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H-Ras and c-Fos Exhibit Similar Expression Patterns During Most Stages of Oral Oncogenesis

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Abstract. *Background: H-ras and c-fos oncogenes interact in signalling pathways but their level and time course of expression during oral cancer development are unclear. The present study used an animal model for the simultaneous investigation of H-Ras and c-Fos expression in sequential stages of oral oncogenesis. Materials and Methods: Three experimental groups of Syrian golden hamsters (A, B and C; 10 animals each) and one control group (7 animals) were used. The buccal pouches of hamsters in groups A, B and C were treated with 0.5% of the carcinogen 9,10-dimethyl-1,2-benzanthracene and were excised at 10, 14 and 19 weeks, respectively. The biopsies, which included tissue stages ranging from normal oral mucosa to moderately differentiated carcinoma, were studied immunohistochemically. Results: A reduction in both H-Ras and c-Fos expression was observed from group A to B and from hyperplasias to early tumour stages, while a simultaneous increase was noted from group B to C and from well-differentiated to moderately-differentiated carcinomas. The H-ras/c-fos expression ratio had a value of approximately (1.09 ±0.21) in five out of seven studied tissue stages. Conclusion: H-Ras and c-Fos exhibit a similar expression pattern throughout most stages of oral carcinogenesis, an observation supported by the known molecular pathway connecting H-ras signalling with subsequent c-fos gene transcription.*

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Oral squamous cell carcinomas (OSCC) comprise the vast majority of cancers developing in the oral cavity and many of them are preceded by potentially malignant oral lesions (1). Despite the substantial developments in both diagnostic and therapeutic possibilities, the prognosis of OSCC remains unfavourable (2). Oral oncogenesis is a multistep procedure that requires the accumulation of several genetic alterations in oncogenes and tumour suppressor genes that results in destabilization of several growth control systems (3).

Of the many tumour-related genes known, the *H-ras* oncogene has been widely studied. It belongs to the *ras* gene family (*H-, K-* and *N-ras*), which appears to be a chief contributor to the development of tumours, since it is associated with about one third of all human cancers (4). The *ras* oncogenes exhibit about 85% sequence homology and encode for a 21 kDa protein (p21). The p21 ras proteins are GDP/GTP binding proteins which are activated when GTP is bound and inactivated after GTP is hydrolysed to GDP (5-7). A missense mutation (mainly in codon 12, 13 or 61) reduces the GTPase activity of the protein resulting in a perpetual activated state of H-Ras (4, 8). Activated H-Ras induces the activation of multiple downstream kinase cascades affecting cellular growth, division and differentiation (9). Such an intracellular cascade is that involving mitogen-activated protein kinases (MAPK) which leads to activation of a known oncogene, *c-fos* (10, 11).

c-fos encodes for a component of activation protein-1 (AP-1), a transcription factor which consists mainly of heterodimers of c-Fos and -Jun proteins (12). AP-1 binds to

promoter and/or enhancer regions, regulating transcription of several genes implicated in cell proliferation, differentiation and apoptosis (10, 13). In addition to activated H-Ras, various other stimuli such as growth factors, cellular stress and UV radiation, induce AP-1 activity (11, 12). Once activated, H-Ras generates the MAPK cascade leading to the activation of either Fos-regulating kinase (FRK) or extracellular signal-regulated kinases 1 and 2 (ERK1 and 2), which in turn activate Jun protein (11, 14). In addition, ERK1 and ERK2 increase expression of *c-fos* gene through phosphorylation of its transcription factor Elk-1/TCF, further stimulating activity of AP-1 (15, 16). In order to become oncogenic, *c-fos* does not require any substantial changes in its coding region, as the overexpression of the normal protein is sufficient (12, 17). Nevertheless, in some types of human cancer underexpression of *c-fos* has been noted, including in salivary gland, papillary thyroid and colonic tumours (18-20).

Both *H-ras* and *c-fos* oncogenes have been implicated in human OSCC (21, 22). Interestingly, a remarkable variation in the mutation rates of *H-ras* has been reported ranging from 5 to 40% in various human populations (23-29). Furthermore, there are conflicting reports in the literature regarding *c-fos* expression. Some studies have reported elevated levels of c-Fos in malignant oral lesions (dysplasia, OSCC), others have noted maintained expression at normal levels during carcinogenesis, while still other studies have reported a high *c-fos* expression in normal oral mucosa followed by gradual decrease at the advanced stages of oral cancer (17, 22, 30, 31).

Given the interaction of H-Ras and c-Fos in signaling pathways and the existing ambiguity in their expression, it seems motivating to study their expression, their role and correlation in sequential histological stages of OSCC formation. For this purpose, we established an experimental model in Syrian golden hamsters. The premalignant oral lesions and OSCC which are chemically induced in these animals resemble both microscopicaly and ultrastructurally those that develop in human oral mucosa exposed to environmental agents, such as tobacco and alcohol (32-34).

Materials and Methods

Experimental carcinogenesis. Thirty-seven male Syrian golden hamsters (*Mesocricetus auratus*), aged five weeks (100 g body weight) were used. The handling of the hamsters was consistent with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (35). The animals were randomly divided into one Control group (n=7) and three experimental groups A, B and C (n=10 each) for carcinogen treatment as described elsewhere (36).

Following ether anesthetization of the animals in groups A, B and C, their left buccal pouches were painted with the carcinogen 9,10-dimethyl-1,2-benzanthracene (DMBA; 0.5%) (Sigma, St. Louis, MO, USA) dissolved in paraffin oil, three times per week until the removal of the treated buccal pouches. The treated buccal pouches were removed: at 10 weeks from the application of the carcinogen (group A), at 14 weeks (group B), at 19 weeks (group C) and a tissue sample from the left buccal pouch of the controls, following a ten-week period without application of the carcinogen as described elsewhere (36).

Pathological evaluation. Each section was examined under light microscopy and all possible different lesion types were evaluated. The tissue profiles were classified into non-cancerous (completely normal oral mucosa), precancerous (hyperkeratosis, hyperplasia, dysplasia) and cancerous conditions (early invasion, well- and moderately differentiated carcinoma).

Immunohistochemical analysis. The surgical specimens were fixed in 10% neutralized formaldehyde solution and embedded in paraffin. Sections of 4μm from each specimen, mounted on Super Frost Plus-coated glass slides (Menzel and Co., Braunschweig, Germany), were used for immunohistochemical detection of normal H-Ras and c-Fos proteins with the Immunoperoxidase Secondary Detection System (Chemicon International, USA) using monoclonal primary antibodies against H-Ras (c-H-ras, Ab-1, Cat No.OP23T, 1:100 dilution; Oncogene Research Products) and c-Fos (c-fos 4:SC-52, 1:200 dilution; Santa Cruz Biotechnology) as described elsewhere (36). For H-Ras detection, antigen retrieval was performed through pepsin application for 5 min at 37˚C. As positive controls normal skin and brain tissue were used for H-Ras and c-Fos respectively. Negative controls were processed by substituting the primary antibody with phosphate-buffered saline. For descriptive purposes, the staining intensity was scored as: 0-5% positive nuclei = negative expression (–), 5-20% = low (+), 20-50% = intermediate (++), >50% = high (+++) expression. Independent review of all slides was performed by two investigators blindly. A pathologist experienced in oral pathology evaluated the consecutive haematoxylin-eosin-stained slides, with no knowledge of H-Ras or c-Fos staining patterns.

Statistical analysis. A two-tailed Student's *t*-test was applied for statistical analysis using the SPSS 10.0 program for Windows™ (SPSS Inc. Headquarters, Chicago, IL, USA SPSS Inc. Headquarters, Chicago, IL, USA). In addition, in every group and every histological category a normal distribution check was performed using the Kolmogorov-Smirnov Z test. If a group or a histological category was not normally distributed, additional statistical analysis was performed with the Wilcoxon test using SPSS.

The mean percentages of positively stained cells were calculated from all the different lesions present in each sample. These values were tabulated for each group of animals (control group, experimental groups A, B, C) and the mean percentage of positively stained cells of one group was compared with the corresponding one of the previous group. In each histological lesion the percentages of positively stained cells from each precancerous or cancerous category were compared with those of the normal tissue. In addition, the percentages of positively stained cells from each tumor category were compared with the average percentage of all three precancerous conditions.

Results

The histological status of biopsies in the four studied groups of animals is shown in Figure 1. A progression towards OSCC formation with increased time of carcinogenesis is

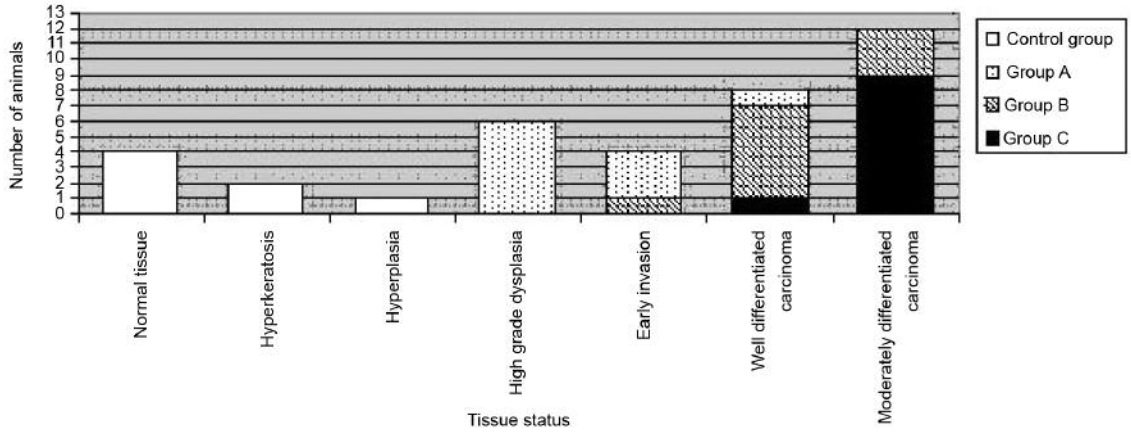

Figure 1. *Tissue status in the control group with no carcinogen treatment and the three experimental groups A, B and C.*

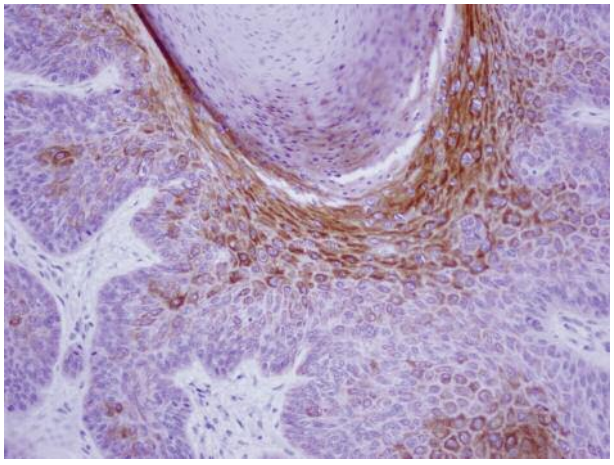

Figure 2. *H-Ras cytoplasmic immunoexpression (++) in well-differentiated oral carcinoma (×100).*

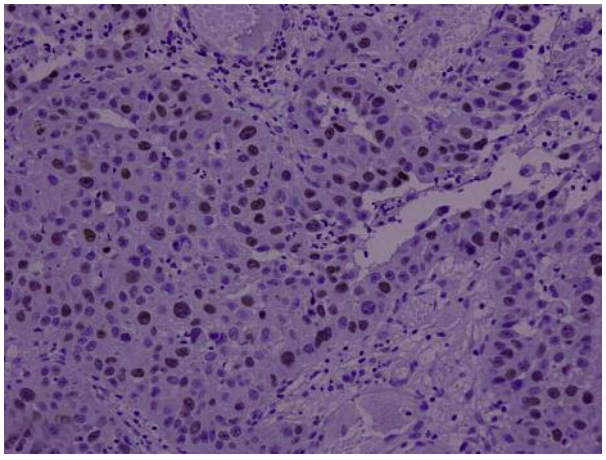

Figure 3. *Strong c-Fos nuclear immunoreactivity (+++) of several nuclei of early invasive squamous cells (×200).*

evident. Therefore, this experimental model seems valid as expected and further analysis of immunostaining data (Figures 2 and 3) was implemented.

The percentages of cells expressing the *H-ras* gene product in the various stages of oncogenesis are shown in Table I. No significant statistical difference was observed in the three subgroups of precancerous lesions in comparison to normal oral mucosa and H-Ras expression was detected at high levels. Accordingly, in early invasion lesions and moderately differentiated carcinomas, no significant statistical difference was revealed compared to precancerous conditions. On the contrary, in well-differentiated carcinoma, the percentages of H-Ras-positive cells were significantly lower in comparison to the respective ones from the precancerous categories ($p<0.01$).

The percentages of H-Ras-positive cells in the control group and the three experimental groups are shown in Table II. There was no statistically significant difference in group A compared to the control group. In contrast, the percentages of H-Ras-positive cells in group B were significantly lower compared to the respective ones of group A ($p<0.05$), while a significant increase was observed again in group C in comparison to group B, reaching the staining levels of the control and group A.

Table I shows the percentages of c-Fos-positive cells in the various histological categories. No statistical difference in c-Fos expression was observed in precancerous lesions compared to normal oral mucosa since the oncogene product was expressed at similarly high levels. In the subsequent stages of OSCC formation, c-Fos expression gradually

Table I. *Percentage of H-Ras and c-Fos positive cells in the various tissue categories.*

		Precancerous			Tumour		
	Normal oral mucosa	Oral mucosa with hyperkeratosis	Oral mucosal hyperplasia	Oral mucosal dysplasia	Early invasion	Well-differentiated OSCC	Moderately differentiated OSCC
H-Ras							
Mean (%) ±S.D.	(N=15) 45.5±27.8	(N=5) 53.2±21.5	(N=16) 52.2±22.8	(N=22) 42.9±21.6	(N=19) 34.1±31.1	(N=24) 29.1±32.8	(N=25) 54.4±36.8
Probability of *t*-test (Precancerous and cancerous to normal)		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Mean percentage (precancerous) (%) ±S.D.			47.5±22.1				
Probability of t-test (cancerous to precancerous mean value)					N.S.	**<0.01**	N.S.
c-Fos							
Mean (%) ± S.D.	(N=15) 48.2±26.9	(N=5) 48.8±19.8	(N=16) 44±36.6	(N=22) 30±27.9	(N=19) 29.5±28.9	(N=24) 39±32.6	(N=25) 51.6±20.8
Probability of t-test (Precancerous and cancerous to normal)		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Mean percentage (precancerous) (%) ± S.D.			31.1±31.1				
Probability of *t*-test (cancerous to precancerous mean value)					N.S.	N.S.	**<0.05**

N.S.: No statistical difference, S.D.: standard deviation.

decreased without revealing statistically detected differences, but it rose again significantly in moderately-differentiated carcinomas, reaching the expression level of normal oral mucosa ($p<0.05$). Finally, statistical analysis of c-Fos expression in the four studied groups did not reveal any statistical difference (Table II).

The ratio of the relative expression values of H-Ras and c-Fos (ratio of percentages of H-Ras-positive cells to c-Fos-positive cells) was calculated for each stage of oral oncogenesis, as shown in a parallel column diagram (Figure 4). In five out of seven tissue categories, this ratio had a value of approximately 1 (1.09±0.21) indicating a similar expression pattern of the two oncogenes in most stages of oral oncogenesis (Figure 4a). The outlying exceptions included the stages of dysplasia and well-differentiated carcinoma, with ratios of 1.43 and 0.75.

The relative expression ratio of H-Ras and c-Fos was also approximately 1 in most animal groups (1.04±0.19, Figure 4b). The only outlying exception was group A, in which fewer cells were c-Fos-positive than H-Ras-positive (ratio 1.29).

Table II. *Percentage of H-Ras- and c-Fos- positive cells in the control group and the three experimental groups, which were treated with a carcinogen and sacrificed at 10 (Group A), 14 (Group B) and 19 weeks (Group C), respectively.*

	Control Group (N=7)	Group A (N=10)	Group B (N=10)	Group C (N=10)
H-Ras				
Mean percentage (%) ±S.D.	47.9±21.6	49.3±21.09	27.5±20.3	51.8±28.3
Probability of *t*-test		N.S.	**<0.05**	**<0.05**
c-Fos				
Mean percentage (%) ±S.D.	51±15.4	38.3±24.5	32.1±25.8	48.3±15.9
Probability of *t*-test		N.S.	N.S.	N.S.

N.S.: No statistical difference, S.D.: standard deviation.

Discussion

In this study, an experimental model of chemically induced tumours of the hamster buccal pouch was used to establish a model for oral carcinogenesis. In this model, the expression levels of oncogenic products of *H-ras* and *c-fos* were investigated by immunohistochemical procedures in various stages of OSCC formation.

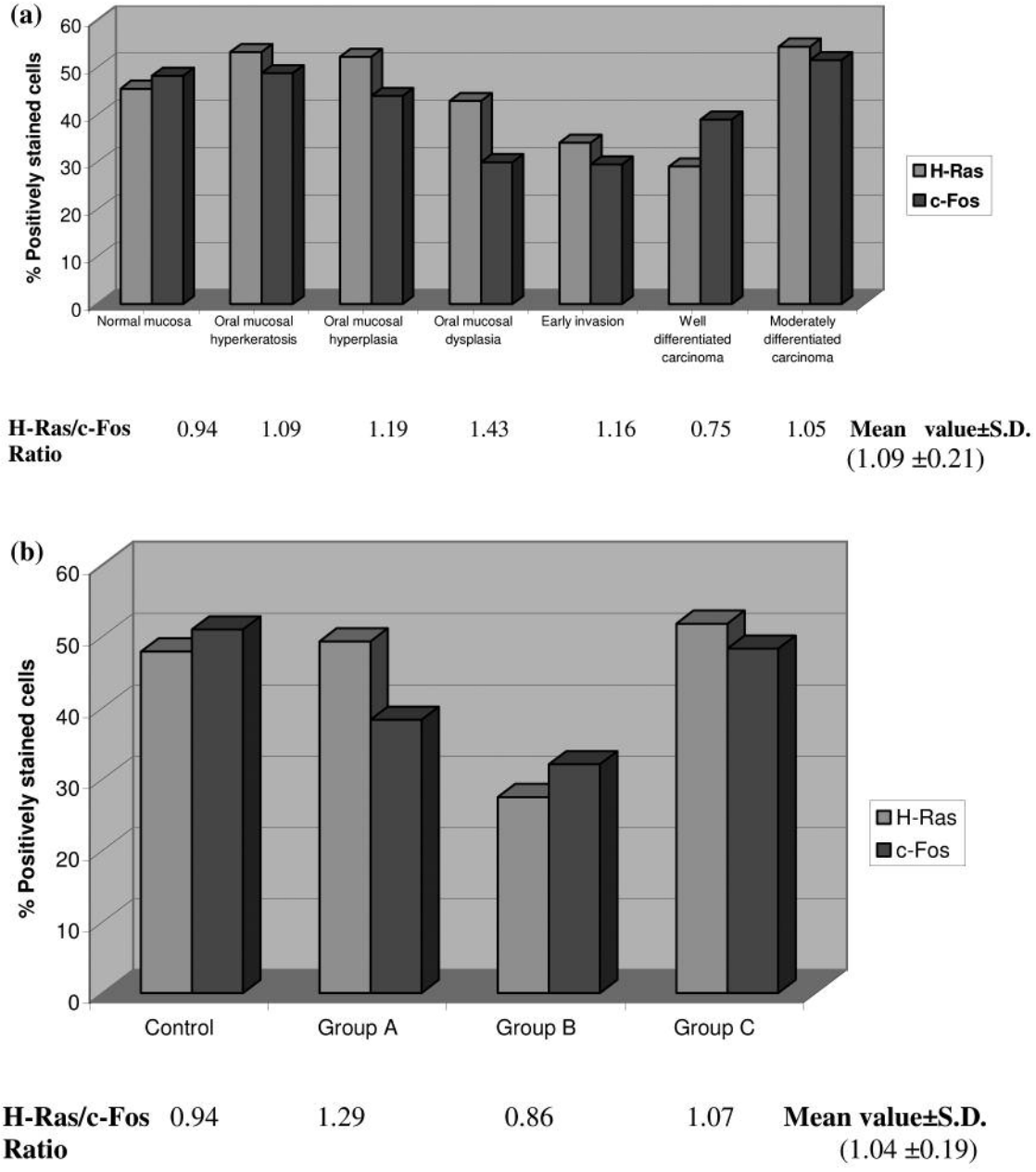

Figure 4. *Parallel expression of H-Ras and c-Fos, and the H-Ras/c-Fos expression ratio in (a) the different tissue categories.*

H-Ras expression was detected at high levels in normal oral mucosa and precancerous lesions, but was significantly lower in well-differentiated carcinomas. This reduction was not persistent further on, as in moderately-differentiated carcinomas, H-Ras expression increased again reaching the high levels of precancerous lesions. In a similar fashion, the *c-fos* oncogene product was expressed at high levels in normal oral mucosa, hyperkeratosis and hyperplasia, while in the subsequent stages of OSCC formation its expression decreased gradually (but not significantly), and then it rose again significantly in moderately-differentiated carcinomas. The fact that the two studied oncogenes had a similar expression pattern during oral oncogenesis is illustrated by the ratio of their relative expression, which had a value of about 1 in most stages. This observation is supported by the known molecular pathway connecting H-Ras signalling with subsequent *c-fos* gene transcription (11, 14).

Interestingly, the reduction of H-Ras levels occurs in the same tissue categories in which increased apoptosis was observed in a study performed by our group using the same experimental model (36). Apoptosis was favoured in the same stages due to a reduction of anti-apoptotic factor Bcl-2 levels. Given the fact that Bcl-2 overexpression immediately affects cellular signaling pathways *via* a direct interaction and up-regulation of H-Ras, the decreased levels of Bcl-2 observed in the initial stages of oral carcinogenesis may influence the reduction of activated H-Ras (36-39). In addition, it is well known that apoptosis will lead to deprivation of growth factors, which in turn may decrease the levels of *ras* activation (9, 40-43).

The increase in H-Ras and c-Fos immunoexpression levels from well-differentiated carcinomas to moderately differentiated carcinomas might also reflect at least a partial contribution of *H-ras* mutations which accumulate at these stages of oral oncogenesis (33). It is known that the consequence of oncogenic mutations in the *H-ras* gene is a constitutively active form of H-Ras. This constitutive activation is due to a severe reduction in GTPase activity, slowing the GTP/GDP cycle *in vivo* and resulting in the accumulation of the active GTP-bound form (44). Given the fact that Bcl-2 causes up-regulation of *H-ras*, this speculation is also supported by the observation that Bcl-2 expression is also increased in the above mentioned stages of oral oncogenesis (36-39).

In the literature, there are many studies of *H-ras* mutations in oral cancer using molecular techniques (25, 26, 32, 33, 45, 46). Interestingly, there is a wide range of reported incidence of *H-ras* mutations depending on the studied population. A low prevalence (<5%) of *H-ras* mutations has been reported in oral cancer from Western patients (25, 26, 29, 44, 47), while a high incidence (35%) was found in patients from India (23, 45). An immunohistochemical study evaluating H-Ras protein expression in oral cancer suggested that there is a trend towards an increase in later stages of OSCC formation indicating that H-Ras could be involved in advanced stages of carcinogenesis (48). Although that study did not compare H-Ras expression of OSCC stages with normal oral mucosa as we did, its findings are in accordance with the increased expression observed in later stages.

Regarding the conflicting reports of c-Fos expression in oral carcinogenesis, the findings of this study seem to be in accordance with previous observations in human precancerous lesions and OSCC (30, 31). One study of different stages of oral oncogenesis by Turatti *et al*. reported high c-Fos expression in normal oral mucosa, which was reduced in dysplasia and increased again in OSCCs (30). Another study compared c-Fos expression in assorted OSCCs at various stages with adjacent normal mucosa and observed no difference (31). The same observation would result in this study if findings of all tumour categories were compared altogether to normal mucosa. Indeed, in this study, the initially lower expression of c-Fos at the earlier tumour stages was not statistically different from that of normal tissue, while it was almost identical to that in moderately differentiated OSCCs.

Interestingly, our study revealed very similar results to those reported by Turatti *et al*. concerning reduced levels of c-Fos in oral mucosal dysplasia compared to normal tissue (30). Since the reduction of H-Ras in this stage is not comparable, it seems that a factor other than H-Ras signaling may negatively affect *c-fos* gene expression levels, but this remains to be elucidated because there is no further evidence in the literature justifying this hypothesis. In regard to the other stage of oncogenesis in which the H-Ras/c-Fos ratio is not about 1, namely in the well-differentiated carcinoma, the extensive increase of c-Fos expression may indicate the contribution of additional signaling factors other than H-Ras. Such a factor implicated in *c-fos* up-regulation of transcription might be the epidermal growth factor receptor (EGFR), the increased levels of which culminate in this particular stage of hamster oral oncogenesis, as we demonstrated previously (49).

In conclusion, this study indicates a close association between H-Ras and c-Fos expression patterns in most stages of oral oncogenesis. Both proteins are found at high levels in normal oral mucosa and in precancerous lesions. When H-Ras expression decreases in early tumour stages, a parallel reduction in c-Fos expression occurs. At later stages of oral oncogenesis, the expression of both proteins increases again to high levels.

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