated the susceptibility of 4-nitroquinoline 1-oxide (4-NQO)-induced tongue carcinogenesis in male CB6F1-Tg-rasH2 @Jcl mice (Tg mice). The Tg mice were administered 4-NQO (20 p.p.m. in drinking water) for 2, 4, 6 or 8 weeks, and thereafter they were untreated up to week 24. At week 24, a higher incidence (80%) of tongue neoplasm with dysplasia was noted in the mice that received 4-NQO for 8 weeks in comparison with the other groups (20% incidence for each) treated with 4-NQO for 2, 4 and 6 weeks. Esophageal tumors also developed in the Tg mice as 4-NQO. Immunohistochemical observation revealed that the EP receptors, especially EP1 and EP2, expressed in the tongue and esophageal lesions induced by 4-NQO, thus suggesting the involvement of prostaglandin (PG) E2 and EP1,2 receptors in the tongue and esophageal carcinogenesis. Using this animal model, we investigated the potential chemopreventive ability of pitavastatin (1, 5 and 10 p.p.m. in diet for 15 weeks), starting 1 week after the cessation of 4-NQO-exposure (20 p.p.m. in drinking water) for 2, 4, 6 or 8 weeks, and thereafter we investigated the potential chemopreventive ability of pitavastatin (1, 5 and 10 p.p.m. in diet for 15 weeks), starting 1 week after the cessation of 4-NQO-exposure (20 p.p.m. in drinking water) for 2, 4, 6 or 8 weeks, and thereafter

Introduction

Oral cancer is the 11th most common human neoplasm and accounts for 3% of all newly diagnosed cancers (1). While this epithelial malignancy mainly developed in the elderly in the past, an increased number of young patients with oral malignancy being seen at present in Europe, North America and India are increased today (2,3). Such prevalence is largely associated with the habit of using chewing tobacco and other related products. More than 90% of oral cancers are histopathologically squamous cell carcinomas (SCCs) (4), with >300 000 new cases being diagnosed every year worldwide (5). Despite efforts to improve the overall outcome, the survival rates of oral cancer patients have not changed for the last 20 years. Since ~50 to 70% of patients die within 5 years due to local recurrence, invasion or metastasis in the esophagus and/or lung, or second primary cancers, generally elsewhere in the oral cavity (‘field carcinization’ theory) (6–8), the prognosis is poor. The detection of many cancers including oral cancer is often too late for successful intervention. Therefore, if appropriate biological markers can identify pre-malignant lesions, then we can detect them and prevent cancer development before they progress to malignancies by the use of chemoprevention modalities or other therapeutics (9,10). The incidence rates for one of the oral pre-malignant lesions, leukoplasia with dysplastic nature, in the Japanese are somewhat higher than those reported from India, since the risk habits of the two countries are both markedly different (11).

The development of oral cancer is a multistep process, which includes hyperplasia, dysplasia and finally neoplasm (benign and malignant) (10). During these steps, the accumulation of genetic alterations, including chromosomal aberrations, DNA mutations, amplifications or deletions and/or epigenetic alterations (methylation) occur (12–15). These events are often influenced by exposure to environmental agents. These include tobacco smoke, alcohol beverages and viruses as the major risk factors (10). In patients, the analysis of these events during the multistep process is hampered by the unavailability of biopsies obtained at all stages (namely hyperplasia, dysplasia and neoplasm) of carcinogenesis. However, animal models of oral carcinogenesis allow the reproducible isolation of all stages, including normal tissues, which are then amenable to pathological, genetic and biochemical analyses. Thus, appropriate animal models are essential for investigating the transition of oral squamous epithelium from normal through dysplastic states and ultimately into SCC. There are several animal models for oral carcinogenesis (16). Among them, the systemic application of 4-nitroquinoline 1-oxide (4-NQO) via drinking water is easily able to induce tongue tumors in rats (10,17) and mice (18). Recent reviews (5,19) have concluded that the rat 4-NQO-induced tongue carcinogenesis is the main model for simulating the process of oral carcinogenesis in humans with a fair degree of reliability. However, in contrast to rats (10), oral and esophageal tumors develop in mice that receive 4-NQO in drinking water (18). This suggests that a mouse model initiated with 4-NQO is useful for investigating field carcinization in the regions (8).

H-ras mutations are implicated in human and murine oral carcinogenesis (20–22). Ras mutations are observed in oral cancer with different frequencies in different countries (20,23). Our recent findings suggest that human c-Ha-ras proto-oncogene-carrying transgenic rats are highly susceptible to a water-soluble carcinogen 4-NQO in their tongue (24), thus suggesting an excellent rat model for investigating oral cancer development and treatment/chemoprevention of this malignancy. Few studies, nevertheless, have addressed the aspect of ras activation, while considering its possible role in mouse oral carcinogenesis. CB6F1-Tg rasH2 @Jcl mice (Tg mice) were developed by Saitho et al. (25) to evaluate the association of chemically induced transgene expression and tumor induction (26,27). Three copies of the human transgene were integrated into the mouse genome in a tandem array through pronuclear injection. The Tg mice are hemizygous for the human c-Ha-ras transgene under control of its endogenous promoter and enhancer sequences. Expression of the transgenic protein is observed in normal tissues and increased ~2-fold in chemically induced tumors (28). Mutation of the endogenous mouse ras genes or of the transgene is infrequent and unpredictable.

Prostaglandins (PGs) are generated via the cyclooxygenase (COX)-1 and -2 and are known to be elevated in the rat tongue following 4-NQO exposure (29). COX-1 and COX-2 both catalyze the first reaction in the conversion of arachidonic acid into PGs, of which prostaglandin E2 (PGE2) is the major product found in the rat tongue exposed to 4-NQO (29). COX-1 is the constitutively expressed isoform, and COX-2 is the inducible isoform (30). Although a number of studies indicate that the inhibition of PGE2 biosynthesis through COX-2 expression contributes to the suppression of cancer development in a variety of tissues (31), including tongue cancer (24,32). COX-2 deregulation is reported in smokeless tobacco-related oral

Abbreviations: COX, cyclooxygenase; PAP, squamous cell papilloma; PBS, phosphate buffer saline; PCNA, proliferative cell nuclear antigen; PG, prostaglandin; PGE2, prostaglandin E2; SCC, squamous cell carcinoma; 4-NQO, 4-nitroquinoline 1-oxide.
carcinogenesis (33,34). COX-1 is also involved in carcinogenesis in the colon (35,36) and the tongue (T. Tanaka, Y. Yasui, M. Kim, in preparation). Therefore, both isoforms are involved in carcinogenesis (37). PGE2 manifests its biological activities through four known G-protein-coupled membrane receptors, EP1, EP2, EP3, and EP4 (38). Recently reported findings suggest that these PGE2 receptors contribute to murine tumorigenesis in the colon (37), skin (39) and mammary gland (40). We have recently reported that an EP3-selective antagonist, ONO-8711, can effectively inhibit 4-NQO-induced tongue carcinogenesis in rats (41), thus suggesting that certain EP receptors are involved in chemically induced tongue carcinogenesis. However, detailed investigations on the expression of all EP receptors are scarce. In the present study, we investigated the susceptibility of 4-NQO-induced tongue carcinogenesis in male Tg mice to inspect our previous finding that the tongue of human c-Ha-ras proto-oncogene carrying transgenic rats is highly susceptible to 4-NQO-induced carcinogenesis. In addition, we immunohistochemically examined the different expression of four EP receptors in normal squamous epithelium, in dysplasia and in neoplasms in the tongue of Tg mice that are adequately evaluated for consideration for carcinogenicity testing of pharmaceutical candidates (42). Subsequently, the chemopreventive potential of a lipophilic statin, pitavastatin (43), against 4-NQO-induced carcinogenesis in the Tg mice was investigated to determine whether this animal model can be utilized for preclinical animal studies for cancer chemoprevention. Pitavastatin affects the expression of p21WDMD that is involved in oral cancer development (21,44), and ras is a target for cancer chemoprevention by statins (45). In addition, pitavastatin is able to inhibit NAD(P)H oxidase activity (46), which is involved in 4-NQO-induced mutagenicity, carcinogenicity and oxidative stress (47). Statins modulate PGs biosynthesis and downregulate COX-2 expression (48). We thus suspected that pitavastatin affects oral carcinogenesis in the Tg mice treated with 4-NQO.

Materials and methods

Animals, diets and carcinogen

Male CB6F1-Tg rasH2@Icl mice (Tg) and non-Tg males bred by CLEA Japan (Tokyo, Japan) at 6 weeks of age were obtained and maintained in plastic cages in an experimental room controlled at 23 ± 2 °C, 50 ± 10% humidity and lighting (12 h light/dark cycle). They were all allowed free access to a powdered basal diet of CRF (Charles River Formula)-1 (Oriental Yeast Co., Ltd, Tokyo, Japan) and tap water. The experiments were conducted according to the ‘Guidelines for Animal Experiments in Kanazawa Medical University’. A carcinogen, 4-NQO (98% pure, CAS no. 56-57-5, Wako Pure Chemical Ind., Osaka, Japan) was used to induce tongue and/or esophageal tumors in this study.

Experimental procedure for developing the rasH2 tongue and esophageal carcinogenesis model

A total of 22 Tg and 24 non-Tg male mice were transferred to the experimental room after a 1 week quarantine. They were given tap water containing 20 p.p.m. 4-NQO for 2 groups (1, n = 5, each of Tg and non-Tg mice), 4 (group 2, n = 5, each of Tg and non-Tg mice), 6 (group 3, n = 5, each of Tg and non-Tg mice) and 8 weeks (group 4, n = 5, each of Tg and non-Tg mice), and thereafter they received no further treatments. Two rasH2 mice (group 5) and four non-Tg mice (group 5) were untreated controls. The animals were killed at 24 weeks to determine the occurrence of preneoplasms and neoplasms in the tongue and esophagus. At killing by exsanguination under deep ether anesthesia, macroscopic observations were carefully performed and the numbers of grossly visible tumors in the tongue and esophagus were recorded, and then these tissues with or without tumors were processed for histopathological examination after being fixed in 10% buffered formalin. The tongues and esophagus with or without lesions were also processed to assess the expression of EP receptors and a cell proliferation biomarker by immunohistochemistry. For a histological examination, the tissue and gross lesions were fixed in 10% buffered formalin, embedded in paraffin blocks and then the histological sections were stained with hematoxylin and eosin. Epithelial lesions (dysplasia and neoplasia) in both tissues were diagnosed according to the criteria described by Kramer et al. (49). To determine the multiplicity of the tongue and esophageal lesions, the tissue specimens were examined for gross lesions without the use of any magnification aid. Tissue specimens from both the tongue and esophagus were cut in half longitudinally and each tissue specimen was fixed in 10% buffered formalin. Each tissue specimen was totally submitted as multiple transverse sections for histological processing. This averaged 5–6 pieces/tissue specimen of the tongue and 10–12 pieces/tissue specimen of the esophagus. The lesions in the tissues were counted on all slides stained with hematoxylin and eosin, and then the sum was divided by the number of slides, and they were expressed as the mean ± SD.

Immunohistochemistry of EP receptors

An immunohistochemical analysis of the EP receptors, EP1, EP2, of the tongue and esophageal specimens from all the mice was done. Four-micrometer-thick paraffin sections of the 10% buffered formalin-fixed tongue and esophagus were mounted on salinized glass and deparaffinized in xylene and descending strengths of ethanol. The sections were washed in 0.05 M phosphate buffer saline (PBS, pH 7.6). The sections were next incubated in 3% hydrogen peroxide in methanol for 5 min at room temperature. After being rinsed with PBS three times for 9 min and exposed to PBS/0.1% bovine serum albumin for 5 min at room temperature to reduce non-specific binding, the slides were incubated overnight at 4°C with rabbit polyclonal antibodies against EP1 (code no. 101740, Cayman Chemical Co., Ann Arbor, MI), EP2 (code no. 101760, Cayman Chemical Co.), EP3 (code no. 101750, Cayman Chemical Co.) and EP4 (code no. 101775, Cayman Chemical Co.), all being diluted at 1:1500 in PBS. The slides were rinsed three times for 9 min in PBS and incubated for 30 min in Dako Envision + Peroxidase Rabbit (K4003, Dako Japan, Kyoto, Japan). The slides were rinsed three times for 9 min in PBS. Thereafter, they were incubated in biotinylated anti-rabbit IgG (1:200 in PBS, 1 h). Finally, the sections were counterstained with Mayer’s hematoxylin. The negative controls were prepared by substituted primary antibody with buffered saline. To estimate the degree of staining of the EP receptors in the lesions, the grading system (grade 0–5) was used: grade 0–10, no immunoreactivity; grade 1, very weak immunoreactivity in 11–20% of cells; grade 2, weak immunoreactivity in 21–30% of cells; grade 3, moderate immunoreactivity in 31–40% of cells; grade 4, moderate immunoreactivity in 41–50% of cells and grade 5, marked immunoreactivity in 51–100% of cells. The results of the immunohistochemical analysis were blindly scored as a ‘normal’ appearing squamous epithelium, severe dysplasia, squamous cell papilloma (PAP) and SCC that developed in all groups.

Preclinical chemoprevention study of pitavastatin using rasH2 male mice

Since no tumors developed in the tongue and esophagus of non-Tg mice, a preclinical chemoprevention study for evaluating possible inhibitory effects of pitavastatin on 4-NQO-induced tumorigenesis was conducted in male Tg mice. Forty-two Tg males were divided into six experimental and control groups. Groups 1 through 4 were given 4-NQO (20 p.p.m. in drinking water) for 8 weeks. Group 1 received no further treatment. Starting 1 week after the carcinogen administration, EP receptors in the tongue and esophagus of the mice were measured. All the remaining animals were given the diet containing pitavastatin at dose levels of 1, 5 and 10 p.p.m. for 15 weeks respectively. Group 5 was given the diet mixed with 10 p.p.m. pitavastatin. Group 6 was an untreated control. At week 24, all mice were killed by exsanguination under deep ether anesthesia, and the macroscopic inspections were carefully performed. After killing, the number of grossly visible tumors in the tongue, esophagus and other tissues were recorded, photos were taken and then the organs with lesions were processed for histopathological examination after fixation in 10% buffered formalin. Each tongue was cut in half longitudinally: one half was used for histopathology and immunohistochemistry and the remainder for PGE2 determination. Five serial sections (4 μm each) were made from the tissue specimens after embedding in paraffin. One section was used for histopathology and the others for immunohistochemistry of the EP receptors, EP1 and EP2, proliferative cell nuclear antigen (PCNA) and cyclin D1.

The histopathological diagnosis was done on the sections stained with hematoxylin and eosin. Epithelial lesions (dysplasia and neoplasia) in the tongue and esophagus were diagnosed according to the criteria described by Kramer et al. (49). The multiplicities of the tongue and esophageal lesions were determined, as described above. An immunohistochemical analysis of EP1 and EP2 was performed as described above. The intensity and localization of the immunoreactivity against the EP1 and EP2 primary antibodies were determined on the tongue sections containing SCC using a microscope (Olympus BX41, Olympus Optical Co., Ltd, Tokyo, Japan) and evaluated for the intensity of the immunoreactivity of EP1 and EP2, primary antibody and cell proliferation in 4–16 fields of view on the tongue sections containing SCC using a microscope (Olympus BX41, Olympus Optical Co., Ltd, Tokyo, Japan) and evaluated for the intensity of the immunoreactivity of EP1 and EP2, primary antibody and cell proliferation in 4–16 fields of view
immunohistochemical analysis kit (Dako Japan). The labeling indices were calculated by counting the PCNA-positive nuclei in at least 100 cells at three different fields of tongue and esophagus from each mouse. Cyclin D1 immunohistochemistry was also performed for the evaluation of cell cycle activity of tongue SCCs. Briefly, 4 µm paraffin-embedded sections from tongue SCCs developed in groups 1 through 4 were deparaffinized with three changes of xylene and hydrated using a graded series of alcohol. Slides were incubated twice in 1 mM ethylenediaminetetraacetic acid (pH 8.0) at 121°C in an autoclave, 5 min each to effect antigen retrieval before staining, then exposed overnight to 1:100 diluted cyclin D1 mouse monoclonal antibody (Novocastra Laboratories, Newcastle upon Tyne, UK). Slides were then developed by the avidin–biotin–peroxidase complex methods. Cells were considered positive for cyclin D1 when definite nuclear staining was identified. The positive cell ratio (percentage) for cyclin D1 was determined by randomly observing 100 cancer cells under magnification ×400 (>25 fields) to score. Positive cell ratios were calculated as numbers per 100 cells.

For the determination of the PGE₂ content, the tongue tissue (~100 mg) without tumors was obtained from groups 1–6 after killing. The samples were then placed into microcentrifuge tubes containing 1 ml of sodium phosphate buffer (10 mmol/l, pH 7.4) and finely minced with scissors for 15 s. The samples were then incubated for 20 min at 37°C in a shaking water bath. Following the incubation period, the samples were centrifuged at 9000g for 30 s and the supernatants collected. The supernatants were then flash frozen in liquid nitrogen and stored at −80°C for subsequent determination of PGE₂ content. The PGE₂ level was assayed using the PGE₂ ELISA kit (Cayman Chemical Co.) according to the manufacturer’s instructions.

Statistical analysis
A statistical analysis of the incidence of lesions was performed using Fisher’s exact probability test, and the other results expressed as the mean ± SD were analyzed by Student–Newman–Keuls multiple comparison test using the GraphPad InStat software (version 3.05, GraphPad Software, San Diego, CA). A level of P < 0.05 was considered to be statistically significant.

Results
Animal model study
General observation. All Tg and non-Tg mice in groups 1–5 showed a good tolerance for 4-NQO-exposure in their drinking water. The growth curves during the study did not significantly differ among the groups with different treatment periods and between the two phenotypes (data not shown). After killing, the mean body, liver and relative liver weights did not significantly differ among the groups (data not shown).

Tongue and esophageal tumors development. Whereas no tumors or dysplastic lesions developed in any organs, including the tongue, esophagus and forestomach, of the non-Tg mice that received drinking water with or without 4-NQO, exophytic tongue and esophageal tumors developed in the Tg mice that received 4-NQO (Figure 1A). One large forestomach tumor, histologically diagnosed as an SCC, developed in a rasH2 mouse that received 4-NQO for 6 weeks. Such neoplasms were not observed in the untreated Tg mice. A large forestomach tumor developed in one mouse of group 3, but no tumors were observed in tissues other than the tongue and esophagus of any animals. The cumulative distribution of the tongue and tumors is illustrated in Figure 1B. When compared with the rasH2 mice that received 4-NQO for 2, 4 or 6 weeks, a number of tongue and esophageal tumors developed, distributed in whole parts of the tissues. The tongue and esophageal tumors were histopathologically PAP and SCC (Figure 1C). As summarized in Figure 2A, the number of tumors increased with the increased duration of 4-NQO-exposure: the highest incidence and multiplicity of tongue (100% incidence with a multiplicity of 2.80 ± 1.30 per tongue) and esophageal (60% incidence with a multiplicity of 1.40 ± 1.67 per esophagus) tumors were observed in the Tg mice given 4-NQO for 8 weeks. Preneoplastic lesions that were diagnosed to be dysplasia with various degrees of atypia also developed in the tongue and esophagus with or without tumors in the Tg mice that received 4-NQO (Figure 2B). The incidence and multiplicity of dysplasia were increased when the duration of the 4-NQO exposure was increased. In addition, the histopathological grade of dysplasia depended on the duration of the 4-NQO exposure.

Statistical analysis
As indicated in Figure 3, the neoplasms and dysplasias that developed in the tongue and esophagus expressed EP (EP1, EP2) receptors, while the expression in the non-lesional and normal squamous epithelium of the tongue and esophagus was quite low or absent. Among the EP receptors, EP1 and EP2 expressed as strongly positive in the dysplastic lesions and neoplasms in these tissues. The expression was observed in the cytoplasm of the cells that composed the lesions.

EP receptors' immunohistochemistry of tongue and esophageal lesions. As indicated in Figure 3, the neoplasms and dysplasias that developed in the tongue and esophagus expressed EP1 and EP2 receptors, while the expression in the non-lesional and normal squamous epithelium of the tongue and esophagus was quite low or absent. Among the EP receptors, EP1 and EP2 expressed as strongly positive in the dysplastic lesions and neoplasms in these tissues. The expression was observed in the cytoplasm of the cells that composed the lesions.

Chemoprevention study
General observation. All Tg mice belonging to groups 1–5 showed a good tolerance of the treatment with 4-NQO and/or pitavastatin. The mean body weight (29.0 ± 5.5 g) of group 2 (4-NQO plus pitavastatin) and the mean body weight (28.4 ± 4.5 g) of group 4 (4-NQO plus 10 p.p.m. pitavastatin) were significantly lower than that (body weight, 35.9 ± 6.1 g; liver weight, 1.81 ± 0.29 g and relative liver weight, 5.07 ± 0.31 g/100 g body wt) of group 1 (4-NQO alone) at week 24 (P < 0.05, for each comparison). However, the values of group 4 (4-NQO plus 10 p.p.m. pitavastatin) were comparable with those of group 1 (data not shown).

Tongue and esophageal tumor development. As summarized in Table I, tongue tumors with a 100% incidence and a multiplicity of 2.20 ± 1.23 developed in rasH2 mice that received 4-NQO alone.
Feeding with pitavastatin reduced the incidence and multiplicity of tongue tumors: 5 p.p.m. pitavastatin in the diet after 4-NQO-exposure significantly lowered the incidence and multiplicity of tongue PAP (P = 0.03316 and P = 0.05, respectively) and 10 p.p.m. pitavastatin in the diet significantly lowered the incidence of tongue PAP (P = 0.0316), SCC (P = 0.0229), total tumors (PAP + SCC, P = 0.0065) and the multiplicity of total tongue tumors (P < 0.05).

Fig. 2. Incidence and multiplicity of (A) tumors and (B) dysplasia that developed in the tongue and esophagus of rasH2 mice that received 4-NQO in their drinking water.

Regarding tongue dysplasia, feeding with 1 and 5 p.p.m. pitavastatin significantly reduced the incidence of mild dysplasia (P = 0.0065 and P = 0.0015, respectively), and 10 p.p.m. pitavastatin in the diet
significantly lowered the incidence of severe tongue dysplasia ($P = 0.0306$, Table I). Similarly, dietary feeding with 5 p.p.m. pitavastatin significantly reduced the multiplicity of mild dysplasia of the tongue ($P < 0.05$), and pitavastatin feeding at all dose levels significantly lowered the multiplicity of total dysplasia (mild, moderate and severe dysplasia) ($P < 0.01$ at 1 p.p.m., $P < 0.001$ at 5 and 10 p.p.m.).

Regarding esophageal tumors, the incidence of SCC was significantly reduced by feeding with 10 p.p.m. pitavastatin ($P = 0.0076$). Although the incidence and multiplicity of esophageal SCC and total tumors (PAP + SCC, Table II) decreased after the administration of pitavastatin at all dose levels, the reduction did not reach statistical significance (Table II). Treatment with pitavastatin at all dose levels lowered the multiplicity of esophageal dysplasia to various degrees; the differences were not significant (Table II).

**Fig. 3.** Immunohistochemical expression of EP$_1$–4 receptors in the normal epithelium and lesions induced by 4-NQO in tongue and esophagus. Expression of EP$_1$–4 receptors is weak in the normal squamous epithelium of both tissues. Expression of EP$_1$ and EP$_2$ is strong in the dysplasia, PAP and SCC, whereas that of EP$_3$ and EP$_4$ is weak in the lesions. nl, normal squamous epithelium; DYS, squamous cell dysplasia. Hematoxylin and eosin stain and immunohistochemistry of EP$_1$–4, original magnification $\times 20$.

**PCNA-labeling index and cyclin D1-positive index of tongue SCC.** The PCNA-labeling indexes of SCCs developed in the Tg mice belonging to groups 1–4 are illustrated in Figure 4. The index of group 1 (Figure 4A) was the greatest and that of group 4 (Figure 4A) was the lowest among the groups: group 1, 74.2 ± 9.9; group 2, 53.6 ± 15.0; group 3, 39.8 ± 7.7 and group 4, 33.4 ± 8 (Figure 4A). The values of groups 2 ($P < 0.01$), 3 ($P < 0.001$) and 4 ($P < 0.001$) were significantly smaller than those of group 1. The index of group 4 ($P < 0.01$) was also significantly lower than that of group 2. As to
Table I. Incidence and multiplicity of tongue dysplasia and neoplasms in the rasH2 mice that received 4-NQO and/or pitavastatin

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment</th>
<th>Dysplasia Incidence</th>
<th>Tumor Incidence</th>
<th>Multiplicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mild DYS* Moderate DYS Severe DYS Total</td>
<td>Mild DYS Moderate DYS Severe DYS Total</td>
<td>PAP SCC Total</td>
</tr>
<tr>
<td>1</td>
<td>4-NQO alone</td>
<td>10/10 (100%) 10/10 (100%) 8/10 (100%)</td>
<td>10/10 (100%)</td>
<td>1.10 ± 0.32* 1.50 ± 0.71 1.40 ± 1.08</td>
</tr>
<tr>
<td>2</td>
<td>4-NQO → p.p.m. pitavastatin</td>
<td>3/8 (38%)</td>
<td>5/8 (63%)</td>
<td>4/8 (50%)</td>
</tr>
<tr>
<td>3</td>
<td>4-NQO → p.p.m. pitavastatin</td>
<td>2/8 (25%)</td>
<td>7/8 (88%)</td>
<td>4/8 (50%)</td>
</tr>
<tr>
<td>4</td>
<td>4-NQO → p.p.m. pitavastatin</td>
<td>4/8 (50%)</td>
<td>5/8 (63%)</td>
<td>2/8 (25%)</td>
</tr>
<tr>
<td>5</td>
<td>10 p.p.m. pitavastatin</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
</tr>
<tr>
<td>6</td>
<td>None</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
</tr>
</tbody>
</table>

*DYS, squamous cell dysplasia.

| Mean ± SD. |

| Significantly different from group 1 based on the Fisher’s exact probability test (c P = 0.0065, e P = 0.0015, h P = 0.0316, i P = 0.0306 and j P = 0.0229). |

| Significantly different from group 1 based on the Tukey–Kramer multiple comparison test (d P < 0.01, f P < 0.05 and g P < 0.001). |

Table II. Incidence and multiplicity of esophageal dysplasia and neoplasms in the rasH2 mice that received 4-NQO and/or pitavastatin

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment</th>
<th>Dysplasia Incidence</th>
<th>Tumor Incidence</th>
<th>Multiplicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mild DYS* Moderate DYS Severe DYS Total</td>
<td>Mild DYS Moderate DYS Severe DYS Total</td>
<td>PAP SCC Total</td>
</tr>
<tr>
<td>1</td>
<td>4-NQO alone</td>
<td>9/10 (90%) 10/10 (100%) 7/10 (70%)</td>
<td>10/10 (100%)</td>
<td>1.50 ± 0.71b 2.60 ± 1.17</td>
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<tr>
<td>2</td>
<td>4-NQO → p.p.m. pitavastatin</td>
<td>6/8 (75%)</td>
<td>8/8 (100%)</td>
<td>6/8 (75%)</td>
</tr>
<tr>
<td>3</td>
<td>4-NQO → p.p.m. pitavastatin</td>
<td>5/8 (63%)</td>
<td>8/8 (100%)</td>
<td>6/8 (75%)</td>
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<tr>
<td>4</td>
<td>4-NQO → p.p.m. pitavastatin</td>
<td>6/8 (75%)</td>
<td>7/8 (88%)</td>
<td>6/8 (75%)</td>
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<tr>
<td>5</td>
<td>10 p.p.m. pitavastatin</td>
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<td>0/4 (0%)</td>
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<tr>
<td>6</td>
<td>None</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
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</tr>
</tbody>
</table>

*DYS, squamous cell dysplasia.

| Mean ± SD. |

| Significantly different from group 1 based on the Fisher’s exact probability test (c P = 0.0076). |
cyclin D1-positive index (mean ± SD or mean) of SCCs, the values of groups 2 (n = 5, 38.00 ± 6.28, P < 0.01), 3 (n = 4, 24.50 ± 9.26, P < 0.001) and 4 (n = 1, 24.50) were smaller than that of group 1 (57.00 ± 8.21, n = 8).

Scores of EP1 and EP2 receptors’ immunoreactivities in the tongue SCC. As shown in Figure 4B, the tongue SCC that developed in groups 1–4 expressed EP1 and EP4 receptors, the strongest being the SCC of group 1. The dietary pitavastatin reduced the score of both EP1 and EP4 reactivity, and the values of group 3 were significantly lower than those of group 1 (EP1 for P < 0.05 and EP2 for P < 0.01). The mean values (2 for EP1 and 1 for EP2) of group 4 were also low, but a statistical analysis could not be done because of the small sample size from group 4.

Tongue PGE2 level. The PGE2 content of the tongue is illustrated in Figure 4C. The PGE2 content (13.60 ± 3.66 pg/mg tissue, P < 0.01) of group 1 (4-NQO alone) was significantly greater than group 6 (untreated, 8.00 ± 1.83 pg/mg tissue). The pitavastatin feeding reduced the level and the value of group 4 (4-NQO → 10 p.p.m. pitavastatin, 9.25 ± 1.67 pg/mg tissue) was significantly smaller than that of group 1 (P < 0.05). The value of group 5 (10 p.p.m. pitavastatin, 8.50 ± 1.29 pg/mg tissue) was comparable with that (8.00 ± 1.80) seen in group 6.

Discussion
In this study, we demonstrated that rasH2 mice are highly susceptible to a genotoxic carcinogen, 4-NQO, in drinking water and many neoplasms developed in their tongue and esophagus within the 24 experimental weeks. Interestingly, the EP receptors, especially EP1 and EP2, were immunohistochemically expressed in the lesions (dysplasia and neoplasm) induced by 4-NQO in these tissue specimens. This novel animal carcinogenesis model is useful to investigate field cancerization in the head and neck regions (oral cavity and esophagus) (6,7). The model also can be used to identify cancer chemopreventive agents in these tissues, as we revealed that dietary pitavastatin was
capable of inhibiting the tumor development in the tissue specimens, especially the tongue.

Similar to the high frequency of p53 mutation in human oral cancers (50), a high percentage of ras mutation is also detected in Indian patients (51), but infrequently in white Caucasian populations (52). Especially, a high frequency of mutation in codons 12 and 61 of the H-ras gene was observed in chewing tobacco-related oral cancers in India (20). Among the ras genes, mutations of the H-ras (28%) and K-ras genes (33%), but not the N-ras gene, were found in oral tumors from the eastern Indian population (51). Therefore, the ras gene mutation is relatively high in oral cancer associated with tobacco chewing, and ras and p53 mutational events might be independent and mutually exclusive (53).

Our previous work (24) demonstrated that c-Ha-ras proto-oncogene transgenic rats are highly susceptible to 4-NQO-induced carcinogenesis in the tongue, but not the esophagus. Other different findings from the c-Ha-ras proto-oncogene transgenic rats are that the tumor tongues distributed throughout the dorsal site of the tongue in this study, whereas the tongue tumors developed in the dorsal site of the root of the tongue of the c-Ha-ras proto-oncogene transgenic rats that received 4-NQO in their drinking water (24). The reasons for this are not known, but the species difference and/or the difference in the distribution of an enzyme (DT-diaphorase) that catalyzes the conversion of 4-NQO to an ultimate carcinogen, 4-hydroxyaminoquinoline 1-oxide (54), in the tongue may reflect the differences observed.

In the current study, tongue and esophageal tumors developed in the rasH2 mice that received 4-NQO (20 p.p.m. in drinking water) for 4–8 weeks. Similar findings have been reported by Tang et al. (18), who were able to induce numerous tumors in these tissues of CBA and C57BL/6 mice when given 4-NQO in drinking water for 16 weeks. Their extensive work also indicated alterations in the expression of intermediate filaments (K14 and K1), proliferation activity by estimating bromodeoxyuridine-positive nuclei and a cell cycle inhibitor, p16. In this study, we observed that 4-NQO-induced tongue and esophageal carcinogenesis depends on the duration of a carcinogen treatment and that 4 weeks of treatment is sufficient to induce tumors in two tissues. As Tang et al. (18) did not observe any tumors in the tissue specimens other than the tongue and esophagus, we found only one forestomach tumor in a rasH2 mouse that received 4-NQO for 6 weeks. In our previous investigation using c-Ha-ras proto-oncogene transgenic rats, no tumors developed in their forestomach when given 4-NQO in drinking water. Recently, Fong et al. (55) have reported interesting findings that rats given 4-NQO in drinking water and fed a zinc-deficient diet developed tumors in the tongue, esophagus and forestomach, whereas those given 4-NQO and a zinc-sufficient diet had tumors occur only in the tongue, suggesting that dietary modulation, including zinc, influences a manifestation of field cancerization.

Regarding mice genetically modified for tongue carcinogenesis, the p53 transgenic mice have been reported to be highly susceptible to 4-NQO-induced oral cancer (56). When the palate of the p53 

\[ \text{Val}^{135}/\text{WT} \]

mouse was treated by the direct application of 4-NQO with a hairbrush, which had been dipped in 4-NQO solution, thrice weekly for 16 weeks and followed by no further treatment for 32 weeks, a greater incidence and multiplicity of squamous cell tumors in the oral cavity, esophagus and forestomach were observed in comparison with the p53 

\[ \text{WT}/\text{WT} \]

mice at 48 experimental weeks. Their microarray data suggest the importance of the p53 mutation, alteration in p53-dependent apoptosis and cell proliferation during the carcinogenesis of these tissues. These findings also supported the belief that the crostalk of apoptosis, cell cycle arrest, transforming growth factor \( \beta \) signaling pathway and Ras-mitogen-activated protein kinase pathway may be involved in tumorigenesis. In the current study, we observed numerous tumors mainly in the tongue and esophagus, occurring as early as 24 weeks, although no p53 mutations were determined. Recently, Caulin et al. (57) developed an interesting mouse model without the use of a carcinogen, in which the focal activation of an oncogenic K-rasG12D allele in the oral squamous epithelium resulted in the development of a number of oral cavity tumors with an altered expression pattern of keratin 16 weeks after the activation. Taken together, ras mutation and/or activation as well as p53 mutation are therefore considered to be involved in oral cancer development.

Using rasH2 mice, we investigated the chemopreventive ability of pitavastatin in 4-NQO-induced tongue and esophageal carcinogenesis. The dietary pitavastatin is therefore considered to have the potential to suppress the development of tongue tumorigenesis, whereas the potential in esophageal carcinogenesis was relatively weak in comparison with that observed in tongue carcinogenesis. The reasons for this are not known, but a different bioavailability of dietary pitavastatin in these tissues is considered to be one possible explanation. In this study, the PGE \(_2\) content in the tongue was lowered by the treatment with pitavastatin. The findings support our previous results that lowering the PGE \(_2\) content by treatment with COX-2 inhibitors (24,32) and a non-steroidal anti-inflammatory drug (58) suppresses 4-NQO-induced carcinogenesis. In this study, the dietary administration of pitavastatin reduced the immunohistochemical expression of EP \(_1\) and EP \(_2\) in the tongue, thus suggesting the involvement of these receptors in tongue carcinogenesis. The inhibitory effects of pitavastatin on carcinogenesis has recently been observed in colon tumorigenesis in our laboratory (43). In addition, an EP \(_2\) antagonist, ONO-8711, in the diet effectively inhibits tongue tumor development in human c-Ha-ras transgenic rats initiated with 4-NQO (41). Besides these effects of pitavastatin, we observed that dietary pitavastatin at all doses significantly lowers the PCNA-labeling index of tongue SCCs, thus suggesting that this drug is able to inhibit the growth of tongue SCCs possibly through affecting events during the tumor progression stage. Additionally, feeding with pitavastatin (groups 2–4) reduced the cyclin D1-positive rates of cancer cells when compared with group 1 (4-NQO alone). The findings are of interest, because H-ras and cyclin D1, a downstream of the Ras, influence the susceptibility of oral cancer (21). Thus, the multiple effects of pitavastatin on the expression of EP \(_1\) and EP \(_2\) receptors, PGE \(_2\) content, proliferation and cell cycle may result in inhibition of tongue carcinogenesis initiated with 4-NQO in rasH2 mice.

Using this model, detailed research on molecular and proteomics events, such as the involvement of inflammation in tongue/esophageal carcinogenesis, could be conducted to fight oral and esophageal epithelial malignancies. In addition, the gene-environment and gene–gene interactions in the carcinogenesis (59) of these tissues can be investigated using this rasH2 mouse model, since the H-ras gene and other members of the ras gene family also appear to be a common target for the coding sequence mutations in the initiation of carcinogenesis at several organ sites and in various species by specific carcinogens (60).

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References
