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**Histology and Histopathology**

*Cellular and Molecular Biology*

# Diabetes enhances the expression of H-ras and suppresses the expression of EGFR leading to increased cell proliferation

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**Summary.** EGFR kinase activity triggers numerous signaling pathways, such as the Ras/Raf/MAPK cascade, leading to the activation of various mitogen activated protein kinases, which are implicated in cell proliferation through induction of several genes, including c-fos. The possible effect of diabetes on the expression of the oncogenes EGFR, H-ras and c-fos was investigated in an experimental model of chemically induced oral oncogenesis in normal and diabetic (type I) Sprague-Dawley rats. Thirteen diabetic and twelve normal rats developed cancer after 4NQO treatment, while six diabetic and six normal animals were used as controls. The biopsies were classified pathologically (ranging from dysplasia to moderately differentiated oral squamous cell carcinoma) and were studied immunohistochemically. Several representative histological regions from each biopsy were analysed in regard to EGFR, H-ras and c-fos expression, and a comparison between normal and diabetic rats was effected. A trend of decreased EGFR expression in diabetic compared to normal rats was revealed throughout oncogenesis, which was significant in the stage of dysplasia ($P<0.05$). On the contrary, a trend of increased H-ras expression was observed in diabetic compared to normal rats during oncogenesis, which rose significantly in early invasion and well differentiated OSCC ($P<0.001$ and $P<0.01$ respectively). Finally, no statistical differences concerning c-fos expression were detected between diabetic and normal animals. In conclusion, it seems that diabetes reduces the expression of EGFR and initiates the Ras/Raf/MAPK signal transduction pathway by enhancing activation of other signalling molecules, such as the diabetes-associated Insulin Receptor Substrate-1, leading to increased cell proliferation without c-fos involvement.

**Key words:** EGFR, H-ras, c-fos, Oral squamous cell carcinoma, Diabetes

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## Introduction

Squamous cell carcinoma is the sixth most common malignancy (Das and Nagpal, 2002). Oral squamous cell carcinoma (OSCC) is identified as a significant public health threat all over the world (Sankaranarayanan et al., 1998). OSCC arises through a multistep process of genetic alterations, including activation of oncogenes and inactivation of tumour suppressor genes, usually as a result of exposure to environmental agents, such as tobacco smoking, alcoholic abuse, and viruses, including human papillomavirus (Forastiere et al., 2001; Sudbo et al., 2001).

Aberrant expression of several oncogenes, such as epidermal growth factor receptor (EGFR), H-ras, and c-fos, is believed to contribute to oral carcinogenesis (Todd et al., 1997; Milde-Langosch, 2005). EGFR is the prototypal member of one family of transmebrane receptors with intrinsic activity of protein tyrosine kinase (RPTK) (Wells, 1999). As a pleiotropic signaller, EGFR interacts with most members of the c-erbB subfamily of RPTK (Wells, 1999). The integrated biological response to EGFR activation varies from mitogenesis to apoptosis, migration to differentiation (Wells, 1999). EGFR kinase activity triggers numerous

downstream signaling pathways, such as the phospholipase C-γ (PLCγ) cascade, the Ras/Raf/MEK cascade leading to the activation of various mitogen activated protein kinases (MAPKs) implicated in cell proliferation, as well as the phosphatidylinositol 3-kinase (PI3K) pathway (Wells, 1997). MAPKs phosphorylate Elk-1, a ternary complex factor, which leads to increased induction of *c-fos* mRNA (Karin, 1995). EGFR oncogene has been implicated in a large variety of malignancies resulting from mutations, gene amplification or protein overexpression (Olayioye et al., 2000). These abnormalities, commonly found in head and neck cancer, have been correlated significantly with tumour size and stage (Santini et al., 1991; Rikimaru et al., 1992; Werkmeister et al., 1996, 2000; Todd and Wong, 1999; Xia et al., 1999; Crowe et al., 2002; Ekberg et al., 2005).

Furthermore, both *H-ras* and *c-fos* oncogenes have been implicated in human OSCC. A remarkable variation in rates of *H-ras* activational mutations has been reported, ranging from 5 to 40% in various human populations, while a trend towards increased *H-ras* protein expression in later stages of OSCC formation has been detected immunohistochemically, indicating that *H-ras* could be involved in advanced stages of carcinogenesis (Saranath et al., 1991; Warnakulasuriya et al., 1992; Yeudall et al., 1993; McDonald et al., 1994; Sakata, 1996; Munirajan et al., 1998; Xu et al., 1998; Das et al., 2000). Regarding *c-fos* expression, there are conflicting reports in the literature. Some studies have reported elevated levels of *c-fos* in oral dysplasia or OSCC, while other studies have reported a high *c-fos* expression in normal oral mucosa followed by a gradual decrease in advanced stages of oral cancer (De Sousa et al., 2002; Ohyama et al., 2004; Turatti et al., 2005).

Diabetes is traditionally correlated with a variety of oral conditions (Finney et al., 1997; Kawamura et al., 1998). Interestingly, recent epidemiological studies have incriminated diabetes mellitus as a risk factor for the development of OSCC, as well as oral premalignant lesions (Ujpal et al., 2002, 2004). Several studies have reported that EGFR is differentially affected in various tissues obtained from diabetic animals. Particularly, EGFR has been reported to be elevated in the gastric mucosa and the kidney tubules of diabetic rats (Ziyadeh and Goldfarb, 1991; Khan et al., 1999), while decreased in the liver, placenta and pancreas of the same animals (Korc et al., 1984; Sissom et al., 1987; Okamoto et al., 1988). There are rare data in the literature concerning H-ras and c-fos expression in diabetic states. One study suggested that H-ras levels are increased in the retina in diabetes and in retinal endothelial cells incubated in high glucose (Kowluru et al., 2004). In addition, one study suggested increased c-fos mRNA in diabetic rats compared to controls in the myocardium (Wang et al., 1999).

To our knowledge, the first report suggesting a molecular basis of the association between diabetes and oral cancer was published by our group, indicating that a possible mechanism linked to both diseases involves insulin receptor substrate-1 (IRS-1) and focal adhesion kinase pp125 (FAK), based on an experimental rat model of oral oncogenesis (Goutzanis et al., 2007). Therefore, we used the same experimental model of induced OSCC in diabetic (type I) and normal rats, in order to examine the expression of EGFR, H-ras and c-fos in normal oral mucosa tissues and in various tissues exhibiting both precancerous and cancerous lesions.

## Materials and methods

Thirty-seven female Sprague-Dawley rats purchased from the Hellenic Pasteur Institute (Athens) at the age of six weeks and weighing approximately 135g each, were used in this study. The rats 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 four groups. A) Group D (n=6): Diabetic rats without carcinogenesis, B) Group Dc (n=13): Diabetic rats used for induced carcinogenesis, C) Group N (n=6): Normal rats without carcinogenesis, D) Group Nc (n=12): Normal rats used for induced carcinogenesis.

The induction of diabetes was performed in 19 previously (overnight fasted animals) by a single intraperitoneal injection of streptozotocin (STZ) dissolved in saline buffer at a dose of 70 mg/kg of weight (ZANOSAR, Pharmacia & Upjohn Co., USA) and determined by glucose levels in blood after three weeks, as described elsewhere (Goutzanis et al., 2007). The diabetogenic action of streptozotocin results primarily from its highly specific cytotoxic action on the pancreatic ß-cells of the islets of Langerhans with rapid and irreversible necrosis (Junod et al., 1969). Oral cancer was induced in Dc and Nc animals by application of carcinogen 4-nitroquinoline N-oxide (4NQO) at a concentration of 5% in propylene glycol 3 times per week for 5 months in the rats' hard palate (Fluca AG Chemische Fabrik, Switzerland), as described elsewhere (Goutzanis et al., 2007). Clinical signs of oral lesions putatively tumor-related were observed within 6 months after last application of carcinogen. After sacrification of animals by ether treatment, the oral regions with cancer (mainly palate and tongue) of Dc and Nc rats and the respective regions of D and N rats were excised for immunohistochemical analysis (Goutzanis et al., 2007).

### *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 in the following categories: normal mucosa, hyperplasia, dysplasia, early invasion, well- and moderately

differentiated carcinoma. In every sample all possible different lesions were evaluated.

### *Immunohistochemical analysis*

Surgical specimens were fixed in 10% neutralized formaldehyde solution and embedded in paraffin. Four sections of 4 $\mu$m 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 hematoxylin and eosin for routine histological evaluation, while the other three were used for immunohistochemical detection of EGFR, H-ras and c-fos proteins as described elsewhere (Derka et al., 2006) with monoclonal primary antibodies against EGFR (EGFR 1005: sc-03, dilution 1: 100, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), H-ras (H-ras F235: sc-29, dilution 1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and c-fos (c-Fos 4: sc-52, dilution 1: 200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). For EGFR antigen retrieval was performed by incubation of the sections with trypsin for 15 min. Each section was studied immuno-histochemically using monoclonal antibodies against EGFR, H-ras and c-fos proteins, and various representative histological regions in each section were analysed.

Breast carcinoma, colon and brain tissue which express strongly EGFR, H-ras and c-fos respectively were used as positive controls. Negative controls of human brain and tonsils were processed in the same manner, using Phosphate Buffered Saline (PBS) instead of the primary antibody. All slides were independently reviewed by two investigators blindly. The consecutive hematoxylin-eosin-stained slides were evaluated by a pathologist experienced in oral pathology, without knowing the EGFR, H-ras and c-fos staining patterns.

### *Statistical analysis*

For each biopsy obtained, we evaluated the percentages of EGFR, H-ras and c-fos expression in all contained possible lesions. All lesions were classified into the following categories: “normal mucosa, hyperplasia, dysplasia, early invasion, well- and moderately differentiated carcinoma”. Statistical analyses were performed using the two-tailed Student's t-test for each group of animals and for each category of accumulated histologically similar lesions. The percentage of positively stained cells from each non cancerous or precancerous condition (hyperplasia, dysplasia) were compared to normal tissue, while the percentage of positively stained cells from each tumor (early invasion, well- and moderately differentiated carcinoma) were compared to the mean value of percentages of the non-cancerous and precancerous conditions. In all cases with no normal distribution, the results of both the Wilcoxon test and two-tailed student's t-test provided the same level of significance.

## Results

The histological status of biopsies observed in various regions of rats from all groups (N, Nc, D, Dc) after induced oral oncogenesis is summarized in Fig. 1. The experimental model seems valid since several normal, non-cancerous, precancerous and cancerous regions in the various tissue biopsies were collected, and further analysis of immunostaining data was implemented (Figs. 2, 3, 4). In all cases with no normal distribution, the results of both the Wilcoxon test and two-tailed student's t-test provided the same level of significance.

The percentages of positive expression of EGFR, H-ras and c-fos, in the various histological categories for non-diabetic animals from groups N and Nc are shown in Table 1. EGFR expression was progressively increased during all stages of oral oncogenesis, although statistical differences were found only in the stage of dysplasia ($P<0.05$). Similarly, H-ras expression was found to be increased in sequential stages of oral carcinogenesis, with significant differences observed in the stages of early invasion ($P<0.01$), well differentiated OSCC ($P<0.001$) and moderately differentiated OSCC ($P<0.001$). On the other hand, c-fos expression was absent in most stages and was present only in the tumour stages of well and moderately differentiated OSCC ($P<0.01$).

The percentages of positive expression of EGFR, H-ras and c-fos in the various histological categories for non-diabetic animals from groups D and Dc are shown in Table 2. In diabetic animals, both EGFR and H-ras expression was found to be progressively increased during all stages of oral oncogenesis, with significant differences (ranging from $P<0.05$ to $P<0.001$). Nevertheless, c-fos expression was completely absent in

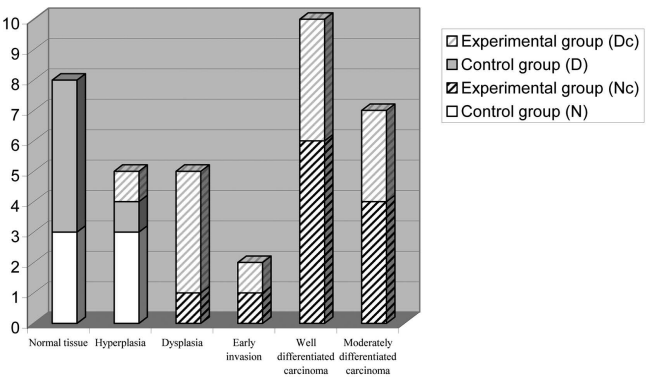


**Fig. 1.** Histological status of biopsies in groups N, D, Nc and Dc. D: diabetic rats without carcinogenesis; Dc: diabetic rats with induced carcinogenesis; N: normal rats without carcinogenesis; Nc: normal rats with induced carcinogenesis.

diabetic animals in all stages of carcinogenesis.

The comparison of EGFR, H-ras and c-fos expression percentages between normal (N and Nc) and diabetic (D and Dc) rats is shown in Table 3. This comparison revealed significantly decreased EGFR expression in the stage of dysplasia in diabetic rats compared to normal rats ($P<0.05$), significantly increased H-ras expression in early invasion and well differentiated OSCC ($P<0.001$ and $P<0.01$ respectively) in diabetic rats compared to normal rats, and finally, no

**Table 1.** Percentage of EGFR, H-ras and c-fos positive cells in the various categories of tissue status for normal rats (N and Nc).

| | | Non-cancerous and Precancerous | | Tumor | | |
|---|---|---|---|---|---|---|
| | Normal oral mucosa | Oral mucosal hyperplasia | Oral mucosal dysplasia | Early invasion | Well differentiated OSCC | Moderately differentiated OSCC |
| EGFR | | | | | | |
| Mean of percentages | 2.25 (N=11) | 3.11 (N=31) | 7.5 (N=12) | 5 (N=11) | 7 (N=27) | 8.75 (N=20) |
| Mean of non-cancerous and precancerous conditions | | 5.4 | | | | |
| Probability of t-test (P value) | | N.S[a] | <0.05 | N.S[a] | N.S[a] | N.S[a]. |
| H-ras | | | | | | |
| Mean of percentages | 2 (N=11) | 2.3 (N=31) | 6.1 (N=12) | 6.67 (N=11) | 10.74 (N=27) | 19.5 (N=20) |
| Mean of non-cancerous and precancerous conditions | | 3.24 | | | | |
| Probability of t-test (P value) | | N.S[a] | N.S[a] | <0.01 | <0.001 | <0.001 |
| c-fos | | | | | | |
| Mean of percentages | 0 (N=11) | 0 (N=31) | 0 (N=12) | 0 (N=11) | 5 (N=27) | 3.75 (N=20) |
| Mean of non-cancerous and precancerous conditions | | 0 | | | | |
| Probability of t-test (P value) | | N.S[a] | N.S[a] | N.S[a] | <0.01 | <0.01 |

[a]: No statistical difference.

**Table 2.** Percentage of EGFR, H-ras and c-fos positive cells in the various categories of tissue status for diabetic rats (D and Dc).

| | | Non-cancerous and Precancerous | | Tumor | | |
|---|---|---|---|---|---|---|
| | Normal oral mucosa | Oral mucosal hyperplasia | Oral mucosal dysplasia | Early invasion | Well differentiated OSCC | Moderately differentiated OSCC |
| EGFR | | | | | | |
| Mean of percentages | 0 (N=22) | 0.78 (N=33) | 0.95 (N=21) | 4.25 (N=4) | 4.47 (N=23) | 10 (N=11) |
| Mean of non-cancerous and precancerous conditions | | 0.85 | | | | |
| Probability of t-test (P value) | | <0.05 | <0.001 | <0.001 | <0.001 | <0.001 |
| H-ras | | | | | | |
| Mean of percentages | 0.17 (N=22) | 2 (N=33) | 8 (N=21) | 15 (N=4) | 15.4 (N=23) | 18.6 (N=11) |
| Mean of non-cancerous and precancerous conditions | | 4.3 | | | | |
| Probability of t-test (P value) | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| c-fos | | | | | | |
| Mean of percentages | 0 (N=22) | 0 (N=33) | 0 (N=21) | 0 (N=4) | 0 (N=23) | 0 (N=11) |
| Mean of non-cancerous and precancerous conditions | | 0 | | | | |
| Probability of t-test (P value) | | N.S[a] | N.S[a] | N.S[a] | N.S[a] | N.S[a] |

[a]: No statistical difference.

**Table 3.** Comparison of EGFR, H-ras and c-fos percentages between normal (N and Nc) and diabetic rats (D and Dc).

| | Non-Cancerous and Precancerous | | | Tumour | | |
|---|---|---|---|---|---|---|
| | Normal oral mucosa | Oral mucosal hyperplasia | Oral mucosal dysplasia | Early invasion | Well differentiated OSCC | Moderately differentiated OSCC |
| EGFR diabetic rats versus EGFR normal rats per stage | | | | | | |
| P value (T-test) | N.S[a] | N.S[a] | <0,05 | N.S[a] | N.S[a] | N.S[a] |
| H-ras diabetic rats versus H-ras normal rats per stage | | | | | | |
| P value (T-test) | N.S[a] | N.S[a] | N.S[a] | <0.001 | <0.01 | N.S[a] |
| c-fos diabetic rats versus c-fos normal rats per stage | | | | | | |
| P value (T-test) | N.S[a] | N.S[a] | N.S[a] | N.S[a] | N.S[a] | N.S[a] |

[a]: No statistical difference.

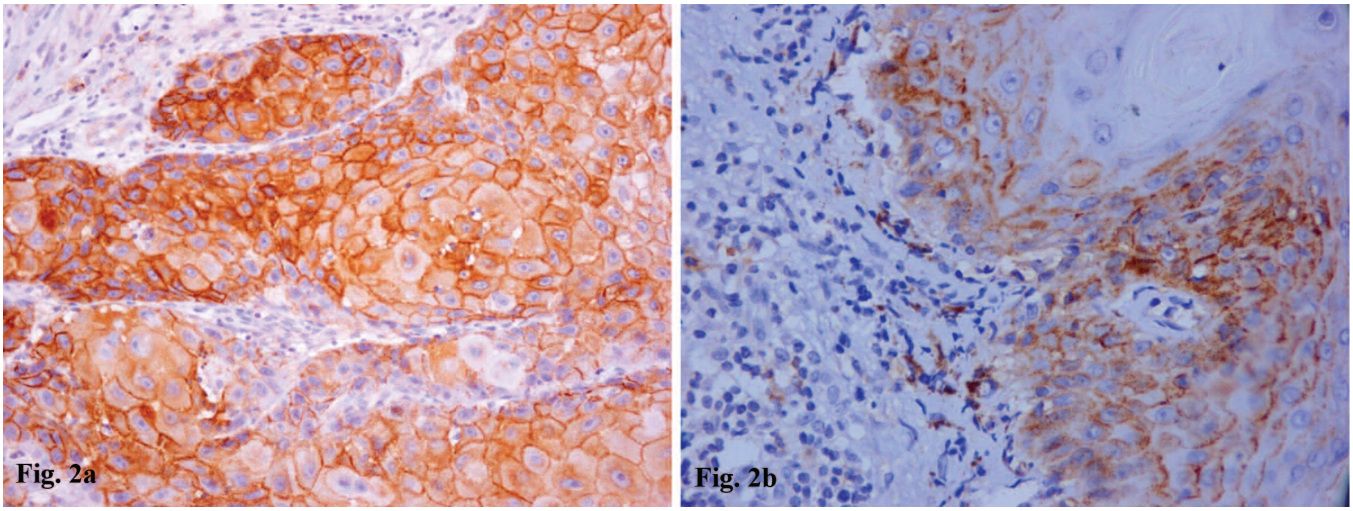


**Fig. 2. a.** Intense EGFR immunoreactivity in the majority of cancer cells from a moderately differentiated squamous cell carcinoma in a diabetic rat; **b.** Evident EGFR immunostaining in a dysplastic area from the oral mucosa of a non diabetic rat. a, x 200; b, x 400.

statistical differences concerning c-fos expression between diabetic and normal rats.

## Discussion

In this study, an experimental system of chemically induced diabetes mellitus (type I) and its effect on oral carcinogenesis was investigated in rats. As previously described (Goutzanis et al., 2007), the status of diabetes was established with STZ treatment, based on higher glucose levels and decreased body weight detected in diabetic rats in comparison to normal controls. Although theoretically STZ depresses cell-mediated immune responses (Fisher et al., 1998), no growth advantage of tumors was observed in Dc rats versus Nc rats, since there were no major differences in histopathological stages of oral cancer biopsies obtained from diabetic and normal animals, as previously described (Goutzanis et al., 2007). Based on these data, diabetes does not seem to confer an earlier and more aggressive effect on carcinogenesis in the oral region, if a carcinogen is continuously applied for five months. Nevertheless, it cannot be excluded at present that this robust approach of induced carcinogenesis might mask a subtler increase in cancer susceptibility of diabetic versus normal rats.

In the present study, the expression of EGFR, H-ras and c-fos was investigated in an experimental system of chemically induced oral cancer in sequential stages of oral oncogenesis, varying from normal oral mucosa to moderately-differentiated oral carcinomas in diabetic and normal rats, in order to delineate the molecular pathway linking diabetes and oral cancer. In normal

animals, EGFR and H-ras expression was found to be progressively increased during all stages of oral oncogenesis, in accordance with previous studies (Santini et al., 1991; Saranath et al., 1991; Rikimaru et al., 1992; Warnakulasuriya et al., 1992; Yeudall et al., 1993; McDonald et al., 1994; Sakata, 1996; Werkmeister et al., 1996; Munirajan et al., 1998; Xu et al., 1998; Todd and Wong, 1999; Xia et al., 1999; Das et al., 2000; Werkmeister et al., 2000; Crowe et al., 2002; Ekberg et al., 2005).

It is obvious that the two oncogenes, EGFR and H-ras, exhibit similar expression levels throughout oral carcinogenesis, an observation supported by the known molecular pathway connecting EGFR with Ras/Raf/MEK cascade, leading to the activation of various MAPKs implicated in cell proliferation (Wells, 1997). On the other hand, c-fos expression was completely absent throughout the whole process of carcinogenesis, except for a substantial increase during the stages of well and moderately differentiated carcinoma. Nevertheless, there are conflicting reports in the literature regarding c-fos expression, including either elevated or decreased levels of c-fos in malignant oral lesions in comparison to normal oral mucosa (De Sousa et al., 2002; Ohyama et

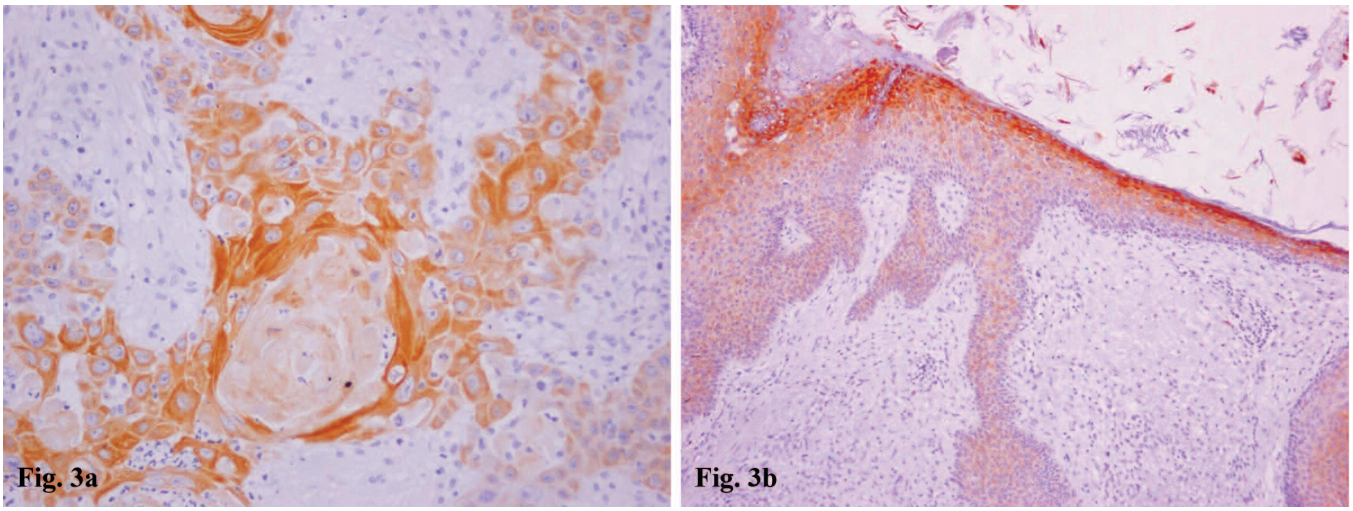


**Fig. 3. a.** Intense H-ras cytoplasmic immunoreaction in malignant cells of a well differentiated OSCC from a diabetic rat; **b.** H-ras immunostaining confined to the upper layers of a hyperplastic mucosa of a non diabetic rat. a, x 200; b, x 100.

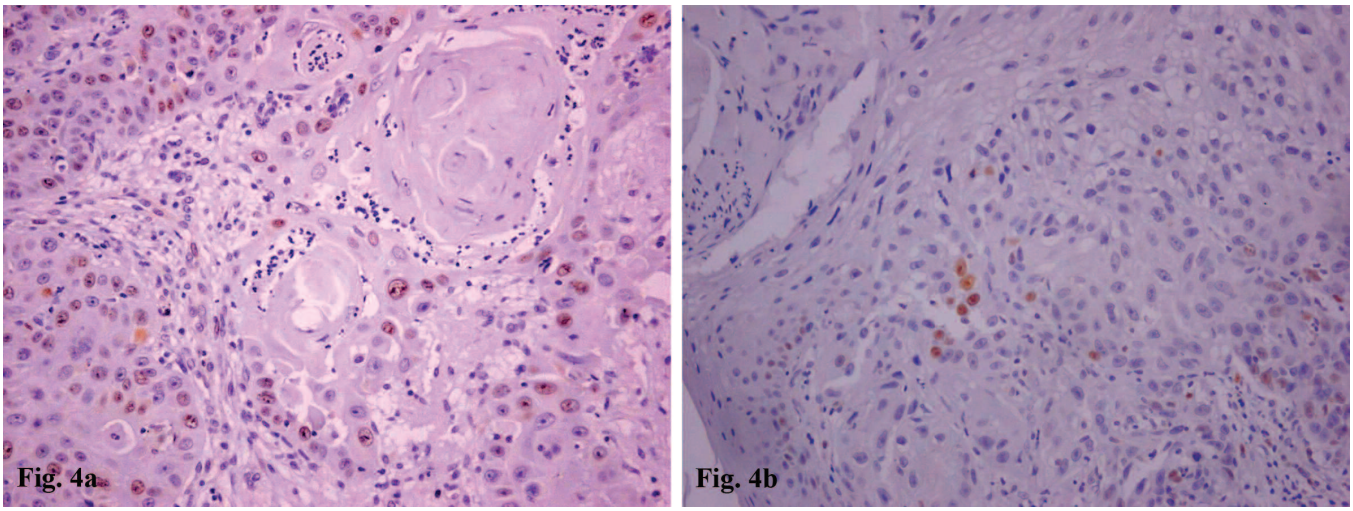


**Fig. 4. a.** Increased c-fos immunostaining in a well differentiated OSCC of a diabetic rat. Decreased c-fos immunoreactivity in a moderately differentiated OSCC in a non diabetic rat; **b.** Decreased c-fos immunoreactivity in a moderately differentiated OSCC in a non diabetic rat. x 200.

al., 2004; Turatti et al., 2005). Interestingly, the only study in rats among them investigated 4NQO-induced tongue oncogenesis and suggested that c-fos expression was undetected in normal mucosa, weak in dysplasia and intense in tongue OSCC (Ohyama et al., 2004). These results are in accordance with our present study since we used the same carcinogen (4NQO) in order to induce oral carcinogenesis, the same animals, and we also found increased expression of c-fos only in oral cancer. It seems that the elevation of c-fos levels plays an important role in rat oral oncogenesis and is associated with the establishment of OSCC.

The comparison between normal and diabetic animals indicated lower levels of EGFR in diabetic rats compared to normal rats with a significant decrease noted in the stage of dysplasia in diabetic rats (Fig. 5a). The findings of the present study are in accordance with several studies which demonstrated decreased EGFR levels in the liver, placenta and pancreas of diabetic animals (Korc et al., 1984; Sissom et al., 1987; Okamoto et al., 1988). It has also been demonstrated that even a modest attenuation of EGFR signalling leads to a severe defect in the growth of beta-cells, resulting in the development of diabetes (Miettinen et al., 2006). On the other hand, in our study higher levels of H-ras were noted in diabetic rats compared to normal rats, with a significant elevation observed specifically in the stages of early invasion and well differentiated OSCC (Fig. 5b). These findings are in accordance with one study which suggested that H-ras levels are increased in diabetic retinas and in retinal endothelial cells incubated in high glucose (Kowluru et al., 2004).

It is known that autophosphorylation and subsequent activation of Insulin Receptor as well as other types of cell receptors, such as EGFR, leads to the recruitment of signalling molecules, such as IRS-1, to the cell membrane, and the initiation of signalling cascades, including the Ras/Raf/MAP kinase cascade and PI3 kinase cascade (Rogers et al., 2005). A previous experimental study by our group suggested that the expression of IRS-1 was significantly higher in diabetic than normal animals (Goutzanis et al., 2007). IRS-1 overexpression may be attributed to an attempt of the cell to cope in an environment of scarce insulin. It has been suggested that the abundance of IRS-1 might facilitate its activation by the relatively few receptors that bind to minimally available insulin (Friedman et al., 1997). Given the association between IRS-1 and H-ras, it follows that in diabetes, H-ras is elevated due to increased levels of IRS-1, leading to the activation of MAP kinase cascade. It seems that diabetes promotes the activation of Ras/Raf/MAPK pathway leading to

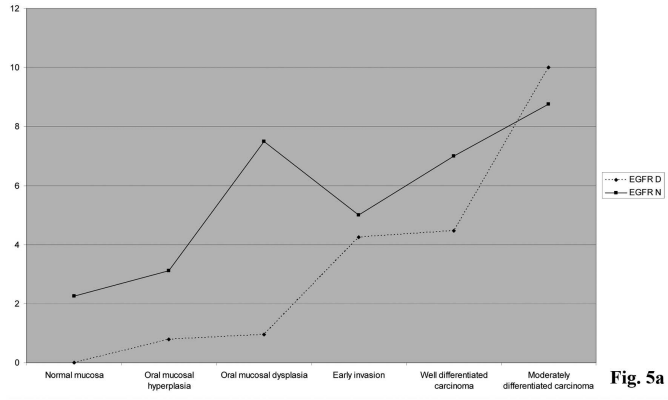


Fig. 5a

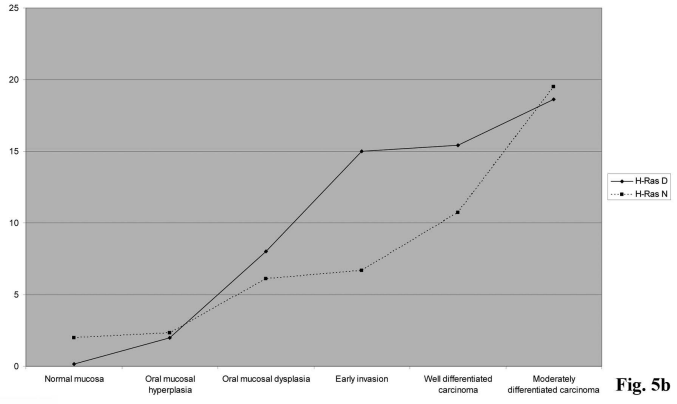


Fig. 5b

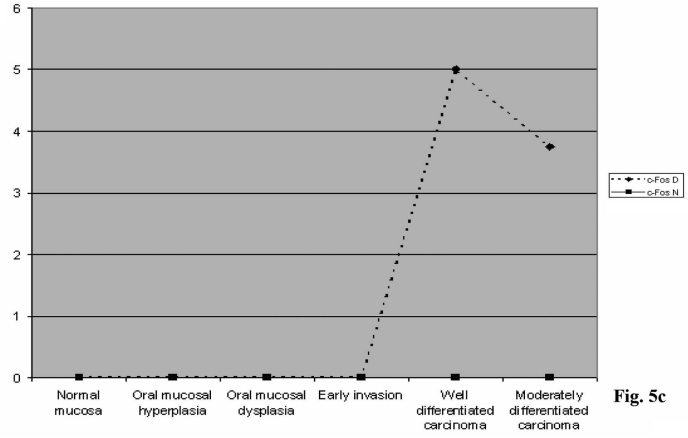


Fig. 5c

**Fig. 5.** Expression of EGFR (**a**), H-ras (**b**) and c-fos (**c**) in the different tissue categories in normal (N) and diabetic (D) rats.

increased cell proliferation. These findings are also supported by another previous study by our group performed in the same experimental system, in which diabetes was found to increase both N-ras and ets-1 expression during oral oncogenesis, resulting in enhanced cell proliferation and metastatic potential (Vairaktaris et al., 2007). Interestingly, in the present study, EGFR exhibited reduced levels in diabetes, a finding which in association with the simultaneous elevated expression of both IRS-1 and H-ras implies that EGFR in rat oral mucosa might not be implicated in the activation of IRS-1 or H-ras molecules and consequently in the stimulation of Ras/Raf/MAPK signal transduction pathway.

On the other hand, c-fos did not produce any significant findings concerning diabetes (Figure 5c). Nevertheless, there is one study which suggested increased c-fos mRNA in diabetic rats compared to controls in the myocardium (Wang et al., 1999). It may be suggested that c-fos is not implicated in the diabetes-related oncogenesis in the oral region of the present experimental animal system.

In conclusion, it seems that diabetes enhances the expression of H-ras and reduces the expression of EGFR, leading to increased cell proliferation via the activation of Ras/Raf/MAPK signal transduction pathway. In addition, diabetes might not influence c-fos expression during oral oncogenesis. Therefore, animal models may be an extremely useful tool in understanding important mechanisms affected by diabetes in oral oncogenesis.

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