INCREASED EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE FOR HUMAN BUCCAL SQUAMOUS-CELL CARCINOMAS: IMMUNOHISTOCHEMICAL, REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (RT-PCR) AND IN SITU RT-PCR STUDIES

Yuk-Kwan Chen, DDS, MS, Shui-Sang Hsue, DDS, MS, Li-Min Lin, DDS, PhD

Oral Pathology Department, School of Dentistry, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung, Taiwan. E-mail: k0285@ms22.hinet.net

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Abstract: *Background.* The inducible nitric oxide synthase (iNOS) is involved primarily in inflammatory and carcinogenesis processes. An enhanced expression of iNOS at the protein level has been reported previously for human oral squamous cell carcinoma; however, the expression of iNOS at the mRNA level has not yet been demonstrated. Furthermore, no studies have addressed whether iNOS expression at mRNA level correlates with cervical lymph node metastasis.

Methods. Specimens of the squamous cell carcinoma of the buccal mucosa obtained from tissue samples of surgically resected tumors from 25 male patients were evaluated with immunohistochemical assessment of iNOS protein and *IS*-RT-PCR, as well as RT-PCR for iNOS mRNA. We also analyzed the iNOS expression status with clinical parameters to determine whether it had any prognostic significance in this homogenous population.

Results. Inducible NOS protein (16 of 25, 64%) and mRNA (13 of 25, 53%) activities were detected for the oral carcinoma specimens examined in this study. Cytoplasmic and/or nuclear stainings were observed in the specimens of both well-differentiated SCC (10 of 15, 67%), and moderately to poorly differentiated SCC (6 of 10, 60%). The cellular location of iNOS mRNA was noted to be consistent with the finding using immunohistochemical technique (cytoplasm and/or nuclei stainings of the tumor islands). Using in situ RT-PCR, iNOS mRNA activity was detected in nine specimens of well-differentiated squamous cell carcinoma (9 of 15, 60%) and four specimens of moderately to poorly differentiated squamous cell carcinoma (4 of 10, 40%). With RT-PCR, an electrophoretic band corresponding to a 907-bp PCR product was observed for nine specimens of well-differentiated squamous cell carcinoma (9 of 15, 60%) and four specimens of moderately to poorly differentiated squamous-cell carcinoma (4 of 10, 40%). Neither iNOS protein nor mRNA was noticed in the samples of normal buccal mucosa or in the negative control samples. There was a significant relationship between iNOS expression (at both protein and mRNA levels) and whether patients had nodal disease.

Conclusions. We have demonstrated an enhanced expression of iNOS protein and mRNA in a number of human buccal carcinomas compared with normal buccal mucosa. Such an observation suggests that the iNOS protein and mRNA expression is chiefly derived from a subset of oral cancerous tissues. Our observation also indicates that iNOS expression correlates with cervical lymph node metastasis in oral squamous cell carcinomas. Therefore, it may also indicate that a subset of oral cancers with greater metastatic potential, as evidenced

Correspondence to: L-M. Lin
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by increased expression iNOS protein and mRNA, is identified. © 2002 Wiley Periodicals, Inc. *Head Neck* **24:** 925–932, 2002

Keywords: iNOS; squamous cell carcinoma; oral; human; immunohistochemistry, mRNA, RT-PCR; in situ RT-PCR

Nitric oxide (NO) mediates a diverse array of pathologic phenomena associated with inflammatory processes and cancer formation.[1] It is a small, highly reactive radical produced from the amino acid L-arginine by the enzyme NO synthase (NOS; E.C.1.14.13.39).[2,3] Nitric oxide synthase exists in three different isoforms: two constitutively produced (cNOS) and an inducible form (iNOS).[4] The constitutive form cNOS (endothelial and neuronal NOS) contributes to the maintenance of normal body physiology in the nervous and cardiovascular system and is both calcium- and calmodulin-dependent.[4] The inducible form of iNOS is involved principally in inflammatory and carcinogenesis processes and is both calcium- and calmodulin-independent.[5]

NO has been postulated to have both beneficial and detrimental roles.[6] In examples of neoplastic tissue, NO has been implicated in the demonstration of a positive role in permitting tumor growth, including mutagenicity, angiogenesis, and metastasis. NO has also been implicated in the cytotoxicity of macrophages toward tumor cells and immunosuppression.[6] Furthermore, NO reacts with the superoxide anion to form a peroxynitrite anion, a highly toxic molecule causing DNA damage and protein modifications.[7] In addition, NO can also react directly with a variety of enzymes and other proteins to either activate or inhibit their functions by oxidizing SH groups, complexing with metal ions, or reacting with tyrosine.[8]

Although an enhanced expression of iNOS at the protein level for patients with oral squamous cell carcinomas has already been reported,[9–11] the corresponding expression of iNOS at the mRNA level has not, to the best of our knowledge, been demonstrated previously for human oral carcinomas. Also, whether there is a known or suspected disconcordance between the mRNA level and the expressed protein for iNOS in human oral cancerous tissues remains to be determined. Furthermore, the relationship between iNOS expression at mRNA level and lymph node metastasis and the degree of differentiation in oral carcinomas has not yet been studied. Therefore, the goal of this study is to investigate the correlation between mRNA and protein expression of iNOS in tumor samples from 25 patients with buccal squamous cell carcinomas using immunohistochemistry, in situ reverse transcription–polymerase chain reaction (IS RT-PCR) and RT-PCR studies. We then analyzed the iNOS expression status (at both protein and mRNA levels) with clinical parameters (degree of differentiation and cervical lymph node status) to determine whether it has any prognostic significance in this homogenous population.

MATERIALS AND METHODS

Study Population. Specimens of the squamous cell carcinoma of the buccal mucosa were obtained from tissue samples of surgically resected tumors from 25 male patients aged between 32 and 80 years (mean age, 51 years), who visited the Oral Pathology Department at the Kaohsiung Medical University Hospital. All of the patients were betel-quid chewers. Cases were taken from patients who had undergone elective neck dissection; 17 of them had histologically confirmed cervical lymph node involvement. The buccal carcinomas involved in this study comprised well-differentiated squamous cell carcinomas (15 samples) and moderately to poorly differentiated squamous cell carcinomas (10 samples). Normal buccal mucosal tissue was taken from five healthy individuals between 22 and 65 years (mean age, 47 years), none of whom chewed betel-quid or smoked cigarettes, which was included as control tissue. All tissues (including the normal tissues) were obtained with the patients' informed consent and with the approval of the Ethics Committee for Scientific Research on Human Beings of this institution. A portion of the surgically resected buccal tissue was immediately frozen in liquid nitrogen for subsequent RNA extraction. Another portion was fixed in 10% neutral buffered formalin solution for about 24 h, dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin for immunohistochemistry and IS RT-PCR studies.

Immunohistochemistry of iNOS Protein. Paraffin-embedded, 4-μm-thick tissue sections were stained for the iNOS protein using a primary rabbit polyclonal anti-iNOS antibody obtained from Calbiochem-Novabiochem Corporation (Cat. no. 482728). This antibody recognizes iNOS protein in humans, rats, and mice and exhibits no

cross-reactivities with eNOS and nNOS (information from manufacturer). The specificity of the antibody has been established in a previous study.[12] Deparaffinization of all sections was performed through a series of xylene baths, and rehydration was performed through graded alcohols. To retrieve the antigenicity, tissue sections were treated three times with microwaves in a 10 m*M* citrate buffer (pH 6.0) for 5 min each. The sections were then immersed in methanol containing 0.3% hydrogen peroxidase for 30 min to block the endogenous peroxidase activity and were incubated in normal goat serum to reduce nonspecific binding. Sections were incubated for 30 min at room temperature with primary anti-iNOS antibody (1:200). The sections were then processed using standard avidin-biotin immunohistochemistry in accordance with the manufacturer's recommendations (Vector Laboratories, Burlingame, CA).[13] Diaminobenzidine was used as a chromogen, and commercial hematoxylin was used for counterstaining. Immunohistochemical staining was classified as negative if no staining or positive staining was present in <25% of the cells or positive if >25% of the cells stained positively.[11] Negative controls for the specificity of anti-iNOS antibody were included by omitting the primary antibody.

IS RT-PCR of iNOS mRNA. RNase-free conditions were used throughout slide preparation and the IS RT-PCR procedure. After deparaffinization and dehydration, the section was pretreated with 10 μg/mL proteinase K (Sigma, St. Louis, MO) for 1 min at room temperature, at which time enzyme activity was prevented with 0.1 M glycine in PBS.

The Titan one-tube RT-PCR system (Boehringer Mannheim, Indianapolis, IN) was used to perform this IS RT-PCR reaction. The final concentration for the RT-PCR reaction mixture was as follows: 200 μ*M* each of deoxyribonucleotide (dATP, dCTP, dGTP), 180 μ*M* dTTP, 40 μ*M* Dig-11-DTP, 0.4 μ*M* downstream primer, 0.4 μ*M* upstream primer, 5 m*M* dithiothreitol solution (DTT), 1.5 m*M* $MgCl_2$, and 1 μL Expanol high-fidelity enzyme mix. All of the reagents were obtained from Boehringer Mannheim, with the exception of primers. The oligonucleotide primers specific for iNOS were purchased from Genset Corp. (La Jolla, CA) (Table 1). The primer pairs were chosen from the published cDNA sequences of iNOS[14] (GenBank accession no. D14051). Hybaid Sure-Seal (Hybaid Instruments, Holbrook, NY) was placed around the sample on the slides. The reaction mixture was then carefully pipetted onto each tissue section. After coverslips were applied, slides were placed on the block of a thermal cycler (TaKaRa MP, Tokyo, Japan). Reverse transcription was carried out at 50°C for 30 min. PCR amplification was carried out with an initial denaturing step at 94°C for 2 min and then 20 cycles of amplification with denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 68°C for 2 min, and a final extension of 68°C for 7 min.

The DIG nucleic acid detection kit (Boehringer Mannheim) was used to detect the digoxigenin-tagged IS RT-PCR amplified products. After rinsing the slides with PBS, they were incubated in anti-digoxigenin antibody conjugated with alkaline phosphatase diluted at 1:500 for 2 h. A subsequent enzyme-catalyzed color reaction solution with 5-bromo-4-chloryl-3-indolyl phosphate and nitroblue tetrazolium salt was applied on the slides to produce an insoluble purple coloration. Color development was checked under the microscope. Slides were washed with Tris buffer containing EDTA to terminate the reaction, counterstained with nuclear Fast Red, and cover slipped with a water-soluble mounting medium. The positivity of iNOS mRNA of each section as purple staining was observed using a light microscope. The protocol has been reported in detail by Zhang et al.[14] Negative control experiments were performed for each specimen on the same slide by omitting the

Table 1. Oligonucleotide primers used to amplify iNOS and β-actin cDNAs.

Oligonucleotide primers	cDNA positions	Sequences	PCR products
iNOS sense	529–550	5′-TCG AAA CAA CAG GAA CCT ACC-3′	907 bp
iNOS antisense	1414–1435	5′-ACA GGG GTG ATG CTC CCG GAC-3′	
β-actin sense	628–657	5′-AAC CGC GAG AAG ATG ACC CAG ATC ATG TTT-3′	350 bp
β-actin antisense	1048–1077	5′-AGC AGC CGT GGC CAT CTC TTG CTC GAA GTC-3′	

primers to exclude endogenous priming of degraded DNA.

RT-PCR of iNOS mRNA. Total RNA was extracted by homogenizing the buccal tissue specimens in guanidium isothiocyanate followed by ultracentrifugation in cesium chloride, as described previously.[15] The RNA concentration was determined from the optical density at a wavelength of 260 nm (by using an OD_{260} unit equivalent to 40 μg/mL of RNA).

Isolated total RNA (1 μg) was reverse transcribed to cDNA in a reaction mixture (with a final volume of 20 μL) containing 4 μL of $MgCl_2$ (5 m*M*), 2 μL of 10× reverse transcription buffer (10 *mM* Tris-HCl, [pH = 9.0], 50 m*M* KCl, 0.1% Triton X-100), 2 μL of dNTP mixture (1 m*M* each), 0.5 μL of recombinant RNasin ribonuclease inhibitor (1 μ/μL), 15 units of avian-myeloblastosis-virus (AMV) reverse transcriptase (High Conc) (15 μ/μg), 0.5 μg of oligo$(dT)_{15}$ primer (Promega, catalogue no. A3500, WI). The reaction mixture was incubated for 15 min at 42°C. The AMV reverse transcriptase was inactivated by heating for 5 min at 99°C and then incubated at 0−5°C for a further 5 min.

All oligonucleotide primers were purchased from Genset Corp. (La Jolla, CA). The primer pairs were chosen from the published cDNA sequences of iNOS[14] (GenBank accession no. D14051), and β-actin[16] (GenBank accession no. X-00351). Oligonucleotide primers used for PCR reactions are shown in Table 1.

The 20-μL first-strand cDNA synthesis reaction product obtained from the reverse transcriptase reaction was diluted to 100 μL with nuclease-free water. The PCR amplification reaction mixture (with a final volume of 100 μL) contained 20 μL of diluted, first-strand cDNA reaction product (<10 ng/μL), 2 μL of cDNA reaction dNTPs (200 μ*M* each), 4 μL of $MgCl_2$ (2 m*M*), 8 μL of 10× reverse transcription buffer (10 m*M* Tris-HCl, pH = 9.0, 50 m*M* KCl, 0.1% Triton X-100), 50 pmol of upstream primer, 50 pmol of downstream primer, and 2.5 units of *Taq* DNA polymerase (Promega, catalogue no. M7660).

The PCR steps were carried out on a DNA thermal cycler (TaKaRa MP, Tokyo, Japan). Thermocycling conditions included denaturing at 94°C for 1 min (one cycle), then denaturing at 94°C (60 s), annealing at 55°C (60 s) for iNOS or at 60°C (60 s) for β-actin, and extending at 72°C (60 s) for 30 cycles, and a final extension at 72°C for 7 min. The β-actin primers were used as positive controls. Negative controls without the presence of RNA and without reverse transcriptase were also performed. Amplification products were analyzed by electrophoresis in a 2% agarose gel along with the relevant DNA molecular-weight marker (Boehringer, Mannheim, Germany) containing ethidium bromide. The PCR products were visualized as bands with a UV transilluminator. Photographs were taken with a Polaroid DS-300 camera. The PCR products were then sequenced to confirm their identities using a T7 Sequenase version 2.0 kit (Amersham International, Little Chalfont, UK).

Statistical Analysis. Fisher's exact test was used to analyze the association between categorical variables. All tests were two sided. $P < .05$ was considered to be statistically significant.

RESULTS

Immunohistochemistry of iNOS Protein. Cytoplasmic and/or nuclear stainings were observed in the specimens of both well-differentiated SCC (10 of 15, 67%, Figure 1), and moderately to poorly differentiated SCC (6 of 10, 60%, Figure 2). Inducible NOS staining was also found in cells of the tumor stroma that were presumed to be macrophages. No iNOS activity could be detected in any of the specimens of normal buccal mucosa. Omission of the primary antisera in control sections disclosed negative findings for iNOS activity in all specimens.

IS RT-PCR of iNOS mRNA. The cellular location of iNOS mRNA was noted to be consistent with the preceding finding using the immunohistochemical technique (cytoplasm and/or nuclei stainings of the tumor islands). Inducible NOS mRNA activity was detected for about half (13 of 25, 52%) of the buccal-carcinoma specimens examined in this study: nine specimens of well-differentiated squamous cell carcinoma (9 of 15, 60%, Figure 3), and four specimens of moderately to poorly differentiated squamous cell carcinoma (4 of 10, 40%, Figure 4). Three cases were negative on IS RT-PCR but were positive for iNOS on immunohistochemistry. Inducible NOS mRNA activity could not be found in the normal buccal mucosa. Omission of the primers in control sections dis-

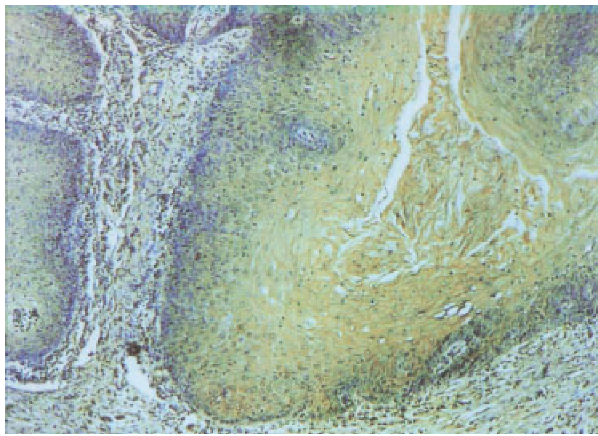

FIGURE 1. Both cytoplasmic and nuclear stainings of iNOS protein were observed in a representative sample of well-differentiated squamous cell carcinoma (original magnification ×100).

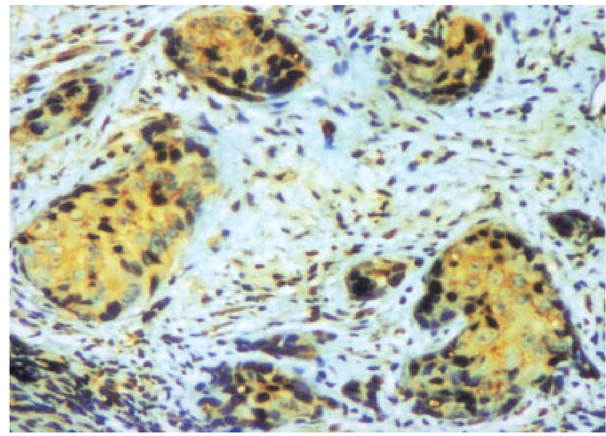

FIGURE 2. Inducible NOS protein was noted in a representative sample of moderately to poorly differentiated squamous cell carcinoma (original magnification ×100).

closed negative findings for iNOS mRNA activity in all specimens.

RT-PCR of iNOS mRNA. The results obtained from RT-PCR were very consistent with those from IS RT-PCR. A band corresponding to a 907-bp PCR product was observed for nine specimens of well-differentiated squamous cell carcinoma (9 of 15, 60%) and four specimens of moderately to poorly differentiated squamous cell carcinoma (4 of 10, 40%). On direct sequencing, this 907-bp band was confirmed to be part of the iNOS gene. No such bands were noticed in any of the samples of normal buccal mucosa, or the negative control samples (Figure 5). All samples apart from the negative control samples revealed bands of β-actin (350 bp) (Figure 5). Three cases were negative on RT-PCR but were positive for iNOS on immunohistochemistry.

Statistical Analysis. Tables 2 and 3 show the relationship between iNOS expression and cervical lymph node metastasis. There was a significant correlation between iNOS expression (at both protein and mRNA levels) and whether patients had nodal disease. No association between degree of differentiation and iNOS protein or mRNA expression was noted.

DISCUSSION

The results of this study are consistent with those of previous reports on iNOS protein expression in human oral carcinomas.[9–11] To the best of our

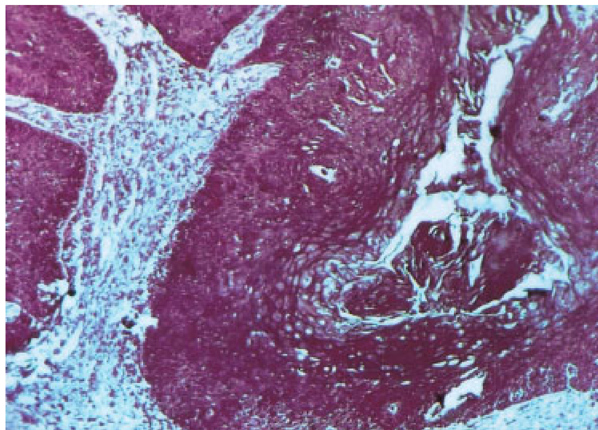

FIGURE 3. The corresponding iNOS mRNA expression for the same sample as in Figure 1 (original magnification ×100).

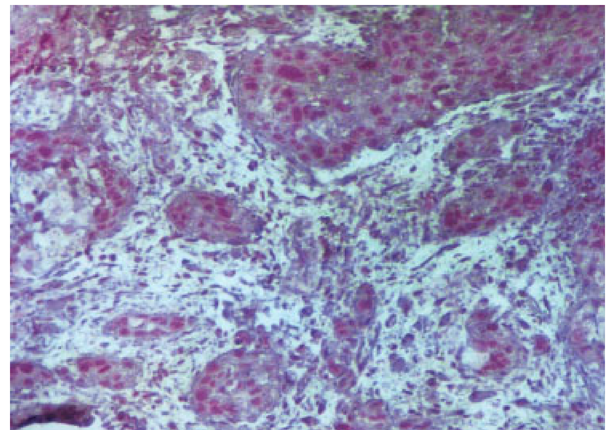

FIGURE 4. The corresponding iNOS mRNA expression for the same sample as in Figure 2 (original magnification ×100).

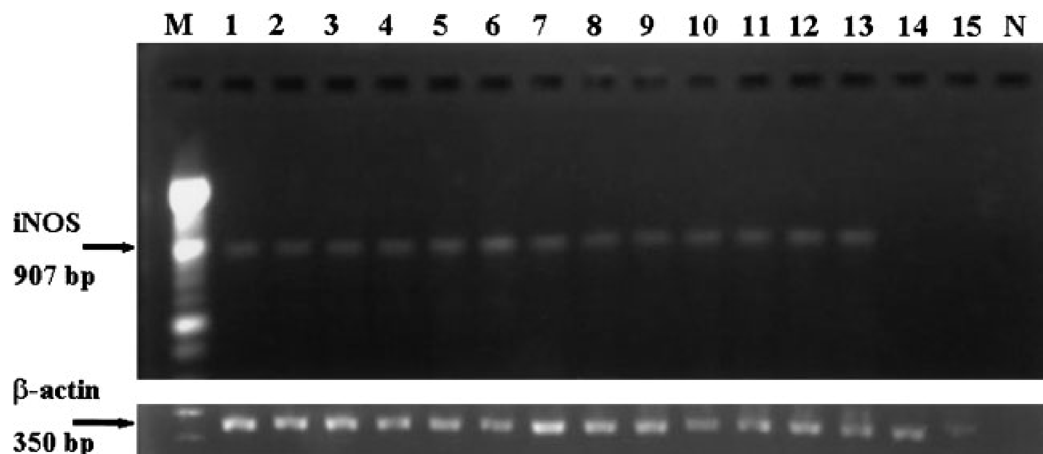

FIGURE 5. Expression of iNOS mRNA in buccal carcinomas using RT-PCR. A band corresponding to a 907-bp PCR product was observed for nine specimens of well-differentiated squamous cell carcinoma (lanes 1–9), and four specimens of moderately to poorly differentiated squamous cell carcinoma (lanes 10–13). No bands were noticed for normal buccal mucosa (lanes 14–15), and the negative control (lane N) samples. All samples (lanes 1–15) except the negative control sample (lane N) revealed bands of β-actin (350-bp). Lane M is the DNA molecular-weight marker.

knowledge, there were no published reports that have investigated iNOS mRNA expression in human oral squamous cell carcinomas. Here, using RT-PCR, we report the presence of iNOS mRNA among a number of human squamous cell carcinomas. We also identify the cellular localization of iNOS mRNA for these squamous cell carcinomas using IS RT-PCR. In addition, we find that the protein expression of iNOS is consistent with mRNA expression in most of the samples examined. Only three cases were negative on RT-PCR and IS RT-PCR but were positive for iNOS on immunohistochemistry. We think that this discrepancy between RT-PCR/IS RT-PCR and immunohistochemistry could be due to mRNA degradation during the tissue processing or to topographic diversity of iNOS expression within the same tumor.

At present, we do not completely understand whether the overexpression of iNOS protein and mRNA is a result of carcinoma or caused by interaction with cytokines or mediated by way of hypoxia in tumor tissue; however, it seems reasonable to suspect that the escalated activity of iNOS protein and mRNA may be, at least in part, implicated in human oral carcinogenesis. Further study is apparently required to explain the exact of role of NO/iNOS in human oral carcinogenesis. As is the case for many other tumors, the expression of mRNA of iNOS is not a ubiquitous finding in oral cancers. The presence of iNOS mRNA has already been found among patients exhibiting gastric carcinomas.[17]

It is noteworthy that the exposure of normal human hepatocytes to a high level of NO initiates a cascade of events that rapidly lead to damage of both nuclear and mitochondrial DNA. Such damage subsequently leads to cell-cycle arrest and mitochondrial dysfunction, ultimately resulting in the pathologic consequences associated with the development of hepatocellular carcinoma.[18] Therefore, by analogy, the prolonged exposure of human oral keratinocytes to NO, as reflected by the enhanced iNOS mRNA activity as noted in this report, may contribute to human oral cavity carcinogenesis.

A feedback loop between NO production and p53 tumor suppressor gene has been reported

Table 2. Correlation of iNOS protein expression in the primary buccal squamous cell carcinoma and status of cervical lymph node metastasis (Fisher's exact test, $p = .01$).

	iNOS protein (+)	iNOS protein (−)
Buccal squamous cell carcinoma with lymph node metastasis	14	3
Buccal squamous cell carcinoma without lymph node metastasis	2	6

Table 3. Correlation of iNOS mRNA expression in the primary buccal squamous cell carcinoma and status of cervical lymph node metastasis (Fisher's exact test, $p = .01$).

	iNOS mRNA (+)	iNOS mRNA (−)
Buccal squamous cell carcinoma with lymph node metastasis	12	5
Buccal squamous cell carcinoma without lymph node metastasis	1	7

previously.[19] If iNOS produces high levels of NO resulting in DNA damage, such activity leading to the activation of the wild-type p53 gene, the presence of wild-type p53 gene in a feedback loop might suppress the expression of NO. However, for cancer cells that have a mutant p53 gene or lack the gene completely, dysregulation of NO and hence an up-regulation of NO will subsequently occur, with the potential to lead to a cancerous state.[20] Given that mutation of the p53 gene is a frequent event in human oral cancerous tissues,[21] it therefore seems reasonable to suggest that oral mucosa tissue with a high level of iNOS protein and mRNA, as demonstrated in this study, could be implicated in the development of cancer.

Consistent with a previous report,[11] there was a significant correlation of iNOS protein expression with cervical lymph node metastasis in oral squamous cell carcinoma. In this study, we further demonstrate that iNOS expression at mRNA level correlates with nodal status in oral carcinomas. Our results were also compatible with previous studies in breast cancer,[22] gastric cancer,[17] and head and neck cancer[23] in which raised iNOS expression was found to be significantly associated with lymph node metastasis.

To our knowledge, no studies have been reported to determine the effects, if any, of betel-quid chewing on iNOS expression in cancer. All patients in this study were betel-quid chewers. Therefore, it would be interesting to know whether betel quid has any impact on NO/iNOS expression. A more corroborative conclusion could be achieved by investigating the NO/iNOS activity in those oral cancer patients without the habit of betel-quid chewing and those individuals who had engaged in betel-quid chewing that did not induce disease in the buccal mucosa. On the other hand, the high expression of iNOS in human oral carcinomas examined in this study may imply a therapeutic avenue through the inhibition of tumor growth with a novel inhibitor of iNOS such as N-(3-(aminomethyl)benzyl)acetamidine (1400 W).[24]

In conclusion, we have demonstrated an enhanced expression of iNOS protein and mRNA in a number of human buccal carcinomas compared with normal buccal mucosa. Such an observation suggests that the iNOS protein and mRNA expression are chiefly derived from a subset of oral cancerous tissues. Our observation also indicates that iNOS expression correlates with nodal status in oral carcinomas. Therefore, it may also indicate that a subset of oral cancers with greater metastatic potential, as evidenced by increased expression iNOS protein and mRNA, is identified.

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