Department of Oncology-Pathology
Karolinska Institutet, Stockholm, Sweden

MOLECULAR PROFILING FOR PREDICTING TUMOR PROGNOSIS, TREATMENT OUTCOME AND PROGRESSION OF SQUAMOUS CELL CARCINOMA

Darawalee Wangsa

Stockholm 2009
ABSTRACT

Squamous cell carcinoma is the most common histological tumor type in the cervix uteri and oral tongue. Although both cancers are diagnosed at an early stage in the majority of cases, cervical cancer has a better prognosis despite similarities in treatment. The aim of this thesis is to increase our knowledge of tumor progression in squamous cell carcinoma at the molecular level, and to use this knowledge to explore the clinical implications of this knowledge in the development of therapeutic regimens.

We collected archived tissues from squamous cell carcinoma of the cervix uteri and oral tongue (OTSCC) and applied immunohistochemistry (IHC), DNA cytometry and fluorescence in situ hybridization (FISH) to paraffin-embedded tissues.

Proliferative activity and genomic instability are two important factors in tumor progression. To identify patients with a high risk for locoregional recurrences we investigated Ki-67 expression (by means of IHC) and DNA ploidy (using DNA image cytometry) in 76 pretreatment OTSCC biopsy specimens. We found Ki-67 expression to be associated with an increased risk for locoregional recurrence in surgically-treated Stage I cancer patients (P=0.028). Ninety-seven percent of OTSCC specimens were aneuploid.

Overexpression of epidermal growth factor receptor (EGFR) is associated with poor prognosis in head and neck cancer, but information on EGFR status in OTSCC is limited. We analyzed EGFR protein expression (IHC) and gene copy number (FISH) in 78 pretreatment OTSCC samples. We found EGFR gene copy numbers to be significantly associated with EGFR protein expression (P=0.002). EGFR was overexpressed in all OTSCC, suggesting that patients with this cancer type may benefit from EGFR targeting treatment. Non-smokers showed higher EGFR gene copy numbers and protein overexpression than did smokers.

The presence of lymph node metastases is a strong prognostic factor in early stage cervical cancer and OTSCC. LAMP3, PROX1, PRKAA1 and CCND1 are genes associated with carcinogenesis. We analyzed these gene copy numbers using FISH probes in pretreatment cervical biopsies from LN positive and LN negative Stage IB-IIA cervical cancer patients (N=31) to explore their role in predicting LN metastasis. A combined marker panel consisting of amplified probes for LAMP3, PROX1 and PRKAA1 provided a significant (P=0.001) predictor for LN metastasis and needs to be evaluated in larger studies.

To further explore genetic alterations in OTSCC, and inspired by the association between smoking habits and EGFR gene copy numbers, we applied five FISH probe markers (TERC, CCND1, EGFR, p53, CEP®4) to 65 pretreatment OTSCC specimens. CCND1 displayed the highest copy number of all markers and highest levels of this gene correlated significantly with better prognosis in Stage II OTSCC (P=0.03). Non-smoking habits were significantly related to higher copy numbers in all five markers (P=0.002).
LIST OF PUBLICATIONS

Ki-67 expression predicts locoregional recurrence in stage I oral tongue carcinoma.  

Clinical value of EGFR protein expression and gene copy number in oral tongue squamous cell carcinoma.  
*Eur J Cancer*, 2009: (In press)

FISH markers for detection of cervical lymph node metastases.  
Submitted to *Am J Pathol*

Multiple FISH markers in oral tongue squamous cell carcinoma.  
*Manuscript*

*Authors contributed equally*
ADDITIONAL PUBLICATIONS


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPK</td>
<td>5′-AMP-activated protein kinase</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>CCND1</td>
<td>Cyclin D1</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinases</td>
</tr>
<tr>
<td>CEP®4</td>
<td>Centromere 4 probe</td>
</tr>
<tr>
<td>CEP®7</td>
<td>Centromere 7 probe</td>
</tr>
<tr>
<td>CGH</td>
<td>Comparative genomic hybridization</td>
</tr>
<tr>
<td>CIN</td>
<td>Cervical intraepithelial neoplasia</td>
</tr>
<tr>
<td>DFI</td>
<td>Distance from ideal</td>
</tr>
<tr>
<td>dim</td>
<td>Double minute chromosomes</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>FDG</td>
<td>18F-fluoro-2-deoxy-D-glucose</td>
</tr>
<tr>
<td>FHIT</td>
<td>Fragile histidine triad gene</td>
</tr>
<tr>
<td>FIGO</td>
<td>International Federation of Gynecology and Obstetrics</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FNAC</td>
<td>Fine needle aspiration cytology</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and Neck Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papilloma virus</td>
</tr>
<tr>
<td>hsr</td>
<td>Homogenously staining regions</td>
</tr>
<tr>
<td>HTX</td>
<td>Hematoxylin-eoxin</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>LAMP3</td>
<td>Lysosomal-associated membrane protein 3</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative predictive value</td>
</tr>
<tr>
<td>OSCC</td>
<td>Oral squamous cell carcinoma</td>
</tr>
<tr>
<td>OTSCC</td>
<td>Oral Tongue Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>PAP</td>
<td>Papanicolaou test</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive predictive value</td>
</tr>
<tr>
<td>pRB</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>PROX</td>
<td>Prospero-related homeobox 1</td>
</tr>
<tr>
<td>PRKAA1</td>
<td>Protein kinase, AMP-activated, alpha 1 catalytic subunit</td>
</tr>
<tr>
<td>PROX1</td>
<td>Prospero-related homeobox 1</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operator characteristic</td>
</tr>
<tr>
<td>RSSFIA</td>
<td>Tumor suppressor genes inactivated by exonic deletion and hypermethylation</td>
</tr>
<tr>
<td>RT</td>
<td>Radiotherapy</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>SKY</td>
<td>Spectral Karyotyping</td>
</tr>
<tr>
<td>TERC</td>
<td>Human telomerase RNA component gene</td>
</tr>
<tr>
<td>TK</td>
<td>Tyrosine kinase</td>
</tr>
<tr>
<td>UICC</td>
<td>International Union against Cancer</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel-Lindau</td>
</tr>
<tr>
<td>χ² MH</td>
<td>Mantel-Haenszel chi-square test</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS

1. INTRODUCTION .................................................................................................................................................. 1
   1.1 Squamous Carcinoma of the Cervix Uteri and Oral Tongue ................................................................. 2
       1.1.1 Etiology and Risk Factors .................................................................................................................. 2
       1.1.2 Carcinogenesis ...................................................................................................................................... 3
       1.1.3 Diagnosis and Staging ......................................................................................................................... 5
       1.1.4 Treatment ............................................................................................................................................ 7
       1.1.5 Prognosis ............................................................................................................................................. 9
   1.2 Genomic Instability ....................................................................................................................................... 10
   1.3 Metastatic Potential ................................................................................................................................... 12
   1.4 Molecular Markers ..................................................................................................................................... 13

2. AIMS OF THE THESIS ...................................................................................................................................... 17

3. MATERIALS AND METHODS ........................................................................................................................... 18
   3.1 Patients ....................................................................................................................................................... 18
   3.2 Tissue Preparation and Methodology ....................................................................................................... 20
       3.2.1 Immunohistochemistry ......................................................................................................................... 22
       3.2.2 DNA Cytometry ................................................................................................................................... 22
       3.2.3 Fluorescence in situ Hybridization ...................................................................................................... 23
   3.3 Statistical Analysis ...................................................................................................................................... 26

4. RESULTS AND DISCUSSION .......................................................................................................................... 28
   4.1 Paper I ....................................................................................................................................................... 28
   4.2 Paper II ....................................................................................................................................................... 30
   4.3 Paper III ..................................................................................................................................................... 32
   4.4 Paper IV .................................................................................................................................................... 34

5. GENERAL CONCLUSION .................................................................................................................................. 36

6. ACKNOWLEDGEMENTS ................................................................................................................................. 38

7. REFERENCES ..................................................................................................................................................... 40
1. INTRODUCTION

Cancer development is a multistep process in which genetic and cellular alterations lead to excessive and uncontrollable proliferation. The aggressiveness of a cancer due to chromosomal and genetic alterations can affect tumor progression, treatment and prognosis.\textsuperscript{1-3}

Genetic and other biomarkers can potentially play important roles as diagnostic markers and prognostic markers (for recurrence and survival), and in the prediction of treatment outcome, thus enabling individualized treatment and thereby avoiding over- and under-treatment. In addition, biomarkers may serve as targets for new treatment modalities. Advances in research and technology suggest that the discovery of new biomarkers can transform the application of molecular biologic techniques in human diseases, including cancer, and several such biomarkers have already been developed.\textsuperscript{4} Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) are two of the more common techniques for evaluating biomarkers.

The interphase FISH technique produces direct visualization of chromosomal aberrations in cell nuclei using fluorescently-labeled DNA probes. This allows for the simultaneous identification of numerical and structural chromosomal anomalies in histologically and morphologically well-defined tissue. Since interphase FISH can be applied to paraffin-embedded, formalin-fixed tissues, retrospective analyses and correlation of chromosomal alterations with clinical endpoints are possible. As an in situ technique, interphase FISH is also useful for the identification of chromosomal aberrations in small subsets of cells that are often difficult to track and that are likely to escape detection with methods that rely on extracted tumor DNA, such as polymerase chain reaction (PCR) and comparative genomic hybridization (CGH).

IHC-based biomarker analysis detects proteins by the use of antibodies raised against specific targets. IHC is a user-friendly, low-cost method which can be applied to a variety of patient materials, including paraffin-embedded formalin-fixed tissues. As an in situ technique, it has advantages similar to those of FISH, such as the detection of small cell populations displaying changes from the normal.\textsuperscript{5} Its drawbacks include variations in staining quality and subjectivity in the evaluation of staining patterns, problems that can be overcome with the use of an automated staining procedure and other evaluation methodologies.

This thesis focuses on the investigation of squamous cell carcinomas of the uterine cervix and the oral tongue (OTSCC). Both of these tumor entities are epithelial cancers which, due to their location, can be easily accessed for cytology or biopsy sampling, therefore making them good model systems for early detection and prediction of tumor progression, and for monitoring response to treatment.

We applied the techniques of DNA cytometry measurement, immunohistochemical detection of proliferation markers, and FISH probe panels using tumor-specific gene probes, to archived tissue from well-defined patient cohorts displaying carcinomas of the uterine cervix or the oral tongue. The goal was to determine whether or not any of these markers, either alone or in combination, can be useful in identifying specific patient subgroups, thereby making possible the development of improved and individualized treatment options.
1.1 SQUAMOUS CARCINOMA OF THE CERVIX UTERI AND ORAL TONGUE

Cervical cancer is globally the second most common tumor in women, with more than 80% of cervical cancer cases occurring in developing countries, including those in Southeast Asia, Sub-Saharan Africa, the Caribbean and South and Central South America. Worldwide, cervical cancer accounted for 493,000 incident cases, 1.4 million prevalent cases and 273,000 deaths in 2002. The incidence and mortality rates in Sweden have markedly decreased since the introduction of the PAP (Papanicolaou) smear screening program in the 1960s. Approximately 450 new cases are diagnosed in Sweden each year.

Oral cancer accounts for 274,000 new cases and 145,000 deaths annually worldwide, with half of them occurring in developing countries. In Scandinavia, the incidence of squamous cell carcinoma of the tongue in young adults has increased 5-fold in men and 6-fold in women during the study period of 1960-1994. Annually, around 150 new cases of tongue cancer are diagnosed in Sweden.

Squamous cell carcinoma remains the most common histopathologic tumor type in both cervical and oral tongue cancers with occurrence seen in 85% and 95% of all cases, respectively.

1.1.1 Etiology and Risk Factors

Cervical Cancer

Human papilloma virus (HPV) is present in virtually all cervical cancers. Sexually transmitted persistent HPV infection is a major etiological factor in cervical cancer, with two HPV types (16 and 18) accounting for a majority of the HPV-induced cervical cancer in the world. High-risk HPVs like HPV 16 and HPV 18 induce carcinogenesis by expressing viral oncogenes E6 and E7, which inactivate the tumor suppressor genes p53 and pRB, as reviewed by Doorbar et al.

Most cervical abnormalities caused by HPV infections regress spontaneously indicating that other factors are involved. An increased risk of cervical cancer has been found to be associated with smoking, possibly by allowing HPV to proliferate in the cervical tissues and by immunosuppression.

HPV types associated with cervical cancer are shown on Table 1.
Table 1: HPV type associated with cervical cancer

<table>
<thead>
<tr>
<th>GROUP</th>
<th>HPV TYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Risk</td>
<td>16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82</td>
</tr>
<tr>
<td>Probable High Risk</td>
<td>26, 53, 66</td>
</tr>
<tr>
<td>Low Risk</td>
<td>6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81</td>
</tr>
<tr>
<td>Undetermined Risk</td>
<td>34, 57, 83</td>
</tr>
</tbody>
</table>

Adapted from Kisseljov et al. and Munoz et al.\(^{14,15}\)

Oral Tongue Cancer

Tobacco usage and alcohol consumption are well known risk factors for cancers of the oral cavity.\(^{16}\)

In the literature, most studies on head and neck cancer combine the results for tumors from different sub-sites but sub-site differences exist. One difference is the prevalence of HPV. HPV infection has been identified as an etiologic agent in certain oral cancer sub-sites, including those of the oropharynx, tonsil, and base of tongue.\(^{17-19}\) The occurrence of HPV was found to be a favorable prognostic marker in the tonsil and base of tongue cancers.\(^{17,18,20}\) Although HPV is prevalent in base of tongue cancers it is not frequent in oral tongue cancers, which show an incidence of approximately 10%, as determined in a study by our group.\(^{17,21}\)

1.1.2 Carcinogenesis

Cervical Cancer

The development of cervical cancer involves cellular changes in the cervical tissue that ultimately lead to cancer. Premalignant stages can be distinguished by the development of cellular dysplasia, cervical intraepithelial neoplasia (CIN). CIN is graded on a scale based on severity (CIN 1–3). CIN3 is used synonymously with severe dysplasia/carcinoma in situ of the cervix.\(^{15}\)

Genomic abnormalities also play an important role in inducing cervical carcinogenesis and evidence from experimental systems suggest that HPV infection alone may not be sufficient to induce transformation and tumor progression.\(^{22}\) Gain of 1q, 3q, 5p and 8q are frequently detected, as well as loss of 2q35-qter.\(^{1,23-25}\)

Research has shown that a gain of chromosome 3q is required for the transition from premalignant lesions to invasive cervical carcinoma.\(^{1,24,26}\) The gain of chromosome 3q was found through the utilization of CGH, a technique which provides the ability to screen the number of DNA copies in a tumor making it a useful tool in analyzing chromosomal imbalances in tumor material. Using a FISH marker for the TER C gene on Thinprep cervical samples, the gain of 3q as a diagnostic marker has been shown by our group and has subsequently been verified in two other independent laboratories.\(^{27-29}\) Additional chromosomal aberrations acquired during progression to advanced-stage disease include the gain of chromosome arms 1q and 5p, as well as the loss of chromosome bands 2q36-37.\(^{24}\)
Although there are additional chromosomal gains and losses in advanced cervical cancer, the gain of chromosome 3q persists.

**Oral Tongue Cancer**

Oral cancer may develop through distinct histopathological stages, starting with hyperplasia, and then progressing to dysplasia, carcinoma in situ and finally, invasive cancer. The evolution of oral cancer is dependent upon an individual’s predisposition and exposure to environmental carcinogens, as well as genetic and epigenetic alterations.31

In the oral cavity, a large number of chromosomal aberrations are seen, as shown in the hypothetical progression model in figure 1. Frequently gained areas include 3q, 7p, 8q, 9q, 11q13, 20q, 20p and 5p.32-34 Loss of chromosomal regions were frequently observed in 3p, 8p, 18q and 11q14-pter.32, 34-36

![Hypothetical progression model of oral cancer](image)

Increased loss of heterozygosity (LOH), a mechanism by which genetic loci containing tumor suppressor genes are eliminated, was seen in the 3p and 9p chromosome arms in oral cancer.39 The loss of chromosomal region 9p21, a common genetic alteration in head and neck squamous cell carcinoma (HNSCC), occurs in approximately 70 to 80 percent of dysplastic lesions of the oral mucosa, implying that this could be an early event in oral carcinogenesis.31, 40, 41 The chromosomal region 9p21 encodes the tumor suppressors p16 and p14ARF. Loss of chromosome arm 3p, an additional early event in oral carcinogenesis, deletes the *FHIT* (fragile...
histidine triad gene) and \textit{RSSFIA} (tumor suppressor gene inactivated by exonic deletion and hypermethylation) genes.\textsuperscript{41, 42} Other common genetic events associated with oral tumorigenesis include \textit{p53} mutation and, overexpression of Cyclin D1 (CCND1) and the epidermal growth factor (EGFR).\textsuperscript{43}

Genetic aberrations differ among different head and neck cancer sub-sites.\textsuperscript{36} Studies specifically of OTSCC have shown LOH of the \textit{von Hippel-Lindau} (\textit{VHL}) gene in chromosome 3p.\textsuperscript{44, 45} According to CGH studies of OTSCC, gains in DNA copy number have been detected predominantly in 7q, 3q, 11q13, 16p and 20q.\textsuperscript{46} One study investigating metastatic versus non-metastatic OTSCC found high copy number gains of 3q23-qter, 5p, 12p, and 13q21-q22 specifically associated with metastatic tumors.\textsuperscript{47} The gain of 3q was also prevalent in a majority of OTSCC cases.\textsuperscript{47} Frequently amplified oncogenes in OTSCC included \textit{CCND1} and \textit{EGFR}.

\subsection{1.1.3 Diagnosis and Staging}

The diagnosis of cervical cancer and OTSCC is based on histopathologic evaluation, most commonly from a biopsy. The staging system for cervical cancer (International Federation of Gynecology and Obstetrics; FIGO) and OTSCC (International Union against Cancer; UICC) is clinical and relies on physical examination under general anesthesia and basic imaging techniques;\textsuperscript{48, 49} See Table 3 and 4. For OTSCC, ultrasound guided fine needle aspiration cytology (FNAC) is used to confirm regional spread to the neck. Imaging studies such as CT/MRI and PET are only used to guide treatment decisions.

Although lymph node (LN) metastasis is one of the most important prognostic factors in cervical cancer, LN status is not included in the staging system. The risk of LN metastasis increases with stage. The incidence of pelvic LN metastasis in stage IB ranges from 11.5\% to 21.7\% and in stage IIA from 10\% to 26.7\%.\textsuperscript{50} LN metastasis tends to spread in a sequential manner, with drainage first to the pelvic nodes.

Since the presence of LN metastasis influences primary treatment modality, preoperative detection of LN metastasis by imaging studies have been advocated. Recently, 18F-fluoro-2-deoxy-D-glucose (FDG)-positron emission tomography (PET) has emerged as a technique for evaluating LN involvement. In a prospective study comparing MRI and PET/CT for presurgical detection of LN metastasis the sensitivity for MRI was 30.3\% and for PET/CT 57.6\%, while specificity was 92.6\% for both groups.\textsuperscript{51} The accuracy rates were 72.6\% for MRI and 85.1\% for PET/CT. There is thus a need to improve modalities in assessment, either by diagnostic procedures on a molecular level, or by technologic advances in imaging.
Table 3: Carcinoma of the Cervix Uteri FIGO Staging Classification*

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Carcinoma in situ, cervical intraepithelial neoplasia Grade III.</td>
</tr>
<tr>
<td>I</td>
<td>The carcinoma is strictly confined to the cervix (extension to the corpus would be disregarded).</td>
</tr>
<tr>
<td>Ia</td>
<td>Invasive carcinoma which can be diagnosed only by microscopy. All macroscopically visible lesions—even with superficial invasion—are allotted to Stage Ib carcinomas. Invasion is limited to a measured stromal invasion with a maximal depth of 5.0 mm and a horizontal extension of not &gt;7.0 mm. Depth of invasion should not be &gt;5.0 mm taken from the base of the epithelium of the original tissue—superficial or glandular. The involvement of vascular spaces—venous or lymphatic—should not change the stage allotment.</td>
</tr>
<tr>
<td>Ia1</td>
<td>Measured stromal invasion of not &gt;3.0 mm in depth and extension of not &gt;7.0 mm</td>
</tr>
<tr>
<td>Ia2</td>
<td>Measured stromal invasion of &gt;3.0 mm and not &gt;5.0 mm with an extension of not &gt;7.0 mm.</td>
</tr>
<tr>
<td>Ib</td>
<td>Clinically visible lesions limited to the cervix uteri or preclinical cancers greater than Stage Ia.</td>
</tr>
<tr>
<td>Ib1</td>
<td>Clinically visible lesions not &gt;4.0 cm.</td>
</tr>
<tr>
<td>Ib2</td>
<td>Clinically visible lesions &gt;4.0 cm.</td>
</tr>
<tr>
<td>II</td>
<td>Cervical carcinoma invades beyond uterus, but not to the pelvic wall or to the lower third of vagina.</td>
</tr>
<tr>
<td>IIa</td>
<td>No obvious parametrial involvement</td>
</tr>
<tr>
<td>IIb</td>
<td>Obvious parametrial involvement</td>
</tr>
<tr>
<td>III</td>
<td>The carcinoma has extended to the pelvic wall. On rectal examination, there is no cancer-free space between the tumor and the pelvic wall. The tumor involves the lower third of the vagina. All cases with hydronephrosis or nonfunctioning kidney are included, unless they are known to be due to other causes.</td>
</tr>
<tr>
<td>IIIa</td>
<td>Tumor involves lower third of the vagina, with no extension to the pelvic wall.</td>
</tr>
<tr>
<td>IIIb</td>
<td>Extension to the pelvic wall and/or hydronephrosis or nonfunctioning kidney.</td>
</tr>
<tr>
<td>IV</td>
<td>The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum. A bullous edema, as such, does not permit a case to be allotted to Stage IV.</td>
</tr>
<tr>
<td>IVa</td>
<td>Spread of the growth to adjacent organs.</td>
</tr>
<tr>
<td>IVb</td>
<td>Spread to distant organs</td>
</tr>
</tbody>
</table>

*FIGO nomenclature. FIGO Annual report Vol. 26. Quinn et al.48
**Molecular Profiling for Squamous Cell Carcinoma**

Table 4: Oral Tongue Carcinoma Staging Classification

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>The cancer is less than 2 centimeters in size, and has not spread to lymph nodes in the area (lymph nodes are small almond shaped structures that are found throughout the body which produce and store infection-fighting cells).</td>
</tr>
<tr>
<td>Stage II</td>
<td>The cancer is more than 2 centimeters in size, but less than 4 centimeters, and has not spread to lymph nodes in the area.</td>
</tr>
<tr>
<td>Stage III</td>
<td>Either of the following may be true: The cancer is more than 4 centimeters in size. The cancer is any size but has spread to only one lymph node on the same side of the neck as the cancer. The lymph node that contains cancer measures no more than 3 centimeters.</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Any of the following may be true: The cancer has spread to tissues around the lip and oral cavity. The lymph nodes in the area may or may not contain cancer. The cancer is any size and has spread to more than one lymph node on the same side of the neck as the cancer, to lymph nodes on one or both sides of the neck, or to any lymph node that measures more than 6 centimeters. The cancer has spread to other parts of the body.</td>
</tr>
</tbody>
</table>

*UICC nomenclature⁴⁹

1.1.4 Treatment

Treatment for squamous cell carcinoma of the cervix uteri and oral tongue depends on the stage and overall health of the patient.

In general, patients with cervical cancer stage IB1 or less are treated with surgery, whereas those with stage IB2 or higher are treated with chemoradiation. The surgical treatment includes a radical hysterectomy and pelvic lymphadenectomy to assess metastasis to the pelvic LNs. The histopathological evaluation of the surgical specimens provide prognostic information regarding the risk of recurrence and, when poor prognostic factors (LVI, depth of tumor spread, tumor size and LN metastasis) are present, adjuvant (chemo)radiotherapy may significantly reduce the cancer recurrence.⁵², ⁵³ For patients displaying LN metastasis on clinical presentation, primary radio-chemotherapy is advocated in order to avoid combinatorial treatment with surgery and radiation since the combined approach can cause morbidity and does not seem to provide any survival benefits. Preoperative brachytherapy is applied in some institutions to tumors with stage IB-IIA in order to increase local control.⁵⁴

Similar treatment strategies exist for OTSCC. In early stage (Stage I-II) treatment consists of surgery alone, preoperative radiotherapy followed by surgery or radiotherapy alone. Patients with more advanced OTSCC (Stage III-IV) receive a combined treatment regimen including surgery and/or radiotherapy and/or chemotherapy depending on the size and spread of the tumor. See table 5 and 6 for more detailed treatment options.

OTSCC treatment varies in differing centers worldwide.
Table 5: Treatment algorithm for Cervical Cancer

<table>
<thead>
<tr>
<th>Stage</th>
<th>Treatment</th>
</tr>
</thead>
</table>
| IA1   | If patient desires fertility, conization of cervix  
       | If patient does not desire fertility, simple hysterectomy |
| IA2   | Radical hysterectomy with pelvic lymphadenectomy  
       | Radiotherapy |
| IB1   | Radical hysterectomy with pelvic lymphadenectomy  
       | Radiotherapy |
| IB2-IVA | Radical hysterectomy with pelvic lymphadenectomy  
        | Chemoradiotherapy |
| IIB-IVA | Chemoradiotherapy |

Table 6: Treatment algorithm for OTSCC*

<table>
<thead>
<tr>
<th>Stage</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Surgery</td>
</tr>
<tr>
<td>II</td>
<td>Pre-operative Radiotherapy and Surgery</td>
</tr>
<tr>
<td>III**</td>
<td>Chemoradiotherapy and/or Surgery</td>
</tr>
<tr>
<td>IV</td>
<td>Chemoradiotherapy and/or Surgery or Palliative treatment</td>
</tr>
</tbody>
</table>

**T<sub>3</sub>N<sub>0</sub>M<sub>0</sub> is treated with pre-operative radiotherapy and surgery.
1.1.5 Prognosis

The overall five-year survival rates for cervical cancer and OTSCC are approximately 70 and 50 percent respectively. LN metastases is one of the most important prognostic factors in both squamous cell carcinoma of the cervix and oral tongue.

The principal cause of cervical cancer treatment failure is recurrence. Recurrence usually occurs 2-3 years after treatment. The presence of LN metastases significantly increases the risk of dying, by a factor of four in stage Ib1, constituting the strongest prognostic factor for survival in cervical cancer. Overall 5-year survival rate for stage IB1 cervical cancer without LN metastasis has been reported to be 94.5% versus 75.9% for patients with LN metastasis.

The major type of treatment failure in OTSCC patients is regional failure. In OTSCC, LN metastasis is discovered in up to 35% in early stage disease (T1 and T2), and approximately 63% in higher stages (T3 and T4). In patients with early OTSCC, 2-year survival rates decrease by approximately 50% with the discovery of cervical nodal metastasis.
1.2 GENOMIC INSTABILITY

Genomic instability and proliferative activity are important factors in tumor progression and metastatic growth. Although genomic instability refers to a broad topic, this thesis focuses upon the areas of chromosomal instability, gene amplifications, and aneuploidy.

Chromosomal Instability
Cancer cells often exhibit varying degrees of chromosomal abnormalities, ranging from numeric changes, such as loss or gain of entire chromosomes, to structural rearrangements involving one or more chromosomes. Structural alterations range from simple balanced rearrangements to complex abnormalities (Figure 2). This altered chromosomal state affects the genomic stability of the cell. Useful methods for determining structural chromosomal abnormalities include spectral karyotyping and FISH using chromosome-specific DNA probes.

Figure 2: Spectral Karyotyping (SKY) depicting chromosomal aberrations in DLD-1, a colon cell line and two head and neck cancer cell lines, WSU-HN6 and WSU-HN8, derived from the base of tongue and epiglottis, respectively. The white stars annotate chromosome 11 in each cell line. Chromosome 11 is not involved in chromosomal rearrangements in the near diploid DLD-1 cell line and therefore shows two normal copies in that cell line. In contrast, both head and neck cell lines show different derivative chromosomes 11. WSU-HN6 has a der(11)t(2;11)(p14;q22.3) and a der(16)t(11;16)(p11;q11). WSU-HN8 shows an chromosome 11 derivative of der(11)t(5;11)(p11;q11) and der(5)t(5;16)(q11;p11).
Gene Amplification
Amplified gene regions can be observed cytogenetically as either double minute chromosomes (dmin’s) or homogenously staining regions (hsr’s). In HNSCC, amplification of the 11q13 region which harbors the \textit{CCND1} gene and the 7p12 region which harbors the \textit{EGFR} gene are frequently observed.\textsuperscript{62, 63} Gene amplification of \textit{CCND1} as observed in HNSCC cell lines are shown in Figure 3.

Figure 3: Amplification of \textit{CCND1} (yellow) is observed in both WSU-HN6 and WSU-HN8 with the use of FISH, annotated by the white arrows. Whole chromosome paints (WCP) of 11 are seen in green while WCP 16 (HN6) and WCP 5 (HN8) are observed in red. WSU-HN6 contained one amplified \textit{CCND1} signal on t(11;16) and another on a shortened chromosome 11. WSU-HN8 presents with two amplified \textit{CCND1} signals, in the two copies of chromosomes with t(5;11).

Aneuploidy
Aneuploidy refers to numerical chromosomal imbalances, commonly observed in cancer, and is one of the most prevalent genetic changes seen in tumors. Numerical chromosomal imbalances can encompass chromosomal segments or whole chromosomes leading to altered DNA quantity. Classic cytogenetic studies were limited in their ability to study aneuploidy; however, improved methodologies have emerged with advances in technology. One such method is CGH, a technique that can be used to analyze a tumor genome for specific chromosomal imbalances by hybridizing differentially labeled tumor and normal DNA onto normal metaphase chromosomes or onto DNA microarrays.\textsuperscript{64} Crude DNA content measurements can be done by flow or image cytometry which assesses the ploidy of the cell in comparison to a corresponding control.
Aneuploidy detected by flow or image cytometry has been linked to poor prognosis in various cancers.\textsuperscript{65–67} Identification of the various oncogenes and tumor suppressor genes on the gained and lost chromosomes have provided evidence that chromosomes involved in aneuploidy play a major role in tumorigenesis.\textsuperscript{68} Acquisition of extra chromosomal copies and/or loss of others can often create a genetic configuration that gives cancer cells a growth advantage.\textsuperscript{69}

1.3 METASTATIC POTENTIAL

Architecturally, tissues are organized with relatively thin sheets of epithelial cells sitting above the extracellular matrix (ECM). The ECM provides the structural support and is made up of proteins. Metastasis initiation requires that tumor cells break away from the primary tumor, where they subsequently degrade the protein making up the ECM.

Metastasis requires a series of inter-related processes: local invasion, entrance of the tumor cells into the bloodstream (intravasion), survival in the circulation, exit of tumor cells from the capillary beds into the parenchyma of the organ (extravasion), and finally colonization (Figure 3).\textsuperscript{70} These processes entail the contribution of essential genetic and epigenetic events in order to progress to a metastatic state. In addition to the tumor initiating events, Nguyen et al. have discerned three general classes of metastasis genes: metastasis initiation, metastasis progression, and metastasis virulence.\textsuperscript{70} Metastasis-initiation genes include genes that provide the primary tumors an advantage in order to pave a path into circulation.\textsuperscript{70} Metastasis-progression genes include those that perform rate limiting functions and other specialized activities.\textsuperscript{70} Metastasis-virulence genes are those that provide an advantage at the metastatic site but not at the primary site.\textsuperscript{70}

![Figure 3: Proposed steps to metastasis and hypothetical classes of metastasis genes. Adapted from Nguyen et al.\textsuperscript{70}](image_url)
1.4 MOLECULAR MARKERS

In cervical and oral tongue cancers, past research by members of our group have uncovered several potential molecular markers that have led to further investigations. One important finding in one of our cervical cancer studies was that laminin-5 detection could be used to facilitate the identification of microinvasive cervical lesions. An additional important observation was that the vast majority of cervical cancers harbored a gain of chromosome 3q.71 Our group developed a FISH probe for a candidate gene on 3q, whose gain was subsequently shown to be able to discriminate high-grade from low-grade cervical Pap smears with high sensitivity and specificity. In a retrospective study we were then able to show that the 3q FISH assay could differentiate cervical lesions that progressed from those that regressed.30 In the oral cavity, our group discovered that high HPV levels are a more favorable prognostic factor for base of tongue cancer patients and that high nuclear DNA content predicts stage I tongue cancer recurrence.17,72

PRKAA1 and LAMP3

The protein kinase, AMP-activated, alpha 1 catalytic subunit (PRKAA1) and the lysosomal-associated membrane protein 3 (LAMP3) are two potential candidate genes for cervical metastasis. Both genes are located in chromosomal areas which have been shown to be frequently gained in cervical cancer.23,24,75-77

The gene, PRKAA1, maps to chromosome 5p13, and covers 39kb on the reverse strand. PRKAA1 is the catalytic subunit of the 5′-AMP-activated protein kinase (AMPK). High expression of PRKAA1 may help cancers establish their malignant nature through cellular resistance to cell death and by overcoming their hypoxic condition.73,78 Overexpression of PRKAA1 was found to be significantly associated with cervical carcinogenesis and was predominantly observed in the basal layer, an area of active proliferative activity.73

LAMP3 is located on chromosome 3q26.3-q27 and is approximately 40kb in size. LAMP3 was first cloned as a gene expressed specifically in lung tissues, named TSC403.79 It belongs to the LAMP family along with LAMP1 and LAMP2. LAMP1 and LAMP2 are expressed constitutively on the cell surface of some colon cancer cell lines, notably those derived from highly metastatic colon carcinoma.80 LAMP3 is located in a chromosomal region that is frequently involved in gains or amplifications in cervical cancer.1,26 LAMP3 overexpression was reported to be associated with metastatic potential both in vitro and in vivo in cervical cancer.74 Although the function of LAMP3 is still relatively unknown, it has been proposed that LAMP3 may be involved in tumor cell migration into surrounding lymph vessels.74

PROX1

The lymphatic system is a system composed of lymphatic vessels, LNs, and lymphocytes. The lymphatic system is responsible for balancing bodily fluid levels and to defend the body against infections. In cancer, the lymphatic system may transport cancer cells between various parts in a process called metastasis.
Lymphatic development is characterized by the expression of prospero-related homeobox 1 (PROX1), a homeobox gene product seen exclusively in cells committed to lymphatic lineage.\textsuperscript{81} In mice, inactivation of PROX1 was shown to result in embryonic lethality and prevention of the development of the lymph vasculature.\textsuperscript{81, 82}

PROX1 overexpression in human endothelial cells suppresses many blood vascular-specific genes and upregulates lymphatic endothelial cell markers (podoplanin and endothelial growth factor receptor-3).\textsuperscript{83, 84} PROX1 also causes upregulation of cyclin E1 and E2 mRNAs, as well as other cyclin E promoters in various cell types.\textsuperscript{84} A recent study in mice has shed light on the mechanism by which PROX1 induces lymph vasculature growth. The overexpression of Sox18, a developmental transcription factor, in blood vascular endothelium induces the expression of PROX1 and other lymphatic endothelial factors.\textsuperscript{85}

The gene, PROX1, is located on chromosome 1q41. In colon cancer, overexpression of PROX1 was found to induce colon cancer progression by promoting a transition from benign to a highly dysplastic phenotype.\textsuperscript{86} In cervical cancer, PROX1 lies within a region of frequent gain and amplification, suggesting that PROX1 copy number gain may function similarly in cervical cancer as it does in colon cancer.\textsuperscript{21, 76} Decreased PROX1 expression has been found to be associated with hepatocellular carcinoma.\textsuperscript{86-88}

**Cyclin D1**

CCND1, an important cell cycle regulator of the G1 to S phase, is located on chromosome 11q13. Its expression has been observed to be altered in many cancers. CCND1 protein functions by activating cyclin-dependent kinases (CDK) 4 and 6, leading to pRb phosphorylation consequently depressing E2F-mediated transcription and promoting cell cycle progression.\textsuperscript{89, 90}

In HNSCC, CCND1 amplification and overexpression are seen in approximately 20-40% and 40-80% of cases, respectively.\textsuperscript{91} These abnormalities have been associated with cancer development, progression, aggressiveness, poor prognosis and metastasis.\textsuperscript{91-96} CCND1 amplification has also been correlated with tumor specific sub-sites.\textsuperscript{97} Nimeus et al found that CCND1 amplification frequencies differed among HNSCC sub-sites, with tumors from the hypopharynx, larynx and oropharynx showing higher rates of amplification as compared to tumors from the oral cavity and epipharynx.\textsuperscript{97} An association between CCND1 amplification and DNA non-diploidy was also observed in HNSCC with the use of flow cytometry.\textsuperscript{97}

Poor prognosis has been frequently observed with CCND1 overexpression and amplification in HNSCC and cervical cancer.\textsuperscript{62, 98-101} Although CCND1 overexpression and gene amplifications have been observed in OTSCC, determination regarding the prognostic value of CCND1 in this patient group is difficult due to the incorporation of tumors, regardless of stage, in all studies.\textsuperscript{100, 102}
Cisplatin is a commonly used drug for systemic treatment in both squamous cell carcinomas of the cervix uteri and oral cavity. However, resistance to cisplatin often develops and is a major cause of chemotherapy failure. Research in OSCC has suggested that increased CCND1 expression may be associated with cisplatin resistance, with CCND1 inhibition increasing the patients' sensitivity to cisplatin. CCND1 suppression has been found to have an apoptosis-inducing affect in various squamous carcinoma cell lines, including head and neck, leading to growth arrest.

**p53**

One of the most studied gene mutation is the **TP53** tumor suppressor gene, and deletions and mutations are commonly seen in human tumors. This gene encodes a protein responsible for regulating the cell cycle and apoptosis. A deletion or mutation in **p53** causes the cell cycle to continue unrestrained, thereby producing damaged DNA. With continued division, the damaged DNA replicates which can lead to cancer. A damaged or missing **p53** gene severely reduces tumor suppression.

**p53** gene mutations occur in approximately 70% of oral cancers. Overexpression of **p53** and the presence of **p53** mutations were found to be associated with early recurrence and lower overall survival in HNSCC patients. Carcinogens such as benzo(a)pyrene in cigarette smoke have been found to induce **p53** mutations by undergoing a G:C to T:A mutation in lung and liver cancer. A history of tobacco and alcohol use was also found associated with high frequencies of **p53** mutations in HNSCC patients. The incidence of **p53** mutation among smokers (present and former) was significantly higher than non-smokers. In addition, Liligou et al found that patients with **p53** mutations (nine of 18 patients) had less genetic damage as measured by fractional allele loss (FAL). FAL was measured by dividing the number of chromosome arms where allelic imbalance was observed by the number of chromosomal arms in the normal cells using 145 microsatellite markers on 39 chromosome arms. **p53** deletion, by means of FISH, has been observed in a few head and neck cancers including those of the lingual, salivary glands and, thyroid.

**TERC**

The human telomerase RNA component gene (**TERC**) is located on chromosome 3q26.2 and its gain and/or amplification, have been shown to be linked to progression in cervical cancer. Gain of 3q was observed in both squamous cell carcinoma and adenocarcinoma of the cervix. Telomerase is involved in the maintenance of chromosomes by providing telomere stability and regulating telomere length. Telomerase is made up of two components, **TERC** and **TERT**. **TERC** serves as a template for telomere addition while **TERT**, a protein subunit, acts to catalyze telomere synthesis.
EGFR

EGFR is located on chromosome 7p12.3-p12.1 and has been linked to poor outcome in many cancers, making therapies aimed at blocking EGFR function of great interest.\textsuperscript{117} EGFR overexpression is particularly high in both head and neck cancers and cervical cancers. EGFR is a tyrosine kinase (TK) receptor, and its activation stimulates angiogenesis, proliferation, protection from apoptosis, loss of differentiation, migration and invasion. Overexpression is thought to be the main mechanism for the EGFR signaling increase observed in many cancers; however its function is relatively unknown.

EGFR overexpression was shown to be correlated with radiation resistance and prognosis in head and neck squamous cell carcinoma (HNSCC).\textsuperscript{117, 118} Moreover, it was shown that radiotherapy (RT) induces EGFR nuclear translocation and internalization, improving DNA repair through DNA protein kinase activation.\textsuperscript{35} In a phase III trial with locally advanced HNSCC treated with radiotherapy (70Gy in 35 fractions), EGFR overexpression was found to be a strong predictor of higher locoregional recurrence and diminished overall survival.\textsuperscript{117} Therefore, if EGFR signaling protects tumors from radiation damage, then EGFR inhibition would enhance tumor response to radiation. Evidence supporting this theory was observed in a phase III trial in which 424 locoregionally advanced HNSCC patients were subjected to RT alone or RT in combination with cetuximab, a monoclonal antibody against EGFR.\textsuperscript{119} Results of this trial revealed significant increase of locoregional control and survival with a combined treatment of RT and cetuximab.\textsuperscript{119} Cetuximab or C225, a monoclonal EGFR inhibitor, has shown promising radiosensitivity enhancement due to amplification of radiation-induced apoptosis in tumor specimens.\textsuperscript{120}

Overexpression of EGFR is seen in approximately 90% of head and neck cancer making it an important target for therapy.\textsuperscript{121-123} EGFR expression has been suggested as a prognostic indicator in head and neck cancer; conflicting results, however, were observed in oral cancers.\textsuperscript{63, 123-125} EGFR genetic alterations and protein overexpression have been observed in oral cancer, but a correlation between the two has never been shown in OTSCC.\textsuperscript{126-128} The combination of mixed tissue types, including anaplastic thyroid cancer, esophageal cancer and laryngeal cancer in previous studies has made it difficult to determine the effect of EGFR alterations in specific sub-site head and neck cancers.\textsuperscript{129-131}

In cervical cancer, EGFR overexpression has been found in approximately 50% to 72% of patients in the reported studies.\textsuperscript{132-134} Overexpression of EGFR in cervical cancer cells have been linked to poor prognosis and tumor aggressiveness, despite some evidence to the contrary.\textsuperscript{132-135}
2. AIMS OF THE THESIS

The aim of this study was to analyze a panel of molecular markers in well-defined patient cohorts to determine their usefulness for the prediction of metastatic potential, prognosis and response to therapy in cervical and oral tongue cancer. The following techniques, fluorescence in situ hybridization (FISH), immunohistochemistry, and DNA cytometry were applied to archival specimens.

Specific Aims

- Paper I: To investigate whether Ki-67 expression and ploidy measurements can be of clinical use for prediction of locoregional recurrence in primary OTSCC.
- Paper II: To examine the association between EGFR gene and protein expression levels and determine whether or not EGFR expression can be of clinical value as a prognostic or predictive marker in OTSCC.
- Paper III: To explore whether molecular markers (PROX1, PRKAA1, LAMP3, CCND1) in pretreatment biopsies can predict the presence of LN metastasis in Stage IB-IIA cervical cancer patients. We also aimed to pinpoint specific genomic changes (gains/losses) between cervical carcinomas and their corresponding LN metastases and to analyze clonality or clonal changes between the primary tumors and their LN metastases.
- Paper IV: To evaluate the utility of molecular markers to predict response to therapy and determine prognosis in oral tongue cancer using five specific markers (TERC, CCND1, EGFR, p53, CEP®4).
3. MATERIALS AND METHODS

3.1 PATIENTS

All patients with both cervical cancer and OTSCC consented to study participation. Ethical approval was granted approved by the Research Ethical Review Board (Dnr: 01-269; 2005/431-31/4; 2005/1330-32) at Karolinska Institutet.

Patients with OTSCC (Papers I, II, and IV)

Patients included in paper I, II and IV were diagnosed with OTSCC and treated at the Department of Oto-Rhino-Laryngology, Head and Neck Surgery, Karolinska University Hospital (Stockholm, Sweden) from January 2000 to December 2004. Clinical data for all patients were obtained from medical records and included: age, gender, smoking status, tumor grade, treatment modality, treatment response and follow-up. The UICC classification was used to stage the patients and the differentiation grade used was that of the WHO international histological classification of tumors.

A total of 78 patients, UICC Stages I-IV (Stage I, n=23; Stage II, n=34; Stage III, n=8; Stage IV, n=13) were initially included in papers I, II and IV. Gender distribution among the patients consisted of 33 females and 45 males. The mean age at diagnosis was 58 years, with a range from 21 to 96 years of age. Thirty-seven patients received preoperative radiotherapy: there were eight responders and 29 non-responders to the radiotherapy treatment. In early stage patients (Stage I and II), 23 had locoregional recurrences and one had distant metastasis.

**Paper I**

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-representivity. An additional four patients were excluded from DNA cytometry studies since their specimens were not measureable.

**Paper II**

All 78 patients were included in this study. However, for FISH studies, thirteen patients were eliminated due to tissue limitations, resulting in the utilization of only 65 patients (Stage I, n=15; Stage II, n=30; Stage III, n=7; Stage IV, n=13).

**Paper IV**

Sixty-five patients (Stage I, n=15; Stage II, n=30; Stage III, n=7; Stage IV, n=13) were included in this study. As mentioned above, for FISH studies, material limitations necessitated the elimination of thirteen patient samples.
Patients with cervical cancer (Paper III)

Patients included in paper III were diagnosed with cervical cancer and treated at the Department of Gynaecologic Oncology, Radiumhemmet (Stockholm, Sweden), from January 1994 to December 1997. Clinical information, including age, stage, histology, tumor size, grading, LN metastasis, treatment modality, and follow-up status were retrieved from medical records. Staging was made using the FIGO staging classification.

A total of 31 patients with Stages IB-IIA primary cervical cancer were included in the study. All patients were surgically treated with radical hysterectomy and pelvic lymphadenectomy, with or without preoperative brachytherapy. Sixteen patients (cases) had pelvic LN metastasis and 15 patients (controls) had no pelvic LN metastases. The stage distribution was 25 patients in Stage IB and six in Stage IIA. Tumor characteristics, including stage, tumor size, grade, histology, lymph vascular invasion and pre-operative bracytherapy treatment were matched between the cases and controls.

In the 16 LN positive cases, the mean age was 45 years and ranged from 29 to 66 years of age. Histological distribution consisted of 10 squamous cell carcinomas, two adenocarcinomas, and four adenosquamous carcinomas. Of the 16 cases, seven (43%) died during the study follow-up period.

In the 15 controls, the mean age at diagnosis was 47 years and ranged from 34 to 69 years of age. Distribution of histology was 10 squamous cell carcinomas, two adenocarcinomas, and three adenosquamous carcinomas. In the control patients, no one died during follow-up period.
3.2 TISSUE PREPARATION AND METHODOLOGY

Papers I, II, and IV
Paraffin-embedded, formalin-fixed pretreatment biopsy samples were obtained from all OTSCC patients as described in papers I, II and IV. Eleven additional surgical specimens were collected from Stage II patients following preoperative radiation for comparison between the corresponding biopsy specimens already collected (paper I).

Paraffin samples were cut into sections with a 4µm hematoxylin-eosin (HTX) section made before and after each cut (Figure 6A). A pathologist confirmed the OTSCC diagnosis on the HTX sections to confirm tumor representativity. A scheme of the different tissue sections used for each study and the tissue thickness requirement are shown in Figure 6A.

Four µm sections were used for immunohistochemistry studies (papers I and II) and 8µm sections for DNA cytometry studies (paper I). In paper II and IV, 6µm sections were used for FISH studies. A pathologist confirmed tumor representativity and marked the tumorous area on the slide.

Figure 6: Scheme of tissue sectioning for (A) cervical cancer and (B) OTSCC paraffin embedded blocks

Paper III
Paraffin-embedded, formalin-fixed pretreatment biopsy samples were obtained from 31 cervical cancer patients, as described in paper III. Four 6µm sections were cut for FISH studies, with a HTX (4µm) made before and after to determine tumor representativity (Figure 6B). One 6µm section was disintegrated using the Hedley method to produce slides used for FISH.
The distributions of tumor specimens were divided according to the planned experimental design (Figure 7).

Figure 7: Pictorial representation of the tumor material and experimental design of all four papers.
3.2.1 Immunohistochemistry

The Benchmark XT system, a product of Ventana Medical Systems was used to automatically standardize, prepare and stain the 4µm Ki-67 sections and the 4µm EGFR sections using the monoclonal MIB-1 antibody (DAKO, Glostrup, Denmark) and the monoclonal Zymed EGFR, clone 31G7 antibody (Invitrogen, Carlsbad, CA, USA) respectively. For both immunohistochemical staining procedures, all slides were placed in the machine at the same time to avoid discrepancies in staining. Tonsilar cancer served as a positive control for Ki-67 staining while cell line A431 served as the positive control for EGFR 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 pathologist using a low power (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 \(^{136, 137}\). A light microscope (40X magnification) was used to evaluate the staining in four representative, independent areas as determined by a pathologist. Tumor samples were scored as weak, moderate, or intense staining, with assessments compared with the negative control (breast tissue), which provided a baseline staining evaluation.

3.2.2 DNA Cytometry

Histologic 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 \(^{138}\). DNA measurements were made in relation to a corresponding control, which was used to indicate the normal DNA (diploid) content at 2c. All specimens were then divided according to their histograms into diploid or aneuploid tumors. A diploid tumor corresponded with histograms having stem lines in the 2c region, without any cells exceeding the 4c region. Aneuploid tumors had histograms with one or more peaks outside the 2c area and a substantial number of cells with peaks exceeding the 4c region. Approximately 100 cells were analyzed in each sample.
3.2.3 Fluorescence in situ Hybridization

**Probe Preparation**

In the cervical FISH paper (Paper III) two FISH probe panels consisting of a total of five probes were designed based on previous CGH results and information about potential lymphangiogenic markers. The FISH marker panels used for the cervical paper consisted of \( LAMP3 \), \( PROX1 \), \( PRKAA1 \) and \( CCND1 \). Centromere 7 (CEP®7) was included in all cervical cancer cases as a ploidy control since numerical changes are rarely seen for this chromosome in cervical cancer.

In the OTSCC FISH paper (Papers II, IV) two FISH probe panels were designed according to CGH data for oral tongue cancer found in the literature. The FISH marker panels included \( TERC, CCND1, EGFR, p53 \) and CEP®4.

Probes used in the studies and their location on the chromosome are shown below in figure 5 for cervical cancer and figure 6 for OTSCC studies.

![Figure 5](image-url)

Figure 5: Scheme of FISH markers and the corresponding fluorochrome color used for the cervical cancer studies in the thesis.
Bacterial artificial chromosome (BAC) contigs comprised of 4-5 overlapping BAC sequences specific to the region of interest were made for all FISH markers. BAC clones were streaked, inoculated, and grown overnight for 12-16 hours in a shaking incubator at 37°C before being extracted using the plasmid maxi kit (Sigma Aldrich). Centromere specific probes for chromosome 7 (CEP®7) and chromosome 4 (CEP®4) were obtained from Abbott Molecular Incorporated, Abbott Park, IL, USA. All centromere specific probes were labeled in aqua. The BAC clone contigs for the cervical probes (paper III) were labeled by nick-translation with Spectrum Orange-dUTP for LAMP3, Rhodamine Green-dUTP for PROX1, Spectrum Orange-dUTP for PRKAA1 and Rhodamine Green-dUTP for CCND1. OTSCC probes (papers II and IV) were labeled by nick-translation with Spectrum Orange-dUTP for TERC, Rhodamine Green-dUTP for CCND1, Spectrum Orange-dUTP for EGFR, and Rhodamine Green-dUTP for p53.

All probes were denatured for 5 minutes at 80°C, followed by preannealing at 37°C for two hours, with the exception of CEP®4 which does not require preannealing.
Slide preparation (Paper III)

The tissue was deparaffinized using xylene (three times for 10 minutes) and rehydrated using an ethanol series. The tissue was then centrifuged at 14000 rpm. Excess 50% ethanol was removed prior to a 20-minute incubation in sterile water at room temperature. The cells then underwent digestion by adding 0.1% Protease (Type XXIV, Sigma) in 1X PBS at 45°C for 45 to 60 minutes. Once an optimal single cell suspension was achieved, the reaction was stopped with 1X PBS. The cells were then placed on the slides via centrifugation. Finally, the slides were fixed in ethanol for ten minutes and air dried.

The slides then underwent pepsin digestion (0.04%) for two hours (200µl), followed by formaldehyde fixation for 10 minutes at room temperature. The slides were then put through an ethanol dehydration series and air dried. After the slides were completely dried, they were denatured in 2.7% formaldehyde at 80°C for two minutes, followed immediately by a final dehydration series using cold ethanol. The slides were again air dried.

Slide preparation (Paper II and IV)

The 6µm paraffin tissue sections were deparaffinized using xylene (three times for 10 minutes) and then rehydrated in an ethanol series. The slides next underwent pepsin digestion (0.04%) for four hours (400µl) followed by formaldehyde fixation for 10 minutes at room temperature. The slides were dehydrated in an ethanol series before being air dried. They were then denatured with 2.7% formaldehyde at 80°C for two minutes, followed immediately by a final dehydration series using cold ethanol. The slides were air dried.

Hybridization

Once the probes were preannealed, they were added to the dried, denatured slides and covered by 18 x 18 mm or 22 x 22 mm coverslips for overnight hybridization at 37°C.

Detection

After overnight hybridization, the slides were washed in 2X SSC (three times for 3 minutes) followed by a dehydrating ethanol series (three times for 3 minutes each). The slides were then air dried. They were counterstained with a 4,6-diamidino-2-phenylindole (antifade) solution and mounted with a coverslip.

The slides were then ready for viewing with the Leica DM-RXA fluorescence microscope (Leica, Wetzlar, Germany) equipped with custom optical filters and a 40X objective. The Leica Q-Fluoro was used to acquire multifocus images for each filter used. Ten to 15 images were taken in areas of optimal cell density with minimal overlapping cells and cellular clumps. Excluded nuclei included those that could not be evaluated due to various reasons, including cellular overlaps and insufficient hybridization. Approximately two hundred and fifty cells were analyzed for each case.

Observations

Observations regarding differences between paraffin-embedded, formalin-fixed tissue from cervical cancer and OTSCC were made. Although both tissue types were squamous cell carcinomas, it was observed that cells in OTSCC were larger and more spread out than that of cervical cancer. During FISH hybridization, the conditions for pepsin digestion, which
improves hybridization signal in cells, differed for the two tissue types. It was observed that cervical cancer tissues required much less aggressive pepsin pretreatment than did OTSCC tissues. The tissue and cellular architecture of cervical cancer is possibly more sensitive and less resilient than OTSCC.

### 3.3 Statistical Analysis

**Paper I**
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 made 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 used in paper I, 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.

**Paper II**
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 (FISH and IHC), 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.

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

**Paper III**
The $\chi^2$ MH was used to test patient characteristics against all FISH scores. The receiver operator characteristic (ROC) plot was used to compare FISH markers and their sensitivity and specificity for identifying specimens with LN metastasis. The distance from ideal (DFI) plot depicts the distance from the ideal point on the ROC plot as a function of threshold, with minimums identifying optimal threshold values. $P$-value < 0.05 indicated statistical significance.
FISH markers (LAMP3, PROX1, PRKAA1, and CCND1) were compared against the corresponding CEP®7 probe in each cell and a ratio was calculated (FISH marker divided by CEP®7). A gained signal consisted of a ratio per cell of greater than one. An amplified signal consisted of a ratio per cell of more than two. Calculations were made according to the percentage of cells with gained or amplified signals within each case. The FISH composite marker was based on the percentage of cells with amplified signals, relative to optimized thresholds established for each single FISH marker. Cases with scores above the optimized threshold were considered a positive test for single FISH markers. Cases exhibiting a positive test for all three markers (LAMP3, PROX1, and PRKAA1) were considered a FISH composite marker with a positive test.

Clonal patterns were determined by comparing single FISH markers against the CEP®7 probe in each cell using calculated ratios (FISH marker divided by CEP®7). Gained (G) signals correspond to ratios greater than one and no gained (N) signals correspond to ratios of one or less than one. Clonal patterns depict the percentages of cells with the observed gained (G) or not gained (N) signal combination for the four FISH markers (LAMP3, PROX1, PRKAA1 and CCND1).

**Paper IV**

The Gene Cluster 3.0 and the Java Tree program were used to produce the heat map. The heat map grouped cases according to the mean signal number per marker in each sample. After comparing the mean for all markers, the heat map assigns a color to each mean signal number per marker, with green signifying a relatively low mean signal number compared to a red color, where the mean signal is high. The χ² MH was used to compare patient characteristics with FISH scores. Survival was made using the Kaplan-Meier method with a 60-month cutoff and the Wilcoxon test was used to analyze survival associations. Statistical significance was determined for all P-values from a 2-sided test, with a P-value <0.05 indicating statistical significance.

FISH markers were categorized according to the percentage of cells with copy number increases. Copy number increases of more than three signals were considered elevated signals, while copy number increases of more than eight signals were considered high copy number increases.
4. RESULTS AND DISCUSSION

4.1 PAPER I

Poor prognosis in OTSCC is partly due to metastatic disease. Given that detection of occult metastases is difficult, prognostic markers used to indicate metastatic potential in primary diagnostic tumor specimens are thus highly desirable. Genomic instability and proliferative activity, using Ki-67 expression and ploidy measurements, were applied in order to examine the prediction of locoregional recurrences exclusively in primary OTSCC.

Genomic instability, as measured by DNA image cytometry, was measured in 72 tumors. Seventy tumors (97%) of cases were found to be aneuploid while only two tumors (3%) were characterized as diploid. The two patients whose tumors were characterized as diploid remained alive at the end of follow-up. However, of these two patients, one had a secondary primary tumor while the other had no recurrence, with proliferative activities of 32% and 49%, respectively. Studies have shown that highly aneuploid tumors have reduced disease free survival times as compared to patients with less unstable tumors (e.g., breast and colorectal cancer). Genomic instability and its association with overall survival were difficult to assess in our study given that 97% of our tumors were aneuploid. Ploidy studies in oral cancers have yielded varying degrees of aneuploidy ranging from 50% to 70% in most studies. Our study reflected a high genomic instability in our material. The difference in findings could be due to the inclusion tumors of the entire oral cavity for most studies whereas our study encompasses only OTSCC material.

Proliferative activity, as determined by expression levels of the Ki-67 nuclear antigen, could be detected in the pretreatment biopsy specimen of all 76 patients and in the 11 additional surgical specimens. The percentage of Ki-67 positive cells ranged from 17% to 95% with a median of 56%. The median number of positive Ki-67 cells also increased between Stage I and Stage IV tumors. The first Ki-67 category, divided the 76 tumors into 29 and 47 samples, at 0-50 and 51-100, respectively. The second Ki-67 category, divided the specimens into 10 and 66 samples, at 0-32 and 33-100, respectively.

High proliferative activity was related to an elevated recurrence risk after surgery in Stage I primary tumors. This association was found using both the continuous Ki-67 variable (P=0.030) and the second categorical division (P=0.028). Therefore, a higher Ki-67 expression in Stage I correlated with a higher number of locoregional recurrences in two of three assessments. However, Ki-67 levels were not associated with locoregional recurrence in Stage II-IV tumors. After adjusting for stage and recurrence in a multivariate analysis, a significant correlation was not detected. Earlier studies have revealed conflicting results concerning Ki-67 expression in locoregional recurring oral cancers although two studies in oropharyngeal cancer are in agreement with our results. One study focused on OTSCC demonstrated that a lower Ki-67 expression was associated with a six-times-greater risk of recurrence, which was contradictory to our results. These apparent contradictions could be due to the incorporation of both Stage I and II patients in that study; our findings were based only on Stage I patients. In addition, our study employed an automatic staining and counting method which decreases variability and increases objectivity in the analysis of the Ki-67 expression.
Proliferative activity was not associated with specific clinical characteristics such as stage, radiotherapy response, resection status or genomic instability (as determined by DNA cytometry). However, Ki-67 expression levels were evaluated before and after radiotherapy treatment in eleven Stage II patients. These patients were classified as non-responders to radiotherapy. The proliferative activity decreased in all eleven patients in comparison to their pretreatment specimens. The mean percentage of Ki-67 positive cells decreased from 59 to 38 after radiotherapy. This decrease in proliferative activity may signify radiotherapy treatment response in the patient. It mirrors the fact that the individual response to radiotherapy varies and most certainly depends on many different factors which makes it difficult to predict response.

Proliferative activity and survival was assessed in all stages with no significant differences observed. A trend was observed in Stage I, with patients exhibiting low proliferative expression (Ki-67≤32%) tending to fare better than patients with Ki-67 expression above 32%. This trend was not observed in any other stage or categorical group. Although conflicting results regarding proliferative activity and its impact on survival were found, several studies have also confirmed that lower Ki-67 expression was associated with better patient survival in oral cancer.150-153

In summary, our data suggest that OTSCCs are highly unstable cancers. High Ki-67 expression was found to be associated with locoregional recurrence in Stage I tumors, making it a potential marker for additional therapy in patients. However, additional studies in a larger cohort of patients are warranted before Ki-67 can be used in a clinical setting.
4.2 PAPER II

Novel therapeutic agents targeting the EGFR have been developed but information concerning the status of EGFR in OTSCC is limited. EGFR status was thus investigated using IHC and FISH in OTSCC patients.

EGFR expression as determined by IHC, showed that all tumors were positively stained for EGFR. A majority (72%) of patients had intensely stained EGFR protein expression while only 5% were weakly stained. *EGFR* copy number gain, as determined by FISH, separated patients into positive and negative groups. Fifty-four percent (35 out of 65) were FISH positive while 30 tumors were characterized as FISH negative. The number of tumors in each FISH pattern is shown on Table 7.

Table 7: Classification and description of FISH patterns according to positive and negative groups

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

*EGFR* FISH positive results were significantly (*P*=0.003) higher in advanced tumors (Stage II, III, and IV) in comparison to Stage I tumors.

Comparisons were made between the two methods, IHC and FISH, and a significant association (*P*=0.004) was seen between EGFR protein expression levels and EGFR gene copy number in all patients. Correlation analyses also confirmed this significant correlation in tumors [Spearman correlation coefficient of 0.385 (*P* = 0.002)]. Although a majority of tumors with high *EGFR* gene copy number had intense EGFR protein expression in our study, a correlation between the two methods has never been shown in OTSCC. Earlier studies on EGFR using IHC and FISH in head and neck cancers have combined various tissue types (anaplastic thyroid cancer, esophageal cancer and laryngeal cancer).129-131 Two studies found a significant correlation between FISH and IHC in head and neck tumors although Lee et al did not observe any *EGFR* gene copy number increase,130,131
Intense EGFR protein expression and high gene copy number tended to be higher in 29 (n=37) tumors characterized as non-responders to radiotherapy (non-pCR), though the results were not significant. Studies have shown that cells exposed to EGF are protected from radiation damage (more radioresistant), which implies that EGFR may be regulating cellular response to radiation in order to protect it from radiation damage. A high EGFR expression in the diagnostic pretreatment tumor biopsy may predict a worse radiotherapy effect and could possibly identify patients who would benefit from the addition of an EGFR blocker to the therapeutic regimen.

The impact of smoking habits on EGFR expression and gene copy number increases were also examined. To determine the association between EGFR levels in non-smokers and smokers, comparisons were made according to stage (see table 4, paper II). Significant associations were found in early stage tumors (Stage I and II) for both IHC (P<0.001) and FISH (P=0.009) in non-smokers. Non-smokers tend to have both high EGFR expression and copy number gains. High EGFR expression was seen in 83% of non-smokers with the immunohistochemistry technique and 79% of patients with the FISH method. Smoking habits may thus be important when considering the addition of an EGFR inhibitor treatment. In previous studies, certain clinical parameters such as history of never smoking, female gender, Asian ethnicity and adenocarcinoma histology have shown EGFR inhibitor sensitivity in lung cancer. Lung cancer investigations have revealed that mutations in the TK domain of the EGFR gene are associated with EGFR inhibitor sensitivity, making mutations a predictor for EGFR inhibitor treatment response. Pao et al. reported that non-smokers associated with EGFR inhibitor sensitivity in lung cancer also displayed EGFR domain mutations in exons 19 and 21. In addition, Miller and colleagues reported that non-smokers were more sensitive than smokers to gefitinib treatment in non-small-cell lung cancer. High EGFR expression in non-smokers may therefore indicate the presence of EGFR mutations which could relate to EGFR inhibitor sensitivity. EGFR mutations are reported as rare in HNSCC although no studies have been reported for OTSCC. Future studies may therefore reveal the importance of using EGFR as a marker to determine patient response to EGFR inhibitors in OTSCC.

No association was detected between EGFR expression (P = 0.494) or copy number gain (P = 0.683) and patient survival. Our results concurred with several studies which showed no association between EGFR protein expression and survival. Patients displaying intense EGFR protein expression did have a longer median survival time (22 months; range: 3 to 60 months) compared to patients with tumors expressing weak EGFR protein staining (14 months; range: 7 to 59 months). Median survival time for FISH positive and negative groups were 21 (range: 3 to 59 months) and 28 (range: 4 to 60 months) months, respectively.

In conclusion, EGFR overexpression was found to be associated with EGFR gene copy number gains in this study. EGFR is an interesting area for the exploration of new therapeutic agents targeting the EGFR receptor.
4.3 PAPER III

The presence of LN metastasis constitutes the strongest prognostic factor for survival in cervical cancer making LN prediction using molecular markers highly desirable. FISH markers including LAMP3, PROX1, PRKAA1 and CCND1 were thus used to explore their role as potential predicting factors for LN metastasis in Stage IB-IIA cervical cancer.

Clinically, almost half (44%) of the LN positive patients died, while all of the LN negative patients remained alive during the ten year follow-up period. Of the seven patients who died, lymph vascular invasion was observed in two patients.

A combined FISH marker consisting of amplified (a ratio per cell of more than two signals) probes for LAMP3, PROX1 and PRKAA1 proved to be a significant ($P=0.001$) predictor of LN metastases. The composite FISH markers result was not related to tumor histology. To our knowledge, no other study has performed FISH analyses to predict LN metastases in cervical cancer. Previous studies seeking to predict lymph node metastases have used techniques such as immunohistochemistry, RT-PCR, and gene expression profiling and the findings have been conflicting. The prediction of LN involvement in cervical cancer will assist in developing adequate treatment options since histologically undetectable micrometastases in the lymphatic system may account for cervical cancer recurrence. Histologic examinations are not fail-safe methods since examinations only comprise a small portion of LNs, with estimates indicating that pathologists have a one percent chance of detecting a micrometastatic focus within a three-tumor-cell diameter.

The sensitivity and specificity of the composite marker indicated that 75% of LN positive patients were correctly identified with a positive test, while 87% of LN negative patients were correctly identified with a negative test. The positive predictive value (PPV) and negative predictive value (NPV) of the composite FISH probes represented the proportion of test positive patients (86%) who actually had the disease while the negative predictive value (NPV) at 76% represented the percentage of test negative patients who were correctly identified with a negative test result (see table 3, paper III). The ROC curves depict the relationship between the sensitivity and specificity of the FISH composite marker (see figure 4a, paper III). The DFI curve illustrates the single FISH markers used to determine the composite FISH marker and supports the selected threshold values.

Observations of all four FISH markers revealed copy number gains in all 31 biopsy specimens. The only marker for which amplified cells were observed in all cases and controls was LAMP3, with an average of 17% of cells containing an amplified signal. When comparing amplified signals in LN positive and negative cases, a larger percentage of amplified nuclei were seen in LN metastases samples, especially the LAMP3 marker. The LAMP3 marker lies on an area frequently gained in cervical cancer. In addition, 3q gain is of interest since the consistent gain of 3q has been found to predict the development of cervical cancer. In this study, LAMP3 is consistently gained and amplified, which supports the proposal that 3q copy number gain is ubiquitous in cervical cancer. Studies with the remaining three markers (PROX1, PRKAA1, and CCND1) resulted in tumor cases or controls with no observable amplified signals. CCND1, as a single marker, provided borderline significance ($P=0.052$), with most positive values having low percentages approaching zero. The FISH methodology, which offers the ability to visualize several genes of interest within the individual cells, has not previously been reported for the three genes of interest (LAMP3, PROX1 and PRKAA1), in cervical cancer.
The examinations of clonal patterns in tumor biopsies and the corresponding LN metastases provided evidence that in certain cases the proportions of different clones can vary between the primary tumor and the metastatic LN. In our study, several cases showed the exact same clonality and clonal distribution in the primary tumor and LN metastasis, indicating that the major clone(s) of the primary tumor was also the most successful clone(s) to establish LN metastasis. In other cases, altered clonality between the primary tumor and LN metastasis indicated that a pre-existing but minor clone in the primary tumor had growth advantages for establishing metastases in the LNs compared to the major clone of the primary tumor. This difference in patterns may reflect different mechanisms in the development of LN metastases in cervical cancer. To our knowledge, this is the first study to investigate differing clonal patterns using FISH markers between primary and synchronous LN metastases in cervical cancer.

In summary, a composite marker, comprised of \textit{LAMP3}, \textit{PROX1}, and \textit{PRKAA1}, distinguished between LN positive and negative patients in our study. However, further studies with additional patients are warranted to better determine the composite marker's potential in the clinical setting.
4.4 PAPER IV

Genetic alterations in are frequently present in head and neck cancers but studies on these alterations in OTSCC and their correlation with clinical and histological parameters have been limited. In order to better understand the etiology of OTSCC, a more extensive examination using FISH with five markers (TERC, CCND1, EGFR, p53 and CEP®4) was carried out.

To our knowledge, this is the first study investigating five FISH markers exclusively in OTSCC. Copy number gain could be seen in all five markers for all 65 tumors. CCND1 had the highest copy number gain in comparison to the other markers used in this study. The percentage of cells with high CCND1 copy number gain (greater than eight signals) increased with advancing stage, from 7% in stage I to 18% in stage IV. In addition, dense clusters were seen in CCND1 and EGFR in eight and seven cases, respectively. In one case, clusters for both CCND1 and EGFR were observed together. Multiple numerical and structural chromosome changes have been observed in OTSCC, including gains in chromosome arms 3q, 7p, and 11q13.46, 47

Higher copy number in all five markers was found to be significantly (P=0.002) related to smoking habits. An increased mean signal number in all five markers (Group 3) corresponded to a higher number of non-smokers while low mean signal numbers corresponded to smokers, as shown by the heat map. Elevated CCND1 signals were also found to be associated (P=0.001) with non-smokers, especially in early stage OTSCC. Genomic instability, using DNA image cytometry, also corroborated the discovery of increasing copy number association. In the heat map (see figure 2, paper IV), increasing mean copy number gain by heat map group showed increasing 2.5-5c and greater than 5c exceeding rates. The results are shown below in table 8.

Table 8: DNA Cytometry results in relation to heat map grouping

<table>
<thead>
<tr>
<th>Heat Map</th>
<th>DNA Image Cytometry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5-5c exceeding rates</td>
</tr>
<tr>
<td>Group 1</td>
<td>61.9%</td>
</tr>
<tr>
<td>Group 2</td>
<td>83.6%</td>
</tr>
<tr>
<td>Group 3</td>
<td>88.7%</td>
</tr>
</tbody>
</table>

We observed a correlation between multiple high FISH copy number gains in all five probes and smoking habits. Although exposure to carcinogens such as tobacco is a known risk factor for oral cancer, the exact genetics of how these carcinogens instigate cancer remains unknown. Our results imply that different mechanisms in the development of OTSCC may exist for patients exposed to tobacco and those who are not. A significant correlation between survival and FISH markers was found only for the CCND1 marker. In Stage II OTSCC, patients with elevated CCND1 copy numbers (greater than three CCND1 signal numbers) in at least five percent of cells correlated significantly (P = 0.03) with a better prognosis. This correlation was not seen for all patients. Survival rates were not found to be significant among the three heat map groups. Previous studies on CCND1 and survival in oral cancers are conflicting; several publications have shown that high CCND1 infers a worse survival.62, 100, 102 Studies confined specifically to OTSCC have found conflicting results as well, with one study finding no prognostic factor related to CCND1 copy number.101 Other studies presenting CCND1 as a poor
prognostic marker in OTSCC have either employed the methodology or combined all patients regardless of stage.\textsuperscript{100,102}

In conclusion, our results provide a better understanding of the genetic alterations related to OTSCC. Higher FISH copy number was found to be related with non-smoking habits in this study. Our observations lead us to believe that differences in genetic alterations may pertain to the causal mechanism of the initiation of OTSCC.
5. GENERAL CONCLUSION

The studies presented in this thesis have increased our knowledge of tumor progression in squamous cell carcinoma of the uterine cervix and the oral tongue at the molecular level. Several of our findings might have potential clinical implications which could improve patient care in the future. Specifically, we are able to conclude the following:

- High proliferative activity was found to be associated with locoregional recurrence in Stage I OTSCC, suggesting that Ki-67 may be a potential marker for additional therapy.

- High EGFR copy number gain and overexpression was found in OTSCC indicating that this patient group is likely to benefit from the application of therapeutic agents targeting the EGFR receptor.

- A FISH marker panel comprised of LAMP3, PROX1, and PRKAA1, was able to discern LN positive from LN negative cervical cancer patients in our study making it a potential marker for the prediction of LN metastasis. Additional, larger scale studies are warranted.

- Clonal patterns between cervical primary carcinomas and their respective lymph node metastasis were often the same or very similar, indicating that the major clone(s) observed in the primary tumor metastasized further to the lymph nodes. However, cases with lymph node signal patterns differing from the ones seen in the primary tumor were observed. This switch of aberrations is likely to reflect an adaptation of the tumor cells to the lymph node environment and its exploration might be useful for finding more optimized treatment options in this patient group.

- Higher FISH copy numbers were found to be significantly related to non-smoking habits in OTSCC patients, indicating that OTSCC may develop along different pathways in smoking and non-smoking patients.
6. ACKNOWLEDGEMENTS

This work was supported by the Cancer Society of Stockholm (Cancerföreningen), Laryngfonden, Karolinska Institutet, the Intramural Research Program of the NIH, National Cancer Institute and the National Library of Medicine.

I would like to express my sincere gratitude to all those who have supported or contributed to the completion of this work. Special thanks to:

Assistant Professor Elisabeth Åvall Lundqvist, my supervisor, for your guidance, recommendations and tremendous encouragement throughout my entire thesis.

Professor Eva Munck-Wikland, my co-supervisor, for your supervision and passion in the field which lead to the completion of my thesis.

Professor Gert Auer, for your great advice and wisdom in the field of science.

Thomas Ried, my supervisor in the US, for paving the way for my academic education and for your continuous support and advice. I sincerely appreciate your allowing me access to your expertise and resources.

Kerstin Heselmeyer-Haddad, my supervisor in the US, for your never ending enthusiasm, support and guidance which have impacted me in more ways than I can say.

Docent Richard Kuylenstierna, Head of the Department of Oto-Rhino-Laryngology, Head and Neck Surgery, for being my external mentor.

Professor Tina Dahlianis, the Head of the Department of Oncology-Pathology

Michael Ryott, my coworker and collaborator during this thesis: We made a great team together and without your help and hard work, this thesis wouldn't be what it is.

Patricia Ried, your friendship throughout the years has been dear to me and I wanted to express my appreciation for all the recommendations you have given me.

Turid Knutsen: I cannot thank you enough for your help throughout this thesis process. Your kindness in helping me has been tremendous.

Renee Gamborg, thank you again all the support and for your continuous assistance throughout the years. You were always there when I needed you.

Koneti Rao, you introduced me to the field of Cytogenetics and Cancer which has led me to the completion of this thesis. Thank you!

Marianne Poruchynsky, a big thank you for the support and encouragement you've given from the beginning.
Past and present coauthors and collaborators: Göran Elmberger, Fredrik Petersson, Johan Lindholm, Elina Eriksson, Juhua Luo, Alejandro A. Schäffer, Larry E. Morrison Sonia Andersson, Ann-Cathrin Hellström, Catherine Beskow and Eva Lindblad

All the past and present members of the Ried Lab in Bethesda, especially: Buddy, Jana, Priya, Mike, Kundan, Hesed, Nicole, Jordi, Amanda, Diana, Joseph, Tri, Fong, Lissa, Chanelle, Marian and Jens. I just wanted to thank each of you for your friendship and support! Going into the lab each morning was like stepping into my second home.

Bryan Linares for your information technology support.

All past and present members of the Karolinska Biomic Center (KBC):
Thank you all for including me in all the KBC activities. I really felt like a part of the group! A special thanks to Birgitta, my office mate who provided me with assistance and friendship throughout these past years. Suzanne and Marta, thank you for your great company and friendship.

Angie, Nancy, Jennifer, Jana, Buddy, Eileen and Mai for all the great times we had together.

Görel Söderblom, for nice times together.

Anki and Evi, for assistance regarding Ph.D. matters throughout my thesis.

Anne Wompa, for your excellent assistance.

Gail, for your friendship and positive attitude

My friends and colleagues at CCK: Jeremy, Magda and Juan. Liss, Maggan, Margareta Waern and Ann Ohlsson for technical support.

All my friends and colleagues in the Head and Neck Surgery Department: Lalle, Pelle, Mathias, and Linda

A special thanks to all my close friends at Karolinska Institutet, Bettina, Jin, Nimesh, Betzy and Dali. You guys are the best!

Jaid, for your encouragement to do my Ph.D. and your support throughout the entire thesis.

My family, especially my parents, Danny and Kitty Wangsa, my brother Don, and my Aunt Pom….. who I would like to thank for their everlasting love and support during the long process of my thesis.
7. REFERENCES


Molecular Profiling for Squamous Cell Carcinoma


151. Myoung H, Kim MJ, Lee JH, Ok YJ, Paeng JY, Yun PY. Correlation of proliferative markers (Ki-67 and PCNA) with survival and lymph node metastasis in oral squamous cell carcinoma:


