Targeting Mammalian Target of Rapamycin by Rapamycin Prevents Tumor Progression in an Oral-Specific Chemical Carcinogenesis Model

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Abstract The increased molecular understanding of cancerous growth may now afford the opportunity to develop novel therapies targeting specific dysregulated molecular mechanisms contributing to the progression of each cancer type. In this regard, the aberrant activation of Akt/mammalian target of rapamycin (mTOR) pathway is a frequent event in head and neck squamous cell carcinomas (HNSCC), thus representing a potential molecular target for the treatment of HNSCC patients. The ability to translate this emerging body of information into effective therapeutic strategies, however, has been hampered by the limited availability of animal models for oral malignancies. Here, we show that the administration in the drinking water to mice of 4-nitroquinoline-1 oxide, a DNA adduct-forming agent that serves as a surrogate of tobacco exposure, leads to the progressive appearance of preneoplastic and tumoral lesions in the tongue and oral mucosa, with 100% incidence after only 16 weeks of carcinogen exposure. Remarkably, many of these lesions evolve spontaneously into highly malignant SCCs few weeks after 4-nitroquinoline-1 oxide withdrawal. In this model, we have observed that the activation of the Akt-mTOR biochemical route represents an early event, which is already detectable in dysplastic lesions. Furthermore, we show that the inhibition of mTOR by the chronic administration of rapamycin halts the malignant conversion of precancerous lesions and promotes the regression of advanced carcinogen-induced SCCs. Together, these findings support the contribution of the mTOR signaling pathway to HNSCC progression and provide a strong rationale for the early evaluation of mTOR inhibitors as a molecular-targeted strategy for HNSCC chemoprevention and treatment.

With approximately 500,000 new cases annually, squamous cell carcinomas of the head and neck (HNSCC), the vast majority of which arise in the oral cavity, represent the sixth most common cancers in the world (1). This disease results in nearly 11,000 deaths each year in the United States alone (2). The most prevalent risk factors involved in the development of these highly aggressive malignancies, such as alcohol and tobacco use, betel nut chewing, and infection with the human papillomavirus, have been long well recognized (3–7). However, the 5-year survival rate after diagnosis for HNSCC remains low, approximately 50%, which is considerably lower than that for other cancers, such as those of colorectal, cervix, and breast origin (2). This poor prognosis of HNSCC patients is likely due to the fact that most patients are diagnosed at advanced disease stages and often fail to respond to available treatment options (3, 4). However, emerging knowledge on the molecular mechanisms underlying HNSCC progression may provide unique opportunities for the development of novel molecular-targeted strategies for the prevention and treatment of HNSCC.

Like most cancers, HNSCC progression involves the sequential acquisition of genetic and epigenetic alterations in genes encoding tumor suppressors and oncogenes, together with the aberrant activity of signaling networks controlling cell proliferation, differentiation, migration, survival, and death. The most frequent genetic alterations in HNSCC include loss of heterozygosity and promoter silencing of the *p16* tumor suppressor gene, and inactivating mutations in the *p53* tumor suppressor gene (3, 4). HNSCCs often overexpress the epidermal growth factor receptor (EGFR) and some of its active variants, such as the truncated mutant form EGFR variant III, which causes its constitutive activation (5). These findings have provided a rationale for current clinical efforts aimed at targeting EGFR for HNSCC treatment (6). HNSCC lesions harbor activating mutations in the *ras* oncogene with variable frequency, ranging from 3% to 5% in Western countries (3) to around 30% to 40% in India (7), and often

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overexpress cyclin D and exhibit alterations in the nuclear factor-κB, signal transducers and activators of transcription, Wnt/β-catenin, transforming growth factor-β, and phosphatidylinositol 3-kinase-Akt-mammalian target of rapamycin (mTOR) signaling pathways (8). However, the ability to translate this emerging body of information into effective therapeutic approaches to prevent HNSCC development has been hampered by the limited availability of appropriate animal models for oral malignancies. Indeed, the search for novel molecular-targeted therapies for HNSCC has primarily relied on the use of xenotransplanted human HNSCC tumors in immunocompromised mice. In this regard, chemically induced tumors in immunocompetent mice often reflect better the heterogeneity and more complex and challenging situation of the clinical setting (9, 10). Thus, it stands to reason that the development of suitable oral-specific chemical carcinogenesis animal models may help understand the molecular mechanisms driving the progression of these tumoral lesions and may facilitate the identification of novel chemopreventive strategies and therapeutic approaches.

Here, we have optimized the use of a DNA adduct-forming agent, 4-nitroquinoline-1 oxide (4NQO), which serves as a surrogate of tobacco exposure (see below; refs. 11, 12), and show that its oral administration to mice leads to a 100% incidence of heterogeneous oral lesions, many of which evolve spontaneously into highly malignant invasive SCCs a few weeks after carcinogen withdrawal. In this model, we observed that the activation of the Akt-mTOR pathway is an early event, already detectable in dysplastic lesions. Furthermore, we show that the inhibition of mTOR by the chronic administration of rapamycin can cause the regression of carcinogen-induced SCCs and halts the progression to malignancy of early oral cancerous lesions. These findings support the contribution of the Akt-mTOR pathway to HNSCC development and suggest that mTOR may represent a potential target for HNSCC chemoprevention and treatment.

Materials and Methods

Reagents

4NQO obtained from Sigma-Aldrich was dissolved in propylene glycol (Sigma-Aldrich) as stock solution (4 mg/mL), stored at 4°C, and diluted in the drinking water to a final concentration of 50 μg/mL. Water was changed weekly. Rapamycin was purchased from LC Laboratories dissolved in 100% ethanol to a 20× concentration and stored at −80°C. Before its i.p. administration (5 mg/kg), this concentrated stock of rapamycin was diluted in an aqueous solution of 5.2% Tween 80 and 5.2% polyethylene glycol (U.S. Biochemical and Polysciences, Inc., respectively) as reported (13).

Experimental model

All animal studies were carried out according to NIH-approved protocols, in compliance with the Guide for the Care and Use of Laboratory Animals. Female C57Bl/6 mice (Harlan Sprague-Dawley, National Cancer Institute at Frederick, Frederick, MD), 4 to 6 wk old and weighing 18 to 20 g, were housed in appropriate sterile filter-capped cages and fed and given water *ad libitum*. Ten mice per group were given either water (control) or 4NQO in the drinking water for 16 wk, after which all animal cages were reverted to regular water and mice were monitored until week 22. This schedule was selected based on preliminary studies in which we observed that this approach limits nonneoplastic-related toxic effects of 4NQO and leads to a very low incidence of esophageal neoplasias but a 100% incidence of oral cancer. All animals underwent a biweekly full oral cavity examination under anesthesia [2-5% isoflurane (Baxter) mixed with oxygen], and any observed pathologic changes were documented. Animals were euthanized on either week 16 or week 22 for tissue retrieval. Complete autopsies were done, and tissues were immediately collected and fixed in buffered zinc formalin (Z-fix, Anatech) at room temperature for 12 h. The tissues were then transferred to 70% alcohol and processed for paraffin embedding for histopathologic diagnosis and further studies. For the analysis of the effect of rapamycin on tumor development, mice (10 mice per group) were exposed to the carcinogen for 16 wk, examined, and randomly distributed into treatment and control groups, which received daily i.p. injections with rapamycin (5 mg/kg/d) or an equal volume of diluent (an aqueous solution of 5.2% Tween 80 and 5.2% polyethylene glycol), respectively, as previously described (14). In this regard, recently available evidence suggests that lower doses of rapamycin may be sufficient to block mTOR activity in tumor tissues *in vivo* (15, 16), hence suggesting that lower doses of rapamycin may be considered for future analysis in this animal model of oral cancer. All animals were monitored daily for general behavioral abnormalities, signs of toxicity, illness, or discomfort. All animals were euthanized on week 22, and tissue retrieval was done as described above.

Immunohistochemistry

All antibodies used were from Cell Signaling Technology, Inc., except for anti-cyclooxygenase-2 (COX-2), anti-bromodeoxyuridine (BrdUrd; BD Transduction Laboratories and Accurate Chemical, respectively), Ki-67 (Dako), and anti-EGFR (Santa Cruz Biotechnology, Inc.). The antibodies were used in the following concentrations: p53 rabbit monoclonal antibody, 1:40; phospho-Akt (Ser^{473}) rabbit monoclonal antibody, 1:50; phospho-S6 monoclonal rabbit antibody, 1:100; COX-2 mouse monoclonal antibody, 1:100; BrdUrd rat monoclonal antibody, 1:10; anti-mouse Ki-67 rat monoclonal antibody, 1:30; anti-phospho-EGFR (Tyr^{1173}) rabbit monoclonal antibody, 1:200; and anti-EGFR rabbit polyclonal antibody, 1:100. Tissue slides were dewaxed in Safeclear II, hydrated through graded alcohols and distilled water, and washed thrice with PBS. Antigen retrieval was done using an unmasking solution (Vector Laboratories) or 10 mmol/L citric acid boiled in a microwave for 20 min (2 min at 100% power and 18 min at 10% power). The slides were allowed to cool down for 30 min at room temperature, rinsed twice with PBS, and incubated in 3% hydrogen peroxide in PBS for 10 min to quench the endogenous peroxidase. The sections were then sequentially washed in distilled water and PBS and incubated in blocking solution (2.5% bovine serum albumin in PBS) for 30 min at room temperature. Excess solution was discarded, and the sections were incubated with the primary antibody diluted in blocking solution at 4°C overnight. After washing with PBS, the slides were sequentially incubated with the biotinylated secondary antibody (1:400; Vector Laboratories) for 30 min and with the avidin-biotin complex, reconstituted according to the instruction of the manufacturer in PBS (Vector Stain Elite, ABC kit, Vector Laboratories), for 30 min at room temperature. The slides were developed in 3,3-diaminobenzidine (Sigma FASTDAB tablet, Sigma Chemical) diluted in distilled water under microscopic control. Development was stopped in distilled water. The slides were thoroughly washed, counterstained with Mayer's hematoxylin, dehydrated, and mounted. Whole slide images were acquired using an Aperio ScanScope at ×20 magnification. Relevant areas were converted into jpeg format, quantified using the Northern Eclipse Image Analysis Software (Empix Imaging), and expressed as the percentage of positive cells with respect to the total number of cells in each histologically defined area. BrdUrd is expressed as mitotic index = number of positive cells/total number of cells.

Statistical analysis

One-way ANOVA followed by Bonferroni's or Newman-Keuls multiple comparison tests was used to analyze the differences of

tumor mass volume between experimental groups and differences between immunohistochemical quantification of each group. Mann-Whitney test was used to evaluate differences in total surfaces and tumor multiplicity. Data analysis was done using GraphPad Prism version 4.00 for Windows (GraphPad Software); P values of <0.05 were considered statistically significant.

Results

To explore early molecular events in HNSCC progression, we have evaluated several experimental oral chemical carcinogenesis models, which may help identify new targets for the prevention and treatment of this disease. Among them, we focused on the use of 4NQO, a synthetic water-soluble organic compound that forms DNA adducts, thereby causing adenosine for guanosine substitutions (17), and induces intracellular oxidative stress resulting in mutations and DNA strand breaks (11). These changes in the cellular DNA induced by 4NQO administration are typical from those provoked by tobacco carcinogens, hence serving as a surrogate of tobacco exposure (11, 12). The extensive optimization of the experimental conditions using a variety of mouse strains led us to establish a general procedure for the administration of 4NQO depicted in Fig. 1A, which resulted in the progressive appearance of tumoral lesions in the tongue and oral mucosa that were preceded by clearly identifiable preneoplastic events.

In this study, we used C57Bl/6 mice, one of the most frequently used mouse strains, and provided water (control) or 4NQO (50 μg/mL) in the drinking water for 16 weeks, a procedure that required minimal intervention. Water was replaced weekly, and mice were monitored before each water change. After 16 weeks, the administration of the carcinogen was suspended, and all mice received regular water. As expected, none of the control animals developed any lesions through the end of the study. However, ~50% of the mice exposed to 4NQO developed tumors in the tongue and other areas of the oral mucosa as early as week 12 (Fig. 1B and C). The lesions were usually papillomatous-like and could be distinguished from the adjacent mucosa by its irregular growth and whiter appearance. At week 16, at the end of the carcinogen exposure, most mice presented visible oral tumoral lesions. The number and size of the tumors continued growing progressively even after switching to regular water without 4NQO (Fig. 1C). Indeed, most mice developed remarkable tumoral lesions in the tongue, and we also observed discolorations and hypertrophic growth in the palate as well as in the floor of the mouth, some of which evolved into full papillary lesions (Fig. 1B). Overall, at the end of the study on week 22, all mice exposed to 4NQO developed one or more large oral cavity tumors but not in other anatomic sites in repeated experimental groups, supporting the usefulness of this chemical carcinogenesis model to investigate oral cancer development.

Of interest, the highest incidence of tumors in this animal model occurred in the tongue, a location that represents 20% to 40% of all oral cancers in humans (18). Thus, we conducted a detailed histologic analysis of the resulting cancerous lesions in the entire tongue from each animal at the end of the study (week 22; Fig. 2A and B). Besides readily visible tumors, there were also a large number of clearly identifiable precancerous lesions in each tongue, which were classified into low grade and high grade, following classic tissue architectural and cytologic features (19). Whereas all papillary lesions were squamous carcinomas, all these preneoplastic lesions were flat, resembling human oral lesions in which premalignant lesions rarely exhibit a papillary aspect, with the exception of proliferative verrucous leukoplakias (20). Microscopically, all malignant tumors examined were identified as SCCs with different degrees of keratinization (Fig. 2B). There was an absence of significant inflammatory infiltration, with inflammation becoming evident only in grossly papillary lesions. All dysplasias, microcarcinomas, and SCCs were quantified. As shown in Fig. 2C, on week 16, at the end of the carcinogen treatment, we found that every mouse had one or multiple dysplastic lesions, with overall four times fewer carcinomas than dysplasias per tongue. However, at week 22, all mice had one or more large SCC in their tongue, and nearly the same number of dysplastic than SCC lesions (Fig. 2C). This suggests that 4NQO may provoke the accumulation of genetic changes in the oral epithelial cells, which leads to few malignant but numerous premalignant lesions at the end of the carcinogen exposure, but that many of these lesions may then evolve spontaneously into large carcinomas during the follow-up period after carcinogen withdrawal.

We then investigated the proliferative status of these dysplastic and tumoral lesions (Fig. 3). There were very few isolated BrdUrd-positive nuclei in the tongue mucosa in control animals, but there was an increased BrdUrd incorporation in proliferating epithelial cells even in the nonneoplastic areas of the tongue in 4NQO-treated mice. In every case, however, the proliferating cells were confined to the basal layer. Aberrant proliferation was evident instead in dysplastic tissues, with cells incorporating BrdUrd in focal areas, some of which involved suprabasal cells. All SCCs displayed a massive growth of tumoral cells, which included both basal and suprabasal regions. We also investigated the levels of COX-2 expression to assess the possible contribution of proinflammatory pathways in tumor progression in this chemical carcinogenesis model. COX-2 expression was absent in normal as well as in hyperplastic 4NQO tissues, a feature that was associated with the lack of stromal inflammatory infiltration, as previously noted (21). Increased expression of COX-2 was detectable in the early dysplastic lesions, and this was even more remarkable in SCCs. However, in both cases, COX-2 expression was restricted to the epithelial cells, suggesting that in this model, cell inflammation involves intrinsic mechanisms triggered in tumoral cells, and not as part of a secondary inflammatory response provoked by the stroma. Remarkable changes also occurred in the immunodetection of p53, which often reflects the presence of mutations in the *p53* tumor suppressor gene, a highly prevalent alteration in HNSCC (22). Immunoreactive p53 was absent from normal tongue tissues, but isolated foci of p53-positive cells were evident in nonmalignant epithelium of 4NQO-treated mice, particularly in areas of hyperplasia, reflecting an early compromise of this tumor-suppressive protein in the development of tongue SCC. The number of immunoreactive cells increased in precancerous lesions and was maximal in SCC, with most cells expressing p53 located in the basal and parabasal layers. A progressive dysregulation of the Akt-mTOR pathway was also evident in this carcinogenesis model, as judged by the fact that as the lesions progress to malignancy, there was an increase in the number of positive cells for the phosphorylated species of Akt, $pAkt^{S473}$,

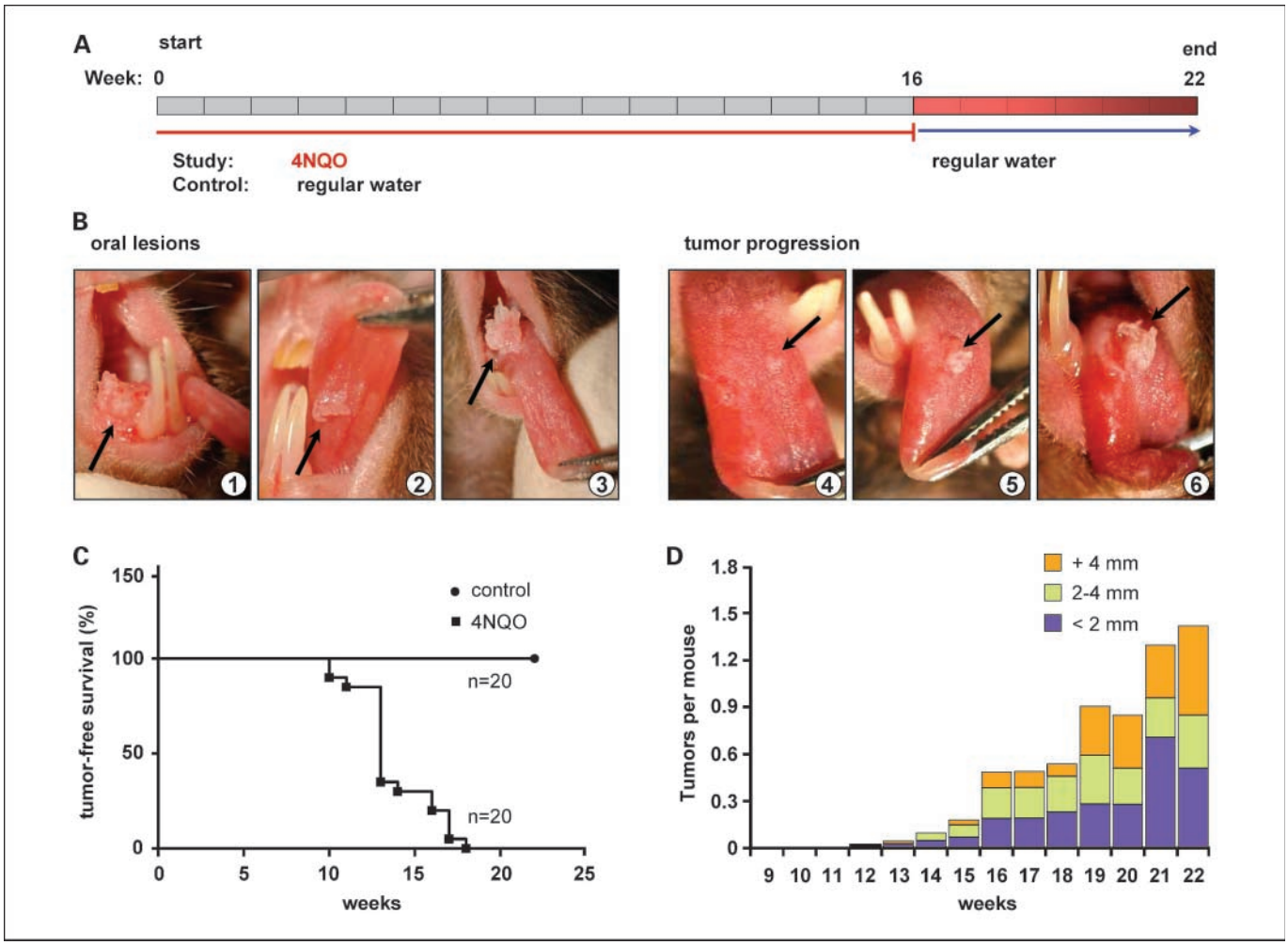

Fig. 1. Administration of 4NQO in the drinking water leads to rapid oral tumor formation in mice. *A*, experimental scheme. Mice were given either water (control) or 4NQO (50 μg/mL) in the drinking water for 16 wk, after which all animals were reverted to regular water until week 22. All animals underwent a biweekly full oral cavity examination, and any pathologic changes were documented. *B*, oral lesions in 4NQO-treated mice. Examples of large papillomatous lesions in the lower right gum (*1*) and tongue (*2* and *3*) are depicted. A typical example of clinical tumor progression from a flat, likely preneoplastic stage, to SCC (*4-6*) is also shown. *C*, tumor-free survival curves for control and 4NQO-treated groups. The percentage of mice lacking any macroscopic tumoral lesions on oral examination is represented. *D*, tumor progression in the 4NQO-treated group. The number of tumors per animal each week and the distribution of their corresponding tumor sizes (color coded) are represented as indicated ($n = 20$). Note that the 4NQO treatment was stopped at week 16, although tumors continued growing after the carcinogen withdrawal. No tumors were observed in control mice.

and S6, pS6, the latter representing one of the most downstream targets of mTOR (23). An interesting finding was the observation that most cells positive for pS6 and pAkt^{S473} in normal and dysplastic lesions involved parabasal cells, which most likely represent nonproliferating cells undergoing differentiation. In contrast, SCC cells displayed elevated pS6 and pAkt^{S473} in multifocal areas throughout the tumor, similar to what we and others have reported in human HNSCC lesions (13, 24, 25). This includes areas that show elevated levels of EGFR but not of its phosphorylated active form (Supplementary Fig. S1), suggesting that Akt-mTOR activation in this animal model of oral SCC involves EGFR-dependent and EGFR-independent mechanisms, reflecting prior studies in human HNSCC (24).

The availability of an oral chemical carcinogenesis model reflecting many pathologic features often found in human HNSCCs provided an opportunity to explore chemopreventive strategies aimed at reducing the conversion of dysplastic lesions into frank carcinomas. In particular, we explored the consequences of blocking mTOR function with its specific inhibitor, rapamycin (23), in this oral SCC model system. Initially, animals exhibiting large SCC tumors at week 22 were treated with rapamycin for 3 consecutive days, which resulted in a dramatic decrease of the levels of pS6 and pAkt^{S473} (Fig. 4), both targets of mTOR. We did not detect the accumulation of apoptotic cells at this time point but observed a decrease in cell proliferation, particularly in suprabasal and tumoral cells as shown by Ki-67 immunostaining (Fig. 4). Of interest, whereas pS6 is a downstream target of mTOR complex 1, which is blocked by rapamycin, the phosphorylation of Akt in its Ser^{473} represents a target of mTOR complex 2, which is indirectly inhibited on prolonged exposure to rapamycin (26, 27). In both cases, only a limited residual immunodetectable pS6 and pAkt^{S473} was observed in the most differentiated areas of the tumor, suggesting that mTOR may be more resistant to inhibition in this cell population, whereas the less

differentiated and proliferating cells are highly sensitive to this novel antineoplastic agent. Similarly, we observed a reduction of pS6 and pAkt^{S473} in the stroma of all tumoral tissues, as recently reported (14). Overall, we confirmed that rapamycin treatment blocked the elevated mTOR activity in the stroma and the proliferative cell compartment of these oral cancerous lesions.

We next asked whether rapamycin could interfere with tumor development when its administration was initiated at the end of the 4NQO exposure (week 16), thus avoiding any possible interactions between 4NQO and rapamycin, which could prevent the appropriate interpretation of the emerging results. Indeed, following a treatment scheme as depicted in Fig. 5A, we observed that the effects of rapamycin were quite remarkable. At gross examination, differences between the rapamycin-treated and control groups were readily evident in both the size and number of tumoral lesions (Fig. 5B). Multiple sections from each tongue were systematically examined as described for Fig. 2, and the number and size of each SCC lesion were quantitated and used to reconstruct a topographic map of compromised surfaces in the rapamycin-treated and control groups. Cartoons for each tongue depicted the shape of their actual pictures, with the red areas indicating the presence of SCCs, as judged by their histologic examination (Fig. 5C). A highly significant difference was observed in the number of SCCs in each tongue between the rapamycin-treated and control groups. When the compromised areas in each tongue were digitally quantified, we found that rapamycin dramatically diminished both the tumor multiplicity and the overall affected tumor surface area (Fig. 5D and E, respectively), thus supporting the effectiveness of blocking mTOR in preventing the development of oral SCC lesions once initiated by chemical carcinogens.

Discussion

The emerging molecular understanding of normal and cancerous growth has afforded the opportunity to explore the development of novel therapies targeting specific molecular mechanisms whose dysregulation contributes to the

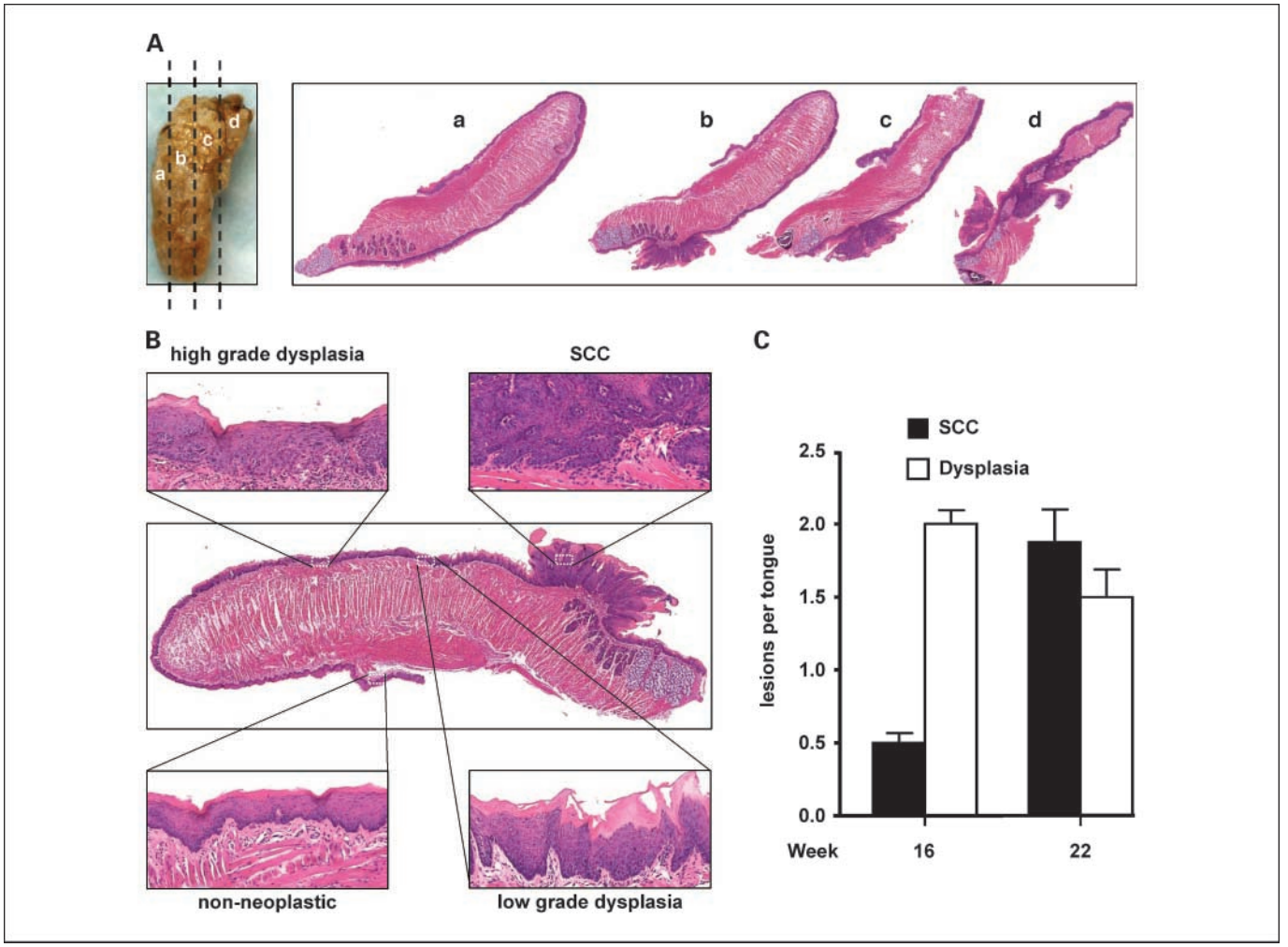

Fig. 2. Histopathologic analysis of 4NQO-induced oral tumoral lesions. *A,* after fixing, each tongue was cut into four sections of approximately the same thickness, following its major axis, as depicted. These sections were embedded in a single paraffin block and multiple sections were cut and stained with H&E for diagnostic purposes and for immunohistochemical analysis. Representative H&E-stained sections are shown. *B,* section b from above is shown in higher magnification to illustrate the presence of multiple dysplastic and cancerous lesions in the same tongue. *C,* quantitative analysis of the number of dysplasias and SCCs per tongue at the end of the 4NQO treatment (week 16) and at the end of the observation period (week 22). The increase in the number of cancerous lesions at week 22 reflected the progression of the carcinogenesis process.

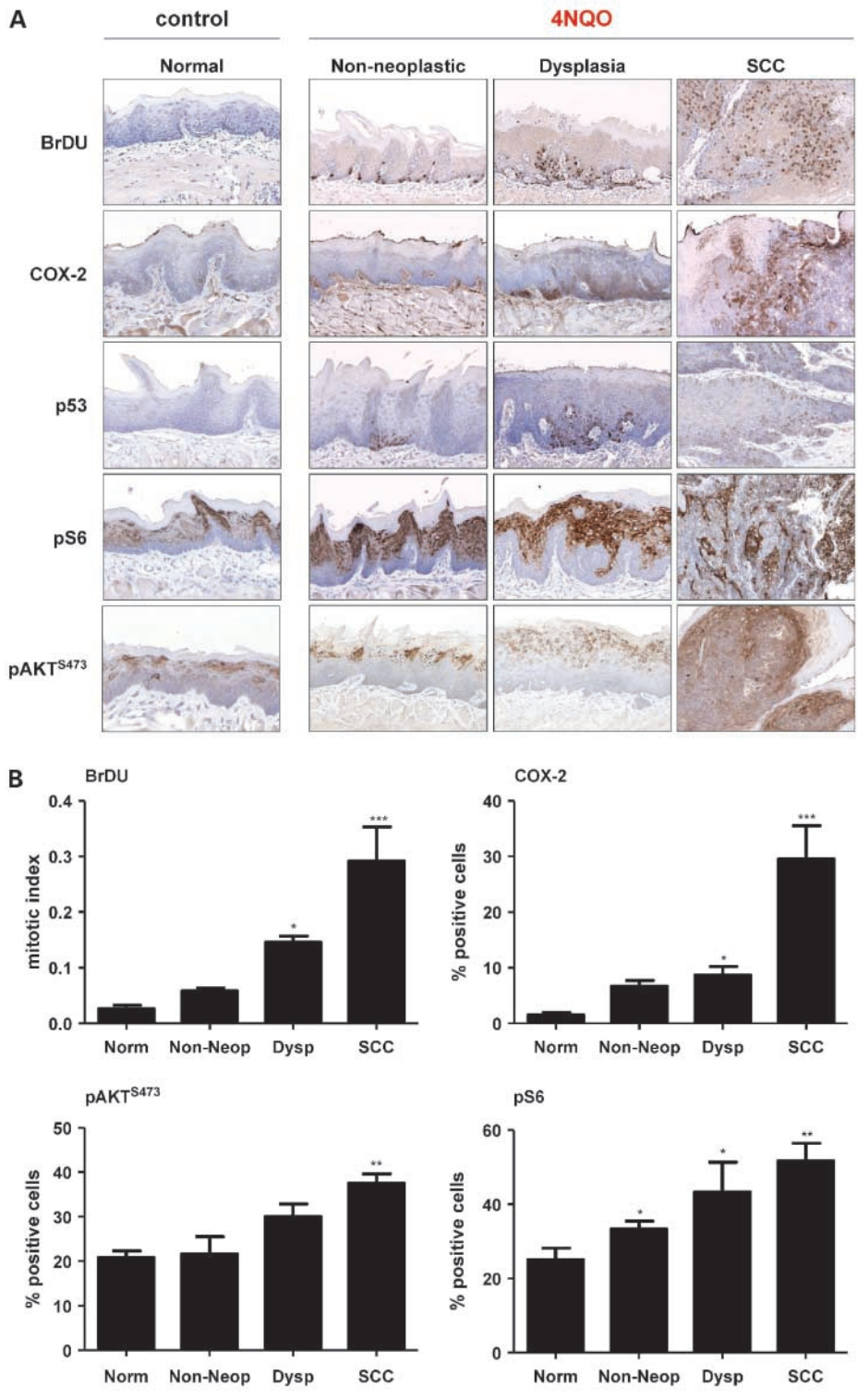

Fig. 3. *A,* molecular changes characterizing tumor progression in 4NQO-induced oral lesions. Representative tissue sections from the tongue of control mice and nonneoplastic, dysplastic, and cancerous (SCC) regions of mice exposed for 16 wk to 4NQO and euthanized on week 22 were analyzed for their proliferative status (BrdUrd, BrDU), expression of COX-2, and the accumulation of immunoreactive forms of p53 and the phosphorylated species of S6 (*pS6*) and Akt (*pAkt*^{S473}), as indicated. Note the increased number of proliferating cells, and the distinct pattern and increased levels of COX-2, p53, pS6, and pAkt^{S473}, as the tumors progress. See text for details. All tumoral lesions displayed staining for nuclear p53. *B,* quantification of molecular changes associated with tumor progression. Tissue sections from the tongue of control mice and nonneoplastic, dysplastic, and cancerous (SCC) regions of mice exposed for 16 wk to 4NQO and euthanized on week 22 were immunostained for BrdUrd, COX-2, pS6, and pAkt^{S473} and quantified as described in Materials and Methods. *Columns,* average of four to six immunostained sections; *bars,* SE. Nonneoplastic (*Non-Neop*), dysplasias (*Dysp*), and SCC were compared with the corresponding staining in normal tissues (*Norm*). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

progression of each cancer type. In the case of HNSCC, the activation of the Akt-mTOR signaling pathway represents an early and widespread event, likely independently of the mutational status of p53, hence raising the possibility of using mTOR inhibitors for HNSCC treatment (13, 24, 25, 28). In this regard, however, efforts should be made to examine the effectiveness of mTOR inhibitors and other candidate drugs in animal models reflecting the complexity of HNSCC better than classic xenograft models. Here, building on prior studies (11, 12), we have now optimized the oral delivery to mice of a DNA adduct-forming agent, 4NQO, which induces changes in the cellular DNA that are typical from those provoked by tobacco carcinogens. We show that this procedure results in the progressive appearance of preneoplastic and tumoral lesions in the tongue and oral mucosa with 100% incidence after 16 weeks of carcinogen administration, and that many of these lesions then evolve spontaneously into highly malignant SCCs few weeks after 4NQO withdrawal. We also observed that the

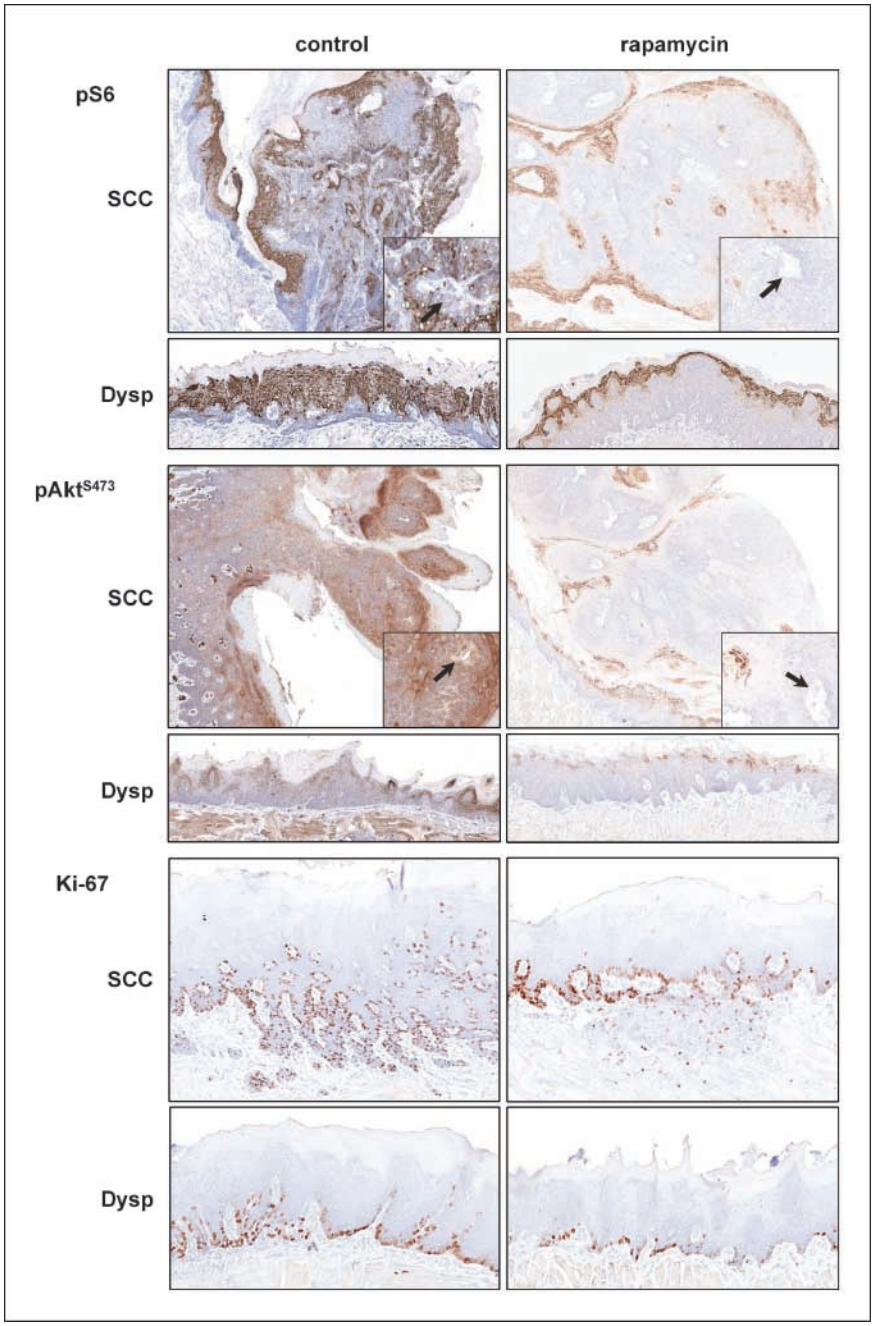

Fig. 4. Administration of rapamycin decreases the activity of mTOR in 4NQO-induced cancerous lesions. The treatment for 3 d with rapamycin (5 mg/kg/d; i.p.) caused a remarkable reduction in the levels of both pAkt^{S473} and pS6 in both SCC and dysplasias. Although few cells remained positive for pS6 and pAkt^{S473} after the rapamycin treatment, they were limited to the upper, most differentiated layers, but absent from the proliferative basal layers (*arrows*, rapamycin group), compared with their high levels in the basal layers of the control, vehicle-treated, 4NQO-induced SCCs (*arrows*, control). A similar finding was observed in the basal and parabasal areas in preneoplastic lesions. A decrease in the proliferation marker Ki-67 is seen in rapamycin-treated tissues in both SCC and dysplasias. A lower number of immunoreactive cells are evident, and they are mostly confined to the basal areas, similar to the staining patterns of the normal squamous epithelium.

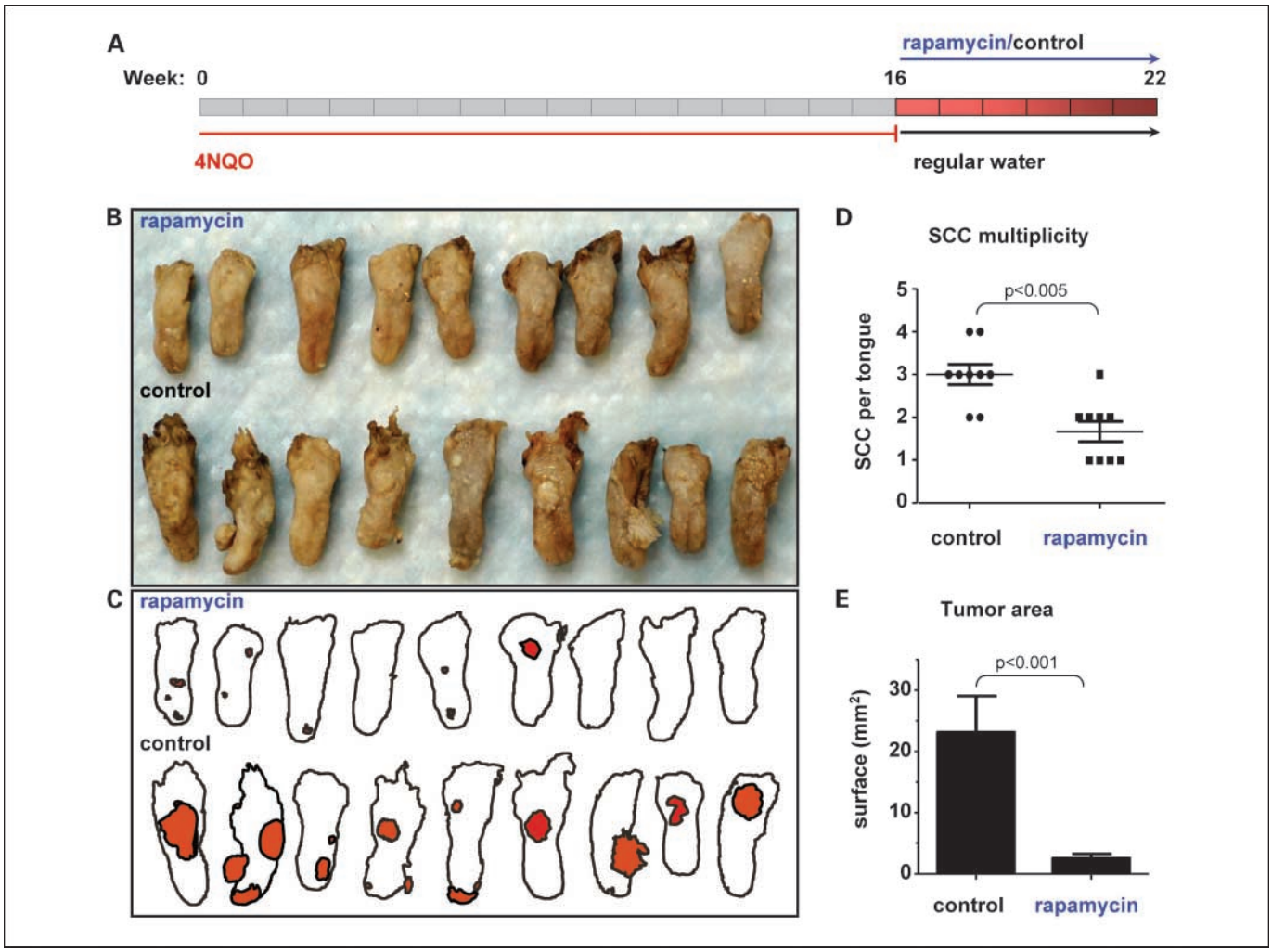

Fig. 5. The treatment with rapamycin reduces the tumor burden in the 4NQO-induced oral carcinogenesis model. *A,* experimental scheme. Mice were given 4NQO (50 μg/mL) in the drinking water for 16 wk, and then divided into a rapamycin-treated group (5 mg/kg/d; i.p.) and a control group (vehicle control; i.p.), and reverted to regular water until week 22. All animals were euthanized on week 22, and complete autopsies were done. *B,* pictures of representative tongues of control and rapamycin-treated mice, showing a decreased tumor burden in the rapamycin-treated group. *C,* histologic analysis of each tongue as described in Fig. 2A enabled the digital representation of each of the SCC lesions in the dorsal tongue, which are shown as red areas. *D,* multiple tissue sections from each tongue were examined, and the number of SCC lesions, including large SCCs and microcarcinomas, per tongue was represented individually for both the control and rapamycin-treated groups. There was a significant reduction in the number of SCC lesions on rapamycin treatment. *E,* the compromised areas in each tongue were digitally quantified. *Columns,* average of the surface of the affected area per tongue for the control and rapamycin-treated group; *bars,* SE. A highly significant reduction in the overall size of the oral SCC lesions caused by 4NQO was observed in the rapamycin-treated group. Similarly, we observed a significant reduction in the surface area of high-grade preneoplastic lesions (control, 0.45 ± 0.05 mm^2; rapamycin, 0.28 ± 0.03 mm^2; $P < 0.05$).

activation of the Akt-mTOR pathway is an early event in this oral-specific chemical carcinogenesis model, which can be detected in dysplastic lesions, and that the blockade of mTOR with rapamycin causes the regression of the large squamous carcinomas and prevents the progression of their premalignant lesions. Thus, these findings suggest that mTOR may represent a suitable target for HNSCC chemoprevention and treatment.

Surgery is one of the treatments of choice in the management of oral preneoplastic lesions, although several interventional studies have shown that despite its beneficial effects, their surgical excision may not significantly reduce the risk of recurrence in the same anatomic location or the appearance of cancerous lesions in other oral mucosal sites (3, 29). This reflects the fact that the prolonged exposure to risk factors, such as tobacco and alcohol consumption, may cause multiple related molecular events throughout the entire oral mucosa (3, 30), which may progress to cancer in a multifocal fashion by a lengthy multistep process (3, 31). In this regard, opportunities exist to explore chemopreventive strategies to reverse, suppress, or prevent the progression of these lesions to invasive HNSCC. For example, multiple promising compounds have been used in clinical chemoprevention trials in HNSCC, ranging from vitamin A and retinoids, flavonoids, curcumin analogues, to polyphenols found in natural products (32, 33), which may promote the differentiation of precancerous cells or prevent procarcinogenic mechanisms. Particularly promising are the approaches aimed at inhibiting the effects of proinflammatory mediators that contribute to tumor progression, and the activation of growth factor receptors and their downstream pathways that promote aberrant cell proliferation (34). The main challenge for any chemopreventive agent, besides its

demonstrable efficacy, is that it should lack any significant toxicity (35, 36). In particular, the limited ability to stratify individuals who have lesions most likely to progress to carcinomas may prevent an educated decision about those patients that may benefit the most from available chemopreventive approaches, including those that may harbor some risk of toxicity (36). In this regard, the use of rapamycin has been approved by the Food and Drug Administration in 1999 to prevent renal transplantation rejection (37) and several trials involving thousands of renal transplant patients have shown its relative safety and limited incidence of secondary effects (38). In addition, many new analogues of rapamycin have been recently developed, some of which have increased bioavailability and are well tolerated with reduced side effects, including lack of immunosuppressive activity at doses that are effective in blocking mTOR (23, 37, 39–41).

Overall, we can conclude that the extensive clinical experience with rapamycin (38), the promising antitumoral activity of rapamycin and its analogues, CCI-779 (temsirolimus) and RAD001 (everolimus), in breast cancer, glioblastoma, and mantle cell lymphoma patients (37, 42), the enhanced sensitivity to mTOR inhibitors of cells harboring mutant *p53* (43), a frequent event in HNSCC (22), the recent approval by the Food and Drug Administration of the use of temsirolimus in advanced renal carcinoma, and our present findings provide a strong rationale for the early evaluation of mTOR inhibitors as a molecular-targeted approach to treat HNSCC. Furthermore, the remarkable inhibitory effect of rapamycin on the progression of chemically induced oral tumors suggests that the chronic treatment with mTOR inhibitors may prevent the development of HNSCC, particularly in those at-risk patients presenting premalignant lesions that exhibit elevated mTOR activity. In addition, the availability of suitable oral-specific chemical carcinogenesis animal models reflecting better the complexity of human HNSCC progression may facilitate the identification of biological relevant markers to monitor whether new mechanism-based chemopreventive strategies hit their intended molecular targets in the preneoplastic and cancerous lesions, and it may also help identify biomarkers predicting treatment response. Ultimately, further studies using genetically defined and chemically induced oral-specific carcinogenesis models may accelerate the evaluation of the effectiveness of novel mTOR inhibitors and other molecular-targeted approaches to halt HNSCC development.

Disclosure of Potential Conflicts of Interest

The authors have filed a patent application for the use of mTOR inhibitors in the chemoprevention of head and neck squamous cell carcinoma.

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Cancer Prevention Research

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