

## The Co-expression of c-myc and p53 Increases and Reaches a Plateau Early in Oral Oncogenesis

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**Abstract.** *Background: The balance between cell proliferation and apoptosis plays a significant role in cancer development. The expressions of the p53 and c-myc genes, both strongly related to cell proliferation and apoptosis, were studied in sequential histological grades of oral carcinogenesis in an animal model. Materials and Methods: Thirty-seven hamsters were divided into three groups (A,B,C), which were treated with 9,10-dimethyl-1,2-benzanthracene and sacrificed at 10,14 or 19 weeks, respectively, after treatment. The histological status of the oral lesions in the experimental groups corresponded well with tumour advancement (from dysplasia to moderately-differentiated carcinoma). Tumour sections were studied immunohistochemically. Results: The expressions of both p53 and c-myc increased significantly in precancer stages and then reached a plateau. The same pattern was observed in the animal groups with the culmination of expression of both genes in group A. Conclusion: The co-expression of p53 and c-myc proteins in the earlier stages of oral oncogenesis may be used for the early detection of premalignant lesions.*

Oral carcinogenesis is a molecular and histological multistage process, which involves enhanced function of several oncogenes and/or the deactivation of tumour suppressor genes, resulting in the loss of cell cycle checkpoints (1, 2). The accumulation, rather than the sequence, of

genetic events determines the progression to malignancy, which includes sequential pathological alterations ranging from hyperplasia through dysplasia to carcinoma *in situ* and invasive carcinoma (1).

One of the tumour suppressor genes most frequently involved in human OSCC is p53 (1, 3-5). The wild-type (WT) p53 protein controls growth by interacting with a great number of cell proteins in order to regulate gene transcription, arrest the cell cycle facilitating DNA repair and trigger apoptosis (6-8). Inactivation of the p53 gene by mutations hinders the growth control pathways and confers genetic instability, facilitating the subsequent progression to malignancy (3, 4, 8). The inactivated mutant p53 protein is more stable, therefore its accumulation seems to occur early in oral neoplastic development and gradually increases through the sequential oncogenic stages from hyperplasia to OSCC (9, 10). The extent of p53 abnormalities in oral biopsies is associated with poor survival of the patients (1, 10, 11).

Among the oncogenes which are frequently involved in oral cancer is c-myc (12-14). Normally, it promotes cell replication in response to extracellular signals (15, 16), but also contributes to cell metabolism, differentiation and apoptosis (17-22). Tumorigenicity of the c-myc protein is ascribed to the promotion of cell proliferation and inhibition of apoptosis (16, 23). Amplification and overexpression of c-myc has been observed in 10-40% of human OSCC (24) and was correlated with progressive cell transformation in oral cancer lesions (14). Vora *et al.* observed that tongue SCC exhibited the highest incidence of c-myc among oral tumours (75%) and was related to significantly poor survival (3).

Interestingly, c-myc and p53 have been shown to collaborate in the regulation of both cell proliferation and apoptosis (15, 16, 20, 21, 25). Deregulated expression of c-myc is required for the induction of both these biological processes

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and the promotion of tumour formation, but the mechanism that controls which of these two processes will be favoured is not yet fully understood.

Regarding the apoptotic function of c-myc, previous studies indicated that c-myc-induced apoptosis may involve heterogeneous mechanisms with or without p53 (26). The p53-dependent apoptotic pathway involves the induction of *p19<sup>ARF</sup>* by c-myc, which in turn inhibits the repressor of p53 Mdm2, resulting in the elevation of p53 expression and triggering of apoptosis (25, 27). At the same time, the c-myc protein causes further cell growth stimulating transcription of the rRNA and tRNA genes. A polymerase III-specific transcription factor, TFIIB, plays a pivotal role in this process, since p53 and c-myc compete for its repression or activation, respectively (28).

Hence, the interactive involvement of c-myc and p53 with cell proliferation and apoptosis, when altered by mutational events, seems to be a key regulatory element of oncogenesis. In order to study their expression and define their roles in the sequential histological stages of OSCC formation, an experimental model was established in Syrian golden hamsters. Chemical carcinogenesis in the hamster buccal pouch is known to induce premalignant oral lesions and OSCC, resembling, both microscopically and ultrastructurally, those that occur during development in human oral mucosa exposed to environmental agents, such as tobacco (9, 29, 30).

## Materials and Methods

**Experimental carcinogenesis.** Forty male Syrian golden hamsters (*Mesocricetus auratus*) were used in this study. They were purchased from the Hellenic Pasteur Institute (Athens, Greece) at the age of 5 weeks and weighed approximately 100 g each. The hamsters were handled in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animals were randomly divided into three experimental groups for carcinogen treatment (A, B and C) and one control group (n=7). Three hamsters in the experimental groups died unpredictably during the chemical carcinogenesis and were excluded from the study.

The animals in groups A (n=10), B (n=10) and C (n=10) were anaesthetized three times per week with ether and their left buccal pouches were painted with 0.5% 9,10-dimethyl-1,2-benzanthracene (DMBA) (Sigma, St. Louis, MO, USA) dissolved in paraffin oil, using a #4 camel's hair brush. The amount of carcinogen delivered to each animal was quite uniform using the "wiped-brush" method (31). It is known that numerous metabolic defects and several histological alterations occur within hours, days or weeks following exposure of target tissues to DMBA (32). Treatment with this carcinogen was effected on the group A animals for 10 weeks and on the animals of groups B and C for 14 weeks. The pouches of all the animals were examined weekly in order to observe the growth of tumours on the mucosa.

All the animals were sacrificed by an overdose of ketamine hydrochloride (>30 mg/kg), given intraperitoneally, and the treated buccal pouches were removed either at 10 weeks from the application of the carcinogen (group A), at 14 weeks (group B) or at 19 weeks (group C). Following a 10-week period without application of the

Table I. Tissue status in the control and the three experimental groups.

Tissue status	Control group	Group A	Group B	Group C
Normal tissue	4			
Hyperkeratosis	2			
Hyperplasia	1			
Dysplasia		6		
Early invasion		3	1	
Well-differentiated carcinoma		1	6	1
Moderately-differentiated carcinoma			3	9

carcinogen, a tissue sample from the Control animals' left buccal pouch was taken. The size of most tumours was approximately 1-2 cm, with the exception of three large samples in group C, which were about 5 cm. After their excision, the tumours from the animals in groups A, B and C and the buccal pouches of the control group were numbered and examined blindly.

**Pathological evaluation.** The histological status of the lesions was defined after examination of the complete section under light microscopy and the tissue profiles were classified into the following categories: normal, hyperkeratosis, hyperplasia (acanthosis), dysplasia, early invasion, well-differentiated carcinoma and moderately-differentiated carcinoma. In every sample, all different lesions were evaluated separately.

**Immunohistochemical procedures.** The biopsies from the 37 animals were fixed in 10% neutralised formaldehyde solution and embedded in paraffin. Three 4-µm sections were prepared from each specimen and were mounted on Super Frost Plus-coated glass slides (Menzel and Co., Braunschweig, Germany). One section was stained with haematoxylin and eosin for routine histological evaluation, while the other two were used for immunohistochemical detection of p53 and c-myc gene products. The sections were incubated with monoclonal primary antibodies against p53 (NCL-p53-D07; Novocastra, Newcastle, UK; diluted 1:200) and c-myc (p-c-Myc (Thr 58/Ser 62)-R: sc-8000-R; Santa Cruz Biotechnology, Inc.; diluted 1:100) using standard immunohistochemical methodology, as described previously (33). The monoclonal antibody NCL-p53-DO7 detects both wild-type and mutant forms of p53 (34), while the antibody for c-myc recognises only the normal forms. A breast carcinoma with strong p53 expression and mouse uterus tissue with strong c-myc expression were included as positive controls. Negative controls for both antibodies were processed in the same manner, using PBS instead of the primary antibody. All the samples were independently reviewed by two investigators blindly.

**Statistical analysis.** In each sample, the mean value of percentages of positively-stained cells was calculated from all the different lesions present. These values were tabulated for each group of animals (control group, experimental groups A, B, C) and were compared by the two-tailed Student's *t*-test. In order to evaluate the pattern of antibody expression in relation to the histological status, the various lesions were divided into three categories according to tumour progression: i) normal tissue, ii) precancer condition (hyperkeratosis, hyperplasia, dysplasia) and iii) tumour (early invasion, well-differentiated carcinoma, moderately-differentiated carcinoma).

Table II. Percentage of p53- and c-myc-positive cells in the various categories of tissue status.

	Precancer				Tumour		
	Normal oral mucosa	Oral mucosa with hyperkeratosis	Oral mucosal hyperplasia	Oral mucosal dysplasia	Early invasion	Well-differentiated OSCC	Moderately-differentiated OSCC
<i>p53</i>							
Mean value of percentages	0 (N=15)	0 (N=5)	21.9 (N=16)	35.7 (N=22)	36.3 (N=19)	21 (N=24)	33.9 (N=25)
Mean value of percentages in precancerous lesions			26.5				
Probability of <i>t</i> -test		N.S.	$p < 0.001$	$p < 0.001$	N.S.	N.S.	N.S.
<i>c-myc</i>							
Mean value of percentages	36.1 (N=15)	64.6 (N=5)	83.1 (N=16)	76.6 (N=22)	73.6 (N=19)	59.1 (N=24)	66.2 (N=25)
Mean value of percentages in precancerous lesions			77.6				
Probability of <i>t</i> -test		N.S.	$p < 0.001$	$p < 0.001$	N.S.	N.S.	N.S.

N.S.: No statistical difference.

Using the two-tailed Student's *t*-test, the percentages of positively-stained cells from each precancer category were compared with those from the normal tissue, while the percentages of positively-stained cells from each tumour category were compared with the percentages of all the three precancer conditions together.

## Results

The histological status of the biopsies in the control group and in the three experimental groups is shown in Table I. A progression towards OSCC formation in correlation to increased time of carcinogen application was evident. Therefore, as expected, this experimental model seemed valid and further analysis of the data was implemented.

The percentages of cells positively stained for p53 and c-myc proteins in the various histological categories are shown in Table II. A significantly increased percentage of mutant p53-positive cells ( $p < 0.001$ ) was observed in oral mucosal hyperplasia and dysplasia compared to normal mucosa (Table II, Figure 1). Interestingly, no statistical difference was observed in the three tumour categories compared to the precancer lesions. The same pattern of expression in the precancer conditions was revealed for c-myc (Table II, Figure 2). Again, no statistical difference was observed in the three tumour categories compared to the precancer lesions.

The percentages of cells positive for p53 and c-myc in the control group and in the three experimental animal groups are

shown in Table III. There was a significant difference between the control group and group A in the percentage of p53-stained sections ( $p < 0.001$ ), which remained virtually unchanged, with no significant statistical difference between groups A and B or groups B and C. Regarding c-myc, there was a significant increase in group A compared to the control group ( $p < 0.05$ ), practically no difference between groups A and B and a significant decrease in c-myc-positive cells in group C compared to group B ( $p < 0.05$ ).

## Discussion

An experimental animal model of oral carcinogenesis was established in the study and the expressions of the tumour suppressor gene *p53* and the oncogene *c-myc* were studied. Both genes displayed the same pattern of increased expression in the precancer stages of oral mucosal hyperplasia and dysplasia and remained virtually at the same level in the following stages of OSCC formation, indicating their close correlation with oral oncogenesis. Other authors reported that p53 protein accumulation occurred frequently and early in carcinogenesis, at the stage of premalignant lesions in hamster buccal pouch tumours (9, 29) in accordance with our results. No previous study has systematically investigated the expression of the *c-myc* gene in hamster oral tumours.

Conflicting results have been reported regarding p53 immunopositivity in precancer and tumour stages. Some

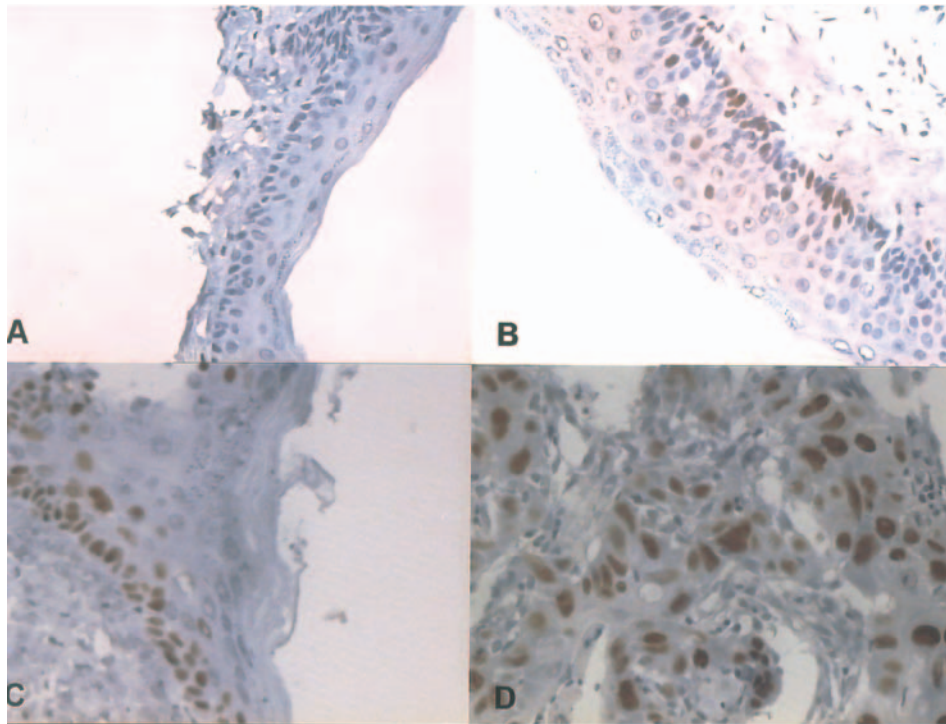


Figure 1. Immunohistochemical staining of oral tissues using the p53 antibody is shown: (A) p53-negative (0-5% positive nuclei) normal oral mucosa; (B) p53-stained + (5-20% positive nuclei) oral mucosa with mild dysplasia; (C) p53-stained ++ (20-50% positive nuclei) oral mucosa with severe dysplasia; (D) p53-stained +++ (>50% positive nuclei) moderately-differentiated oral carcinoma, x 40.

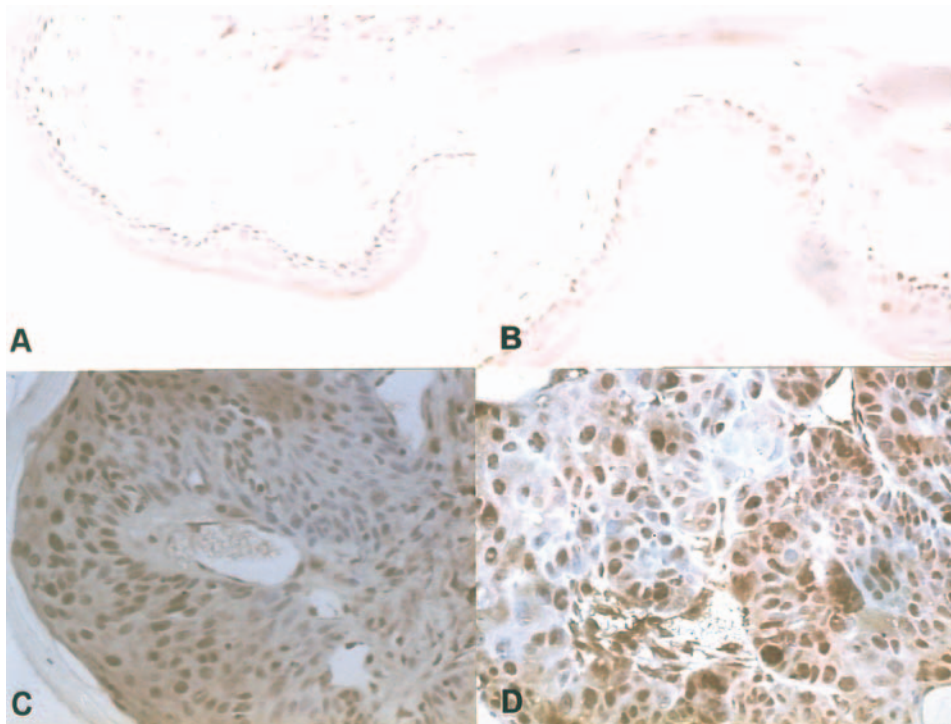


Figure 2. Immunohistochemical staining of oral tissues using the c-myc antibody is shown: (A) c-myc-negative (0-5% positive nuclei) normal oral mucosa; (B) c-myc-stained + (5-20% positive nuclei) hyperplastic oral mucosa; (C) c-myc-stained ++ (20-50% positive nuclei) area of early invasion; (D) c-myc-stained +++ (>50% positive nuclei) moderately-differentiated oral carcinoma, x 40.

Table III. Percentage of p53- and c-myc-positive cells in the control and the three experimental groups.

	Control Group	Group A	Group B	Group C
<i>p53</i>				
Mean value of percentages	0 (N=7)	33.4 (N=10)	30.1 (N=10)	28.6 (N=10)
Probability of <i>t</i> -test		$p < 0.001$	N.S.	N.S.
<i>c-myc</i>				
Mean value of percentages	46 (N=7)	74.9 (N=10)	79 (N=10)	54.5 (N=10)
Probability of <i>t</i> -test		$p < 0.05$	N.S.	$p < 0.05$

N.S.: No statistical difference.

researchers have suggested a significant increase in p53 expression throughout the histological progression of the disease (34), while others cited a correlation of p53 overexpression only with the early stages of the disease, with a practically stable expression in the later stages (3, 35), as revealed in the present study. The variation in the reported results may reflect differences in experimental approach and interpretation of data. The p53 protein is normally present in cells at very low levels, has a short half-life (about 20 min) and, therefore, can be hard to detect by immunohistochemistry in normal tissue (5). However, the protein may remain longer in tissue either due to a mutation or a defect in the degradation pathway; this inactive protein can be detected with the use of antibodies. Therefore, it is important to note that the immunohistochemical data do not always correspond to the detection of mutant p53 protein (11). Nevertheless, all previous studies agree that there is an increase of p53 immunorexpression at the early stages of tumorigenesis in the oral cavity (3, 34, 35).

This study also revealed a correlation between *c-myc* overexpression and precancer lesions. However, *c-myc* seemed to be suppressed at the last stage of oral cancer. This observation may be explained by a possible activation of some tumour suppressor genes that antagonise *c-myc*, such as the MM-1 protein, which was reported to be present in several types of cancer cells, including oral cancer cells (36).

In light of the synergism between *c-myc* and *p53* in both the induction of apoptosis and tumour formation, the co-evaluation of the data obtained for these two proteins is imperative. It is obvious that a similar pattern of immunorexpression was revealed for both p53 and *c-myc* in the

initial stages of oral oncogenesis, indicating a possible strong correlation between them, which can be further supported by the fact that the human p53 promoter can be directly transactivated by *c-myc*/Max heterodimers, or indirectly through the induction of *p19<sup>ARF</sup>* by *c-myc*, which in turn inhibits the repressor of p53 Mdm2 (25, 27, 37). Co-overexpression of the p53 and *c-myc* proteins was also observed in tobacco and betel-related OSCC, and was linked with advanced stages of oral cancer (38).

The above-mentioned data may be explained by the contribution made by p53 and *c-myc* to the formation of OSCC. If a mutation in a single allele of *p53* that inactivated the tumour suppressor activity were the primary event, then a subsequent deregulation or overexpression of *c-myc* could result directly or indirectly in elevated transcription of the mutant gene. Since mutant p53 proteins generally are more stable than the WT proteins (10), the combined result would be an elevated level of mutant p53 protein in the cell. This would lead to an increased growth advantage for these cells, possibly by virtue of the ability of high concentrations of mutant p53 to complex with and inactivate the remaining WT p53.

Alternatively, if deregulated or elevated expression of *c-myc* were the primary event, then the WT p53, which is normally expressed at very low levels, would be transactivated either directly or indirectly by *c-myc* (25, 27, 37). The increased levels of WT p53 would then trigger the cells to undergo either growth arrest or apoptosis. In this case, the *p53* gene would be behaving as a genuine tumour suppressor. In this light, the co-overexpression of *c-myc* and p53 proteins in the early stages of oral cancer could be explained as an attempt by the cells to induce, through the expression of *c-myc*, the expression of WT p53 and stop cell proliferation. On the other hand, neighbouring cells with other mutant oncogenes would not have to compete for nutrients and have a more aggressive growth advantage. In accordance with this assumption, both cell proliferation and apoptosis rates were observed to be highest during the early stages of oral oncogenesis (33).

The findings of this study suggest that high expression of p53 and *c-myc* signal an oral lesion before significant invasion has occurred and may be used for the early detection of premalignant lesions. The expression of p53 and *c-myc* revealed in the experimental model described here is similar in many respects to that observed in equivalent human lesions of the oral region, thus this model could be a useful tool for determining the contribution of other genes to oral oncogenesis.

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