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PREDICTIVE AND PROGNOSTIC BIOMARKERS IN ORAL TONGUE SQUAMOUS CELL CARCINOMA

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Don’t let what you can’t do stop you from doing what you can do.

*John Wooden*

To Anna, Julian and Leo
ABSTRACT

Oral tongue squamous cell carcinoma (OTSCC) is an aggressive disease frequently associated with poor prognosis due to the high risk of regional failure and mortality rates have been practically unchanged in Sweden the last fifty years, despite advancements in both diagnostics and treatment. Today we lack means to assess the biological aggressiveness of each individual tumor which varies largely. Treatment hinges on the International Union Against Cancer (UICC) TNM classification and comprises surgery with additional radio/chemotherapy in more advanced tumors.

This thesis focuses on molecular biomarker expression in OTSCC. Increased knowledge paves the way to a more individualized cancer treatment aiming for better outcome and less overtreatment and sequele.

The aim of this thesis was to investigate the predictive and prognostic value of quantitative DNA aberration, Ki-67, epidermal growth factor receptor (EGFR) and cyclooxygenase-2 (COX-2) in OTSCC. These biomarkers have all been found to influence carcinogenesis in head and neck cancer but little is known about their potential clinical role in OTSCC. We also wanted to evaluate outcome after elective neck dissection compared with observation of the neck in T1N0 OTSCC patients.

A consecutive material consisting of biopsies taken from 78 OTSCC patients treated between the years 2000-2004 at Karolinska University Hospital in Stockholm Sweden were examined. Quantitative DNA aberration was analyzed using image cytometry. Immunohistochemistry (IHC) evaluating protein expression was performed on Ki-67, EGFR and COX-2. Furthermore EGFR gene copy number was investigated using fluorescence in situ hybridization (FISH).

Results of these studies show that a high proliferative activity (Ki-67 expression) is associated with locoregional recurrence in stage I OTSCC. EGFR and COX-2 was overexpressed by IHC and high EGFR gene copy number was seen in OTSCC. Improved disease free and overall survival was found for patients when elective neck dissection was added to the primary treatment of T1N0 OTSCC.

The high number of regional recurrence observed in patients with small tongue cancer with clinically negative necks resulted in a new treatment protocol. The head and neck group at Karolinska University Hospital has since 2006 included elective neck dissection to tumor resection in patients with T1N0 OTSCC.
LIST OF PUBLICATIONS


IV. **Ryott M, Marklund L, Hammarstedt L, Lundberg B, Munck-Wikland E.** Selective neck dissection versus observation as management of the neck in T1N0 oral tongue squamous cell carcinoma. *Unpublished data - submitted*

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<td>American Head and Neck Society</td>
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<td>BAC</td>
<td>bacterial artificial clone</td>
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<td>CCND1</td>
<td>cyclin D1</td>
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<td>CEP4</td>
<td>centromere 4 probe</td>
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<td>CGH</td>
<td>comparative genome hybridization</td>
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<td>CI</td>
<td>confidence interval</td>
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<td>CN</td>
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<td>cancer stem cell</td>
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<td>fine needle aspiration cytology</td>
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<td>HTX</td>
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<td>ICD</td>
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<td>IHC</td>
<td>immunohistochemistry</td>
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<td>KRAS</td>
<td>V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog</td>
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<td>MAPK1</td>
<td>mitogen-activated protein kinase 1</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<td>NCI</td>
<td>National Cancer Institute</td>
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<td>OTSCC</td>
<td>oral tongue squamous cell carcinoma</td>
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<td>pCR</td>
<td>pathologic complete remission</td>
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<td>RR</td>
<td>relative risk</td>
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<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>UICC</td>
<td>International Union Against Cancer</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<td>$\chi^2$ MH</td>
<td>Mantel-Haenszel chi-square test</td>
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1 INTRODUCTION

Cancer is the term used for a disease that originates from an unregulated proliferation of cells resulting from the accumulation of mutations in a precursor cell. Cancer consists of a population of cells that continue to mutate and that secrete self-perpetuating growth factors and angiogenic factors. The lifetime probability of being diagnosed with an invasive cancer is 44% for men and 38% for women (3).

Cancers of the oral cavity accounted globally for 274,000 cases in 2002, with almost two-thirds in men. Tongue cancer is the most common oral cancer where all subsites are dominated by squamous cell carcinoma (3, 4).

Oral tongue squamous cell carcinoma (OTSCC) is an aggressive cancer frequently associated with poor prognosis due to the high risk of regional failure. Today we have no non-surgical method to detect microscopic foci of metastatic disease. Treatment is determined by the UICC TNM-classification and we rely on primary surgery for the accessible tumors with the addition of postoperative radiotherapy if the histopathological examination reveals unfavorable histological features.

We know that the biological aggressiveness and the sensitivity to radiotherapy of tongue cancer varies but we lack means to evaluate this. Treatment is therefore a balance between being too extensive, with unnecessary sequelae, or insufficient rendering recurrence.

Research on predictive and prognostic biomarkers has ignited hope for individualizing therapy to facilitate better survival and reduce overtreatment in these patients.

We wanted to study the predictive and prognostic value of quantitative DNA aberration, proliferation/Ki-67 expression, EGFR expression and COX-2 expression in oral tongue cancer.

Furthermore evaluate if the addition of elective neck dissection to tumor resection in patients with T1N0 disease has any impact on prognosis.

This thesis focuses on OTSCC and has resulted in a change in department protocol on the treatment of small tongue cancer with clinically negative neck, i.e. without metastatic spread. In the year 2006 our head and neck group at Karolinska University Hospital added elective neck dissection to the primary treatment for patients with T1N0 OTSCC due to the high percentage of regional recurrence seen in the material.
1.1 ORAL TONGUE SQUAMOUS CELL CARCINOMA

1.1.1 The oral cavity

The oral cavity extends from the lips to the palatoglossal folds. The tongue comprises an oral part, i.e. the anterior two thirds to the circumvallate papillae, and the tongue base, the posterior third. The oral tongue is mobile and attached to the floor of the mouth anteriorly by a median lingual frenulum. Its surface is covered by stratified squamous epithelium and contains several types of papillae with taste buds and mucous glands with underlying muscular tissue. The tongue base differs from the oral part with an irregular surface due to the presence of underlying lymphoid tissue forming the lingual tonsil.

Nerve supply to the oral tongue goes via branches from the facial nerve (CN VII) i.e. the chorda tympani and the trigeminal nerve (CN V) i.e. lingual nerve and the muscular innervation is supplied by the hypoglossal nerve (CN XII).

Anatomical subsites of the oral cavity as characterized by UICC, figure 1 (5):

1. Buccal mucosa
   1.1. mucosa of upper and lower lips
   1.2. cheek mucosa
   1.3. retromolar areas
   1.4. bucco-alveolar sulci, upper and lower (vestibule of mouth)
2. Upper alveolus and gingiva (upper gum)
3. Lower alveolus and gingiva (lower gum)
4. Hard palate
5. Tongue
   5.1. dorsal surface and lateral borders anterior to vallate papillae (anterior two-thirds)
   5.2. inferior (ventral) surface
6. Floor of mouth

![Figure 1: Oral cavity subsites.](image)

*Photo: D Danielsson-10*
1.1.2 Lymphatic drainage of tongue

Cervical lymph node status is the most important prognostic factor and crucial when determining treatment for OTSCC. Poirer described the lymphatic flow from the oral tongue in 1908, figure 2. The lymphatic vessels drain mainly into the deep cervical glands lying between the posterior belly of the digastric muscle and superior belly of the omohyoid muscle. The tip of the tongue as well as the bottom surface of the anterior portion will drain through the mylohyoid muscle and end up in the submental glands (1, 6). Studies visualizing the cervical lymphatic system by lymphography or by lymphoscintigraphy have shown the same preferential pathways of drainage (7, 8).

Figure 2: Lymphatic drainage of the oral tongue. Adapted from Poirer and Charpy 1908 (1).
1.1.3 Tongue cancer

Carcinoma of the oral tongue encompasses 75% of all tongue cancer and is the most common intraoral malignancy (9). Squamous cell carcinomas constitute 67-93% of all tumors in the oral cavity (10-12).

The most frequent location for OTSCC is the lateral border. The left side of the tongue is more commonly affected than the right due to the greater number of right-handed smokers inhaling their smoke stream toward this side (9).

Oral tongue cancer is usually detected as a painless lesion that will not heal. Over time the patient will encounter pain and eventually reduced mobility.

Patients with oral tongue cancer usually present early. Approximately 2/3 of all our OTSCC patients were diagnosed with having T1-2N0 tumors, i.e. without evident metastasis. Oral tongue cancer has an aggressive behavior with a high locoregional recurrence rate ranging between 20-50% (13, 14). Regional control is the most important prognostic factor and 5-year survival after successful surgical salvage is low, approximately 30% (15).

1.1.4 Etiology and predisposing factors

OTSCC develops from a complex process involving multiple genetic alterations modulated by genetic predisposition and environmental influences, although the exact mechanism of carcinogenesis is today an unsolved multi factorial molecular mystery.

Common knowledge states that smoking and alcohol use are the two major predisposing factors in oral cancer. Alcohol consumption has been shown to have carcinogenic effect independently (16). The risk associated with smoking in current smokers as compared to never smokers in oral cancer is RR = 3.43 (95% CI 2.37–4.94) (17, 18).

Other factors related with oral cancer include viral infections i.e. HPV16 and 18 and socioeconomic circumstances (19, 20). In Southeast Asia where oral cancer is a major health problem established risk factors are betel quid and areca nut (17, 21).
1.1.5 Epidemiology

There is a considerable global variation in the incidence of oral cancer (22). Oral cancer is the 7th most common cancer in men in the U.S. with an estimation of approximately 36,540 new cases in 2010 (3). In south-central Asia, it ranks among the three most common types of cancer (17).

Figure 3: Age standardized incidence rates of oral tongue cancer (ICD-7 code 141.7, 141.8 and 141.9) and oral cancer, tongue excluded (ICD-7 code 143 and 144) in Sweden according to the population of year 2000 (2).
In Sweden the incidence rates of oral cancer and tongue cancer differ from each other both by gender and over time. Between the years 1970-2006 there was an increase in oral tongue cancer in both males and females whereas in the same period the incidence of oral cancer in males was stable while females had only a slight increase, figure 3. In comparison there was a slight increase in the incidence of OTSCC in the U.S. while all other oral cancers have remained constant since the seventies. The rise was most pronounced in the younger age group 20-44 years where a significant increase from 0.09 to 0.48 per 100 000 person-years was seen between the years 1973-2001 (23). These figures compare well with those Annertz et al. has reported from Scandinavia where the incidence of OTSCC increased 5-fold, from 0.06 to 0.32 per 100 000 person-years in men and 6-fold, from 0.03 to 0.19 in women in the age group 20-39 years between the years 1960-1994 (12).

The mortality rates for oral cancers have begun to decline slightly over the years and figures from the U.S. show a decrease of approximately 10% the last 30 years (24). An unpublished report from our research group on the Swedish population will however show that mortality rates for OTSCC have stayed practically the same since the sixties despite advancements in diagnostic tools and treatment modalities (25). In congruency with these findings, NORDCAN database shows nearly the same mortality figures for tongue cancer in the Nordic population between years 1953-2007 (26).

Recent figures show that OTSCC has a five year relative survival in the U.S. of 64% in the age group 20-44 years and 51% for those older than 44 years (23). The corresponding survival figures in the Scandinavian population is 66% for ages 20-39, 48% for ages 40-64 and 43% for ages 65-79 (12).

1.1.6 Diagnosis and classification

The diagnosis of OTSCC is based on the histopathological evaluation from a tumor biopsy. The UICC TNM classification stage of the tumor, table 1, is determined with CT/MRI, ultrasound guided fine needle aspiration cytology (FNAC) and palpation under general anesthesia at Karolinska University Hospital. A conference between head and neck surgeons, oncologists and pathologists will follow this assessment and a consensus regarding disease classification and treatment will form for each patient.

The World Health Organizations (WHO) International Classification of Diseases (ICD) codes used for defining the oral tongue in this theses were for ICD-7: 141.7, 141.8 and 141.9 and for ICD-10: C02.0, C02.1, C02.2, C02.3 and C02.8 (5).
1.1.7 Treatment

Globally standard treatment for OTSCC usually involves primary surgery with the option of postoperative radiotherapy when adverse histopathological features in the surgical specimen are encountered or when regional spread is at hand. Concomitant treatment with anti-cancer drugs, i.e. cytostatics and specific receptor blockers are becoming more in use as different studies verify their positive effects. Treatment varies between different centers in the world and probably the most controversial issue at the
moment is if elective neck dissections are warranted in treating T1-2N0 OTSCC patients.

In our department treatment for OTSCC is dependent on the UICC TNM classification stage and patient performance status.

Patients with stage I OTSCC were treated with local resection alone and stage II treatment included preoperative radiotherapy against the tumor and ipsilateral neck followed by hemiglossectomy. Patients diagnosed with OTSCC Stage III and IV have been treated individually with respect to tumor size and spread, with surgery and/or radiotherapy and/or chemotherapy.

Standard treatment protocol was modified during the study period for stage I as well as for stage II patients due to the high percentage of regional recurrences seen in these two groups, 27% and 20% respectively. During the year 2006, ipsilateral elective neck dissection was added for patients with stage I disease. An evaluation of this adjustment is presented in paper 4. Stage II patients have since 2006 been treated with primary surgical resection including elective neck dissection followed by postoperative radiotherapy. The majority of the elective neck procedures have been selective neck dissections (SND), including level IB-IV.

1.1.8 Neck dissection classification

Neck dissections, first described by Crile in 1906 have been performed since the beginning of the twentieth century (27). During this century a number of different surgical techniques were used with the emphasis of radical extirpative surgery. In the 1960s new therapeutic strategies that aimed toward preserving function for patients with head and neck cancer were introduced (28). Years went by and there was no standardization making communication/terminology difficult. To eliminate potential misinterpretation the Academy’s Committee of the American Society of Head and Neck Surgery developed a classification system in 1991 for dissection procedures of the neck (29). A system that today has been widely accepted.

The classification is based on the following concepts: 1) radical neck dissection is the fundamental procedure with which all other neck dissections are compared, 2) modified radical neck dissection denotes preservation of one or more nonlymphatic structures, 3) selective neck dissection denotes preservation of one or more groups of lymph nodes and 4) extended radical neck dissection denotes removal of one or more additional lymphatic and/or nonlymphatic structure.
This 1991 classification has been updated twice, in 2002 and 2008 by the American Head and Neck Society (AHNS) (30, 31). The latest modification concerns markers to define the boundaries between the levels I-V. The change was intended to increase awareness of the subtle difference of the patterns of lymph node metastases based on tumor origin and to improve treatment accordingly.

Treating OTSCC, delicate matters concern the boundary between subzones IB and IIA, which frequently are inadequately described in the literature. In the year 2002 AHNS defined the boundary that separates sublevel IB from sublevel IIA as the border of the stylohyoid muscle which is an impractical anatomical landmark both during clinical examination and radiological imaging. The new consensus released 2008 defined an alternative border between levels IB and IIA: the vertical plane defined by the posterior edge of the submandibular gland, figure 4. From the surgeons viewpoint resecting level IIA while preserving level IB, the dissection plane typically used to separate the two levels is along the fascia overlying the posterior aspect of the submandibular gland (31).

Terminology regarding elective surgical approaches to the N0 neck include supraomohyoid with dissection of level I-III, “expanded” supraomohyoid with the dissection of levels I-IV, lateral with dissection of levels II-IV and selective with dissection of level IB-IV. The supraomohyoid dissections include the submandibular gland in level I and all approaches preserve the spinal accessory nerve, internal jugular vein and the sternocleido muscle.
1.2 MOLECULAR BIOMARKERS

The National Cancer Institute (NCI) defines a biomarker as a biological molecule found in blood, other body fluids, or tissue that is a sign of a normal or abnormal process, or of a condition or disease. A biomarker may be used to see how well the body responds to a treatment for a disease or condition. Also called molecular marker and signature molecule (32). The three basic types of molecules that can be extracted from these sources include: DNA, RNA and protein.

The material reported on biomarkers in OTSCC exclusively is scarce because most studies performed have investigated biomarkers in head and neck tumors or oral cancer as a group and not subsite specific.

Study on biomarkers give hope for a more individualized cancer treatment aiming for better outcome and reduction of overtreatment.

1.2.1 Quantitative DNA aberration

There is a variation of genomic changes which are considered important factors for tumor progression and metastatic growth in cancer. Changes can consist of single mutations in the nucleotide sequence of DNA up to large chromosomal variations (33).

Aneuploidy, the presence of an abnormal number of chromosomes may involve either gain or loss of one or more chromosomes through errors in mitosis. Aneuploidy causes imbalance in gene dosage and has been implicated to be the most prevalent genetic change (34). Whether aneuploidy is a cause or consequence of cancer has long been debated. Studies have associated aneuploidy with different biologic properties of tumor cells such as loss of hormone dependence and metastatic potential (35).

Aneuploidy has also been shown to be a prognostic marker in head and neck cancer (36-38).
1.2.2 Ki-67 protein

The Ki-67 protein is expressed in all proliferating cells and is detectable during the active phases of the cell cycle (G1, S, G2 and mitosis). Resting cells in G0 phase do not express Ki-67, figure 5. During interphase, the Ki-67 antigen can be exclusively detected within the cells nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes (39). Since it is strictly associated with cell proliferation it is widely used as a proliferation marker to determine the growth rate of specific cells/diseases.

The fraction of Ki-67 positive tumor cells i.e. the Ki-67 labeling index, is often associated with the progression of a disease. Numerous studies, among them multivariate analysis covering more than 4000 cases conclude Ki-67 to be an independent prognostic factor in both prostate and breast cancer (39). Studies performed on oral cancer have been incongruous but support in general that a high proliferative activity is correlated with poor prognosis (40).

1.2.3 Epidermal growth factor receptor

The epidermal growth factor receptor (EGFR) belongs to the erbB receptors. A family of transmembranous tyrosine kinase cell surface receptors with four members: erb1/Her1/EGFR, erbB2/Her2-neu, erbB3/Her3, and erbB4/Her4.

Several ligands, including epidermal growth factor (EGF) and transforming growth factor (TGF)-α bind to the extracellular domains on EGFR (41). Upon doing this, EGFR undergoes dimerization, which stimulates phosphorylation of tyrosine kinase residues in the cytoplasm. EGFR is then responsible for the activation of numerous growth factor signaling pathways which modulate many functions in the cell, including proliferation, migration, angiogenesis and apoptosis of normal as well as malignant EGFR-expressing cells as illustrated in figure 6 (42).
In a large variety of cancer, including oral cancer, high levels of EGFR protein or increased gene copy number has been observed and these findings frequently correlate with poor prognosis (43-46).

Inhibitory therapies directed against different domains on EGFR are being developed and have been evaluated in clinical trials the last decade. The most well-known, cetuximab (Erbitux®) has been shown to improve survival when administered together with radiotherapy in advanced head and neck cancer (47, 48).

Figure 6: Schematic presentation of EGFR pathway.
(Source: Wikimedia commons – public domain)
1.2.4 Cyclooxygenase-2

Cyclooxygenase (COX) catalyses the rate limiting step in the conversion of arachidonic acid to prostaglandins, figure 7. There are two known isoenzymes; COX-1 is constitutive and is expressed in most cells for the maintenance of homeostatic function and COX-2 which is inducible and usually undetectable in tissues under normal physiological conditions (49). COX-2 is responsible for increased prostaglandin production associated with inflammation and disease (50, 51).

Carcinogenesis may evolve as a progressive chain of specific intracellular and molecular events in response to induction of COX-2 and the prostaglandin cascade (52, 53). In carcinogenetic animal models it has been observed that overexpression of COX-2 is enough to transform normal cells to malignant neoplasms (49, 50). COX-2 has been shown to inhibit apoptosis, promote angiogenesis and enhance the invasiveness of malignant cells and elevated levels have been found in various tumors, oral cancer included (41, 54-56).

COX inhibitors have been used to enhance cancer treatment. Harris et al has shown that both selective and non-selective inhibition against COX-2 has strong potential for chemoprevention in colon-, breast-, and lung cancer (52). Unfortunately the enthusiasm for adding specific COX-2 inhibitors to cancer treatment subsided by the discovery of serious side effects including risk of cardiovascular disease (57).
2 AIMS OF THE THESIS

The aim of this thesis was to investigate the predictive and prognostic value of molecular biomarkers in OTSCC and to evaluate the effect of change in treatment protocol of stage I OTSCC.

Specific Aims

- **Paper 1**: To investigate the predictive and prognostic value of DNA content/ploidy measurements and Ki-67 expression in primary OTSCC.

- **Paper 2**: To examine the association between EGFR gene copy number and protein expression levels and to determine the prognostic value of this biomarker in OTSCC.

- **Paper 3**: To evaluate the predictive and prognostic value of COX-2 in OTSCC.

- **Paper 4**: To compare outcome after elective neck dissection versus observation as management of the neck in T1N0 OTSCC.
3 MATERIALS AND METHODS

The Research Ethical Review Board in Stockholm has approved the work presented in this thesis (Dnr: 2001/233, 2005/417-31/1, 2009/1278-31/4, 2010/1117-32). All patients have consented to study participation.

3.1 MATERIAL

For paper 1-3 the material consists of biopsy and/or surgical specimen from patients diagnosed with OTSCC, independently of UICC TNM stage, at the Department of Oto-Rhino-Laryngology and Head and Neck Surgery at Karolinska University Hospital in Sweden consecutively between January 1, 2000 and Dec 31, 2004. Clinical data was obtained from the hospital medical records. Patients were staged according to the UICC TNM stage classification and the differentiation grade used was that of WHO international classification of tumor (5, 58).

118 patients were diagnosed with OTSCC between the years 2000-2004. We were able to retrieve 78 surgical samples. The discrepancy depends on insufficient material due to small diagnostic biopsies.

For paper 4 medical records were reviewed, with special consideration to type of surgical treatment and outcome, for all patients treated for OTSCC stage I between the years 2000-2009.

**Paper 1**: Seventy-six patients (stage I, n=22; stage II, n=33; stage III, n=8; stage IV, n=13) were included in this study. Two patients, from stage I and II, were excluded from the original 78 patients in the Ki-67 study due to tumor non-representativity. An additional four patients were excluded from DNA cytometry studies since these specimen were not possible to measure. Furthermore, eleven randomly retrieved surgical samples from stage II patients following preoperative radiation were analyzed for Ki-67.

**Paper 2**: Seventy-eight patients (stage I, n=23; stage II, n=34; stage III, n=8; stage IV, n=13) were included in this study. However, for FISH studies, thirteen patients were eliminated due to tissue limitations, resulting in 65 patients (stage I, n=15; stage II, n=30; stage III, n=7; stage IV, n=13).
**Paper 3:** Seventy-six patients (stage I, n=23; stage II, n=33; stage III, n=7; stage IV, n=13) were included in this study. Two patients, from stage II and III, were excluded from the original 78 patients due to tumor non-representativity. In addition, twelve randomly retrieved surgical samples from stage II patients following preoperative radiation were analyzed.

**Paper 4:** Seventy-four patients (stage I) were included in this study. They were separated into two groups depending on their surgical treatment of the neck. Forty-four patients were observed (group 1) and thirty patients had elective neck dissection (group 2).

A schematic outlay of the study displaying the distribution of tumor specimen is shown in figure 8.

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**Figure 8:** Display of tumor specimen and patient distribution in paper 1-4.
3.2 METHODS

All surgical samples (papers 1-3) were collected as paraffin-embedded formalin-fixed blocks from the Department of Pathology at Karolinska University Hospital. The paraffin blocks were cut into sections as illustrated in figure 9. 4 µm hematoxylin-eosin (HTX) sections were made before and after each cut for our head and neck pathologist to confirm the OTSCC diagnosis and to confirm tumor representativity.

3.2.1 DNA image cytometry

DNA-image cytometry is a quantitative analysis of the single cell DNA content by means of light absorption. It has been introduced for diagnosis of malignant transformation of squamous epithelial cells as an adjuvant tool to the cytologic examination (59).

Histological sections (8µm) were Feulgen stained to measure tumor cell DNA content. The staining, internal standardization, and tumor cell selection were based on previously described methods (60). DNA measurements were made in relation to an internal control (lymphocytes), which was used to indicate the normal diploid DNA content at 2c. All specimens were then divided according to their histograms into diploid or aneuploid tumors. Diploid tumors correspond with histograms having stem lines in the 2c region, without any cells exceeding the 5c region. Aneuploid tumors

Figure 9: Tissue preparation.

Histological sections (8µm) were Feulgen stained to measure tumor cell DNA content. The staining, internal standardization, and tumor cell selection were based on previously described methods (60). DNA measurements were made in relation to an internal control (lymphocytes), which was used to indicate the normal diploid DNA content at 2c. All specimens were then divided according to their histograms into diploid or aneuploid tumors. Diploid tumors correspond with histograms having stem lines in the 2c region, without any cells exceeding the 5c region. Aneuploid tumors
have histograms with one or more peaks outside the 2c area and a substantial number of cells with peaks exceeding the 5c region, figure 10. Approximately 100 cells were analyzed in each sample.

Figure 10: DNA image cytometry showing diploid and aneuploid measurements.

(Source: measurements from paper 1 – Ryott/Wangsa)
3.2.2 Immunohistochemistry

Immunohistochemistry (IHC) is the localization of antigens or proteins in tissue sections by the use of labeled antibodies as specific reagents through antigen-antibody interactions usually visualized by a fluorescent marker.

The Benchmark XT system, a product of Ventana Medical Systems was employed to automatically standardize, prepare and stain the 4µm Ki-67 sections, the 4µm EGFR sections and the 4 µm COX-2 sections using the monoclonal MIB-1 antibody (DAKO, Glostrup, Denmark), the monoclonal Zymed EGFR, clone 31G7 antibody (Invitrogen, Carlsbad, CA, USA) and the monoclonal mouse anti-COX-2 antibody (Zymed Laboratories Inc., San Francisco, CA, USA) respectively. Immunohistochemical staining procedures were done simultaneously, twenty slides/batch to avoid variations in staining. Tonsilar cancer served as a positive control for Ki-67 staining, cell line A431 served as the positive control for EGFR staining and colon cancer served as a positive control for COX-2 staining. Staining reproducibility was verified.

Ki-67 stained slides were then analyzed using the VIAS workstation, a Ventana Image Analysis System. Four representative tumor areas were selected by a head and neck pathologist using a light microscope, 40X magnification on the workstation followed by a quantification of positively stained cells. Approximately 1000 cells were evaluated and graded from 0% (no nuclear staining) to 100% (total nuclear staining) for each case.

EGFR stained slides were scored, according to similar publications, by assessing the intensity of the membranous region (55, 56). A light microscope, 40X magnification was used to evaluate the staining in four representative, independent areas as determined by our pathologist. Staining was scored as weak, moderate, or intense, with assessments compared with the negative control (breast tissue), which provided a baseline staining evaluation.

COX-2 stained slides were evaluated in four representative, independent areas by our pathologist using a light microscope, 40X magnification. The intensity of staining was graded in accordance to previously published work from 0-3; 0: negative, 1: weak, 2: moderate, 3: strong (57, 58).
3.2.3 Fluorescence in situ Hybridization

Fluorescence in situ hybridization (FISH) is a cytogenetic technique used to detect and localize the presence or absence of specific DNA sequences on chromosomes. This technique uses fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence similarity.

Dual color FISH was performed using a centromere-specific gene on 7p12 locus.

Figure 11: EGFR gene on 7p12 locus.
(Source: pubmed/NCBI)

Figure 12: FISH slides viewed in fluorescence microscope. CEP4 labeled with Spectrum Aqua (above), EGFR labeled with Spectrum Orange-dUTP (below). (Photo: D Wangsa)
probe for chromosome 4 and a contig of three overlapping bacterial artificial chromosome (BAC) clones that contained the EGFR gene on the 7p12 locus, figure 11. The FISH probe for EGFR was designed according to comparative genomic hybridization (CGH) data for oral tongue cancer found in the literature.

The centromere-specific probe for chromosome 4 (CEP4) (Abbott Molecular Inc.: Des Plaines, IL, USA) was labeled with Spectrum Aqua. The EGFR contig was labeled by nick-translation with Spectrum Orange-dUTP (Abbott Molecular Inc.: Des Plaines, IL, USA), figure 12.

Hybridized FISH slides were viewed using a fluorescence microscope. Fifteen to 25 images were taken in areas of optimal cell density with minimal cellular clumps and overlapping cells. Nuclei that could not be evaluated due to various reasons, including overlaps and insufficient hybridization, were excluded. Centromere 4 functioned as the control for each case, with nuclei lacking the centromere probe, not being evaluated.

Two hundred and fifty cells were counted for each case.

EGFR FISH patterns were classified into four different groups: disomy, trisomy, low-level gains and high-level gains, table 2. Disomy consisted of 6 two gene copies in more than 90% of the cells. Trisomy is described as three gene copies in more than 10% of cells and ≥ four gene copies in less than 15% of cells. Low-level gains consisted of ≥ four gene copies in ≥ 15% of cells but less than 30% of cells. High-level gains included ≥ four gene copies in ≥ 30% of cells. Tumors classified as disomy and trisomy were considered FISH negative. Tumors showing low-level gains and high-level gains were considered to be FISH positive. The categories used were similar to those published by Hirsch et al. (61).

<table>
<thead>
<tr>
<th>Category</th>
<th>FISH Patterns</th>
<th>Description</th>
<th>Tumors n=65</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FISH Negative</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disomy</td>
<td>≤ 2 gene copies in &gt; 90% of cells</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>Trisomy</td>
<td>3 gene copies in &gt; 10% of cells and ≥4 gene copies in &lt; 15% of cells</td>
<td></td>
<td>19</td>
</tr>
<tr>
<td><strong>FISH Positive</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Level Gains</td>
<td>≥ 4 gene copies in ≥ 15% but less than 30% of cells</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>High Level Gains</td>
<td>≥ 4 gene copies in ≥ 30% of cells</td>
<td></td>
<td>20</td>
</tr>
</tbody>
</table>

Table 2: Classification and description of FISH patterns according to positive and negative groups. (Wangsa/Ryott-2010)
3.3 STATISTICAL ANALYSIS

**Paper 1:** The Mantel-Haenszel chi-square test ($\chi^2$ MH) was used to analyze the association between Ki-67 categorical variables and clinical data. When analyses were made between Ki-67 continuous variable and clinical data, the Kruskal-Wallis test was used. Survival analysis was done using the Kaplan-Meier survival curves with a 60-month cutoff and analyzed by the log rank test. All p-values were from a 2-sided test with a p-value < 0.05 considered to indicate statistical significance.

Two cut-off levels were evaluated in paper 1, in addition to the continuous variable (0-100). The first Ki-67 categorical division at 0-50 and 51-100 was chosen as being half the value of the continuous variable. The second Ki-67 categorical division at 0-32 and 33-100 was chosen for statistical purposes according to a similar study by Davies et al. (62).

**Paper 2:** The $\chi^2$ MH was used to test the association between clinical data with EGFR FISH and IHC scores. To test for correlations between the two methods the Spearman correlation was used. Survival analysis was done using the Kaplan-Meier method. Analysis of survival was made at a 60-month cutoff with the use of the Wilcoxon test. P-values from 2-sided tests were used to determine significance, with a p-value < 0.05 indicating statistical significance.

**Paper 3:** The $\chi^2$ MH tested patient characteristics with COX-2 immunohistochemistry scores. Fisher’s exact test examined the association between COX-2 expression and OTSCC stage. The signed rank sum test compared COX-2 expression results before and after radiotherapy. Survival was defined as disease free survival calculated from the date of tumor diagnosis until the time of event, where event was defined as locoregional recurrence, using the log rank test. Kaplan-Meier survival curves were made to compare survival differences but not presented due to non significant data. All p-values were from a 2-sided test with a p-value < 0.05 used for statistical significance.

**Paper 4:** Overall survival was calculated from date of diagnosis to date of death of any cause. Disease free survival was calculated from date of surgery to time to recurrence. Cases lost to follow up and dead of other cause were censored. Survival proportions were estimated using the Kaplan-Meier method and hazard ratios were calculated using Cox proportional hazards model. Statistical tests were 2-sided and results with a p-value < 0.05 were considered statistically significant.
4 RESULTS

Approximately fifty percent of stage I-IV patients were dead following a minimum 3-year follow-up, with a median survival time of 22 months and about forty percent of stage I and II had a locoregional recurrence.

4.1 PAPER 1

DNA image cytometry
Image cytometry revealed 97% aneuploid and 3% diploid tumors. The fact that the majority of cancers were aneuploid prevented a meaningful statistical analysis of a potential association between genomic instability and recurrence.

Immunohistochemistry
Ki-67 nuclear staining could be detected in 100% of analyzed cases. The percentage of Ki-67-positive cells within a tumor sample ranged from 17 to 95% with a median of 56%.

Recurrence
A significant correlation was detected between high Ki-67 expression in stage I OTSCC and locoregional recurrence.

Radiotherapy response
In stage II, 82% (27/33) patients received preoperative radiotherapy. Radiotherapy response resulted in 26% (7/27) pathological complete responders (pCR) and 74% (20/27) non-pCR. We could not detect any significant differences in Ki-67 expression between pCR and non-pCR cases.
In all eleven stage II patients, all non-pCR, evaluated before and after radiotherapy, a decrease in Ki-67 levels was detected post radiotherapy.

Survival
There was no significant correlation found between Ki-67 expression and survival. In stage I a trend was observed, with patients exhibiting $\leq 32\%$ Ki-67 expression tending to fare better than patients with Ki-67 expression above 32%.
4.2 PAPER 2

Immunohistochemistry

IHC analysis showed that all tumors were positively stained for EGFR. Protein expression was categorized as intense in 72% and weak in 5% of patients.

Fluorescence in situ Hybridisation

FISH analyses were performed on interphase cells from 65 patients. Fifty-four percent (35/65) of tumors were FISH positive and had high gene copy numbers. In the positive group, there were 20 tumors with high level gains and 15 tumors with low level gains. In the FISH negative group, 19 were considered trisomy while 11 were disomy. EGFR FISH results were higher in stage II tumors in comparison to tumors in Stage I.

IHC vs FISH

EGFR protein expression levels were significantly associated with EGFR FISH categories in all patients.

Radiotherapy response

Out of 37 patients treated with preoperative radiotherapy, 8 showed pCR and 29 non-pCR. Intense EGFR protein expression and high gene copy number tended to be higher in non-pCR tumors.

Smoking habits

To evaluate the association between EGFR protein and gene expression levels in non-smokers and smokers, comparisons were made according to stage. Significant associations were found in Stage I and II for both IHC and FISH, with high protein expression and high gene copy number in non-smokers. High EGFR expression was seen in 83% of non-smokers using immunohistochemistry and 79% of patients when using FISH.

Survival

No association was detected between EGFR IHC or FISH and survival.
4.3 PAPER 3

Immunohistochemistry
All tumors displayed COX-2 immunostaining. COX-2 staining was localized mainly in the cytoplasm of tumor cells. Nonspecific reactivity was seen in superficial parts of ulcerated tumors and in some cases in the invasion front associated to epithelial-mesenchymal transition. The staining was based on expression in the bulk of the tumor. Adjacent to the carcinoma cells, in histopathologically normal tissue, COX-2 staining was observed in a few specimen. A significant correlation was found with COX-2 intensity and increasing tumor stage.

Radiotherapy response
In twelve stage II patients, it was possible to measure COX-2 expression levels before and after radiotherapy. Fifty percent of specimen showed a decrease in immunostaining post radiation and fifty percent showed no change. The patients which samples showed a decrease in COX-2 expression post radiotherapy seem to fare better with 2/6 having locoregional recurrence as compared with 4/6 in the group where no change in COX-2 expression was seen.

Survival
The well-known correlation between UICC stage and survival was seen. COX-2 expression did not have any predictive or prognostic value in OTSCC in our material.

4.4 PAPER 4

Seventy-four patients matched inclusion criteria. Forty-four stage I patients were observed (group 1) and thirty stage I patients underwent elective neck dissection (group 2). Follow up time was limited to 60 months with a mean follow up of 40 months (range 7-60 months). One patient was lost to follow up.

Occult metastasis
Postoperative adjuvant radiotherapy was given to 14% (6/44) in group 1 and 40% (12/30) in group 2. Occult metastases were found in 17% (5/30) resulting in a reclassification of four patients as pT1N1 and one patient as pT1N2b. In addition one patient was reclassified as pT2N0.

Recurrence
Group 1 had a local control rate of 89% and group 2 of 97%. The regional control rate was significantly better for group 2 with 75% and 93% respectively. Prognosis was negatively influenced by regional recurrence. Eighteen percent (13/74) had regional recurrences.
recurrence after a mean time of 9 months. Surgical salvage was attempted for all, but 69% (9/13) of these patients are today dead of disease. Neck levels affected by lymph node recurrence were predominately level IIA (n=9) and III (n=5). Recurrence also occurred in level IV (n=2) and level V (n=1). In all but two cases the recurrence was a solitary metastasis. Contralateral recurrence was seen in level IIA (n=2). No recurrence was seen in level I.

Survival
Disease free improved significantly after elective neck dissection figure 13. Overall survival was 80% for group 1 and 90% for group 2.

Figure 13: Presentation of disease free and overall survival in group 1 (resection) vs. group 2 (resection+selective neck dissection) (Source: results from paper 4)
5 GENERAL DISCUSSION

A PubMed search October 2010 on the terms *oral cancer biomarkers* reveals 4274 articles and when you exclude *oral* and instead search for *cancer biomarkers* you will find 176270 hits. Despite all this research in the past two decades, the number of molecular biomarkers that have emerged as clinically useful are very few. Many studies show inconclusive as well as contradictory results for the same marker. The comprehensiveness of a single marker is difficult to assess. Discrepancies can be explained by multiple factors. The majority of studies are performed on retrospective materials creating problems with missing covariate data creating potential bias, if handled inappropriately (63). Study size and different methodological differences are always influential issues (64, 65). Publication and selective reporting bias has also been implicated to inflict on results (66).

Considering this we still believe that these and further studies are warranted because studies on molecular biomarkers fill a valuable role in presenting trends and associations, paving the way to prospective randomized trials.

Oral cancer comprises a wide spectrum of heterogeneous neoplasms for which molecular biomarkers are needed to aid in early diagnosis, risk assessment and therapy response. Ideal biomarkers should be specific, objective, and cost effective. The biomarkers we chose to explore have all been implicated to have a role in head and neck cancer.

With our unique material we have now, through these studies had the opportunity to enhance molecular understanding of the most aggressive tumor of the oral cavity, OTSCC.

**Quantitative DNA aberration**

Aneuploidy has been associated with oral epithelial dysplasia and the progression to malignant epithelial lesions in the oral cavity (67, 68). The degree of aneuploidy in oral cancers varies in the literature between 50-70% (38, 69, 70). In our material 97% of the OTSCC samples were aneuploid reflecting a high genomic instability. The discrepancy may be caused by methodological differences as well as the fact that we only examined OTSCC whereas the other studies included tumors from different subsites of the oral cavity.

**Ki-67**

Studies on Ki-67 in oral tongue have shown both consistent as well as conflicting results compared with our findings. Kim et al showed that high Ki-67 expression is associated with worse prognosis in tongue cancer (71). An opposing study by Davies et al found that a low gradient (0-33%) of staining in the leading edge of the tumor is correlated with shorter time to recurrence (62). This study included a small sample size but in contrast to ours included both stage I and II tumors. There was also a clear distinction in methodology where we used an automatic staining and counting method.
that decreases variability and increases objectivity while Davies relied on visual estimation. We observed that the proliferative activity, as the mean percentage of Ki-67 expression, decreased after radiotherapy which may indicate the radiotherapy treatment response. Because of the scarce and conflicting data we cannot draw any conclusions regarding the role of Ki-67 in clinical practice.

**EGFR**
Studies evaluating EGFR protein expression as a prognostic marker for head and neck cancer patients show conflicting results. Our results showed no association between EGFR protein expression and survival.

This study showed that IHC and FISH results were in conformity indicating that both methods can be used to evaluate the EGFR expression of OTSCC in clinical practice. IHC may be the more cost-efficient method.

EGFR is today a well established biomarker in head and neck cancer. In 2006 the Food and Drug Administration (FDA) in the U.S. approved cetuximab (Erbitux®), a specific monoclonal antibody (mAbs) against the extracellular domain on the EGFR for the use in combination with radiotherapy in advanced head and neck cancer (48, 72). Gefitinib (Iressa®) is another inhibitor (EGFR-TK), blocking the intracellular tyrosine kinase enzyme in EGFR, approved in the year 2009 for treating advanced non-small cell lung cancer (73).

Unfortunately clinical records demonstrate that several patients are resistant to EGFR-inhibitory treatment, underlining that high EGFR protein expression is not a reliable predictor of response to therapy. Mutations on the EGFR have been characterized and are implicated to affect patients refractory to inhibitor therapy (74).

Associations between smoking habits and EGFR inhibitor sensitivity has been postulated in a number of studies. Patients with tobacco associated lung cancer with a history of never smoker or ≤ 20 pack years have a higher prevalence of mutations in the EGFR-TK gene making these patients fare better when treated with EGFR-TK inhibitors. This also suggesting that EGFR-mutations may initiate an alternative carcinogenic pathway in lung cancer (75, 76).

In our limited material non-smokers tended to have higher gene copy number when compared with smokers possibly indicating the presence of EGFR mutations. These results should be interpreted with great caution since smoking data was collected retrospectively.

EGFR mutations are reported as rare in head and neck cancer although no studies have been performed on OTSCC exclusively (77). We believe that further genetic studies on EGFR in head and neck cancer are warranted.
COX-2

COX-2 expression was found in all our tumor samples in agreement with the documentation on overexpression of COX-2 in head and neck cancer (78-80). Interestingly staining intensity increased with more advanced stage implying that COX-2 may be upregulated with the growth of the tumor and therefore influencing prognosis. Also 50% of tumor specimen, despite very few samples, showed a decrease in COX-2 expression post radiotherapy. The same patients tended to fare better with fewer recurrences. These findings warrant additional studies in a larger cohort of patients.

COX-inhibitors strong potential for chemoprevention should render more interest despite the adverse effects noted. A lower dosage of COX-inhibitors with presumably less side effects may show sufficient effect.

Activation of either EGFR signaling or increased production of COX-2 derived prostaglandins can impact on several mechanisms that have been linked to carcinogenesis. Cross-talk between them both may potentially amplify the carcinogenic process, figure 14 (53, 81, 82). This cross-talk has been implicated to influence smokers negatively. The proposed model states that tobacco smoke-mediated induction
of COX-2 is dependent on activation of EGFR. Tobacco smoke stimulates the release of EGFR ligands from the plasma membrane resulting in activation of the EGFR pathway which in turn leads to increased COX-2 transcription and stimulation of prostaglandin E\textsubscript{2} (53).

**Elective neck dissection**

The high prevalence of occult metastases and the knowledge that we have no non-surgical method of detecting microscopic foci of metastatic disease justifies elective neck dissection in patients with T1N0 OTSCC. The successful surgical salvage after regional failure is low, around 30% why elective neck dissections no longer should be considered controversial (83).

Which neck levels should be included in the surgery is also a disputed subject. The lymph drainage from the tongue has been examined by a scintigrafic study concluding that the preferential pathway of drainage is level II-IV (8). Awareness should be considered to where the tumor is situated as some authors imply that the tip of the tongue as well as the bottom surface of the anterior portion will drain through the mylohyoid muscle and end up in the submental glands level IA (1, 6).

Delicate issues are the boundaries of level IB and IIA which I believe are very difficult to distinguish in the surgical field. The fact that the neck levels are often inadequately described both in the literature and in the surgical case notes complicates things even further.

We stress the importance of an established terminology with clear borders of the neck. The American Head and Neck Society (AHNS) released in 2008 a new consensus on neck classification which clarifies this issue (31).

Elective neck dissection in T1N0 patients resulted in better disease free survival and overall survival. If this is a consequence of extended surgery and/or an effect of more postoperative treatment seen in group 2 is difficult to assess. Follow up time was longer for group 1 which also may influence the results.

Based on previously mentioned studies regarding lymphatic flow, the uncertainty in the literature describing the level IB-IIA border and that we have not had any recurrences in level I, aware of the material being limited we recommend that levels IIA, III and IV are dissected in the elective neck dissection, emphasizing that all submandibular lymph nodes are resected. If the surgical field does not permit the latter we advocate that the submandibular gland is excised. Prospective studies support that it is extremely rare that level IIB is affected by metastases in oral cancer, why this level can be excluded in the neck dissection (84, 85). This will minimize complications associated with the spinal accessory nerve.
6 CONCLUSIONS

➢ High proliferative activity measured by Ki-67 expression is associated with locoregional recurrence in stage I OTSCC.

➢ Overexpression of EGFR and high copy number gain was found in OTSCC without any predictive or prognostic implication related.

➢ A correlation exists between EGFR gene copy number by FISH and protein expression by IHC in OTSCC indicating that both methods can be used in clinical practice.

➢ All OTSCC samples expressed COX-2 protein without any predictive or prognostic associations.

➢ Selective neck dissection improves regional control as well as overall survival when included in primary treatment of stage I OTSCC.
7 FUTURE PERSPECTIVES

Cancer research is multifaceted but has always the final endpoint to increase survival. It ranges from intracellular experiments to large scale epidemiological studies and many different areas are constantly being explored. Studies on molecular biomarkers fill a valuable role in presenting trends and associations, paving the way to prospective randomized trials, despite their frequent lack of statistical power. Recent years advantages in genetics, epigenetics and cancer stem cells (CSC) have presented us with new insights regarding carcinogenesis.

Genetics
Mutation analysis of receptors and their downstream regulators show promising results. A recent paper on genomic analysis has concluded that the most frequent chromosomal alteration in oral cancer is the amplification of 7p11.2 which was seen in 31% of patients (86). The same study showed that downstream effectors of EGFR, including KRAS, MAPK1 and CCND1 were also mutated or amplified resulting in an activation of EGFR signaling in 55% of oral cancer patients. The author suggests that anti-EGFR therapy would benefit patients carrying the 7p11.2 amplification. A correlation between EGFR protein expression and gene copy number, in accordance to our study, was also shown.

Epigenetics
Defined as the stable inheritance of information based on gene expression levels without change to the underlying genetic code, has impacted our understanding of cancer biology and is currently having an upswing in cancer research (87). These modifications are not completely understood but clearly refer to alterations in DNA methylation and histone modifications of the DNA molecule, or “how the DNA is packaged” in the nucleus, influencing gene expression (88, 89). Researchers have been able to create panels of promotor hypermethylation markers and demonstrated their ability to detect epigenetic changes in salivary rinses and serum in head and neck cancer patients (90). Promotor hypermethylation of p16 is a common occurrence in head and neck cancer resulting in gene silencing leading to low p16 protein expression (91, 92).

Cancer stem cells
Accumulating evidence supports the existence of CSC’s. They are a subpopulation of cells that can self-renew and produce differentiated cells that form the bulk of a tumor (93, 94). This hypothesis opens a new era in understanding the initiation and progression of cancer. The theory implicates that adult stem cells maintain themselves through a process known as self renewal. Disruption of this regulation may result in expansion of self-
renewal stem cells ultimately leading to the development of cancer. It is suggested that aneuploidy directly contributes to carcinogenesis by disrupting the asymmetric division of adult stem cells (95).

Two different CSC’s have been described by Brabletz et al. (96, 97). Stationary CSC’s (s-CSC) that are embedded in epithelial tissues and migrating CSC’s (m-CSC) that are derived from the s-CSC by acquiring a transient epithelial-mesenchymal transition (EMT).
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9 REFERENCES

Predictive and prognostic biomarkers in oral tongue squamous cell carcinoma


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