Tissue Expression of inducible Nitric Oxide Synthase (iNOS) and p53 in Oral Squameous Cell Carcinoma Patients

[1]Ahmed Khafagy and [2]Manal El-Mahdy

[1]Department of Otorhinolaryngology, Faculty of Medicine, Ain Shams University
[2]Department of Pathology, Faculty of Medicine, Ain Shams University
ahmedkhafaagy@yahoo.com

Abstract: Objectives: To determine tissue expression levels of inducible nitric oxide synthase (iNOS) and p53 in specimens of pathologically documented oral squameous cell carcinoma (OSCC) in comparison to specimens of normal healthy oral tissue. **Patients & Methods:** The study included 24 patients; 18 males and 6 females with mean age of 60.7±10.1 years. Seventeen patients had tongue lesion, 4 had mouth floor lesion and 3 had buccal mucosa lesions. Mean diameter of lesions was 3.6±1.3; range: 1.2-5.2 cm. All patients underwent full history taking, complete clinical examination and grading according to American Joint Committee of Cancer and underwent preliminary biopsy taking for confirmation of diagnosis and to grade the degree of tumor differentiation. All patients underwent surgical resection with safety margin and the resected specimens were examined immunohistochemically for nuclear staining for p53 and cytoplasmic staining for iNOS. **Results:** Fourteen specimens were positive, while 10 negative for iNOS; 8 specimens stained positive were of pathological grade I, 5 specimens were grade II and only one specimen was of grade III with significantly higher frequency of positivity for iNOS among specimens of grade I. Six specimens of patients had nodal involvement were stained positive for iNOS, while the other 2 specimens of patients had nodal involvement were stained negative for iNOS with significantly higher frequency of positive staining for iNOS among patients had nodal involvement and a positive significant correlation between nodal involvement and positive staining for iNOS. Thirteen specimens of those iNOS positive were positive for p53, 8 specimens were negative for both proteins, while one specimen positive for iNOS was negative for p53. **Conclusion:** Overexpression of both iNOS and p53 is a concomitant finding in tissues of OSCC and positively correlated with each other and with nodal involvement and thus mostly indicate unfavorable prognosis.

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1. Introduction

Oral Squameous Cell Carcinoma (OSCC) is the sixth most common cancer in the world and is one of the leading causes of death in India. Sixty percent of oral cancers are well advanced by the time they are detected and despite innovation being made in surgery, radiation and chemotherapy, the long term survival rate remains to be less than 50%. By the time oral cancer is diagnosed, most individuals have localized or regional disease (37% localized; 43% regional; 10% distant and 10% unstaged). Five year survival rates for all oral cancers cases are 79% for those with localized disease, 42% for regional disease and 19% for disease with distant metastases [1-3].

Current evidence shows that genetic factors play an important role in increasing risk of cancer development; however heredity alone cannot explain the etiology of every cancer. In addition, alteration at the protein level as well as environmental factors can also induce carcinogenesis especially in cancer with known risk factors such as cancer associated with inflammation as oral, gastric and colonic cancers [4, 5].

Several pro-inflammatory gene products mediate a critical role in suppression of apoptosis, proliferation, angiogenesis, invasion, and metastasis. Among these gene products, inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), are the current molecules showing close relation to carcinogenesis and angiogenesis [6, 7].

Nitric oxide (NO) is a short-lived biomolecule that plays a role as a signal molecule in organisms, immunological defense mechanisms and carcinogenesis. Nitric oxide is a product of the conversion of L-arginine to L-citrulline by nitric oxide synthase which has three isoforms. The distribution of NOS isoforms is tissue-specific and is altered by pathological conditions. Inducible NOS activity is Ca^{2+}-independent and is induced in various types of cells by inflammatory cytokines, lipopolysaccharides, and other stimuli [8-10].

Although NO is known to have tumoricidal properties by various mechanisms including direct DNA damage, inhibition of DNA synthesis and inhibition of mitochondrial activity; it has also been implicated in tumor progression and dissemination. Moreover, NO reacts with superoxide anions to form a

peroxynitric anion which is highly toxic molecule causing DNA damage and protein modifications [11, 12].

In normal oral epithelium, p53 is restricted to the proliferative basal cell layer. Overexpression of inactivated or mutated forms of p53 in oral epithelial dysplasia has been associated with high risk for transformation to early stage OSCC. Suprabasal p53 immunoexpression patterns are associated with high grades of dysplasia and correlate with progress to OSCC [13- 15].

The relationship between iNOS and tumor suppressor gene, p53 was extensively studied. In murine model with intact p53 pathway, high concentrations of NO produced by iNOS induces accumulation of wild-type p53 protein, which promotes apoptosis. This results from binding of p53 protein to a promoter site on iNOS gene, which inhibits its transcription. Mutant forms of p53 protein do not have this effect. The expression of iNOS is increased in p53 knockout mice missing the gene for p53 causing early death from formation of multiple tumors [16, 17].

The current prospective comparative study aimed to determine tissue expression levels of iNOS and p53 in specimens of pathologically documented OSCC in comparison to specimens of normal healthy oral tissue.

2. Patients and Methods

The present study was conducted at Departments of Otorhinolaryngology and Pathology, Faculty of Medicine, Ain Shams University since Jan 2008 till Jan 2011 and included 24 patients with pathologically documented OSCC. After obtaining approval of Local Ethical Committee and patients' written fully informed consent; all patients underwent full history taking, complete clinical examination and clinical staging according to American Joint Committee of Cancer. All patients underwent MRI examination for determination of tumoral extent and spread. All patients underwent preliminary biopsy taking for confirmation of diagnosis and to grade the degree of tumor differentiation.

All patients underwent surgical resection under general anesthesia with safety margin that was approved using frozen section and defect was closed according to situation. The resected specimens were formalin fixed and paraffin-embedded blocks of obtained samples were collected. Tissue expression was evaluated using monoclonal antibodies to the specific isozymes; iNOS and p53. Nuclear staining for p53 and cytoplasmic staining for iNOS were examined on a semi-quantitative analysis evaluating the percentage of staining within representative areas of each specimen. Immunohistochemical staining for iNOS was classified according to ***Brennan et al.*** [18] as negative if no staining or positive staining was present in <25% of tumor cells; or positive if >25% of tumor cells showed cytoplasmic staining. Nuclear staining for p53 of >20% of the neoplastic cells was considered positive [19].

3. Results

The study included 24 patients; 18 males and 6 females with mean age of 60.7±10.1; range: 45-79 years. All patients presented by oral cavity lesion; 17 patients had tongue lesion, 4 had mouth floor lesion and 3 had buccal mucosa lesions. All tongue lesions were of ulcerative type with evident malignant characters; two buccal lesions were nodular with top ulcerations, one of lesions of floor of mouth appears as a cauliflower mass with evident ulceration, while the remaining lesions were ulcerative lesions. Foul breath odor, bloody salivary spout and bleeding on touch were the commonest symptoms.

Mean diameter of lesions was 3.6±1.3; range: 1.2-5.2 cm in its greatest diameter. All patients had enlarged lymph nodes either cervical or submental or submandibular in different distribution. However, only 8 patients had hard cervical lymph nodes with a picture suggestive of malignant spread. No lesion had distant metastasis.

According to TNM classification, considering absence of distant metastasis or local infiltration no lesions of $T_{1-3}N_{1-3}M_1$ was detected. Seven patients had $T_2N_0M_0$ lesions, 9 patients had $T_3N_0M_0$ lesions, 4 patients had $T_{1-3}N_1M_0$ lesions and 4 patients had $T_{1-3}N_2M_0$ lesions, (Table 1).

Pathological examination of excised specimens defined 12 specimens (50%) of well differentiated SCC (Grade I), 8 specimens (33.3%) of moderately differentiated SCC (Grade II) and 4 specimens (16.7%) of poorly differentiated SCC (Grade III). Nodal involvement was detected in 8 specimens (33.3%), while no nodal involvement was detected in the other 11 specimens (45.8%).

Cytoplasmic positive staining for iNOS was detected in 14 specimens (58.3%), while the remaining 10 specimens (41.7%) were negative for iNOS cytoplasmic staining. The majority of specimens stained positive were of pathological grade I, (n=8; 57.1%), (Fig. 1), 5 specimens (35.7%) were of pathological grade II, (Fig. 2), while the remaining specimen (7.2%) was of pathological grade III, (Fig. 3). On contrary, 3 specimens of pathological grade III, 3 specimens of pathological grade II and 4 specimens of pathological grade I were stained negative, (Table 2, Fig. 4). There was significantly higher (X^2=4.359, p<0.05) frequency of positive cytoplasmic staining for iNOS among specimens of grade I, however, the correlation between cytoplasmic staining and pathological staging was negative non-significant (r=-0.256, p<0.05).

As regards nodal involvement, 6 specimens (75%) of the eight patients had nodal involvement were stained positive for iNOS, while the other 2 specimens

(25%) of patients had nodal involvement were stained negative for iNOS. On the other hand, 2 specimens of patients free of nodal involvement were stained positive for iNOS, while the other 14 specimens of patients free of nodal involvement were stained negative for iNOS, (Fig. 5). There was significantly higher (X^2=8.149, p<0.05) frequency of positive cytoplasmic staining for iNOS among specimens of patients had nodal involvement with a positive significant correlation between nodal involvement and positive cytoplasmic staining for iNOS, (r=0.410, p=0.047).

Nuclear positive staining for p53 was detected in 13 specimens (92.9%) of those showed cytoplasmic positive staining for iNOS, (Figs. 6, 7), one specimen stained positive for iNOS showed nuclear negative staining for p53. Eight specimens (80%) showed cytoplasmic and nuclear negative staining for iNOS and p53, respectively, while two specimens showed positive nuclear staining for p53 were negative for iNOS, (Fig. 8). There was a positive significant correlation between positive nuclear staining for p53 and positive cytoplasmic staining for iNOS, (r=0.720, p<0.001).

Table (1): Patients' distribution according to lesion characters

			Number	%
Site	Mobile tongue		10	41.7
	Posterior tongue		7	29.1
	Floor of the mouth		4	16.7
	Buccal mucosa		3	12.5
Diameter (cm)	1-2		3	12.5
	>2-3		5	20.8
	>3-4		3	12.5
	>4-5		10	41.7
	>5		3	12.5
TNM grade	T	1	4	16.7
		2	9	37.5
		3	11	45.8
	N	0	0	0
		1	20	83.3
		2	4	16.7
	M	0	24	100
Stage	T_1	$T_1N_0M_0$	0	0
	T_2	$T_2N_0M_0$	7	29.1
	T_3	$T_3N_0M_0$	9	37.5
		$T_{1-3}N_1M_0$	4	16.7
		$T_{1-3}N_2M_0$	4	16.7

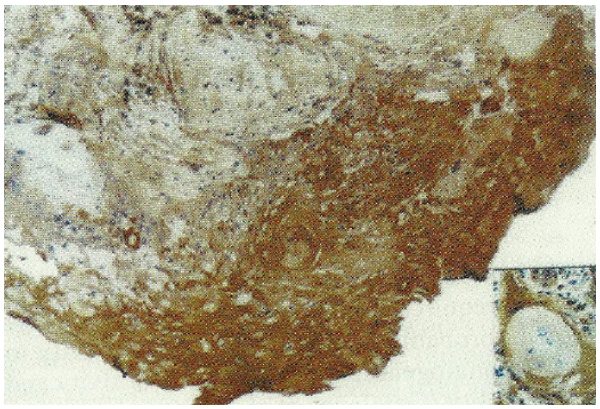

Fig. (1): Well-differentiated SCC showing positive cytoplasmic expression of iNOS protein by tumor cell. Inset: showing staining immediately around the keratin pearl (iNOS, x200).

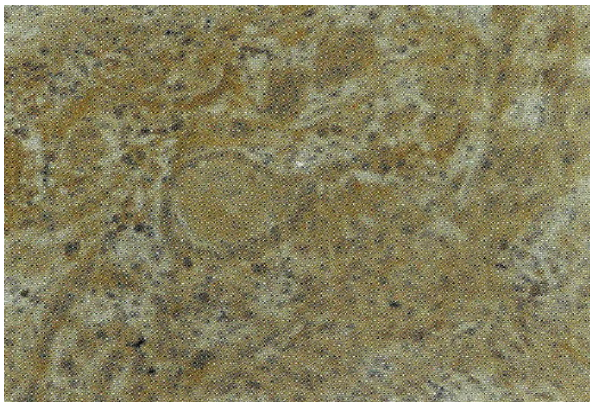

Fig. (2): Moderately differentiated SCC showing positive cytoplasmic expression of iNOS protein by tumor cell (iNOS, x200).

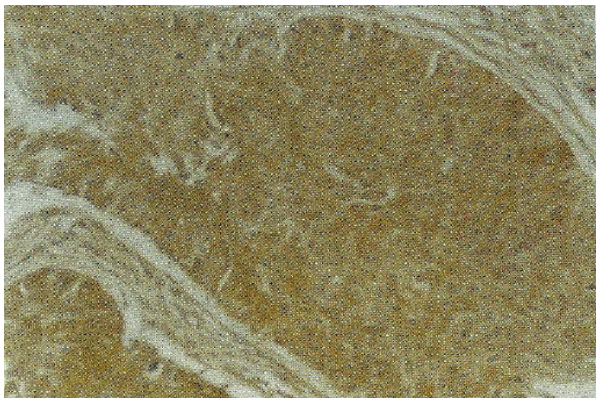

Fig. (3): Poorly differentiated SCC showing positive cytoplasmic expression of iNOS protein by tumor cell, (iNOS, x200).

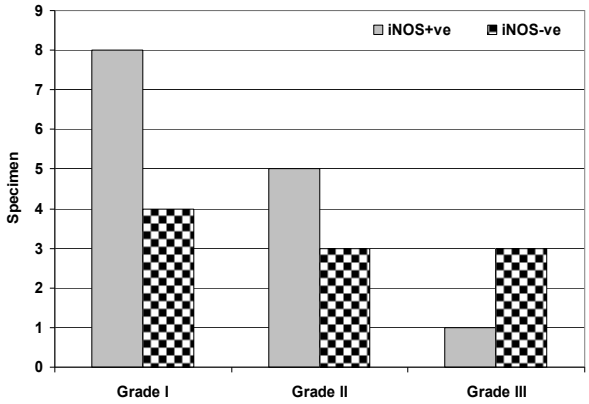

Fig. (4): Distribution of Specimens categorized according to pathological grades according to cytoplasmic staining for iNOS

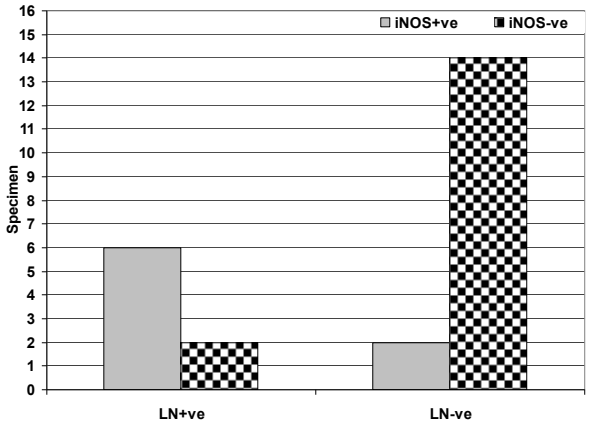

Fig. (5): Distribution of Specimens categorized according to nodal involvement according to cytoplasmic staining for iNOS

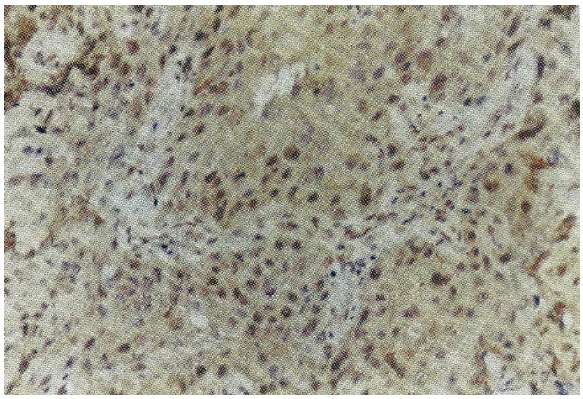

Fig. (6): Moderately differentiated SCC showing positive nuclear expression of p53 protein by tumor cell, (p53, x300).

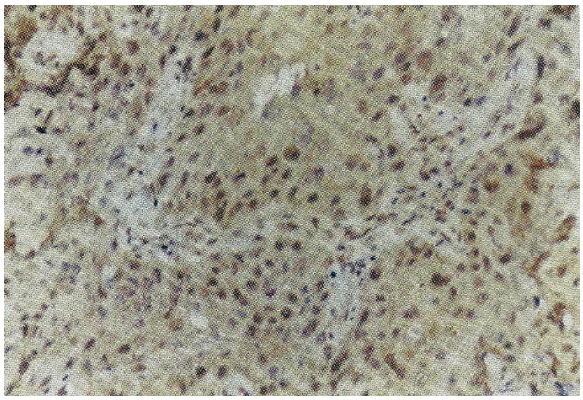

Fig. (7): Poorly differentiated SCC showing positive nuclear expression of p53 protein by tumor cell, (p53, x300).

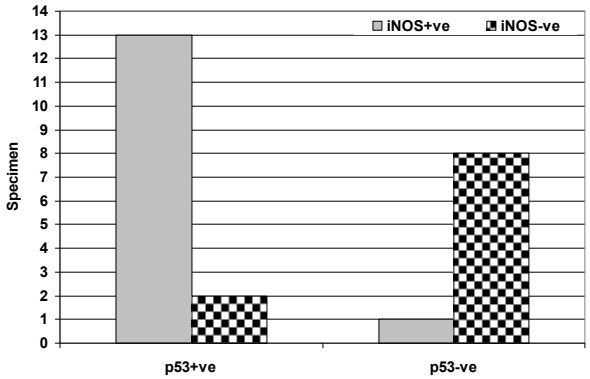

Fig. (8): Distribution of Specimens according nuclear stainng for p53 and cytoplasmic staining for iNOS

4. Discussion

The outcomes of the current study showed the following: firstly, the high prevalence of nuclear and cytoplasmic positive staining for p53 and iNOS, respectively, of tissue specimen of oral SCC with detection rate of 62.5% and 58.3%, respectively. Such finding indicates a strong association between carcinomatous changes in oral tissue and development of positivity of both proteins. Along with this finding, no positivity was detected in control tissue specimens obtained from the safety margin of the same specimen away from the site of cancer. Moreover, the presence of positivity around keratin pearls indicated earlier activation of genes for these proteins and a possible role for both proteins in the progress of dysplastic tissue to invasive carcinoma.

These data supported that previously reported by ***Horta et al.*** [20] who found p53 and p21were overexpressed at the invasive front of lower lip SCC and concluded that p53 and p21overexpression is important in lower lip SCC pathogenesis and with ***Khan et al.*** [21] who reported positive staining for p53 was 59% in oral SCC lesions, 38% in premalignant lesions.

Also, ***Mozet et al.*** [22] reported iNOS protein expression rate of 55% in head and neck SCC and presumed that synthesized NO is able to support angiogenic patterns and facilitate tumor progression and lymphatic spread. ***Safadi et al.*** [23] estimated the positivity of nuclear staining for p53 in various oral lesions and detected that the mean percentages of positive nuclei of p53 was 40.27% in oral lichen planus, 40.5%, 49.78% and 61.36% in mild, moderate and severe oral epithelial dysplasia, respectively and 78.16% in oral SCC with significant difference compared to normal oral epithelium, oral focal keratosis and oral mucositis which showed mean positive percentages of 15.06%, 27.87%, 30.08%, respectively.

Secondly, the current study reported that 8 of 9 specimens negative for p53 were also negative for iNOS with a positive significant correlation between the detection of nuclear and cytoplasmic positivity for p53 and iNOS, respectively; a finding indicating a possible relationship for the expression for both proteins. Multiple studies tried to explore such positive relationship between p53, a tumor suppressor gene and NO which induces angiogensis and tumorgenesis; one possibility is that expression of wild-type p53 protein is increased in response to increased production of NO by iNOS as shown in animal studies which showed that accumulation of p53 protein leads to apoptosis after exposure of cells to high concentration of NO in an attempt to minimize NO-induced DNA damage [24, 25]. Considering the fact that intact p53 pathway would down-regulate iNOS expression, a possibility of a mutant p53 gene resulting in production of stable protein could cause the negative feedback loop to fail and consequently increases iNOS expression with accelerated tumor growth associated with increased vascular endothelial growth factor (VEGF) expression and neovascularization [26].

A new hypothesis for the role of iNOS and p53 in cancer pathogenesis and progression was proposed dependent on the activation of growth factors and subsequent neovascularization; ***Cheng et al.*** [27] reported that the mean labeling indices of VEGF increased significantly from normal oral mucosa (13%), through mild (22%), moderate (24%), and severe oral epithelial dysplasia (32%), to OSCC samples (50%) and higher mean VEGF labeling index was significantly related to oral SCC with positive lymph node metastasis and with more advanced clinical stages. ***Ou Yang et al.*** [28] reported that the positive expression rates of iNOS and VEGF were successively enhanced in well- and poorly differentiated mucoepidermoid carcinoma of salivary glands and microvessel density counts were positively correlated with the expression levels of iNOS and VEGF and the expression of iNOS was positively correlated with the expression of VEGF. ***Nagini et al.*** [29] reported that analysis of markers of cell survival and proliferation revealed increased expression of proliferating cell nuclear antigen (PCNA), glutathione s-transferase protein (GST-P), and nuclear factor-κB (NF-κB) with downregulation of p21, p53 and IkappaB in both human and hamster OSCCs, and both human and hamster oral carcinomas displayed invasive, and angiogenic properties as revealed by dysregulated cytokeratin expression, downregulation of reversion-inducing cysteine-rich protein with Kazal motifs (RECK) which is involved in maturation of vessels, and increased expression of urokinase plasminogen activator (uPA), matrix metalloproteinase-2 (MMP-2) and-9, hypoxia-inducible factor 1- alpha (HIF-1α), and VEGF, and concluded that the aberrant expression of multiple molecules in key signaling pathways in both human oral SCC may underlay development and progression of SOCC and indicated a possible role for angiogenetic factors for cancer progression.

The present study reported significantly higher percentage of cytoplasmic positivity for iNOS in specimens obtained from patients had nodal involvement compared to those who had free lymph nodes and a positive significant correlation between nodal involvement and cytoplasmic positivity for iNOS. These findings go in hand with ***Franchi et al.*** [30] who found that measured iNOS activity in specimens from the tumor periphery correlated strongly with lymphatic vessel density and lymphatic vessel area and indicated that iNOS activity may promote lymphangiogenesis and spread to lymph nodes in HNSCC, with the possible involvement of lymphangiogenic factor vascular endothelial growth factor-C. ***Mozet et al.*** [22] found that iNOS protein expression rates reached higher scores in tumors of patients with lymph node metastasis.

It could be concluded that overexpression of both iNOS and p53 is a concomitant finding in tissues of OSCC and positively correlated with each other and with nodal involvement and thus mostly indicate unfavorable prognosis.

Corresponding author
Ahmed Khafagy
Department of Otorhinolaryngology, Faculty of Medicine, Ain Shams University

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