ANTICANCER RESEARCH *27*: 4185-4194 (2007)

Short Review

Diabetes and Oral Oncogenesis

ELEFTHERIOS VAIRAKTARIS[1], SOFIA SPYRIDONIDOU[1], LAMBROS GOUTZANIS[1], ANTONIS VYLLIOTIS[1], ANDREAS LAZARIS[2], ISMINI DONTA[3], DESPINA PERREA, CHRISTOS YAPIJAKIS[1,4] and EFSTRATIOS PATSOURIS[2]

Departments of [1]Oral and Maxillofacial Surgery, [2]Pathology, [3]Experimental Surgery and [4]Neurology, University of Athens Medical School, Athens, Greece

Abstract. *Oral squamous cell carcinoma (OSCC) is the sixth most common malignancy in humans including type I diabetic and normal rats. Tobacco and alcohol, as well as dysregulation of oncogenes and tumor suppressor genes, epigenetic changes and mitochondrial mutations have been implicated in OSCC development. Recent epidemiological studies have incriminated diabetes mellitus as a risk factor for the development of OSCC, as well as oral premalignant lesions. Recently, an animal model was employed to study the influence of diabetes on signal transduction pathways in every stage of oral cancer development, from normal mucosa to hyperplasia, dysplasia, early invasion, well differentiated OSCC and moderately differentiated OSCC. Diabetes was induced by streptozotocin and chemical carcinogenesis was induced by the carcinogen 4-nitroquinoline N-oxide. The expression of EGFR, erbB2, erbB3, FGFR-2, FGFR-3, c-myc, N-ras, ets-1, H-ras, c-fos and c-jun, the tumor suppressor genes p53 and p16, apoptosis markers Bax and Bcl-2, and the cell proliferation marker Ki-67 in the sequential stages of rat oral oncogenesis was investigated. Diabetes seems to promote the activation of the Ras/Raf/MAPK signal transduction pathway mainly by induction of erbB2 and erbB3 receptors, leading to increased cell proliferation, while there was no difference in apoptosis levels during oncogenesis.*

Correspondence to: Prof. Dr. Eleftherios Vairaktaris, MD, DDS, Ph.D., Department of Oral and Maxillofacial Surgery, University of Athens Medical School, Kifissias and Perikleous Stavrou 9, Athens 11524, Greece. Tel: +30 210 6443035, Fax: +30 210 6443803, e-mail: lvairakt@med.uoa.gr

Key Words: Oral cancer, diabetes, oncogenes, tumor suppressor genes, signal transduction pathways, cell proliferation, apoptosis, animal model, short review.

Animal Models of Oral Squamous Cell Carcinoma

Oral squamous cell carcinoma (OSCC) is the sixth most common malignancy and constitutes a major health problem which is associated with severe morbidity and <50% long-term survival, despite advances in treatment with surgery, radiation and chemotherapy (1, 2). More than 300,000 new cases of OSCC are being diagnosed each year worldwide, but the poor prognosis of oral cancer has not improved significantly over the last four decades (1, 3). The most important risk factors for the development of oral cancer are tobacco and alcohol (4). In addition, dysregulation of oncogenes and tumor suppressor genes, cytogenetic changes, epigenetic changes and mitochondrial mutations have been implicated in OSCC development (5). Therefore, understanding the molecular mechanisms involved in the initiation and progression to malignancy of oral cancer will help to improve its prognosis and in the development of new forms of treatment.

Due to the immediate need for the development of several early diagnostic and prognostic markers of oral cancer, the sequential alterations in molecular mechanisms of OSCC development must be established (6). Oral oncogenesis is a multi-step process which involves various histological changes such as hyperplasia, dysplasia and the development of carcinomas. In humans, the molecular analysis of these multiple steps is hampered by the unavailability of biopsies from all stages of carcinogenesis (6). Therefore, animal models for OSCC development have been generated (including hamster, rat and mouse models) which allow the reproducible pathological characterization of all stages and their comparison with normal tissue (7, 8).

The OSCC induction in animals was first demonstrated in a hamster cheek pouch with the aid of polycyclic aromatic hydrocarbons such as 9,10-dimethyl-1,2-benzanthracene (DMBA), 20 methylcholanthrene

(20MC) and 3,4-benzopyrene (3,4BP) (9). In the early 1970s, the rat model of oral carcinogenesis was introduced, widely using 4-nitroquinoline-1-oxide (4NQO), a synthetic water-soluble carcinogen, to study various stages of oral oncogenesis (10-12). 4NQO exerts potent intracellular stress and its metabolic product binds to DNA predominantly at guanine residues (6). The rat model of 4NQO carcinogenesis produces a spectrum of preneoplastic and neoplastic lesions and, since it parallels OSCC development in humans, it is considered as the preferred model for the study of OSCC (13). Several markers which have the potential to indicate and predict the evolvement of an invasive malignant neoplasm following exposure to 4NQO have been investigated, such as tumor suppressor protein p53, E- and P-cadherin, proliferating markers Proliferating cell nuclear antigen (PCNA) and Bromodeoxyuridine (BrdU), Argyrophilic nucleolar organizer region (AgNOR) parameters, cytokeratin profile, and the nuclear profile of basal epithelial cells (14-20). In addition, the apoptosis-related proteins Bcl-2 and Bax have been shown to be altered in the rat 4NQO model of OSCC (21).

Traditionally, diabetes is correlated with a variety of oral conditions, such as periodontal disease, dental caries, stomatitis, glossitis and sensory changes (22, 23). Nevertheless, it was not considered to be involved in the pathogenesis of oral cancer, although correlations between diabetes and inflammatory oral lesions were established as early as the 19th century (24). Interestingly, recent epidemiological studies have incriminated diabetes mellitus as a risk factor not only for the development of OSCC, but also for oral premalignant lesions such as leukoplakia (25, 26). Type I diabetes, formerly known as either juvenile-onset diabetes (because of the early age of onset) or insulin-dependent diabetes mellitus (because of the clinical need for insulin), is now widely thought to be an organ-specific autoimmune disease resulting from destruction of insulin-producing pancreatic β-cells (27). Insulin deficiency results in reduced amounts of insulin receptor and consequently in cytoskeleton changes and reduced cell adhesion. In a previous study by our group, the molecular basis for the putative association between oral cancer and diabetes and the possible involvement of insulin receptor substrate-1 (IRS-1) and focal adhesion kinase (FAK) was investigated in diabetic (type I) and normal rats with induced oral squamous cell carcinoma (28). Our findings suggested that the IRS-1/FAK pathway is altered by diabetes resulting in reduced cell adhesion and possibly increasing the risk for oral cancer (28). Interestingly, type I diabetic patients not insulin-regulated might have a greater risk for developing oral cancer than do the general population.

The Rat Model of Sequential Oral Oncogenesis

In order to study the molecular pathways involved in the sequential stages of OSCC development and the association between diabetes and oral cancer, an experimental model of OSCC induced in diabetic (type I) and normal rats was used. A brief description of the performed experimental procedures follows, since they have been described in detail elsewhere (28).

Induction of diabetes and experimental carcinogenesis. Thirty-seven female Sprague-Dawley rats purchased from the Hellenic Pasteur Institute (Athens) at the age of six weeks and weighing approximately 135 g 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 confirmed by glucose levels in the blood after three weeks, as previously described (28).

Oral cancer was induced in Dc and Nc animals by application of the carcinogen 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 (28). Clinical signs of oral lesions putatively tumor-related were observed within 6 months after last application of carcinogen. After sacrifice 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 (28).

Pathological and immunohistochemical 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 mucosa, hyperplasia, dysplasia, early invasion, well- and moderately differentiated carcinoma. In every sample, a number of all possible representative histological lesions were analysed. The biopsies from the 37 animals were fixed in 10% neutralized formaldehyde solution and embedded in paraffin. Sections 4 μm-thick were prepared from each specimen and were mounted on Super Frost Plus-coated glass slides (Menzel and Co., Braunschweig, Germany). All sections were used for

detection of Bax, Bcl-2, Ki-67, p53, c-myc, N-ras, ets-1, H-ras, c-fos, c-jun, epidermal growth factor receptor (EGFR), erbB2, erbB3, fibroblast growth factor receptor-2 (FGFR-2), fibroblast growth factor receptor-3 (FGFR-3) and p16 proteins, using a standard immunohistochemical technique (DAB Immunoperoxidase Secondary Detection System) as described elsewhere (29-36).

The mean percentages of positively stained cells were calculated for 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 every group's percentages were compared with those corresponding to the previous group. In order to evaluate the pattern of antibody expression in relation to the histological status, the various lesions were divided according to tumor progression into a) normal tissue, b) non-cancerous and precancerous conditions (hyperplasia, dysplasia), c) tumor (early invasion, well-differentiated carcinoma, moderately differentiated carcinoma). In every lesion, the percentage of positively-stained cells from each non-cancerous and precancerous category was compared with those of the normal tissue, while the percentage of positively stained cells from each tumor category was compared with the average percentage of the non-cancerous and precancerous lesions.

Effect of Diabetes on Signal Transduction Pathways

Deregulation and mutations that augment the function of oncogenes, or the loss or inactivation of tumor suppressor genes, can directly enhance the potential of the cell to grow beyond its normal limits (37). These events can occur at several distinct points in the signal transduction cascades within cells. They include constitutive activation of receptor tyrosine kinase receptors (RTK), changes in the expression of second messengers and mutations in transcription factors which regulate cell proliferation and division, resulting in cancer progression (37).

The most common pathways leading to proliferation act through RTKs (38-40). RTKs bind to extracellular mitogenic growth factors and transduce these positive growth signals to intracellular second messengers (37). The substrates that RTKs phosphorylate belong to a class of proteins having Src homology 2 (SH2) domains (41). Some of these proteins, which play a critical role in cell signalling, are phospholipase-γ (PLCγ), phosphatidylinositol kinase (PI3K) and Grb2 (42, 43).

PLC-γ generates the second messengers diacylglycerol (DAG) and inositol triphosphate (IP3) by hydrolyzing membrane phospholipids (44). PI3K catalyzes the formation of phosphatidylinositol triphosphate (PIP3) resulting in the ultimate activation of protein kinase C (45, 46). Grb2 binds to the RTK and also interact with Son of Sevenless (SOS), which activates Ras (47-51).

Localization of Ras to the inner surface of the cytoplasmic membrane activates several downstream effectors, most notably the kinase Raf, which is the first signalling element in the mitogen activated protein kinase (MAPK) pathway (52). Upon activation, raf protein phosphorylates and thus activates the MAPKs (52). MAPKs in turn lead to the phosphorylation of other kinases eventually resulting in the enhanced expression of genes coding for proteins involved in the regulation of transcription whose products regulate cell proliferation and apoptosis (53).

In the rat model study of oral oncogenesis, the expression of several proteins implicated in these signal transduction pathways was detected by employing immunohistochemical methods. The studied protein factors included tyrosine kinase receptors (EGFR, erbB2, erbB3, FGFR-2, FGFR-3), cytoplasmic proteins (H-ras, N-ras), apoptosis-related proteins (Bax, Bcl-2), cell proliferation markers (Ki-67), nuclear transcriptional factors (p53, c-myc, c-fos, c-jun, ets-1), and cell cycle proteins (p16). The findings of these studies will be presented in regard to the involvement of signal transduction factors in every stage of oncogenesis. The signal transduction pathways that were affected in each sequential stage are presented in Figure 1.

Receptor tyrosine kinases. EGFR, *HER-2/neu* (erbB2) and *HER-3* (erbB3) are included in the EGFR family of tyrosine kinase receptors. Overexpression of EGFR has been described in cases of head and neck cancer (54, 55). In addition, significantly high levels of EGFR have been closely correlated with tumor size and stage in head and neck SCC (56-59). Overexpression of erbB2 is significantly correlated with shorter survival of patients with oral SCC (60, 61). erbB3 Overexpression has been related to the malignant progression of head and neck SCC both in cell lines and clinical studies (61-64). Concerning FGFR-2 and/or FGFR-3 immunoexpression in oral cancer, a study suggested increased intensity of both receptors in dysplasias and squamous cell carcinomas (65).

In the rat model, a trend for reduced EGFR expression in diabetic compared to normal rats was revealed throughout oral oncogenesis (Figure 1) (31). The comparison of diabetic *versus* normal animals revealed that diabetes enhances the expression of erbB2 during nearly all stages of oral cancer development, as well as the expression of erbB3 in non-cancerous and precancerous stages of oral oncogenesis (Figure 1) (35). On the contrary, based on our findings, it seems that diabetes does not affect the FGFR-2 and FGFR-3 pattern of expression throughout the various stages of oral oncogenesis (Figure 1) (34).

Cytoplasmic proteins. Mutations of ras in oral cancer have been studied by a few other groups. It seems that these mutations are quite rare in Western populations and more

frequent in studies conducted with patients from Southeast Asian countries such as India and Taiwan (66). On the other hand, involvement of N-ras is an infrequent occurrence in the malignant progression of oral epithelial cells (67, 68). There are conflicting reports in the literature regarding the increase of N-ras expression at an early or a later stage in oral carcinogenesis (69, 70).

In our experimental model, a trend for increased H-ras expression was observed in diabetic compared to normal rats during oncogenesis; expression rose significantly in early invasion and well-differentiated OSCC (Figure 1) (31). N-ras expression increased with tumor advancement in diabetic rats, while in normal rats N-ras was not detected in the initial stages of oral oncogenesis and increased only in well-differentiated OSCC (Figure 1) (32). It seems that both H-ras and N-ras are positively affected by diabetes during oral oncogenesis.

Apoptosis related-proteins and cell proliferation markers. Bcl-2 and *bax* are two important effector genes during the apoptosis process. Certain studies of human OSCC biopsies reported increased levels of Bcl-2 as well as increased levels of Bcl-2/Bax ratio corresponding to reduced apoptosis in comparison to normal oral mucosa (71, 72). Other studies of human biopsies detected reduced Bcl-2 expression, a reduced Bcl-2/Bax ratio and increased apoptosis in the initial tumor stages, while Bax was not found to be significantly altered during the different stages of oral oncogenesis (73, 74).

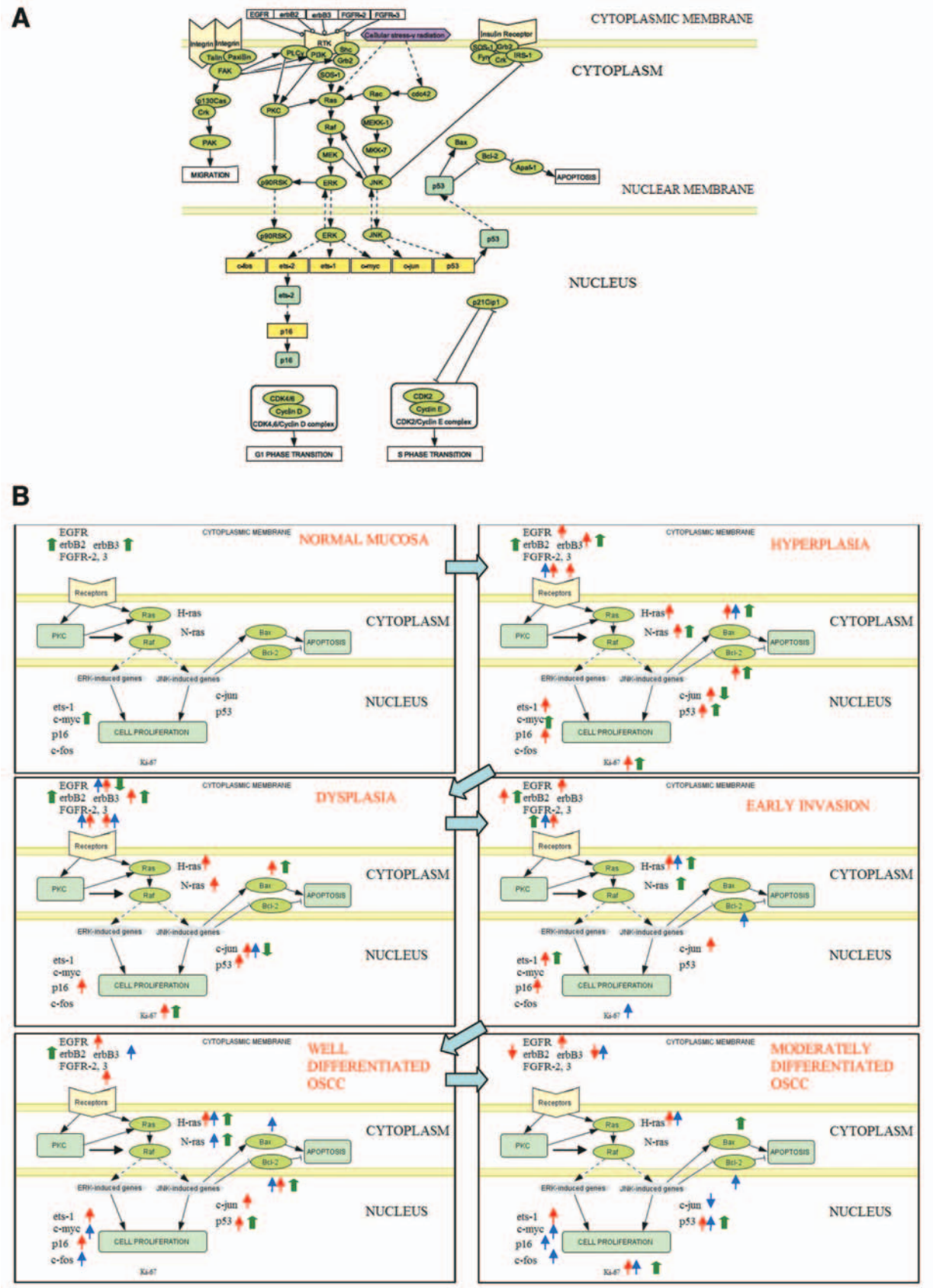
Based on our findings in the rat model, the Bcl-2/Bax ratio was relatively stable during the oncogenesis process in diabetic rats, contrary to normal rats which appear to have an increased Bcl-2/Bax ratio in the stage of moderately differentiated carcinoma, indicating reduced apoptosis levels (Figure 1) (29). On the contrary, the expression of the cell proliferation marker Ki-67 was higher in diabetic rats than in normal ones in almost all stages of oral oncogenesis (Figure 1) (29). It is suggested that diabetes results in increased cell proliferation during oral oncogenesis, but this is accomplished without affecting the Bax/Bcl-2-mediated apoptotic pathways.

Nuclear transcriptional factors. Transcription factors, or proteins that regulate the expression of other genes, are also altered in oral cancer. The transcription factor *c-myc*, which takes part in the regulation of cell proliferation and differentiation, is frequently overexpressed in oral cancers (75, 76). Overexpression due to gene amplification of *c-myc* is most frequently associated with poorly differentiated tumors and with poor prognosis (77). Gene mutations of *p53* have been detected in more than 60% of cases of OSCC (78). The inactivated mutant p53 protein is more stable than the wild-type p53 protein, therefore its accumulation seems to occur early in oral neoplastic development and gradually increases through the sequential oncogenic stages from hyperplasia to OSCC (79, 80). Together *c-fos* and *c-jun* form the composite transcription factor activating protein-1 (AP-1), a mitogen-activated transactivator important for cell proliferation and differentiation (81). There are rare and conflicting reports in the literature regarding *c-fos* and *c-jun* expression in OSCC. In general, *c-jun* is considered as an early marker of differentiation and there are very few studies regarding its role in early carcinogenesis (82). 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 in the advanced stages of oral cancer (83-86). Up-regulation of *ets-1* expression has been documented in many types of human tumors, including oral cancer. Generally, expression levels of ets-1 correlate well with the grade of invasiveness and metastasis (87).

The findings of the animal model suggested that diabetes contributed to increased accumulation of mutations in the *p53* gene contributing to increased proliferation of tumor cells during oral oncogenesis (Figure 1) (30). Additionally, diabetes appeared to enhance c-myc expression only in the initial stages of oral oncogenesis (Figure 1) (30). Higher c-jun levels were observed in non-cancerous and precancerous stages in normal rats compared to diabetic rats, indicating that diabetes might not affect the c-jun *N*-terminal kinase (JNK)/c-jun pathway (Figure 1) (36). Interestingly, no statistical differences concerning c-fos expression were detected between diabetic and normal animals. Therefore, c-fos may not be implicated in the diabetes-related oncogenesis in the oral region of the present experimental animal system (Figure 1) (31). The same

→

Figure 1. *A) Pathways of signaling transduction in normal oral mucosa. Activation of specific protein molecules is depicted with broken or solid lines, depending whether it occurs with or without passage via a membrane, respectively. B) Selected pathways of signaling transduction during sequential stages of oral oncogenesis. The protein expression in non-cancerous and precancerous stages (hyperplasia, dysplasia) was compared to normal oral mucosa, while their expression in tumor stages (early invasion, well differentiated OSCC, moderately differentiated OSCC) was compared to the mean expression in non-cancerous and precancerous stages. The arrows by the studied proteins indicate the significant differences in their expression observed in the rat model. Red arrows indicate significant differences in diabetic rats, blue arrows indicate significant differences in normal rats, while green arrows indicate significant differences between normal and diabetic rats in the same stage. An arrow pointing upwards indicates a significant increase of expression in that stage, while one pointing downwards indicates a significant decrease.*


A
CYTOPLASMIC MEMBRANE
CYTOPLASM
NUCLEAR MEMBRANE
NUCLEUS
EGFR
erbB2
erbB3
FGFR-2
FGFR-3
Integrin
Talin
PaxBin
FAK
RTK
PI3K
Shc
Grb2
SOS-1
Cellular stress-γ radiation
Insulin Receptor
IRS-1
Fyn
Crk
p130Cas
Crk
PAK
MIGRATION
PKC
Ras
Rac
cdc42
Raf
MEKK-1
MEK
MKK-7
p90RSK
ERK
JNK
Bax
Bcl-2
Apaf-1
APOPTOSIS
p53
c-fos
ets-2
ets-1
c-myc
c-jun
p53
p16
p16
p21Cip1
CDK4/6
Cyclin D
CDK4,6/Cyclin D complex
G1 PHASE TRANSITION
CDK2
Cyclin E
CDK2/Cyclin E complex
S PHASE TRANSITION
B
NORMAL MUCOSA
HYPERPLASIA
DYSPLASIA
EARLY INVASION
WELL DIFFERENTIATED OSCC
MODERATELY DIFFERENTIATED OSCC
CYTOPLASMIC MEMBRANE
CYTOPLASM
NUCLEUS
Receptors
Ras
PKC
Raf
H-ras
N-ras
Bax
Bcl-2
APOPTOSIS
ERK-induced genes
JNK-induced genes
c-jun
p53
ets-1
c-myc
p16
c-fos
CELL PROLIFERATION
Ki-67

pattern of elevated ets-1 expression was observed both in diabetic and normal rats, but in cancerous stages this expression was higher in diabetic than in normal rats indicating that diabetes may contribute to enhanced invasion and metastatic potential by increasing ets-1 levels (Figure 1) (32).

Cell cycle regulators. p16, a CDK inhibitor, binds to CDK4 and inhibits cyclin D-CDK4 activity. In addition, p16 is also negatively regulated by pRB (88). Although point mutations of the *p16* gene in head and neck cancer are rare, alternative mechanisms of the abrogation of p16 function involving homozygous deletions or methylation of the 5-CpG promoter region of *p16* have frequently been identified, suggesting that functional inactivation of *p16* is a common event (89-91). In the experimental rat model of oral oncogenesis, the expression of the cell cycle regulator p16 was not found to be affected by diabetes (Figure 1) (33).

Conclusion

It is known that activation of insulin receptor as well as other types of cell receptors (EGFR, erbB2, erbB3, FGFR-2, FGFR-3) 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. A previous experimental study by our group suggested that the expression of IRS-1 was significantly higher in diabetic than in normal animals (28). Given the association between IRS-1 and H-ras, it follows that in diabetes, both H-ras and N-ras are elevated due to increased levels of IRS-1, leading to the activation of MAP kinase cascade. In our study it is obvious that both H-ras and N-ras play a very crucial role in the development of oral cancer in diabetic animals. Therefore, diabetes seems to promote the activation of Ras/Raf/MAPK signal transduction pathway by induction of receptors erbB2 and erbB3 mainly (and not EGFR) leading to increased cell proliferation. Additionally, in diabetic animals the Bcl-2/Bax ratio was found to be relatively stable during the oncogenesis process, indicating that diabetes has no effect on apoptosis levels.

It seems that animal models are a very useful tool towards understanding important mechanisms in oral oncogenesis. The use of carcinogens in animal models in order to study the mechanisms of oral oncogenesis is warranted, since chemical agents appear to be the dominant etiological factors in oral cavity.

Acknowledgements

This work was co-funded by the European Social Fund and National Resources (EPEAEK II "Pythagoras" 70/3/7391) grant to E.V.

References

1 Sudbo J: Novel management of oral cancer: a paradigm of predictive oncology. Clin Med Res *2(4)*: 233-242, 2004.
2 Das BR and Nagpal JK: Understanding the biology of oral cancer. Med Sci Monit *8(11)*: RA258-267, 2002.
3 Greenlee RT, Murray T, Bolden S and Wingo PA: Cancer statistics, 2000. CA Cancer J Clin *50(1)*: 7-33, 2000.
4 Mehrotra R and Yadav S: Oral squamous cell carcinoma: etiology, pathogenesis and prognostic value of genomic alterations. Indian J Cancer *43(2)*: 60-66, 2006.
5 Mithani SK, Mydlarz WK, Grumbine FL, Smith IM and Califano JA: Molecular genetics of premalignant oral lesions. Oral Dis *13(2)*: 126-133, 2007.
6 Kanojia D and Vaidya MM: 4-Nitroquinoline-1-oxide induced experimental oral carcinogenesis. Oral Oncol *42(7)*: 655-667, 2006.
7 Herzig M and Christofori G: Recent advances in cancer research: mouse models of tumorigenesis. Biochim Biophys Acta *1602(2)*: 97-113, 2002.
8 Tang XH, Knudsen B, Bemis D, Tickoo S and Gudas LJ: Oral cavity and esophageal carcinogenesis modeled in carcinogen-treated mice. Clin Cancer Res *10(1 Pt 1)*: 301-313, 2004.
9 Salley JJ: Experimental carcinogenesis in the cheek pouch of the Syrian hamster. J Dent Res *33(2)*: 253-262, 1954.
10 Vered M, Yarom N and Dayan D: 4NQO oral carcinogenesis: animal models, molecular markers and future expectations. Oral Oncol *41(4)*: 337-339, 2005.
11 Liu X, He R and Chen W: A rat model of tongue mucosa squamous cell carcinoma induced by oral administration of 4NQO in drinking water. Zohonghua Kou Qiang Yi Xue Za Xhi 34(6): 354-356, 1999 (in Chinese).
12 Boyd NM and Reade PC: Temporal alterations in cytokeratin expression during experimental oral mucosal carcinogenesis. Carcinogenesis *12(10)*: 1767-1771, 1991.
13 Dayan D, Hirshberg A, Kaplan I, Rotem N and Bodner L: Experimental tongue cancer in desalivated rats. Oral Oncol *33*: 105-109, 1997.
14 Rich AM and Reade PC: Nuclear morphometry in experimental oral mucosal carcinogenesis. Eur J Cancer B Oral Oncol *32B*: 169-175, 1996.
15 Tanaka T, Kojima T, Okumura A, Yoshimi N and Mori H: Alterations of the nucleolar organizer regions during 4-nitroquinoline 1-oxide-induced tongue carcinogenesis. Carcinogenesis *12*: 329-333, 1991.
16 Kaplan I, Engelberg J and Dayan D: The effect of desalivation on 4-nitroquinoline-1-oxide-induced tongue carcinogenesis: a morphometric study of nucleolar organizer regions. J Oral Pathol Med *30*: 48-52, 2001.
17 Kaplan I, Hochstadt T and Dayan D: PCNA in palate and tongue mucosal dysplastic lesions induced by topically applied 4NQO in desalivated rat. Med Oral *7*: 336-343, 2002.
18 Itoh T, Yamamoto Y, Saka T, Inoue I and Takahashi H: Estimation of proliferative activity of experimental tongue carcinoma in rats. Immunohistochemical and DNA cytofluoro-metric analysis. Acta Otolaryngol *113*: 568-574, 1993.
19 Osugi Y: p53 Expression in various stages of 4-nitroquinoline 1-oxide induced carcinoma in rat tongue. J Osaka Dent Univ *30*: 29-35, 1996.

20 Sakaki T, Tamura I, Katoda H and Kakudo K: Changing expression of E- and P-cadherin during rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. J Oral Pathol Med *32*: 530-537, 2003.
21 Nishimura A: Changes in Bcl-2 and Bax expression in rat tongue during 4-nitroquinoline 1-oxide-induced carcinogenesis. J Dent Res *78*: 1264-1269, 1999.
22 Finney LS, Finney MO and Gonzalez-Campoy JM: What the mouth has to say about diabetes. Careful examinations can avert serious complications. Postgrad Med *102(6)*: 117-126, 1997.
23 Kawamura M, Fukuda S, Kawabata K and Iwamoto Y: Comparison of health behaviour and oral/medical conditions in non-insulin-dependent (type 2) diabetics and non diabetics. Austr Dent J *43*: 315-320, 1998.
24 Glickman J: Clinical Periodontology. Philadelphia: Saunders, 1972
25 Ujpal M, Matos O, Bibok G and Szabo G: Incidence of diabetes mellitus in patients with malignant tumors of the oral cavity. Orv Hetil *143(49)*: 2731-2733, 2002 (in Hungarian).
26 Ujpal M, Matos O, Bibok G, Somogyi A, Szabo G and Suba Z: Diabetes and oral tumors in Hungary: epidemiological correlations. Diabetes Care *27(3)*: 770-774, 2004.
27 Jahromi MM and Eisenbarth GS: Cellular and molecular pathogenesis of type 1A diabetes. Cell Mol Life Sci *64(7-8)*: 865-872, 2007.
28 Goutzanis L, Vairaktaris E, Yapijakis C, Kavantzas N, Nkenke E, Derka S, Vassiliou S, Acil Y, Kessler P, Stavrianeas N, Perrea D, Donta I, Skandalakis P and Patsouris E: Diabetes may increase risk for oral cancer through the insulin receptor substrate-1 and focal adhesion kinase pathway. Oral Oncol *43(2)*: 165-173, 2007.
29 Vairaktaris E, Kalokerinos G, Goutzanis L, Yapijakis C, Derka S, Vassiliou S, Spyridonidou S, Vylliotis A, Nkenke E, Lazaris A and Patsouris E: Diabetes enhances cell proliferation but not Bax/Bcl-2 mediated apoptosis during oral oncogenesis. Int J Oral Maxillof Surg 2007 (in press), PMID: 17825529.
30 Vairaktaris E, Goutzanis L, Spyridonidou S, Vassiliou S, Derka S, Kalokerinos G, Yapijakis C, Vylliotis A, Lazaris A and Patsouris E: Diabetes alters expression of p53 and c-myc in different stages of oral oncogenesis. Anticancer Res *27*: 1465-1474, 2007.
31 Vairaktaris E, Goutzanis L, Yapijakis C, Vassiliou S, Spyridonidou S, Vylliotis A, Nkenke E, Lazaris A, Strantzias P and Patsouris E: Diabetes enhances the expression of H-ras and suppresses the expression of EGFR leading to increased cell proliferation. Histol Histopathol 2007 (in press).
32 Vairaktaris E, Goutzanis L, Kalokerinos G, Yannopoulos A, Yapijakis C, Vassiliou S, Spyridonidou S, Vylliotis A, Nkenke E, Lazaris A, Tesseromatis C and Patsouris E: Diabetes increases both N-ras and ets-1 expression during rat oral oncogenesis resulting in enhanced cell proliferation and metastatic potential. In Vivo *21(4)*: 615-621, 2007.
33 Vairaktaris E, Goutzanis L, Nkenke E, Spyridonidou S, Vassiliou S, Derka S, Vylliotis A, Yapijakis C, Lazaris A, Strantzias P, Kalimeras E and Patsouris E: Expression of cell cycle regulator p16 is not affected by diabetes during oral oncogenesis. In Vivo 2007 (in press).
34 Vairaktaris E, Goutzanis L, Nkenke E, Spyridonidou S, Vassiliou S, Derka S, Vylliotis A, Yapijakis C, Lazaris A, Strantzias P, Kalimeras E and Patsouris E: Diabetes does not influence oral oncogenesis through fibroblast growth factor receptors. In Vivo *21(4)*: 623-628, 2007.
35 Vairaktaris E, Goutzanis L, Vassiliou S, Spyridonidou S, Nkenke E, Papageorgiou G, Strantzias P, Lazaris A, Yapijakis C and Patsouris E: Enhancement of erbB2 and erbB3 expression during oral oncogenesis in diabetic rats. J Cancer Res Clin Oncol 2007 (in press), PMID: 17704947.
36 Vairaktaris E, Goutzanis L, Kalokerinos G, Vassiliou S, Spyridonidou S, Avgoustidis D, Strantzias P, Lazaris A, Papageorgiou G, Ragos V, Yapijakis C and Patsouris E: Increased risk for oral cancer in diabetic animals is not associated with c-jun activation pathway. J Craniomaxillofac Surg 2007 (in press).
37 Bell HS and Ryan KM: Intracellular signalling and cancer: complex pathways lead to multiple targets. Eur J Cancer *41(2)*: 206-215, 2005.
38 Carpenter G and Cohen S: Epidermal growth factor. J Biol Chem *265*: 7709-7712, 1990.
39 Ullrich A and Schlessinger J: Signal transduction by receptors with tyrosine kinase activity. Cell *61*: 203-212, 1990.
40 Cross M and Dexter TM: Growth factors in development, transformation, and tumorigenesis. Cell *64*: 271-280, 1991.
41 Margolis B: Proteins with SH2 domains: transducers in tyrosine kinase signaling pathway. Cell Growth Differ *3*: 73-80, 1992.
42 Lemmon MA, Ladbury IE, Mandiyan V, Zhou M, Schlessinger J: Independent binding of peptide ligands to the SH2 and SH3 domains of Grb2. J Biol Chem *269*: 31653-31658, 1994.
43 Sandy JR: Signal transduction. Br J Orthod *25(4)*: 269-274, 1998.
44 Karin M: Signal transduction from cell surface to nucleus in development and disease. FASEB J *6(8)*: 2581-290, 1992.
45 Escobedo IA, Navankasattusas S, Kavanaugh MW, Milfay D, Fried VA and Williams LT: cDNA cloning of a novel 85 kDa protein that has SH2 domains and regulates binding of P13-kinase to the PDGF beta-receptor. Cell *65*: 75-82, 1991.
46 Fantl WJ, Escobedo JA, Martin GA, Turck CW, del Rosario M, McCormick F and Williams LT: Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. Cell *69*: 413-423, 1992.
47 Skolnik EY, Margolis B, Mohammadi M, Lowenstein E, Fischer R, Drepps A, Ullrich A and Schlessinger J: Cloning of P13 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinase. Cell *65*: 83-90, 1991.
48 Lowenstein EJ, Daly RJ, Batzer AG, Li W, Margolis B, Lammers R, Ullrich A, Skolnik EY, Bar-Sagi D and Schlessinger J: The SH2 and SH3 domain containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell *70*: 431-442, 1992.
49 Buday L and Downward J: Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. Cell *73*: 611-620, 1993.
50 Li N, Batzer A, Daly R, Yajnik V, Skolnik E, Chardin P, Bar-Sagi D, Margolis B and Schlessinger J: Guanine-nucleotide-releasing factor hsosl binds to Grb2 and links receptor tyrosine kinases to Ras signaling. Nature *363*: 85-88, 1993.
51 Porfiri E and McCormick F: Regulation of epidermal growth factor receptor signaling by phosphorylation of the ras exchange factor hSOS1. J Biol Chem *271*: 5871 -5877, 1996.
52 Beeram M, Patnaik A and Rowinsky EK: Raf: a strategic target for therapeutic development against cancer. J Clin Oncol *23(27)*: 6771-6790, 2005.

53 Kamer AR, Krebs L, Hoghooghi SA and Liebow C: Proliferative and apoptotic responses in cancers with special reference to oral cancer. Crit Rev Oral Biol Med *10(1)*: 58-78, 1999.

54 Issing WJ, Wustrow TPU and Heppt W: Oncogenes related to head and neck cancer. Anticancer Res *13*: 2541-2552, 1993.

55 Irish JC and Bernstein A: Oncogenes in head and neck cancer. Laryngoscope *103*: 42-52, 1993.

56 Santini J, Formento JL, Francoual M, Milano G, Schneider M, Dassonville O and Demard F: Characterization, quantification, and potential clinical value of the epidermal growth factor receptor in head and neck squamous cell carcinomas. Head Neck *13*: 132-139, 1991.

57 Dassonville O, Formento JL, Francoual M, Ramaioli A, Santini J, Schneider M, Demard F and Milano G: Expression of epidermal growth factor receptor and survival in upper aerodigestive tract cancer. J Clin Oncol *11*: 1873-1878, 1993.

58 Grandis JR and Tweardy DJ: TGF-alpha and EGFR in head and neck cancer. J Cell Biochem Suppl *17F*: 188-191, 1993.

59 Shin D, Ro JY, Hong WK and Hittelman WN: Dysregulation of epidermal growth factor receptor expression in premalignant lesions during head and neck tumorigenesis. Cancer Res *54*: 3153-3159, 1994.

60 Xia W, Lau YK, Zhang HZ, Liu AR, Li L, Kiyokawa N, Clayman GL, Katz RL and Hung MC: Strong correlation between c-erbB-2 overexpression and overall survival of patients with oral squamous cell carcinoma. Clin Cancer Res *33*: 3-9, 1997.

61 Xia W, Lau YK, Zhang HZ, Xiao FY, Johnston DA, Liu AR, Li L, Katz RL and Hung MC: Combination of EGFR, Her-2/neu, and Her-3 is a stronger predictor for the outcome of oral squamous cell carcinoma than any individual family members. Clin Cancer Res *5*: 4164-4174, 1999.

62 Funayama T, Nakanishi T, Takahashi K, Taniguchi S, Takigawa M and Matsumura T: Overexpression of c-erbB-3 in various stages of human squamous cell carcinomas. Oncology *55*: 161-167, 1998.

63 O-charoenrat P, Rhys-Evans PH, Archer DJ and Eccles SA: C-erbB receptors in squamous cell carcinomas of the head and neck: clinical significance and correlation with matrix metalloproteinases and vascular endothelial growth factors. Oral Oncol *38*: 73-80, 2002.

64 Shintani S, Funayama T, Yoshihama Y, Alcalde RE and Matsumura T: Prognostic significance of ERBB3 overexpression in oral squamous cell carcinoma. Cancer Lett *95*: 79-83, 1995.

65 Wakulich C, Jackson-Boeters L, Daley TM and Wysocki GP: Immunohistochemical localization of growth factors fibroblast growth factor-1 and fibroblast growth factor-2 and receptors fibroblast growth factor receptor-2 and fibroblast growth factor receptor-3 in normal oral epithelium, epithelial dysplasias, and squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod *93*: 573-579, 2002.

66 Das N, Majumder J and DasGupta UB: ras Gene mutations in oral cancer in eastern India. Oral Oncol *36*: 76-80, 2000.

67 Matsuda H, Konishi N, Hiasa Y, Hayashi I, Tsuzuki T, Tao M, Kitahori Y, Yoshioka N, Kirita T and Sugimura M: Alterations of p16/CDKN2, p53 and ras genes in oral squamous cell carcinomas and premalignant lesions. J Oral Pathol Med *25*: 232-238, 1996.

68 Yeudall WA, Torrance LK, Elsegood KA, Speight P, Scully C and Prime SS: Ras gene point mutation is a rare event in premalignant tissues and malignant cells and tissues from oral mucosal lesions. Eur J Cancer B Oral Oncol *29B(1)*: 63-67, 1993.

69 McDonald JS, Jones H, Pavelic ZP, Pavelic LJ, Stambrook PJ and Gluckman JL: Immunohistochemical detection of the H-ras, K-ras, and N-ras oncogenes in squamous cell carcinoma of the head and neck. J Oral Pathol Med *23(8)*: 342-346, 1994.

70 Yarbrough WG, Shores C, Witsell DL, Weissler MC, Fidler ME and Gilmer TM: ras mutations and expression in head and neck squamous cell carcinomas. Laryngoscope *104(11)*: 1337-1347, 1994.

71 Chen Y, Kayano T and Takagi M: Dysregulated expression of bcl-2 and bax in oral carcinomas: evidence of post-transcriptional control. J Oral Pathol Med *29*: 63-69, 2000.

72 Loro LL, Vintermyr OK, Liavaag PG, Jonsson R and Johannessen AC: Oral squamous cell carcinoma is associated with decreased bcl-2/bax expression ratio and increased apoptosis. Hum Pathol *30(9)*: 1097-1105, 1999.

73 Tang XH, Knudsen B, Bemis D, Tickoo S and Gudas LJ: Oral cavity and esophageal carcinogenesis modelled in carcinogen-treated mice. Clin Cancer Res *10*: 301-313, 2004.

74 Singh BB, Chandler FW Jr, Whitaker SB and Forbes-Nelson AE: Immunohistochemical evaluation of bcl-2 oncoprotein in oral dysplasia and carcinoma. Oral Surg Oral Med Oral Pathol Oral Radio Endod *85*: 692-698, 1998.

75 Spandidos DA, LamotheA and Field JK: Multiple transcriptional activation of cellular oncogenes in human head and neck solid tumors. Anticancer Res *5*: 221-224, 1985.

76 Field JK: Oncogenes and tumor-suppressor genes in squamous cell carcinoma of the head and neck. Eur J Cancer (B): Oral Oncol *28*: 67-76, 1992.

77 Schantz SP: Basic science advances in head and neck oncology: the past decade. Sem Surg Oncol *11*: 272-279, 1995.

78 Sakai E, Rikimaru K, Ueda M, Matsumoto Y, Ishii N, Enomoto S, Yamamoto H and Tsuchida N: The p53 tumor-suppressor gene and ras oncogene mutations in oral squamous cell carcinoma. Int J Cancer *52*: 867-872, 1992.

79 Tanaka N, Sugihara K, Odajima T, Mimura M, Kimijima Y and Ichinose S: Oral squamous cell carcinoma: electron microscopic and immunohistochemical characteristics. Med Electron Microse *35*: 127-138, 2002.

80 Carlos de Vicente J, Junquera Gutierrez LM, Zapatero AH, Fresno Forcelledo MF, Hernandez-Vallejo G and Lopez Arranz JS: Prognostic significance of p53 expression in oral squamous cell carcinoma without neck node metastasis. Head Neck *26*: 22-30, 2004.

81 Shaulian E and Karin M: AP-1 in cell proliferation and survival. Oncogene 20: 2390-2400, 2001.

82 Szabo E, Riffe ME, Steinberg SM, Birrer MJ and Linnoila RI: Altered c-JUN expression: an early event in human lung carcinogenesis. Cancer Res *56*: 305-315, 1996.

83 De Sousa SO, Mesquita RA, Pinto DS JR and Gutkind S: Immunolocalization of c-Fos and c-Jun in human oral mucosa and in oral squamous cell carcinoma. J Oral Pathol Med *31*: 78-81, 2002.

84 Mangone FR, Brentani MM, Nonogaki S, Begnami MD, Campos AH, Walder F, Carvalho MB, Soares FA, Torloni H, Kowalski LP and Federico MH: Overexpression of Fos-related antigen-1 in head and neck squamous cell carcinoma. Int J Exp Pathol *86*: 205-212, 2005.

85 Ohyama M, Hirayama Y, Tanuma JI, Hirano M, Semba I, Shisa H, Hiai H, Sugihara K and Kitano M: Expressions of junB and c-fos are enhanced in 4-nitroquinoline 1-oxide-induced rat tongue cancers. Pathol Int *54*: 35-40, 2004.
86 Turatti E, Da Costa Neves A, De Magalhaes MH and De Sousa SO: Assessment of c-Jun, c-Fos and cyclin D1 in premalignant and malignant oral lesions. J Oral Sci *47*: 71-76, 2005.
87 Oikawa T and Yamada T: Molecular biology of the ETS family of transcription factors. Gene *303*: 11-34, 2003.
88 Prime SS, Eveson JW, Guest PG, Parkinson EK and Paterson IC: Early genetic and functional events in the pathogenesis of oral cancer. Radiat Oncol Investig *5(3)*: 93-96, 1997.
89 Cairns P, Mao L, Merlo A, Lee DJ, Schwab D, Eby Y, Tokino K, van der Riet P, Blaugrund JE and Sidransky D: Rates of *p16* (*MTS1*) mutations in primary tumors with 9p loss. Science *265(5170)*: 415-417, 1994.
90 Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB and Sidransky D: 5' CpG island methylation is associated with transcriptional silencing of the tumor suppressor *p16/CDKN2/MTS1* in human cancers. Nat Med *1(7)*: 686-692, 1995.
91 Reed AL, Califano J, Cairns P, Westra WH, Jones RM, Koch W, Ahrendt S, Eby Y, Sewell D, Nawroz H, Bartek J and Sidransky D: High frequency of *p16* (*CDKN2/MTS-1/INK4A*) inactivation in head and neck squamous cell carcinoma. Cancer Res *56(16)*: 3630-3633, 1996.

Received July 2, 2007
Revised September 28, 2007
Accepted October 17, 2007