Down-Regulation of Nitric Oxide Synthase-2 and Cyclooxygenase-2 Pathways by p53 in Squamous Cell Carcinoma

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The goal of this study was to analyze the correlation between inducible nitric oxide synthase (iNOS) and COX-2 activities and p53 gene status in head and neck squamous cell carcinomas (HNSCCs) *in vivo* and *in vitro*. In a series of 43 HNSCCs we observed an up-regulation of both iNOS and COX-2 pathways in tumor tissues and both activities were correlated each other ($r_s = 0.612$ and $P = 0.0002$). We also found that p53-mutated HNSCCs (25 cases, 58.1%) showed higher levels of iNOS activity and cGMP in comparison with wild-type p53 tumors (18 cases, 41.9%) ($P = 0.0005$ and $P = 0.01$), as well as higher iNOS immunohistochemical expression ($P = 0.03$). Analogously, higher PgE2 levels were documented in p53-mutated HNSCCs when compared with wild-type p53 tumors ($P = 0.015$) and COX-2 protein expression was higher in p53-mutated HNSCCs ($P = 0.007$). A431 cancer cells expressing a p53 temperature-sensitive mutant showed an ~1.9- and 2.6-fold decrease in spontaneous $\text{NO}_2^-/\text{NO}_3^-$ and PgE2 synthesis at permissive temperature, respectively, when compared with the same cells at nonpermissive temperature ($P \leq 0.001$). Basal levels of iNOS and COX-2 proteins and mRNAs were markedly suppressed by restoration of p53 activity. Our results indicate that p53 gene mutation(s) may be responsible for iNOS and COX-2 up-regulation frequently observed in HNSCCs and suggest that restoration of wild-type p53 expression may interfere with tumor growth by inhibiting iNOS and COX-2 pathways. (*Am J Pathol* 2003, 163:723–732)

Head and neck squamous cell carcinoma (HNSCC) is one of the most common human cancers, representing ~6% of all malignancies in industrialized countries per year. HNSCC is notoriously difficult to treat and has a

high percentage of recurrence. Despite treatment advances, patients with advanced disease have a poor prognosis. More than two-thirds of the individuals with HNSCC at diagnosis will present with stage III and IV disease at primary and/or nodal sites. Despite aggressive therapy, based on combination of surgery and radiotherapy, loco-regional recurrence will develop in 50 to 60% of these patients, and distant metastatic disease in 10 to 20%.¹

The p53 gene is inactivated in the majority of human malignancies,^{2–5} representing the most common specific genetic target involved in human malignant transformation, and its inactivation has been associated in a number of studies with poor clinical prognosis for various types of malignant neoplasms. The gene seems to be also involved in the development and progression of HNSCCs. Depending on the tissue source and method of detection, abnormalities of p53 have been identified in 33 to 100% of HNSCCs.⁶ The presence of abnormal p53 also correlated with a history of heavy smoking and drinking, which represents the primary environmental factors associated with HNSCCs. Moreover, studies suggest that the presence of mutant p53 may also correlate with loco-regional and distant metastases as well as with an increased risk of recurrence in HNSCC patients treated by radiation therapy.^{7–10}

In addition to deregulation of the cell cycle and DNA replication, inactivation of p53 can lead to selective growth advantage and tumor formation^{11,12} by increasing or suppressing the expression of a number of target genes,^{13–15} including positive and negative regulators of neovascularization [eg, vascular endothelial growth factor (VEGF) up-regulation and thrombospondin-1 down-regulation].^{16,17} Angiogenesis is an essential step in tumor growth and metastasis, and several recent studies suggest that tumor vascularization may be regulated, at least in part, by p53.^{18,19} p53 seems also to be involved in the regulation of two key enzymes up-regulated in

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HNSCC: inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).^{20,21}

Release of high amounts of products of iNOS and COX-2 pathways have been reported in several human carcinomas (eg, colon, breast, gastric, ovarian, prostate, and lung mesothelioma cancers),^{22–26} suggesting a central role in the regulation of multiple biological processes responsible for the modulation of tumor growth *in vivo*, such as host immune response, tumor proliferation, and neovascularization.^{27–29}

COX-2 activation induced in epithelial cells by a variety of stimuli seems to be involved in the regulation of tumor angiogenesis by controlling VEGF/vascular permeability factor (VPF), basic fibroblast growth factor, transforming growth factor- β , platelet-derived growth factor, and endothelin-1 release.^{30,31} Analogously, increased expression of iNOS in human cancers has been correlated with a possible role in controlling tumor angiogenesis, mainly influencing VEGF release and fate in carcinoma cell lines.^{32,33}

Recent studies in normal and cancer cells, including a preliminary report on HNSCC from our group,³⁴ suggest a possible role of the p53 gene in controlling iNOS and COX-2 regulation,^{35,36} raising the possibility that disruption of p53 function may influence cancer growth and progression also by an up-regulation of both iNOS and COX-2 activities, however, no definitive data regarding this correlation have been reported. The aim of this study was to examine the possible correlation between the iNOS and COX-2 pathways and p53 gene status in HNSCCs.

Materials and Methods

Patients and Tissue Collection

We studied 43 consecutive HNSCC patients who underwent surgical treatment of the primary tumor and of the neck at the Department of Neuro-oto-ophthalmological Surgical Sciences, Otolaryngology Head and Neck Surgery Division, University of Florence. Clinical, epidemiological, and histopathological characteristics of these patients are shown in Table 1. All tumors were squamous cell carcinomas and were graded as well differentiated, moderately differentiated, and poorly differentiated. Among 43 cases, 17 (39.5%) had histologically confirmed lymph node metastases (N+), whereas the remaining 26 (60.5%) patients had no clinical and histopathological evidence of neck disease (N–).

Tumor sampling was always performed by the same researcher and in the same manner, immediately after surgical specimen removal with a standardized procedure. A central section of the entire specimen including the tumor was collected. The areas of necrosis were excluded. It was then divided by sampling the central area (tumor core), the invasive edge (tumor front), while normal control mucosa was taken 2 to 4 cm away from the tumor margin, either on surgical specimen or outside the excised specimen directly from the remaining tissues. Samples of the tumor edge adjacent to those used for

Table 1. Clinical, Epidemiological, and Histological Characteristics of 43 Head and Neck Cancer Patients

Characteristics	No. (%)
Total no. of patients	43 (100)
Age, years	
Mean 62.3	
Range 50–74	
Sex	
Male	36 (83.7)
Female	7 (16.3)
Smoking history	
No	3 (10.0)
Moderate	15 (34.9)
Heavy	25 (58.1)
Alcohol consumption	
No	9 (21.0)
Moderate	27 (62.7)
Heavy	7 (16.3)
Tumor stage	
I	11 (25.5)
II	13 (30.2)
III	10 (23.3)
IV	9 (21.0)
Tumor site	
Larynx	16 (37.2)
Oral cavity	15 (34.9)
Oropharynx	12 (27.9)
Tumor degree of differentiation	
Well differentiated	15 (34.9)
Moderately differentiated	20 (46.5)
Poorly differentiated	8 (18.6)
Lymph node metastasis	
Yes	17 (39.5)
No	26 (60.5)

molecular studies were also obtained for the histopathological and immunohistochemical assessment.

Analysis of p53 Gene

Analysis of exons 5 to 9 of the p53 gene was performed by polymerase chain reaction (PCR)-single-strand conformational polymorphism (SSCP) on DNA extracted from microdissected paraffin tumor tissue sections.³⁷ Cases with anomalous mobility were sequenced as previously described.³⁷

Assay for iNOS Activity

Fragment of tissues were homogenized at 0 to 4°C in buffer containing 0.32 mol/L sucrose, 20 mmol/L HEPES (pH 7.2), 0.5 mol/L EDTA, and 1 mmol/L dithiothreitol. The homogenates were then processed and the total NOS activity was measured as previously described.²⁰ The activity of the calcium-calmodulin-independent isoform (iNOS) was identified in tumor homogenates by measuring the enzymatic activity in buffer containing 1 mmol/L of EGTA and the calmodulin inhibitor trifluoperazine (100 μ mol/L) (but no calcium) as reported elsewhere.²⁰ For the evaluation of iNOS activity in A431 cells, the cells were stimulated and treated as previously described.²⁰ All determinations were performed in quadruplicate. iNOS activity is expressed as pmol of [³H]citrulline

formed per minute per mg protein in the tissue samples and nmol/ μ g protein in the cells.

Measurement of cGMP Content

The levels of cGMP were measured in the aqueous phase of tissue homogenates extracted from 10% trichloroacetic acid with 0.5 mol/L of tri-*n*-octylamide dissolved in 1,1,2-trichlorotrifluoroethane.²⁰ cGMP was measured with the use of a radioimmunoassay kit and ¹²⁵I-labeled cGMP (Amersham, Buckinghamshire, UK) after acetylation of the samples with acetic anhydride. All determinations were performed in quadruplicate. Values are expressed as fmol of cGMP per mg protein.

PGE2 Measurement

Tissue fragments were homogenized and the homogenates centrifuged at 600 $\times g$ and the supernatants used for PGE2 determination. Five hundred μ l of supernatant of A431 were used for PGE2 determination by a specific radioimmunoassay. Protein concentration in tumor samples and in the cells was determined as described elsewhere.³⁸ Values of PGE2 are expressed as pg/ μ g protein in the cells and μ g/mg protein in the tissue samples.

Immunohistochemical Analysis of iNOS and COX-2 Protein Expression

Immunohistochemical studies were performed by the streptavidin-avidin-biotin technique (Dako S.p.A., Milano, Italy) with diaminobenzidine as chromogen and hematoxylin as counterstain. For COX-2 identification we used a goat polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:30 dilution with incubation lasting for 1 hour at 20°C. iNOS immunostaining was performed using a polyclonal antibody (Biomol Research Laboratories, Plymouth Meeting, PA) at 1:600 dilution, after a microwave antigen retrieval protocol using 10 mmol/L of Tris-HCl buffer (pH 10) and heating for 10 minutes at 500W output.

Fragments of normal human nasal mucosa were used as positive controls for COX-2 and iNOS. Negative controls included substitution of the primary antibody with nonimmune goat or mouse serum and preabsorption of the primary antibody with the antigen. In both conditions the immunostaining was totally or nearly totally abolished.

A semiquantitative system was used to evaluate the level of COX-2 and iNOS expression: immunoreactivity was scored as either absent (–), low (+, less than 25% of positive tumor cells), moderate (+++, 26 to 75% of positive tumor cells), or diffuse (+++, more than 75% of positive tumor cells).

In the attempt to better characterize the source of NO and prostaglandins in tumor specimens, we first evaluated the degree of inflammatory infiltrate present in tumor fragments. In each tumor section, inflammatory cells always represented a minority of total cell population, consisting only up to ~10%. The degree of inflammation was

scored in a semiquantitative manner, as absent, low, moderate, or intense. The inflammatory infiltrate was prevalently detected at the periphery of tumor nests and it was composed of lymphocytes, plasma cells, and macrophages; a small number of neutrophils were identified only in two cases. In 13 HNSCCs (30.2%) there was absent or low inflammatory infiltrate, whereas in the remaining 30 (69.8%) carcinomas inflammation was scored as moderate or intense. In addition, we evaluated the immunohistochemical expression of iNOS and COX-2 in the inflammatory infiltrate using the same score system used for tumor cells.

Cell Culture and Transfection

Human epidermoid carcinoma A431 cells were purchased from European Collection of Cell Cultures-UK (ECACC) and grown at 37°C in 5% CO₂, 10% fetal calf serum Dulbecco's modified Eagle's medium. A431 cells were maintained in Dulbecco's modified Eagle's medium (BioWhittaker, Belgium) supplemented with 10% fetal calf serum (Pool Biological Industries Inc., Logan, UT). Transfection with the p53Val138 plasmid (kindly provided by Prof. N. Tsuchida, Department of Molecular Cellular Oncology and Microbiology, Faculty of Dentistry, Tokyo Medical and Dental University, Japan) coding for a temperature-sensitive p53 (p53TS) were performed with calcium phosphate according to standard protocol. Positive clones for p53TS (A431-p53TS) were identified by PCR. Rescue of p53 activity was confirmed by WAF1 induction assay and growth rate assessment. As negative control we used transfected cells, resistant to neomycin but negative for the presence of p53TS gene (A431-p53TSNeg).

Western Blot Analysis of iNOS, COX-2, and WAF-1 Proteins

Cell pellets were resuspended in 50 μ l of lysis buffer (50 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.5, 5 mmol/L EDTA, 1 mmol/L phenylmethyl sulfonyl fluoride, 0.2 U/ml aprotinin, 0.2 U/ml leupeptin, 0.2 U/ml pepstatin, 1% Triton X-100, 0.5% sodium, and 1 mmol/L sodium orthovanadate) and incubated on ice for 30 minutes. Samples were then clarified by centrifugation (13,000 $\times g$ for 30 minutes at 4°C). One hundred μ g of each cell lysate was mixed with an equal amount of 2 $\times$ sodium dodecyl sulfate sample buffer. The samples were boiled for 5 minutes and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% for COX-2 and iNOS; 10% for WAF-1). The proteins were transferred to nitrocellulose filters that were probed with the following antibodies: anti-COX-2 goat polyclonal (C-20), anti-WAF1 rabbit polyclonal (C19) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and anti-iNOS rabbit polyclonal (Biomol Research Laboratories, Inc.) and developed by the enhanced chemiluminescence system (Amersham, Arlington Heights, IL).

Northern Blot Analysis of iNOS and COX-2 mRNA

Total RNA was extracted from cells by the guanidinium isothiocyanate method. Twenty μg of samples were separated by electrophoresis on 1% agarose gel containing 2.2 mol/L of formaldehyde and transferred to a nylon membrane (Hybond-N; Amersham). After 2 hours at 80°C, the resulting filters were prehybridized for 1 to 2 hours at 42°C in hybridization buffer containing 50% deionized formamide, 5 $\times$ Denhardt's solution, 1 $\times$ sodium chloride/sodium phosphate/EDTA, 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA for 18 to 24 hours at 42°C and washed according to the manufacturer's instructions.

As COX-2 and iNOS cDNA probes we used PCR product fragments obtained by amplification using the following primers: COX-2 forward: 5'-TCACAGGCTTCCATTGACCAAG-3', which gave rise to 644-bp product; COX-2 reverse: 5'-CGCAACAGGAGTACTGACTTC-3'; iNOS forward: 5'-GTCTTGGTCAAAGCTGTGCTC-3'; iNOS reverse: 5'-CAAAGGCTGTGAGTCCTGCA-3' and then purified by the QIAquick gel extraction kit (Qiagen, Valencia, CA). Each probe was labeled with α - ^{32}P dCTP, using random priming reaction (Boehringer-Mannheim, Mannheim, Germany).

We used the GAPDH probe obtained by PCR using primers: GAPDH forward: 5'-CCATGGAGAAGCTGGGG-3'; GAPDH reverse: 5'-CAAAGTTGTCATGGATGACC-3' (196-bp PCR product) as an internal control to adjust for differences in the amount of RNA loaded in each lane. Filters were then exposed for autoradiography to Kodak Xar-5 film (Eastman Kodak, Rochester, NY) for 1 to 6 days at -80°C. The level of expression in cell line samples was performed using an GS-670 Imaging Densitometer (Bio-Rad, Richmond, CA). To exclude possible densitometric signal errors because of background and/or other unspecific phenomenon, the intensity of mRNA expression was compared to the control gene (GAPDH). The densitometric percentage of the autoradiographic signal was evaluated by the comparison between the signal of iNOS or COX2 band and the corresponding hybridization with GAPDH.

Statistical Analysis

Statistical analysis was performed with the use of the Stata Statistic Software (release 5.0; Stata Corp., College Station, TX) and the Number Cruncher Statistical System (version 5.03; J. L. Hintze, Kaysville, UT). Within different tissue samples NOS activity and cGMP and PGE2 levels were compared by use of a paired-value Wilcoxon test. The Wilcoxon rank sum test for unpaired data were used to assess the differences between NOS activity and cGMP and PGE2 levels according to different parameters. The relationship between continuous variables are reported as Spearman product-moment correlation coefficients (rs). Moreover, differences between categorical variables were analyzed by using Pearson's chi-square analysis or Fisher's exact test. Multivariate logistic regression analysis was used to explore the correlation between

p53 mutation and several clinical, histological, and biochemical parameters. Student's unpaired *t*-test was used to assess statistical significance of differences between groups for *in vitro* experiments. All *P* values resulted from the use of two-sided statistical tests. *P* values less than 0.05 were considered to indicate statistically significant differences.

Results

p53 Gene Status in Head and Neck Squamous Cell Carcinomas

An electrophoretic mobility shift indicative of p53 gene mutation was identified in 25 tumor specimens (58%) by SSCP. p53 exon 8 contained 11 of the mutations identified (44%), with 7 mutations detected in exon 5 (28%), 5 in exon 6 (20%), and 2 in exon 7 (8%). The most frequent types of p53 mutation identified in our series were G:C to A:T transitions¹³ and G:C to T:A transversions.⁶

The analysis of the p53 gene status did not show any statistically significant association between p53 gene mutations and sex (*P* = 1.000), tumor site (*P* = 0.333), tumor differentiation (*P* = 0.380), and degree of tumor inflammatory infiltrate (*P* = 1.000). Conversely, tumor stage (*P* = 0.034) and lymph node metastases (*P* = 0.001) correlated with p53 gene mutations. Moreover, a statistically significant difference was found according to alcohol (*P* = 0.036) and tobacco exposure (*P* = 0.049). In fact, all but one p53-mutated cancer was from moderate to heavy smokers.

iNOS and COX-2 Pathways Are Up-Regulated in Head and Neck Squamous Cell Carcinomas

Some of the tumor samples analyzed in this study were included in previous reports.^{20,21} The results of the assays of iNOS and COX-2 pathways and relationships with clinicopathological parameters are summarized in Table 2. We observed a higher iNOS activity in tumor tissue when compared with normal control mucosa (median baseline, 3.67; range, 1.95 to 6.85 pmol min/mg protein for tumor core and median, 4.05; range, 2.041 to 11.80 pmol min/mg protein for tumor edge *versus* median baseline, 1.80; range, 0.60 to 6.85 pmol min/mg protein in normal mucosa) (*P* < 0.0001 in all cases compared with normal mucosa). Analogously, increased cGMP levels were detected in tumor core and tumor edge (median value, 4.82; range, 3.04 to 7.93 fmol/mg protein for tumor core and median, 5.96; range, 3.81 to 16.81 for tumor periphery, respectively) when compared with normal control mucosa (median, 2.93; range, 0.93 to 4.96 fmol/mg protein) (*P* < 0.001).

The levels of iNOS/cGMP detected in tumor tissues, particularly at the tumor front area were significantly higher in patients with stage III to IV disease (rs = 0.41 and *P* = 0.011 for iNOS activity and rs = 0.46 and *P* = 0.003 for cGMP levels) and with histologically confirmed lymph node metastases (N+) (*P* = 0.0002 and *P* < 0.001

Table 2. Correlation of iNOS Activity, cGMP Levels, and PgE2 Levels with Clinicopathological Parameters in 43 HNSCCs

	iNOS activity			cGMP levels			PgE2 levels		
	Normal	Tumor core	Tumor periphery	Normal	Tumor core	Tumor periphery	Normal	Tumor core	Tumor periphery
Site									
Oral cavity	1.84 ± 0.99	4.04 ± 1.46	6.00 ± 3.83	3.10 ± 0.90	5.23 ± 1.45	6.77 ± 2.69	1.09 ± 0.53	2.30 ± 1.06	3.79 ± 1.45
Oropharynx	1.22 ± 0.79	3.28 ± 1.23	4.09 ± 1.90	2.01 ± 0.93	4.88 ± 1.20	6.72 ± 2.71	1.75 ± 1.18	2.97 ± 1.39	4.39 ± 1.98
Larynx	1.87 ± 0.75	3.25 ± 0.96	5.47 ± 2.66	3.93 ± 0.84	4.64 ± 0.98	6.82 ± 2.18	0.94 ± 0.11	1.39 ± 0.48	3.27 ± 2.22
Stage									
I-II	1.37 ± 0.64	3.13 ± 1.03	4.01 ± 1.99	3.01 ± 0.76	4.60 ± 1.25	5.29 ± 1.36	1.04 ± 0.74	1.89 ± 1.16	2.82 ± 1.26
III-IV	1.87 ± 1.04	4.13 ± 1.45	6.30 ± 1.78*	3.09 ± 0.93	5.34 ± 1.36	7.48 ± 2.83*	1.31 ± 0.68	2.59 ± 1.11	4.38 ± 1.55*
Lymph node									
Negative	1.40 ± 0.72	3.16 ± 1.05	4.04 ± 1.81	3.21 ± 0.96	4.70 ± 1.22	5.53 ± 1.28	1.10 ± 0.61	2.16 ± 1.09	3.13 ± 1.24
Positive	2.18 ± 1.08	4.79 ± 1.31*	7.87 ± 4.10*	2.97 ± 0.93	5.71 ± 1.36	8.65 ± 2.99*	1.45 ± 0.82	2.73 ± 1.22	5.23 ± 1.33*
Sex									
Male	1.80 ± 0.97	3.97 ± 1.43	5.95 ± 3.56	3.05 ± 0.92	5.20 ± 1.39	6.94 ± 2.66	1.24 ± 0.75	2.37 ± 1.21	3.87 ± 1.64
Female	1.17 ± 0.64	2.80 ± 0.53	3.10 ± 0.79	3.15 ± 0.69	4.48 ± 1.03	5.68 ± 2.07	1.11 ± 0.39	2.32 ± 0.94	3.88 ± 1.66
Smoking history									
No	1.47 ± 0.6	3.98 ± 1.60	4.47 ± 2.34	3.55 ± 0.96	5.00 ± 1.58	6.25 ± 2.06	1.09 ± 0.37	2.26 ± 0.86	3.31 ± 0.60
Moderate	1.74 ± 1.01	3.12 ± 1.10	4.94 ± 1.93	3.12 ± 1.13	5.15 ± 1.44	6.81 ± 2.04	1.36 ± 0.76	2.51 ± 1.31	4.06 ± 1.78
Heavy	1.77 ± 1.02	4.10 ± 1.41	6.18 ± 4.20	2.90 ± 0.98	5.11 ± 1.31	6.89 ± 2.02	1.17 ± 0.67	2.29 ± 1.18	3.93 ± 1.75
Alcohol consumption									
No	1.76 ± 0.85	3.37 ± 1.07	3.85 ± 1.48	2.75 ± 0.78	4.29 ± 0.95	5.18 ± 1.50	1.14 ± 0.44	2.36 ± 0.93	3.42 ± 1.31
Mild	1.84 ± 1.13	4.15 ± 1.55	6.98 ± 4.35	3.41 ± 0.96	6.09 ± 1.33	8.12 ± 3.10	1.61 ± 0.97	2.80 ± 1.50	4.47 ± 2.01
Heavy	1.50 ± 1.80	3.78 ± 1.46	5.40 ± 2.87	2.93 ± 0.90	4.62 ± 0.92	5.97 ± 1.80	0.85 ± 0.23	1.83 ± 0.72	3.34 ± 0.97
Histologic differentiation									
Well	1.43 ± 0.55	3.24 ± 1.29	6.08 ± 5.99	2.87 ± 1.01	4.93 ± 1.23	6.94 ± 3.77	0.97 ± 0.88	2.62 ± 1.40	3.51 ± 2.02
Moderate	1.86 ± 0.99	4.23 ± 1.45	5.70 ± 2.51	3.22 ± 0.95	5.28 ± 1.40	6.85 ± 2.29	1.30 ± 0.72	2.39 ± 0.94	4.00 ± 1.47
Poor	1.66 ± 0.96	3.39 ± 1.16	4.68 ± 1.79	2.91 ± 0.54	4.62 ± 1.32	6.40 ± 2.05	1.79 ± 0.50	2.04 ± 1.46	3.94 ± 1.73
P53 gene									
Wild type	1.43 ± 0.94	3.50 ± 1.45	3.91 ± 1.97	3.05 ± 0.94	4.97 ± 1.39	5.98 ± 1.89	0.96 ± 0.36	1.97 ± 0.84	2.63 ± 0.98
Mutated	1.91 ± 0.92	4.03 ± 1.35	6.73 ± 3.83*	3.07 ± 0.86	5.19 ± 1.35	7.33 ± 2.92*	1.42 ± 0.82	3.15 ± 1.11	5.12 ± 1.12*

*Denotes significant differences; see text for detailed statistical analysis.

for iNOS activity in tumor core and tumor periphery, respectively, and $P = 0.0002$ for cGMP level at tumor periphery, respectively). Furthermore, considering tumor site, lymph node metastases were observed in four SCCs of the larynx, six of the oral cavity, and seven of the oropharynx. The differences in iNOS activity and cGMP levels between tumor specimens from lymph node-positive and -negative patients were confirmed for oropharyngeal carcinomas ($P = 0.01$). Finally, iNOS activity and cGMP levels at the tumor periphery did not correlate with the degree of tumor inflammatory infiltrate ($P = 0.2$ and $P = 0.18$, respectively).

Cytoplasm staining for iNOS was present in tumor cells of 35 carcinomas (81.4%) and in 30 of 43 cases (69.8%) we identified a moderate or diffuse staining. iNOS protein was also expressed to a variable degree by inflammatory cells infiltrating around and within the tumor, and in endothelial lining of blood and lymphatic vessels. Tumors with moderate or diffuse iNOS expression had higher iNOS activity ($P = 0.09$), as well as significantly higher cGMP levels ($P = 0.01$). Conversely, no correlations were evidenced between iNOS protein expression in the inflammatory cells and iNOS activity ($P = 0.61$) or cGMP levels ($P = 0.54$) at tumor periphery. Similarly to NOS activity, we demonstrated a higher iNOS protein expression in tumor samples from N+ patients when compared with iNOS immunoreactivity detected in carcinomas without neck metastases (N-) ($P = 0.005$).

We then assessed COX-2 activity in specimens obtained from tumor core, tumor edge, and normal control mucosa by determining PgE2 levels (Table 2), and COX-2 protein expression. In specimens from unaffected control mucosa, we detected a median baseline production of PgE2 of 1.04 (range, 0.42 to 3.70) $\mu\text{g}/\text{mg}$ protein. In tumor tissue, PgE2 levels were elevated to 2.09 (range, 0.98 to 4.97) and 3.54 (range, 1.42 to 6.94) $\mu\text{g}/\text{mg}$ protein in tumor core and tumor front area, respectively. These values were significantly higher than those from matched control mucosa ($P < 0.0001$). Accordingly, we found that the extent of PgE2 biosynthesis was significantly different according to the site of sampling within the tumor mass, with specimens at the invasive edge of the tumor exhibiting the highest PgE2 level ($P < 0.0001$).

The levels of PgE2 obtained from tumor periphery were higher in N+ patients ($P = 0.001$) and in more advanced disease (stage III to IV) when compared with those from early stage lesions (stage I to II) ($P = 0.02$). No correlation was found between the degree of tumor inflammatory infiltrate and PgE2 levels ($P = 0.56$) in specimens from tumor periphery.

Immunohistochemical staining of the formalin-fixed paraffin-embedded tissue sections revealed that COX-2 was primarily localized in tumor cells, as well as in tumor-infiltrating inflammatory cells, endothelial cells, and smooth muscle cells of blood vessel wall and striated muscle. Overall, 36 of 43 (83.7%) squamous cell carci-

nomas showed cytoplasm immunoreactivity for COX-2 in tumor cells, 29 (67.4%) of which showed a moderate or diffuse immunostaining. Statistical analysis evidenced a correlation between PgE2 levels and COX-2 immunoreactivity in tumor cells ($r_s = 0.35$, $P = 0.023$), while considering tumor inflammatory infiltrate we were unable to find any significant correlation between COX-2 protein expression and PgE2 levels ($P = 0.53$). Tumors with cervical lymph node metastases showed significantly higher COX-2 expression ($P = 0.007$) in neoplastic cells and COX-2 protein tended to be higher in tumors in advanced stage ($P = 0.1$).

A comparative analysis of iNOS and COX-2 activities showed a statistically significant correlation between iNOS/cGMP and PgE2 levels assessed in normal and tumor tissues (overall $P < 0.01$), particularly in samples from the tumor edge ($r_s = 0.612$ and $P = 0.0002$; $r_s = 0.732$, $P < 0.0001$ for iNOS and cGMP activity, respectively), thus, suggesting a co-regulation of iNOS and COX-2 pathways in HNSCCs. These findings were confirmed by comparative analysis of iNOS and COX-2 protein expression in tumor cells assessed by immunohistochemistry ($P = 0.03$). Conversely, there was no correlation between iNOS and COX-2 expression in the inflammatory infiltrate ($P = 0.7$).

Correlation of iNOS and COX-2 Pathways with p53 Gene Status

When we correlated p53 gene status and COX-2 and iNOS activities in our series, we demonstrated a statistically significant correlation between up-regulation of both activities and p53 gene aberration. In fact, the great majority of tumor specimens obtained from p53-mutated cancers revealed moderate to diffuse immunoreactivity for COX-2 ($P = 0.007$) and iNOS ($P = 0.03$). Accordingly, we found that p53-mutated HNSCCs (25 cases, 58.1%) showed higher levels of iNOS activity, particularly at the tumor edge, and cGMP levels in comparison with wild-type p53 tumors (18 cases, 41.9%) (Table 2, $P = 0.0005$ and $P = 0.01$). Analogously, higher PgE2 levels were documented in p53-mutated HNSCCs, when compared with wild-type p53 tumors, particularly at the tumor periphery (Table 2, $P = 0.015$). Among p53-mutated carcinomas we did not demonstrate any difference in the iNOS and COX-2 pathways according to type of p53 gene mutation (data not shown). Moreover, logistic regression analysis showed that iNOS activity ($P = 0.03$) and PgE2 levels ($P = 0.05$), were independently associated with p53 mutations, whereas age, sex, tumor site, stage, grade, alcohol, and tobacco consumption were not associated.

Wild-Type p53 Expression in A431 Inhibits the Synthesis of Prostaglandins and Nitrite/Nitrate

A431 cells expressing an impaired p53 were transfected with a p53 temperature-sensitive mutant (p53TS).³⁹ An

Figure 1. A: Growth curves of p53 temperature-sensitive-expressing (A431-p53TS) and negative cells (A431-p53TSNEG) after incubation at 32.5°C and 37.5°C, respectively. **B:** Western blot showing the Waf-1 induction after p53 activation in p53 temperature-sensitive-expressing cells (A431-p53TS) versus negative cells (A431-p53NEG). **Left:** A431-p53TSNEG incubated at the indicated temperatures. **Right:** A431-p53TS incubated at the indicated temperatures.

expected PCR product corresponding to the p53TS gene was obtained from 1 of 10 clones after PCR amplification (data not shown). In particular, the p53TS exogenous protein resulted activated by a 37.5 to 32.5°C temperature shift. The A431-p53TS cell line showed a slower growth rate at 32.5°C, as assessed by cell counting (Figure 1A). Despite that A431-p53TS cells grow at approximately the same rate as A431-p53TSNEG at 37.5°C, their growth rate was also slower with respect to the A431-p53TSNEG cells at 32.5°C. Moreover, the apoptotic threshold was lowered by activation of p53 resulting from exposure to increasing concentrations of 5-fluorouracil (data not shown). A431-p53TS cells were also tested for p53 transactivation activity. We performed a WAF-1 induction assay (Figure 1B) by changing the cultivation temperature from 37.5 to 32.5°C. As shown, WAF-1 was undetectable in A431-p53TS cells at 37.5°C and in A431-

Table 3. Prostaglandins and Nitrite/Nitrate Levels in A431-p53TS Cells under Basal or p53-Activating Conditions

Temperature	Prostaglandins/total proteins ($\mu\text{mol}/\mu\text{g}$)	$\text{NO}_2^-/\text{NO}_3^-$ ($\mu\text{mol}/\mu\text{g}$)
37.5°C	76.1	1.470
32.5°C	29.6	0.795

Figure 2. A: Northern blotting analysis of iNOS and COX-2 mRNA expression in p53 temperature-sensitive-expressing cells (A431-P53TS). Temperatures are given on the **top** of the blots; below the same blot probed for GAPDH as control for RNA loading. Results of the densitometry, normalized for the level of the GAPDH in each lane and expressed in arbitrary units were: iNOS: A431-P53TSNEG at 37°C, 4.41; A431-P53TSNEG at 32.5°C, 2.79; A431-P53TS at 37°C, 4.53; A431-P53TSNEG at 32.5°C, 1.25; COX-2: A431-P53TSNEG at 37°C, 6.73; A431-P53TSNEG at 32.5°C, 3.51; A431-P53TS at 37°C, 6.54; A431-P53TSNEG at 32.5°C, 2.19. **B:** Western blot showing the iNOS and COX-2 reduction after p53 activation in p53 temperature-sensitive-expressing cells (A431-P53TS). **a:** Negative A431 cells (A431-P53TSNEG) incubated at the indicated temperatures detected with anti-iNOS Ab. **b:** A431-P53TS incubated at the indicated temperatures detected with anti-iNOS Ab. **c:** A431-P53TSNEG incubated at the indicated temperatures detected with anti-COX-2 Ab. **d:** A431-P53TS incubated at the indicated temperatures detected with anti-COX-2 Ab.

p53TSNeg, while it was strongly induced at 32.5°C only in A431-p53TS. We then proceeded to analyze the effect of p53 activation on nitrite/nitrate and prostaglandin production. As shown in Table 3 prostaglandin and nitrite/nitrate levels were significantly reduced in supernatants when cells were grown at 32.5°C for 24 hours. The increase in the respective levels of prostaglandins and nitrite/nitrate was 2.6- and 1.9-fold.

Restoration of p53 Activity Inhibits iNOS and COX-2 in A431 Cells Both at Transcriptional and Translational Level

We performed Northern blot analysis of total RNA from A431-p53TS and A431-p53TSNeg cell lines, both at 32.5°C and 37.5°C. As shown in Figure 2A the shift to 32.5°C resulted in a dramatic reduction of iNOS and

COX-2 transcripts. Interestingly, we also found a basal expression of both inducible enzymatic isoforms in A431-p53TSNeg and A431-p53TS cells at 37.5°C, according to a transformed phenotype.³³ A similar behavior of both COX-2 and iNOS was observed also at protein level (Figure 2B).

Discussion

In this study we show a simultaneous up-regulation of iNOS and COX-2 activity in HNSCC. The products of these enzymes as well as iNOS and COX-2 protein expression were strongly correlated with lymph node metastases, suggesting that an increased metastatic behavior of these tumors is associated with up-regulation of both iNOS and COX-2 pathways. Moreover, p53-mutated carcinomas released the highest levels of nitrite/nitrate and prostaglandins in our series, suggesting a key role of p53 mutation in the HNSCC up-regulation of both iNOS and COX-2. This hypothesis was confirmed by *in vitro* studies demonstrating that in a p53-mutated cancer cell line (A431) the forced expression of wild-type p53 results in down-regulation of iNOS and COX-2 expression as well as of their products. A431 is a squamous carcinomas cell line able to release spontaneously both iNOS and COX-2 products according to a transformed phenotype.³³ This cell line expresses a mutated impaired p53 protein in which transcriptional activity can be restored after transfection with wild-type p53.⁴⁰ Restoration of p53 activity by transfection with a temperature-sensitive conditional mutant, as assessed by WAF1 induction and reduced growth at the permissive temperature, is clearly inversely related to iNOS and COX-2, both at protein and mRNA levels. More importantly the observed protein and mRNA decreases were paralleled by an analogue lowering in the activities of both enzymes as assessed by NO and PgE2 assays. Thus, our results strongly suggest that iNOS and COX-2 are p53 target genes subjected to p53 repression. Taken together these data evidence that interactions between p53 and both iNOS and COX-2 pathways could be important in regulating tumor growth and angiogenesis in human carcinoma. Indeed, we have previously reported that COX-2 and iNOS pathways are strongly related to tumor angiogenesis in HNSCC, possibly by controlling VEGF release in tumor cells^{20,21}

This is the first report documenting that simultaneous COX-2 and iNOS up-regulation is associated with p53 mutations in HNSCC. This correlation has been previously investigated by other groups with variable results, possibly depending on differences of tumor site. A positive correlation between iNOS, COX-2, and p53 has been recently reported at the protein level in gastric cancer.⁴¹ Conversely, most authors reported a correlation between p53 status and either iNOS or COX-2.^{32,42} In some of these studies, it has been postulated that high NO levels may exert genotoxic effect on DNA, particularly on the p53 gene, thus suggesting a clear correlation between iNOS activity and type of p53 gene mutation.⁴³ On the basis of DNA alteration induced by high NO levels, the high rate of G:C to T:A transition reported in colon can-

cer⁴³ indicates that iNOS up-regulation is an early event in the multistep process of carcinogenesis at different sites. Conversely, in our collection of HNSCCs we were unable to find any correlation between iNOS activity and type of p53 mutation, whereas a clear correlation exists between p53 status and tobacco exposure. Therefore, HNSCC appears to be substantially different from other cancer models, such as colon cancer and non-small-cell lung cancer. It is likely that tobacco carcinogen exposure may be important in the pathogenesis of smoking-related cancer, such as HNSCC, both determining p53 mutation-dependent and/or -independent up-regulation of enzymatic activities like COX-2.⁴⁴ In addition another aspect that appears to be peculiar of HNSCC is the limited contribution of stromal reactive cells to NOS and COX product release in tumor microenvironment, further supporting the recent data by Zweifel and colleagues.⁴⁵ Conversely, in other models a relevant activity of these pathways seems to be related to reactive inflammatory cells,^{46–48} linking chronic inflammation with somatic p53 mutations and increased risk of cancer through the genotoxic effect of NO release by inflammatory cells.

In human carcinogenesis, iNOS and COX-2 products have been implicated in the regulation of immune systems, tumor cell apoptosis, and angiogenesis.^{27–31,33} In several studies the overexpression of iNOS and COX-2 products has been also correlated with the metastatic potential of neoplastic cells showing a potential prognostic significance in human carcinomas.^{24,25,33} We postulated that the loss of p53 wild-type function or the expression of mutant p53 in HNSCC cells, frequently because of the mutagenic effect of tobacco/alcohol carcinogens, would activate several key genes in transformed cells with an up-regulation of both iNOS and COX-2. Therefore, the related increased production of nitric oxide and PGs in tumor cells could have a central role in the release of several angiogenic factors such as VEGF,^{19–21,32} a vital event for the tumor growth sustained by a new vascular network. Together with a positive effect on tumor vascularization, iNOS and COX-2 pathways have a potential role in controlling tumor cell proliferation and apoptosis, thus, it is conceivable that both biochemical activities promote cancer progression by providing a selective growth advantage to tumor cells with mutant p53. This may explain the increased iNOS and COX-2 activities as well as the higher incidence of p53 mutations in advanced HNSCC compared with early cancers. In fact, advanced HNSCC releasing high amounts of nitric oxide and PGs showed a higher rate of p53 mutations. Therefore clonal selection and growth of these p53-mutated tumors could be further supported by the combination of iNOS and COX-2 products induced angiogenesis, increased vascular permeability and host immune suppression, and reduced apoptosis. Accordingly, we have recently suggested a prognostic impact of COX-2 activity in HNSCC⁴⁹ as well as the potential therapeutic implications of these metabolic activities in tumor cells,^{19–21} recently confirmed in colon cancer cells *in vitro*.⁵⁰

Our results also give the opportunity to better clarify some aspects of currently performed gene therapy for head and neck cancer patients. According to a central role of p53 in

HNSCC carcinogenesis, the development of gene transfer technology has allowed the intratumoral administration of an adenovirus vector containing wild-type p53 complementary DNA in several HNSCC patients. In phase I clinical trials repeated intratumoral injections of Ad-p53wt have been well tolerated and resulted in a tumor regression in a subset of patients with advanced disease.⁵¹ These data together with the notion that induction of p53wt can cause cells to undergo apoptosis have suggested that tumor regression after adenovirus replacement of p53wt may be because of a central role of p53 gene in the control of a programmed cell death or apoptosis.⁵² However, this hypothesis was unable to explain the significant bystander effect (killing or growth arrest of nontransduced tumor cells) noted in pre-clinical and clinical studies, whereas it is likely that the *in vivo* anti-tumor effect of the p53wt gene transfer is, at least in part, because of its potent anti-angiogenic effect.⁵³ According to our results, the bystander effect of Ad-p53wt therapy could be at least in part because of a decreased release in tumor microenvironment of proinflammatory and proangiogenic factors derived from COX-2 and iNOS enzymatic activities. These effects might in fact be similar to those induced on tumor cells and angiogenic endothelial cells by COX-2⁵⁴ and iNOS^{32,55} inhibition. Furthermore, Yu and colleagues⁵⁶ have recently reported that mice bearing tumors derived from p53(–/–) cancer cells were less responsive to anti-angiogenic therapy than mice bearing isogenic p53(+/+) tumors, thus suggesting that although anti-angiogenic therapy targets genetically stable endothelial cells in the tumor vasculature, genetic alteration that decrease the vascular dependence of tumor cells can influence the therapeutic response of tumor to this therapy. According to our results and to reported *in vitro* and *in vivo* anti-proliferative and proapoptotic effects of COX-2 and iNOS inhibition on angiogenic endothelial cells,^{49,54,55,57} it is likely to hypothesize that isogenic p53(+/+) tumors might be more resistant to anti-angiogenic therapy also because of protective effects on targeted tumor vessel cells of COX-2 and iNOS enzyme products.

In conclusion, further investigations will be required to determine the exact role of p53, iNOS, and COX-2 in human carcinogenesis as well as the mechanism(s) by which the most frequently mutated tumor suppressor gene and both pathways are involved in the regulation of several biological functions in human cancers, including inflammatory reactions, proliferation, apoptosis, and neo-angiogenesis. However, here we report data about the possibility of a close regulation of iNOS and COX-2 activities by p53, suggesting a possible control of nitric oxide and PG release by human carcinoma cells *in vivo*. These data might have important implications for the therapeutic use of iNOS and COX-2 inhibitors as well as of p53 gene therapy in future anti-cancer therapeutic strategies in p53-mutated malignancies.

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