RADIOINDICTION ORAL SQUAMOUS CARCINOMA CELL LINES: EVALUATION OF APOPTOTIC PROTEINS AS PROGNOSTIC FACTORS

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Accepted 10 July 2006
Published online 12 December 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/hed.20520

Abstract: Background. In this study, we investigated the importance of apoptosis for cell death after radiotherapy, and whether the expression of pro- and anti-apoptotic proteins has any correlation to the radiosensitivity.

Methods. Three oral squamous cell carcinoma cell lines, UT-SCC-2, UT-SCC-9 and UT-SCC-24A, were subjected to radiotherapy. After irradiation, viable and dead cells were counted to determine radiation sensitivity and apoptosis was analyzed by measurement of caspase-3 activity. The expressions of pro- and anti-apoptotic proteins were assessed using western blot analyses.

Results and Conclusion. After irradiation, apoptotic morphology and caspase-3 activity were only detected in cell lines exhibiting high or moderate radiosensitivity. Western blot analysis indicates that survivin, epidermal growth factor receptor, cyclooxygenase-2, and Bcl-xL are critical components in irradiation resistance of the investigated cell lines. Moreover, our results suggest that apoptotic cell death and the balance between pro- and anti-apoptotic proteins are of importance for the outcome of radiotherapy.

Keywords: apoptosis; oral tumors; predictive markers; radiotherapy; radiosensitivity

Cancer of head and neck poses a major health problem in the world and is ranked by prevalence as number 6 of all cancer types. Approximately 600,000 new malignancies are diagnosed each year, about half of which occur in Southern East Asia and China. The majority of all head and neck cancers are squamous cell carcinomas (SCC) originating from epithelial tissues. A combination of radiotherapy and surgery or definitive chemoradiation is the primary mode of treatment of locally advanced squamous cell carcinoma of the head and neck. Radioresistance and local carcinoma recurrences are, however, significant problems following radiotherapy, and therefore there is a paramount need for predictive markers.

Apoptosis is a form of physiological cell death mediated by caspases. It plays a fundamental role in multicellular organisms, and apoptosis is thought to be a critical factor in radiation-induced cell death. Apoptosis can be induced through the receptor or the mitochondrial pathway, and many pro- and anti-apoptotic proteins are involved in its
regulation (see Figure 1). The loss of growth control and a marked resistance to apoptosis are probably major mechanisms driving tumor progression. Most cancers show alterations in 1 or multiple genes that encode proteins involved in the regulation of apoptosis, eg, the tumor suppressor p53, members of the Bcl-2 family, and inhibitor of apoptotic proteins (IAPs). The p53 protein is important in radiation response and is capable of either arresting the cell cycle or inducing apoptosis in a dose-dependent manner. Deregressed expression of proteins controlling apoptosis may suppress the apoptotic deletion of cells that normally follows upon DNA damage. This will contribute to the accumulation of oncogenic mutations and thereby facilitate malignant transformation and tumor development. For example, functionally defective mutant p53 protein or overexpression of the anti-apoptotic protein Bcl-2 result in decreased radiosensitivity of tumor cells. Overexpression of Bcl-2 and Bcl-xL has been shown to prevent apoptosis and to suppress activation of caspases in response to a number of stimuli, which in some tumor cells confer drug resistance.

Furthermore, IAPs are crucial regulators of molecular mechanisms as activity of caspases, a family of cystein proteases central for cellular degradation during apoptosis. Survivin is a recently characterized IAP, and the high expression of this protein has been associated with inhibition of apoptosis. Survivin is undetectable in terminally differentiated adult tissue but becomes expressed in several common human cancers such as stomach, colorectal, lung, breast, pancreatic, and prostate cancer, and in high-grade non-Hodgkin’s lymphomas. Recently, Lo Muzio et al showed that survivin expression may identify cases of oral squamous cell carcinomas with a more aggressive and invasive phenotype.

The epidermal growth factor (EGF) influences cell division, migration, adhesion, differentiation, and apoptosis through a tyrosine kinase pathway. The epidermal growth factor receptor (EGFR) is commonly overexpressed in human epithelial tumors and in tumors from head and neck. Overexpression of EGFR has been shown to correlate with an aggressive malignant progression, resulting in poor clinical outcome. Among SCC patients, a majority have elevated levels of EGFR and its ligand, tumor growth factor-α.

The heat shock protein (Hsp) family consists of both constitutively expressed and stress-induced molecular chaperons that are involved in the regulation of cell growth, transformation, and apoptosis. Hsp70 is highly expressed in the cytosol and plasma membrane of primary tumors of various origins, while its expression is very low and restricted to the cytosol in unstressed normal cells. In vitro studies have suggested that Hsp70 may interfere directly with the apoptotic signaling machinery. Moreover, treatment of oral cancer cells with Hsp70 antisense oligomers resulted in decreased expression of Bcl-2, inhibition of cell proliferation, and consequent increase in apoptotic cell death.

Cyclooxygenase-2 (COX-2), which is normally undetectable in most tissues, is 1 of 2 isoforms that convert arachidonic acid into several eicosanoids such as prostacyclin, prostaglandin, and thromboxins. COX-2 is an inducible enzyme, which
can be stimulated by oncogenes and tumor promoters during malignancy. It has been shown to have a number of cellular effects such as a reduced susceptibility to undergo apoptosis.

The present study was undertaken to evaluate the possibility of using the expression of pro- and anti-apoptotic proteins as predictive factors for the outcome of radiotherapy. It examines the importance of apoptosis for radiosensitivity in 3 oral squamous cell carcinoma cell lines (UT-SCC-2, UT-SCC-9, and UT-SCC-24A).

**MATERIALS AND METHODS**

**Cells and Culture Conditions.** Three recently established oral cavity SCC cell lines were selected for this study to represent different parts of the spectrum of in vitro radiosensitivity. The average intrinsic radiosensitivity measured with the 96-well plate clonogenic assay in a large panel of head and neck SCC cell lines was 1.8 for oral cavity cancers (N = 14) and 1.9 for head and neck cancer of all sites (N = 37). The UT-SCC-2 cell line represents the average intrinsic radiosensitivity (AUC = 1.8), UT-SCC-9 cells are among the most radiosensitive (AUC = 1.4), whereas the UT-SCC-24A cell line is a representative of the most resistant head and neck SCC cell lines (AUC = 2.6) (Table 1). The cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 IU/mL penicillin-G, 50 µg/mL streptomycin, and 10% fetal bovine serum (all from GIBCO, Paisly, UK). The cells were incubated in humidified air with 5% CO₂ at 37 °C and subcultured once a week. Forty-eight hours before the experiments, the cells were trypsinized and seeded into 6-well plates (Costar, Cambridge, MA) at a density of 16,000 cells/cm².

**Irradiation.** Cultures were irradiated with photons from a 4 MV linear accelerator (Varian Clinac 600 C, Varian Medical Systems, Palo Alto, CA) delivering 2 Gy/min and cells were exposed to 15 Gy/day. The field size was 30 × 30 cm, the source surface distance was 100 cm, and 3 cm polymethyl methacrylate was placed above and 10 cm below the cells. Following the first irradiation, cells were brought back to standard culture conditions and then irradiated for a second and third time after 24 and 48 hours (see Figure 2). During the treatment period, cells were cultured in medium as described earlier and control cells (nonirradiated) were run in parallel under the same environmental conditions. Viable and dead cells were counted in control cultures and in the cultures exposed for irradiation using the trypan blue exclusion test. Using this test, cells with permeable plasma membranes are stained blue in necrotic and post-apoptotic necrotic cells.

**Measurement of Caspase Activity.** Caspase-3 activity was measured 28, 52, and 76 hours after the

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**Table 1. Tumor characteristics and intrinsic radiation sensitivity.**

<table>
<thead>
<tr>
<th>Cancer cell line</th>
<th>Tumor location</th>
<th>Type of lesion</th>
<th>TNM*</th>
<th>AUC of cancer cell line6</th>
<th>SF2 of cancer cell line2</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT-SCC-24A</td>
<td>Tongue</td>
<td>Primary</td>
<td>T2N0M0</td>
<td>2.6 ± 0.3</td>
<td>0.51 ± 0.06</td>
</tr>
<tr>
<td>UT-SCC-2</td>
<td>Floor of mouth</td>
<td>Primary</td>
<td>T4N1M0</td>
<td>1.8 ± 0.2</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>UT-SCC-9</td>
<td>Neck</td>
<td>Metastasis</td>
<td>T2N1M0</td>
<td>1.4 ± 0.1</td>
<td>0.19 ± 0.07</td>
</tr>
</tbody>
</table>

*TNM classification according to the Union Internationale Contre le Cancer (UICC, 1977).

1Surviving fraction at 2 Gy (SF2). Data from by Pekkola-Heino et al., Haikonen et al., and Erjala et al.

**FIGURE 2.** Schematic presentation of the experimental design. The tumor cells were trypsinized and seeded into 6-well plates (C), and were thereafter exposed to daily irradiation at 15 Gy for 3 consecutive days (IR). Cultures were sampled at different time points for microscopic analyses, cell counting, and measurement of caspase-3 activity (S).
first irradiation (see Figure 2) using the fluorescent substrate Ac-DEVD-7-amino-4-methyl coumarin (Ac-DEVD-AMC; Becton-Dickinson, Mountain View, CA), which is cleaved by active caspasases with caspase-3-like activity. Measurements were performed according to the manufacturer’s instructions. After incubation for 1 hour at 37°C, the amount of released AMC was analyzed at λ<sub>ex</sub> = 380 nm and λ<sub>em</sub> = 435 nm in an RF-1501 spectrofluorophotometer (Shimadzu, Kyoto, Japan). The amount of released AMC was calculated using a standard curve, and correlated to total protein determined by the method described by Lowry et al.<sup>22</sup> Caspase-3 activity was expressed in nmol AMC/mg protein/hour.

**Transmission Electron Microscopy.** Cultures to be subjected to transmission electron microscopy were prepared as previously described.<sup>23</sup> Briefly, SCC cultures in plastic culture dishes were fixed by adding 2% glutaraldehyde (Agar Scientific, Essex, UK) in 0.1M sucrose-sodium cacodylate-HCl buffer (pH 7.2; Sigma, St Louis, MO) and post-fixed in osmium (Johnson Matthey Chemicals, Royston, UK). Dehydration, followed by en block staining with uranyl acetate (Sigma), dehydration, and embedding in Epon-812 (Fluka AG, Buchs, Switzerland) was also performed in the culture dishes. Thin sections of cured blocks were cut with a diamond knife (Diatome, Bienne, Switzerland), stained with lead citrate (Sigma), and then examined and photographed in a JEOL 1230-EX electron microscope (Tokyo, Japan) at 100 kV.

**Western Blot Analysis.** Cells were washed in phosphate-buffered saline (PBS) and lysed in 63 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.05% bromphenol blue (all from Sigma). The protein concentration was determined,<sup>22</sup> and 100 µg aliquots were separated by 15% or 4% to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories), which was subsequently blocked for 90 minutes at room temperature in Tris-buffered saline (TBS; 50 mM Tris and 0.15M NaCl, pH 7.5) containing 5% skimmed milk and 0.1% Tween-20 (Sigma). After washing in TBS, the membrane was incubated with a mouse anti-Bcl-2 (dilution 1:1,000; Dakopatts, Älvsjö, Sweden), a mouse anti-Bax (1:500), a mouse anti-survivin (1:500), a mouse anti-Hsp70 (1:1,000), a mouse anti-Fas (1:200), a mouse anti-COX-2 (1:200), and a goat anti-PUMA (1:200; all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), a mouse anti-Bad (1:500), a mouse anti-Nip1 (1:250), a mouse anti-Bcl-x<sub>L</sub> (1:500), a mouse anti-hILP/XIAP (1:250), a mouse anti-CAS (1:1,000), a mouse anti-p53 (1:500; all from BD Transduction Laboratories), or a mouse anti-EGFR (1:200; Labvision Corporation, CA), and all antibodies were diluted in TBS containing 0.1% skimmed milk and 0.1% Tween-20 and incubated at 4°C overnight. The membrane was washed and incubated for 1 hour at room temperature with a peroxidase-conjugated anti-mouse (1:1,500; Dakopatts) or a bovine anti-goat antibody (1:1,000; Santa Cruz Biotechnology), and the bands were visualized by Western Blotting Luminol Reagent (Santa Cruz Biotechnology). Equal loading was verified by reprobing the membrane with a goat anti-actin (I-19) (1:1,000; Santa Cruz Biotechnology) followed by a bovine anti-goat antibody (1:1,000; Santa Cruz Biotechnology).

**Statistical Analysis.** All experiments were repeated at least 3 times. Data were statistically evaluated using the Mann-Whitney U-test for comparisons between 2 groups. Values are given as arithmetic means ± standard derivation (SD). Differences were considered significant when p < .05.

**RESULTS**

**Effects of Radiation.** The radiosensitivity of the 3 oral SCC cell lines (UT-SCC-24A, UT-SCC-2, and UT-SCC-9) used in this study was previously tested<sup>24,25</sup> with a clonogenic cell assay.<sup>26</sup> The origin, characteristics, and intrinsic radiosensitivity of these cell lines are summarized in Table 1. To investigate possible correlation between apoptosis frequency and the sensitivity for radiotherapy, we analyzed cell death and apoptosis after fractionated radiotherapy. UT-SCC-24A, UT-SCC-2, and UT-SCC-9 were exposed to daily irradiation at 15 Gy for 3 consecutive days. We chose this relatively high dose in order to be able to measure apoptosis. At a higher dose (20 Gy), the results were an increase of necrotic cell death (assessed by the trypan blue exclusion test), and at a lower dose (10 Gy), the results showed low levels of cell death accompanied by low caspase activity (data not shown). After irradiation, no signs of apoptosis
were found in UT-SCC-24A cultures, but some cells showed an increased number of autophagic vacuoles (Figure 3B), indicating that a repair mechanism had started in these cells. In cultures from UT-SCC-2 and UT-SCC-9, typical signs of apoptosis such as cell shrinkage, fragmented nuclei, and apoptotic bodies were detected 52 hours after the first irradiation using light microscopy (data not shown) and electron microscopy (Figures 3D and 3F). Proliferation and cell death (necrotic and postapoptotic necrotic cell death) were measured by counting viable and dead cells using the trypan blue exclusion test, while apoptosis was analyzed by measurement of caspase-3 activity. In UT-SCC-24A, a decreased proliferation (see Figure 4) but no increase in caspase-3 activity could be detected (see Figure 5). In UT-SCC-2 and UT-SCC-9 cultures, a pronounced decrease in cell number was detected 52 and 76 hours after the first irradiation (see Figure 4), which was accompanied by a signif-

![Figure 3](image-url)  
**FIGURE 3.** Apoptotic morphology of oral squamous carcinoma cells after fractionated radiotherapy. Electron micrographs of cells from the UT-SCC-24A (A and B), UT-SCC-2 (C and D), and UT-SCC-9 (E and F) cell lines. Micrographs A, C, and E show the ultra structure of control cells, and micrographs B, D, and F show cell morphology 52 hours after the first irradiation. Different stages of apoptosis such as cell shrinkage, fragmented nuclei, and apoptotic bodies can be discerned in irradiated cells. Normal nuclei (N) and apoptotic cells (A) are marked in the micrographs. Bar = 5 μm.
significant increase of the caspase-3 activity after 52 hours ($p < .05$; Figure 5).

**Protein Expression: Bcl-2 Family.** Radioresistance and chemotherapy resistance is hypothesized to be due to overexpression of anti-apoptotic proteins like Bcl-xL and Bcl-2 or a decrease in the expression of pro-apoptotic proteins such as Bax, Bad, and Bak. Among the anti-apoptotic Bcl-2 family members, Bcl-xL was highly expressed in the relatively radioresistant cell line UT-SCC-24A, while Bcl-2 was highly expressed both in UT-SCC-24A and UT-SCC-2 as analyzed by Western blot (Figure 6A). On the other hand, the expres-
higher amount in UT-SCC-2 and UT-SCC-24A cells as compared with in UT-SCC-9 cells (Figure 7A).

All cell lines tested were earlier found to exhibit some form of p53 aberration, with the exception of UT-SCC-24A in only 1 allele (Table 2).24,29 Here only cells from UT-SCC-2 cell line showed expression of the p53 protein (Figure 7B).

The expression of the death receptor Fas has been shown to be involved in irradiation-induced cell death30 and was also investigated. However, in these 3 cell lines, no differences in the amount of the receptor were detected (data not shown).

Protein Expression: IAPs, p53, and Fas. High expressions of survivin and XIAP have been associated with inhibition of apoptosis.9 In the studied cell lines, survivin was expressed to a higher degree in the relatively radioresistant cell line UT-SCC-24A compared with in UT-SCC-2 and UT-SCC-9 cells (Figure 6B); however, no differences in Bax expression could be detected between the cell lines (data not shown). PUMA (p53-upregulated modulator of apoptosis), another pro-apoptotic member of the Bcl-2 family for which upregulation is thought to be crucial during p53-dependent apoptosis, was mostly expressed in UT-SCC-2 (Figure 6B).

Protein Expression: EGFR, Hsp70, CAS and COX-2. EGFR, Hsp70, cellular apoptosis susceptibility (CAS), and COX-2 are all shown to be expressed in tumor cells of different origin or to be involved in apoptosis. Here we found EGFR to be highly expressed in the UT-SCC-24A cell line (Figure 7C). Overexpression of Hsp70 has been shown to prevent apoptosis in tumor cells and is implicated in oral tumor genesis. Hsp70 was expressed in all 3 cell lines, although to a lesser extent in UT-SCC-9 (Figure 7C). CAS is found in elevated levels in rapidly dividing cells such as spermatogonia, respiratory tract epithelium, and human tumor cell lines.31,32 CAS was highly expressed in UT-SCC-24A and UT-SCC-2 but only to a low degree in the UT-SCC-9 cell line (Figure 7C). The expression level of COX-2 has been suggested to correlate with radiation tolerance in oral SCC33 and in this study was expressed in the highest amount in the UT-SCC-24A cell line, to a lesser extent in UT-SCC-2, and was hardly detectable in UT-SCC-9 cells (Figure 7C).

DISCUSSION
Apoptosis is a tightly regulated process, and cellular alterations that decrease the ability to activate the apoptotic machinery play a critical role in resistance to radiotherapy and chemotherapy.

<table>
<thead>
<tr>
<th>Cancer cell line</th>
<th>Type of p53 aberration</th>
<th>p53 mutation</th>
<th>Alteration</th>
<th>Second allele present/transcribed</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT-SCC-24A</td>
<td>Splice site mutation</td>
<td>E7–2 A → T</td>
<td>Incorrectly spliced transcript, frame shift</td>
<td>+(WT)/+</td>
</tr>
<tr>
<td>UT-SCC-2</td>
<td>Missense</td>
<td>TGT → TTT</td>
<td>Cys → Phe</td>
<td>−/−</td>
</tr>
<tr>
<td>UT-SCC-9</td>
<td>Loss of transcript</td>
<td>Del exons 2 to 9</td>
<td>−/−</td>
<td></td>
</tr>
</tbody>
</table>

Note: Data from by Pekkola-Heino et al, 1996;24 and Hauser et al, 2002.29
One of our aims was to investigate the role of apoptosis for radiosensitivity in 3 oral SCC cell lines. Irradiation resulted in apoptotic morphology and increased caspase activity in UT-SCC-9 and UT-SCC-2 cell lines, which were earlier found to exhibit high and moderate radiation sensitivity, respectively. This indicates that apoptotic cell death could be important for the outcome of radiation in these cells.

The dynamic balance between pro- and anti-apoptotic proteins is crucial for determining cell fate, survival, or cell death. When looking for predictive factors for radiosensitivity, our hypothesis is that combinations of pro- and anti-apoptotic proteins and probably the balance between them are of higher importance than the expression of single proteins. In the present study, we have chosen to investigate the expression of numerous proteins that are all known to be involved in different parts of the apoptotic process and to be expressed in tumor cells. The results show that among the various experimental parameters studied, enhanced levels of Bcl-xL are found only in the relatively radiosensitive cell line (UT-SCC-24A), while other pro- and anti-apoptotic Bcl-2 family members were expressed in high amounts in UT-SCC-24A and UT-SCC-2 but not in UT-SCC-9 cells. Bcl-2 family members have been demonstrated to regulate the induction of apoptosis at least through the control of mitochondrial functions. In our study, the expression levels of Bcl-2, Bax, Bad, or Nip1 did not indicate any direct relation to radiation-induced apoptosis. UT-SCC-2 cells have an AUC value of 1.8, representing the average intrinsic radiosensitivity of a large number of SCC cell lines (Table 1) and also has a high expression of Bcl-2. One explanation for radiosensitivity of these cells could be that they also have a high expression of the pro-apoptotic protein, PUMA and have a very low expression of Bcl-xL. Furthermore, high expression of PUMA, Bad, and Nip1 give a balance between pro- and anti-apoptotic members of the Bcl-2 family that are similar to the one in UT-SCC-9 cells, which has low expression of both pro- and anti-apoptotic members of the Bcl-2 family and has a relatively higher sensitivity for radiation (AUC = 1.4; Table 1).

The anti-cancer drug carboplatin interacts with DNA and causes DNA lesions and apoptosis. This drug, alone or in combination with irradiation and other chemotherapeutic agents, has been used in the treatment of head and neck cancer. Noutomi et al. have shown enhanced levels of Bcl-xL in a carboplatin-resistant SCC cell line. Furthermore, the levels of Bax and Bcl-2 or the ratio of Bax to Bcl-2 was not critical for drug sensitivity, which is in agreement with our results.

Survivin is the smallest mammalian member of the IAP gene family (16.5 kDa) and has a cell cycle–regulated expression at mitosis. Overexpression of survivin has been associated with inhibition of apoptosis initiated via the extrinsic or intrinsic pathway, and marked overexpression of survivin has been demonstrated in tumors of many different origins. Our results were consistent with earlier reports and indicate that high expression of survivin could be a negative prognostic factor for radiosensitivity in head and neck cancer.

In several studies of head and neck cancer, EGFR expression has been found to correlate to poor prognosis, increased risk of metastasis, and an advanced tumor stage. Blockade of ligand binding to EGFR by C225, a mouse human anti-EGFR antibody, enhances the radiosensitivity and amplification of radiation-induced apoptosis in SCC of the head and neck. Furthermore, a decrease in Bcl-2 expression and concurrent increase in Bax expression was observed after C225 exposure. COX-2 can be induced during pathologic conditions, such as cancer and inflammation, and has been reported in tumors of different origins, including head and neck. Terakado et al. have studied 8 oral SCC cell lines and found a correlation between COX-2 expression and radiation resistance. In this study, EGFR and COX-2 were highly expressed only in the relatively radioresistant cell line UT-SCC-24A. This suggests that overexpression of EGFR and COX-2 can prevent irradiation-induced apoptosis and is a possible prognostic marker for radiosensitivity. Interestingly, as mentioned earlier, COX-2 was expressed highest in the UT-SCC-24A cell line, was found to a lesser extent in UT-SCC-2, and was hardly detectable in UT-SCC-9 cells, which correlate well with these cell lines differing radiosensitivities.

The tumor suppressor gene p53 is known to be mutated in the majority of SCCs of the head and neck. Conflicting results have been published showing both decreased and increased response to radiotherapy related to the p53 status. Our results show no correlation between the expression of mutated p53 protein and radiotherapy response. All cell lines used exhibit aberrations of the p53 gene (Table 2), but only UT-SCC-2 has a high expression of the protein. Interestingly, as
enhanced expression of survivin, EGFR, COX-2, and Bcl-xL in tumor cells are correlated to resistance to radiation. Furthermore, our results indicate that the response to radiation is determined by the balance between multiple pro- and anti-apoptotic factors such as the balance between different members of the Bcl-2 family and not by the expression of a single protein. However, to verify these findings, an increased number of cell cultures from SCCs of head and neck have to be investigated.

REFERENCES