# Detection of Human Papillomavirus in primary site of oral/oropharyngeal cancer and in cervical lymph nodes: Correlation with clinico-pathological parameters and prognostic significance

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October 2005

A thesis submitted to McGill University in partial fulfillment
of the requirement of the degree of Master of Science

*Your file Votre référence*
*ISBN: 978-0-494-22699-5*
*Our file Notre référence*
*ISBN: 978-0-494-22699-5*

## Acknowledgements

I extend my deepest gratitude to my supervisors, Dr. Qutayba Hamid and Dr. Gerard Domanowski, for their mentorship throughout this entire process. I appreciate all of their efforts at directing me through the world of basic research. The lessons I learned have left a mark on me that is indelible.

I am also indebted to my clinical supervisor, Dr. Karen Kost, for her sound advice and guidance throughout this project as well as throughout the early stages of my career through her teachings during the residency.

I would like to acknowledge the contributions made by Dr. Michael Hier and Dr. Martin Black. The hours that they spent supporting me with the clinical research aspects of this project are greatly appreciated and will always be remembered.

My thanks to Valerie Brousseau and Diana Paczesny for their assistance in the recruitment of patients.

I would like to thank Dr. Heberto Ghezzo for his guidance and advice in the statistical analysis of the data. Also to Dr. Abdullah Al-Fiaar and Pierre Fiset who helped do the PCR.

My appreciation goes to the Department of Otolaryngology, the Division of Pathology, McGill University, and Meakins-Christie Laboratories for their support in arranging and carrying out this project, and to the Canadian Institute of Health and Research for their financial support.

Thanks also to Joan O'Malley for her assistance in the preparation of the thesis.

Thanks to my wife Abeer whose unwavering support acts as the foundation for all my dreams.

Finally, to my sons Mohammed and Abdullah, and my daughter Renad, the greatest accomplishments that anyone can attain, I dedicate all that I achieve.

## Abstract

***Objectives:***

1. To Determine the presence of HPV 6, 11, 16, 18, 31, 33, 35, 52b, 58 subtypes in resected oral/oropharyngeal SCCA cancer and associated lymph nodes.
2. To Determine if a relationship exists between koilocytosis, tumor grade, stage, or prognosis.

***Methods:***

Retrospective analysis and pathology review of patients with SCCA of the oral cavity at McGill in the last 5 years was performed. Age at diagnosis, risk factors, tumor stage, grade, koilocytosis, treatment, outcome, and presence of HPV by PCR were analysed.

***Results:***

199 patients included were included in the analyses; 5 years mortality was 18.5%. 146 cases reviewed by pathology revealed 67% koilocytosis. One sample was positive for HPV subtype 35 as determined by PCR. Radiotherapy ($p<0,5$) and complications from radiotherapy ($p<0.5$) significantly affected survival.

***Conclusions***:

Many oral SCCA's do not contain HPV 6, 11, 16, 18, 31, 33, 35, 52b, 58 subtypes. Given the high prevelence of koilocytosis, probe for other subtypes should be utilized. Mortality rates and survival are similar to those published in the literature. The presence of koilocytosis, it is not related grade, stage or prognosis. Only radiotherapy and its complications affect survival.

## Résumé

### Objectifs

1. Déterminer la présence de VPH 6, 11, 16, 18, 31, 33, 35, 52b, 58 lors de cancer épidermoïde de la cavité orale.
2. Déterminer s'il existe une relation entre la koilocytose, le stage tumoral, le grade tumoral et le pronostic chez ces patients.

### Méthodologie

Analyse rétrospective et révision pathologique des patients atteints d'un cancer épidermoïde de la cavité orale à l'université McGill dans les 5 dernières années. L'âge au diagnostic, les facteurs de risque, le stage de la tumeur, le grade de la tumeur, la présence de koilocytose, le traitement reçu, récurrence, mortalité, et présence de VPH par PCR furent analysés.

### Résultats

199 furent identifiés. Le taux de mortalité à 5 ans est de 18.5%. La révision pathologique de 146 patients a révélé une incidence de koilocytose de 67% avec une distribution égale entre les différents stages. Une tumeur est positive pour le VPH de sous-type 35 par PCR. Le taux de survie à 5 ans est de 71.5%. La radiothérapie et les complications qui y sont associées affectent la survie de manière significative.

**Conclusions**

La majorité des cancers épidermoïdes de la cavité orale ne sont pas infectés par le VPH sous-types 6, 11, 16, 18, 31, 33, 35, 52b, 58. Vu la haute prévalence de koilocytose, d'autres sous-types devraient être investigués par PCR. Ces taux de mortalité et de survie sont semblables à ceux publiés dans la littérature. Il y a un haut taux de koilocytose chez les tumeurs épidermoïdes de la cavité orale. Il n'y a pas de lien entre la koilocytose et l'infection au VPH, le grade, le stage ou le pronostic. Seulement la radiothérapie et les complications qui y sont associées affectent la survie.

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## Abbreviations

| | |
|---|---|
| **HPV** | Human Papilloma Virus |
| **PCR** | polymerase chain reaction |
| **HNSCCs** | head & neck Squamous Cell Carcinoma |
| **SCCA** | Squamous Cell Carcinoma |
| **T** | tumor stage |
| **N** | nodal stage |
| **LN** | lymph nodes |
| **Mets** | metastatic |
| **EGFR** | epidermal growth factor receptors |
| **TGF** | transforming growth factor |
| **Bp** | base pairs |
| **HC2** | Hybrid Capture 2® |
| **RFLP** | restriction fragment length polymorphism () |
| **MUHC** | McGill University Health Center |
| **JGH** | Jewish General Hospital |
| **K** | Potassium |
| **LSIL** | Low-grade squamous intraepithelial lesion |
| **LB** | Luria-Bertani |
| **OS** | Overall survival |
| **DSS** | Disease specific survival |
| **RFS** | Relapse-free survival |
| **EBV** | Epstein-Barr virus |
| **ISH** | in situ hybridization |
| **FDA** | federal drug administration |
| **EDTA** | ethyldiacetilic acid |
| **SDS** | Sodium dodecylsulfate solution |
| **Tris HCL** | Tris hydrochloride acid |

# Chapter 1: Introduction

# 1. Introduction

## 1.1 Introduction

Squamous cell cancer of the oral cavity is primarily a disease of men in the sixth to eighth decade of life[1] and is associated with tobacco and alcohol abuse.[2-4] The aims of this study are to determine the prognosis, epidemiology and relevant disease characteristics of patients treated at the McGill University Health Center (MUHC) in the last 5 years as well as to evaluate and review the treatment options offered to these patients at the institution. Furthermore, we wish to investigate the presence of some subtypes of so-called "high risk" [5-11] and so called "low risk"[12,13] of Human Papilloma Virus (HPV) in resected tissue from these patients as well as in the metastasis within the cervical lymph nodes. The results should reveal the potential role of HPV infection in squamous cell carcinogenesis as well as its possible relationship to tumor grade, prognosis, treatment and prevention of disease in this population.

## 1.2 Previous research pertinent to this project

### 1.2.1 Prognosis

Several studies have previously attempted to compare the prognosis of patients who have HPV positive tumors to those with who have HPV negative squamous cell cancer in head and neck. Unfortunately, no consensus has been reached in this debate. Some concluded that the prognosis was worse,[12,14-17]others the same,[5,6,13,18-24] and still others better.[25,26] Our study is unlikely to present an ultimate answer to this question. We nevertheless hope to be able to contribute to the understanding of this disease, and evaluate our management by determining overall survival rate and disease free survival in

this series. Several studies have attempted to define the significant prognostic factors associated with squamous cell carcinoma of the oral cavity. Some of the factors identified include: male gender,[27,28] positive resection margins and smoking,[29] as well as tumor thickness and distance of tumor from resection margins.[30] Furthermore, the presence of positive neck nodes is associated with a poor prognosis and low salvage rate.[30] As with most other cancers, tumor (T) stage, nodal stage (N) and extension to the tonsillar region have been shown to have a significant relationship to survival.[31] Furthermore, perineural invasion has been shown to significantly increase the risk of recurrence.[32] Because epidemiological changes have taken place, notably a significant increase in oral cancer in women,[7] we do not think that gender plays a significant prognostic role in the disease of oral cavity cancer patients. We hope to be able to demonstrate our hypothesis through analysis of risk factors in this series.

### 1.2.2 Etiologic factors

#### 1.2.2.1 Cigarette and alcohol

Chewing betel nut or tobacco, smoking tobacco, and alcohol abuse are all habits that have been identified as risk factors for the development of oral cancer.[8,9,33,34] However, a significant number of patients do not have any of these risk factors. It is this observation, among others, that leads many to suggest other etiologies for cancer in this subpopulation such as immune deficiency,[35,36] genetic factors,[37] diet,[10,38,39] or viruses.[40,41]

#### 1.2.2.2 Genetics

It has been proposed that early exposure to carcinogens,[7] or genetic susceptibility,[11,42,43] may be responsible for carcinogenesis in this population. A number

of genetic alterations have been documented in oral carcinoma such as: increased expression of epidermal growth factor receptors (EGFR) and the erb-b-2 gene, refractoriness to transforming growth factor (TGF, as well as amplification of the oncogene int-2 and genes bcl-1, c-myc, and H-ras.[44] More specifically, p53 has been found to be an important genetic contributor to squamous cell carcinogenesis. Acting as a tumor-suppressor gene, its main role is to control the cell cycle and to trigger apoptosis of damaged cells. Studies have demonstrated the presence of mutations as well as increased expression of p53 in squamous cell cancer.[45] In the case of oral cancer in particular, loss of heterozygosity[46] and mutations[47,48] have been linked to carcinogenesis. Furthermore, the degree of dysplasia[49] and the level of differentiation[50] observed appear to correlate with the degree of expression of the mutant tumor suppressor gene, making it a likely prognostic marker. To emphasize the link between environmental exposure and genetic changes, patients with head and neck cancers who are known tobacco and alcohol users are known to have a high frequency of p53 mutations.[51]

Another example of genetic susceptibility involves a report of familial syndrome of pancreatic cancer and melanoma linked to a CDKN2 tumor suppressor gene mutation. This report also incidentally noted that one proband suffered from early squamous cell tongue cancer.[52] This particular cyclin-dependent-kinase inhibitor mutation is known to be involved with a small portion of head and neck tumors.[53]

Although this study does not focus on specific genetic changes associated with oral cavity cancer, we are well aware of the interplay between genetic predisposition and environmental factors. We will therefore pay special attention to family history, history

of previous benign or malignant oral lesions, previous history of cancer as well as smoking and alcohol consumption during our chart review process. This should help us evaluate their role as prognostic factors and their relationship with HPV infection.

### 1.2.2.3 Viruses

Viruses may contribute to carcinogenesis in several ways: via immunosuppression, modification of host DNA and its expression with or without persistence of viral DNA, induction of oncoproteins, or tumor-suppressor deregulation. Approximately 15% of all cancers are associated with viruses such as HPV, hepatitis B virus, Epstein-Barr virus and human T cell leukemia virus.[54] Given the high prevalence of virus-associated cancers such as cervical and hepatocellular cancers, viruses are considered by some as the second most important risk factor for developing cancer, just following smoking.[54] HPV has long been known to cause warts and mucosal proliferations. A number of these benign lesions have the potential for malignant transformation. Examples include: condylomata acuminata to vulvar, cervical, penile and perianal carcinoma as well as giant condylomata acuminata; laryngeal papillomas, adult and juvenile types, to squamous cell cancer of the larynx or trachea; and epidermodysplasia verruciformis to cutaneous squamous cell carcinoma.[55]

The exact mechanism by which HPV causes malignant transformation is still unclear. However, it has been shown that the E6 oncoprotein from tumor-associated HPV types binds to and triggers the degradation of the p53 tumor-suppressor protein.[56] Allele analysis has also demonstrated that individuals homozygous at a particular site along the p53 gene are up to seven times more likely to develop HPV-induced cancer than

heterozygotes.[56] Furthermore, clinical data has shown a significant inverse relationship between smoking, with or without alcohol consumption, and the presence of HPV in head and neck tumors.[57] These findings clearly underline the importance of the interplay between environmental factors, HPV infection, and genetic susceptibility.

In the context of this research, many suspect viral infections are a factor in head and neck cancer.[58-60] More specifically, HPV-16 has been identified as a risk factor for squamous cell cancer of the head and neck.[59] Studies have identified HPV infection in both premalignant and malignant lesions of the oral cavity. It has been noted that benign lesions contain the viral DNA in a plasmid state[61] whereas malignant lesions have integrated HPV DNA sequences.[62,63] HPV-2 has been found in various oral cavity lesions,[64] HPV-6 and HPV-16 in warts of the oral cavity,[65] and HPV-11 and HPV-16 in oral leukoplakia.[66] Furthermore, HPV-6a[67] and HPV-16[68] have been found in tonsillar carcinoma. Studies suggest that HPV positive oropharyngeal cancers represent a distinct disease with their own molecular, clinical and pathological presentation[58] and that they may have a better prognosis than HPV negative disease. We hope to be able to confirm this hypothesis by analyzing malignant and benign tissue for the presence of HPV and relating both the presence and type of infection with the observed clinical course. A number HPV subtypes have been identified in squamous cell tongue carcinoma: HPV-2,[69] HPV-6,[68] HPV-6/11,[70] HPV-11,[71] HPV-16,[68-70,72] HPV-18.[70,72] Of these studies, only two focused on tongue cancer specifically: all others consist of a selection of oral cancers.

We would like to investigate the presence of both malignant and benign HPV types in malignant oral cavity lesions and in positive cervical lymph nodes. Specifically, we will be testing for HPV-6, -11, -16, -18, -31, -33, -35, -52b, and –58. No study has yet attempted to investigate the presence of such a broad spectrum of HPV types in malignant oral cavity lesions and in positive cervical lymph nodes.

Assuming that HPV plays a significant role in carcinogenesis and knowing that nodular leukoplakia presents a higher risk of malignant transformation compared to homogenous leukoplakia,[73] and that 2-6% of leukoplakias with severe dysplasia lead to invasive oral carcinoma, we would expect a higher rate of HPV infection in nodular lesions than homogenous lesions and would also expect to find the same HPV subtype in patients with nodular leukoplakia who went on to develop frank carcinoma. We would also expect patients with HPV-positive tumors to have different clinical and pathological characteristics as well as better outcomes than patients with HPV-negative tumors. The correlation between previous diagnosis of leukoplakia, clinical and histological tumor characteristics and HPV type will be determined to verify these hypotheses.

Finally, it has been shown that most papillomas of the respiratory tract are caused by genital HPV-6 and HPV-11.[74] Given that the HPV subtypes involved in oral cancer may also be related to the genital tract, sexual practices may be an important factor not only in oral cancer,[60] but also in tongue cancer more specifically. These may play a more prominent role in the population under study and force the medical community to reconsider current prevention programs.

### 1.2.3 Treatment options

The best treatment for oral cancer has been the object of debate for many years. Options include resection with or without neck dissection (followed by reconstruction as needed), or external beam radiotherapy, or brachytherapy alone, or as an adjuvant to surgery. In some rare cases, chemotherapy may also be used as an adjuvant or palliative treatment. Interstitial radium was considered as good as surgery in the local treatment of oral cancer for a very long time.[75] Indeed, radiotherapy treatment results for T1 and T2 disease appear to be similar in younger and older patients[76] and treatment of the primary oral lesion appears to be as effective with radiotherapy or surgery in such patients.[16]

This review should allow us to assess and defend our current practices in light of the literature and our long-term prognostic results. This assessment is critical to ensure that we continue to offer the very best treatment options possible to our patients.

## 1.3 HPV

HPV is a circular, double-stranded DNA virus from the Papovaviridae family. It is nonenveloped, measures 50 to 55 nm in diameter, and has icosahedral capsids together with a genome of about 7900 base pairs (bp). Their genomic organization is simple, consisting of an early (E), late (L), and a non-coding upstream regulatory region. E1 and E2 proteins are responsible for viral DNA replication and regulation of gene expression whereas L1 codes for the major capsid protein and L2 for the minor capsid protein. HPV oncogenicity has been traced to the E6 and E7 genes: the E6 protein via degradation of the p53 tumor-suppressor gene or any product and the E7 protein via binding to the retinoblastoma gene product and related proteins.[77]

There are numerous types of HPV: 85 have been characterized and over 120 other potential types have been partially analyzed.[78] A new type is defined when there is less than 90% homology in specific regions (E6, E7, L1).[79] Numerous types have a high malignant transformation potential which is usually specific to particular epithelia. These include: HPV- 8, -9, -15, -16, -18, -19, -21, -30, -31, -33, -34, -35, -36, -39, -40, -41, -42, -45, -47, -48, -49, - 51, -52, -54, -55, -56, -57, -58, -60, -66, -67, -68, -74.[77]

HPV is a ubiquitous virus that is related to numerous conditions from common warts to laryngeal papillomas. HPV is the most common sexually transmitted infection and the etiology of several diseases of the genital tract, notably condylomata acuminata, the most common sexually transmitted disease in the United States. In addition, more than 95% of all cervical cancers are found to contain some high-risk HPV types. On histopathology, HPV infection is characterized by hyperkeratinization, parakeratosis and koilocytes.

Koilocytosis are cells containing virus and are characteristically large, clear, and contain abnormal nuclei.[80]

### 1.3.1 HPV detection methods

It is not possible to culture HPV in vitro. Given the importance of HPV in cervical cancer, HPV detection and quantification techniques have been developed that rely solely on molecular analysis. There are currently three types of nucleic acid detection methods available: direct nucleic acid probe, hybridization signal amplification, and target amplification methods. The following discussion is condensed from Hubbard.[81]

#### 1.3.1.1 Direct nucleic acid probe methods

The gold standard for HPV genomic analysis is the Southern Blot.[82,83] Another probe method is in situ hybridization in which the presence of antigens is assessed given the histological detection on the pathology samples. The disadvantages of direct probe assays include: low sensitivity; time-consuming techniques; they cannot be performed on previously fixed tissue; and, may require large amounts of highly purified DNA.

#### 1.3.1.2 Hybridization signal amplification methods

Hybridization signal amplification methods are based on direct nucleic acid probe methods but achieve greater sensitivity thanks to proprietary boosting techniques. Unfortunately these techniques are not in the public domain even though the Hybrid Capture 2® (HC2) test manufactured by Digene Corporation Inc (Gaithersburg, MD) is the only FDA approved HPV test for in vitro diagnostic use.

#### 1.3.1.3 Target amplification methods

Target amplification methods refer to PCR techniques where a desired DNA sequence is reproduced serially using known flanking DNA sequences as the starting and ending points. They are the most sensitive and flexible HPV detections techniques available. They may be used for detection, viral load quantification, DNA sequencing and mutation analysis. Furthermore, these methods allow for analysis of multiple strands of DNA at the same time thereby increasing efficiency. PCR techniques are however prone to contamination by previously amplified material which may lead to false positive results. This can be obviated by the use of strict amplicon containment and enzymatic amplicon elimination systems using the uracil-glycosylase (AMPerase) enzyme.

Furthermore, positive and negative controls should always be used to assert that amplification did take place, thereby confirming negative results.

### 1.3.2 Sensitivity and specificity of PCR

In the context of HPV detection, sensitivity of an assay is defined as the lowest amount of DNA required for HPV detection. On the other hand, specificity refers to the accuracy of the detection results. Positive predictive value is the chance that a positive HPV test result is truly positive. Similarly, the negative predictive value is the chance that a negative HPV test result truly represents the absence of HPV in that tissue. Ideally, a test should have high sensitivity and specificity in order to generate high positive and negative predictive values.

When considering the above-mentioned techniques, the relative sensitivities in decreasing order are target amplification techniques followed by signal amplification and direct probe methods. A further advantage of PCR is its capacity to distinguish between single nucleotide sequences allowing identification of specific mutations. This is not possible with the other techniques.

### 1.3.3 PCR and HPV detection

The Pasteur Institute has recently presented Roche Molecular Systems Inc. with an extensive HPV DNA library. In order to assist in HPV detection and typing, the company studied the accuracy and reproducibility of PCR-based HPV detection and typing using PGMY consensus primers targeting the highly conserved HPV L1 gene sequences.[84] The tests were conducted using 109 samples, 29 of which were HPV negative. Tests were performed blindly in triplicate by three separate laboratories and

analysis was performed to determine intralaboratory as well as interlaboratory variation. Intralaboratory agreement ranged from 86% to 98% whereas interlaboratory agreement was greater than 95%. Typically, samples with low numbers of HPV DNA were more likely to lead to divergent results. Finally, overall accuracy varied from 91% to 100% for HPV DNA positivity. Although these tests did not include samples with multiple HPV subtypes, these results clearly demonstrate that PCR is an effective, valid and accurate method for HPV detection. Fisher and von Winterfeld have recently demonstrated the successful use of consensus primers for HPV detection in head and neck cancer.[85] We have chosen PCR based HPV detection for this project because it is rapid, highly sensitive, allows for the testing of multiple HPV subtypes testing at once and is relatively inexpensive.

### 1.3.4 HPV typing

HPV typing is accomplished by analysis of specific regions where type-specific sequence variability is present. These regions are the L1 gene, which encodes a major capsid protein and appears to be the most polymorphic site, as well as the E6 and E7 genes, which endow individual HPV types with varying degrees of oncogenic transformation potential. The genes are first amplified by PCR, thereby allowing for HPV detection. Enzymes targeting type-specific mutation sites along these genes (such as *Bam* HI, *Dde* I, *Hae* III, *Hin*f I and *Pst* I) are then used to digest the PCR products allowing for restriction fragment length polymorphism (RFLP) analysis. The digestion fragments generated are compared to known patterns to identify HPV subtypes. One major inconvenience of this method is the difficulty in interpretation when more than one type

of HPV is present. In this case one may have to resort to DNA sequencing with consensus primers for adequate analysis. Similarly, the presence of extraneous bands may lead to misinterpretation and typing errors. Nevertheless, we have chosen RFLP as our primary method of HPV typing because the method is rapid and less cumbersome than sequencing. In the eventuality where results are equivocal, we will resort to fragment DNA sequencing - time allowing.

### 1.4 Hypothesis and rationale

Although studies have established HPV as a risk factor for oral and oropharyngeal cancer, it is not clear whether HPV affects survival in head and neck malignancies. At present there are no studies demonstrating an association between HPV in cervical lymph nodes and the prognosis of oral and oropharyngeal cancer. We hypothesize that the presence of HPV in malignancies of the oral cavity/oropharynx and lymph nodes may have prognostic value. This thesis will evaluate this hypothesis.

Our expected results should reveal the importance of HPV infection in squamous cell carcinogenesis as well as its possible relationship to tumor grade, prognosis, treatment planning and prevention of disease in this population.

### 1.5 Objectives

The objectives of this study are:

1. Investigate the presence of oncogenic types of HPV (e.g.,16,18) in resected tissues of oral and oropharyngeal cancer, and in metastatic cervical lymph nodes (LN) of patients treated at the MUHC and JGH in the last 5 years (from July 1999 until June 2004).

2. Determine if a relationship exists between koilocytosis, tumor grade, stage, or prognosis.

# Chapter 2: Methods

# 2. Methods

## 2.1 Patients

A list of all patients diagnosed with squamous cell cancer of the oral cavity in a recent five years period was complied from the records of the MUHC and Jewish General Hospital. A total of 199 patients were identified for the present study. The patients' charts were reviewed taking note of the gender, age at diagnosis, family history of head and neck cancer, history of cervical cancer, immune status as determined by looking to the charts, smoking and alcohol consumption, presenting symptoms and findings at diagnosis, pathological report, surgery performed, radiotherapy and/or chemotherapy received, complications and last date of follow-up. A database presenting details on prognostic and staging profile was developed using this information.

## 2.2 Pathological analysis

All slides and pathology blocks (pre-malignant and malignant) for each patient were requested from the various pathology departments at MUHC. A qualified pathologist examined them under light microscopy for grading and koilocytosis using standard criteria( figure 1). Koilocytosis on histological examination is demonstrated by particular nuclear features with perinuclear vacuolization.[86] All tissue samples were re-numbered using a non-descriptive numbering system in order to preserve patient confidentiality and anonymity as well as to reduce bias on the part of the investigators. Only the principle investigator was able to trace the samples back to the patient.

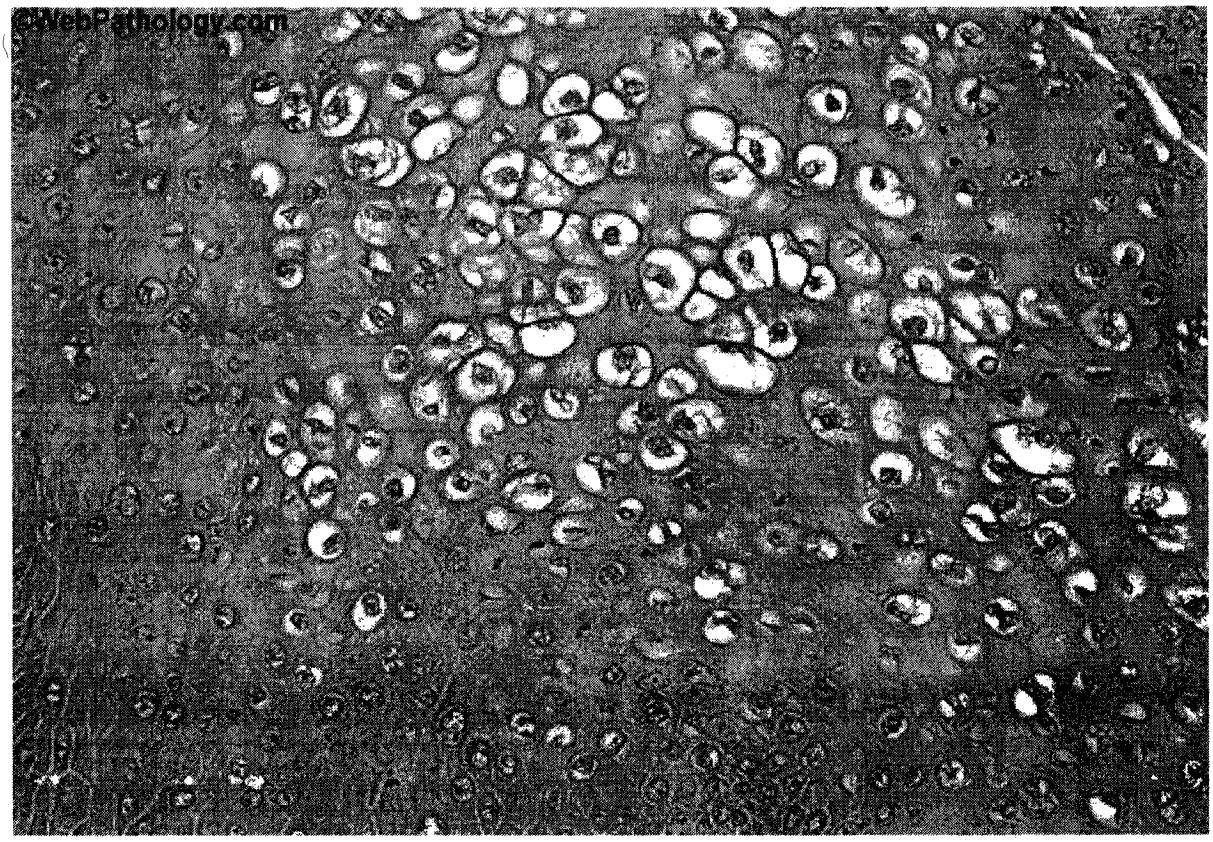


Figure 1: Condyloma Acuminatum showing koilocytosis. Koilocytosis on histological examination is demonstrated by particular nuclear features with perinuclear vacuolization.

The criteria for identifying HPV infected cells is based on The Bethesda System, which is a guideline originally developed for cervical/vaginal cytologic diagnoses. The 2001 version reports the most up to date information about abnormalities of Pap tests and also discusses new techniques for screening based on technological advances made in the last ten years.[87] HPV cytologic effects are included in the category of low-grade squamous intraepithelial lesion (LSIL), a sub-category of squamous cell epithelial cell

abnormalities. According to the 1991 Bethesda criteria, the following are characteristics of HPV effects: well-defined, optically clear, perinuclear cavity associated with a peripheral rim of thickened cytoplasm as well as nuclear alterations.[88] The revised 2001 criteria states that "the cytopathic effects of human papillomavirus (HPV)--nuclear atypia and perinuclear cytoplasmic cavitation or "halo," the morphologic manifestation of a productive HPV infection of low-grade squamous intraepithelial lesion (LSIL). The nucleus is larger than the nucleus of a normal intermediate squamous cell( figure 2)."[89]

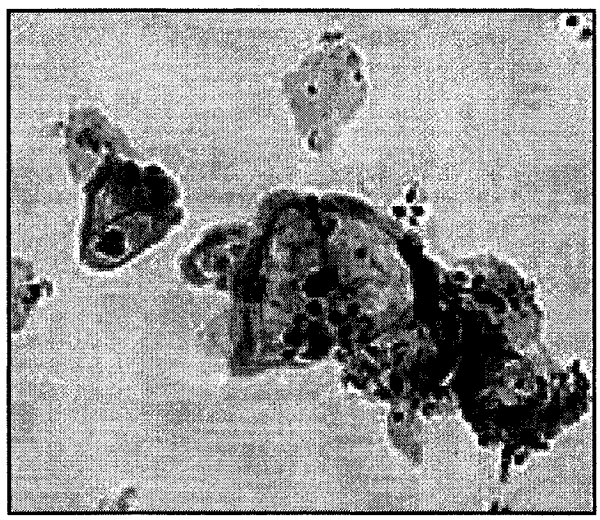

Figure 2: Cytologic effects of HPV. The nucleus is larger than the nucleus of a normal intermediate squamous cell.

**2.3 DNA extraction**

All molecular methods were followed from the instructions provided by the fabricant. Paraffin was removed from the specimens by treating with xylene (twice). The specimens were washed with 100% ethanol (twice) and dried in a desiccator. They were next suspended in a 5 mM EDTA, 0.5% SDS and 10mM Tris HCL (pH 8.0) solution and

digested for 12 hours or overnight with 50 µg/ml proteinase K at 37°C. After digestion, 300 µl of phenol/chloroform solution was added and mixed. The specimens were centrifuged at 12,000 rpm for 10 minutes, the aqueous layer was collected into a fresh tube (upper layer). 300 µl of phenol/chloroform solution was added a second time and the aqueous layer was collected into a fresh tube. The aqueous layer was incubated at -20°C for 1 hour (or 30 minutes at -70°C) with 600 µl of ethanol and 30 µl of 3M CH3COONa. The samples were centrifuged at 12,000rpm for 10 minutes and the precipitate was rinsed with 80% ethanol and dried under the fume hood. The precipitate was then resuspended in water and quantified using spectrophotometry ($A_{260}$ of 1.0 = 50 µg/ml) and aliquots of DNA were prepared at 0.1 µg/ml for the PCR.

**2.4 PCR reaction for HPV detection**

HPV detection was performed by PCR using the TAK 6603 HPV typing set by Takara Mirus Bio120 (obtained from Fisher Scientific). This primer kit uses two pairs of consensus primers to amplify the E6 and E7 HPV regions from malignant HPV-16, 18, 31, 33, 35, 52b, and 58, as well as benign HPV-6 and 11.

| | | 5' Nucleotide | Mismatches | Amplification |
|---|---|---|---|---|
| HPVpU-1M | 5'-TGTCAAAAACCGTTGTGTCC-3' | | | |
| HPV6 | -----C------C-----GA | 420 | 4 | • • |
| HPV11 | -----C--G---------GA | 420 | 4 | • • |
| HPV16 | --------G--AC------- | 419 | 3 | • • |
| HPV18 | --C--G---------AA--- | 426 | 4 | • • |
| HPV31 | -------G---------- | 423 | 1 | • • |
| HPV33 | -------G---T-------- | 424 | 2 | • • |
| HPV35 | ----------C------- | 425 | 1 | • • |
| HPV52b | -------CG--A--A----- | 418 | 4 | • • |
| HPV58 | -------G---A-------- | 425 | 2 | • • |
| HPVpU-31B | 5'-TGCTAATTCGGTGCTACCTG-3' | | | |
| HPV6 | ---------- | 400 | 0 | • • |
| HPV11 | --T-------T--T------ | 400 | 3 | • • |
| HPV16 | --T-----A----TATTAAC | 399 | 9 | • • |
| HPV18 | -AT----AA-----CTG-G- | 406 | 8 | • • |
| HPV31 | --T-----A----TATAAC- | 403 | 8 | • • |
| HPV33 | -AT-----A----TATTA-A | 404 | 9 | • • |
| HPV35 | -AT-----A----TATTACA | 405 | 10 | • • |
| HPV52b | -AACT---A-A--TATAA-T | 398 | 12 | • • |
| HPV58 | -AT-----A-A--TATTA-T | 405 | 10 | • • |
| HPVpU-2R | 5'-GAGCTGTCGCTTAATTGCTC-3' | | | |
| HPV6 | --------TAC--------- | 627 | 3 | • • |
| HPV11 | --------TTC--------- | 627 | 3 | • • |
| HPV16 | --------AT---------- | 656 | 2 | • • |
| HPV18 | TCTGA---------- | 693 | 5 | • • |
| HPV31 | ---------GG--------- | 654 | 2 | • • |
| HPV33 | --------A---------- | 667 | 1 | • • |
| HPV35 | --------A-AC-------- | 656 | 3 | • • |
| HPV52b | --------A-C--------- | 648 | 2 | • • |
| HPV58 | --------A-A--------- | 668 | 2 | • • |

Table 1: Alignment of the consensus primer sequences and HPV subtype homology.

Consensus primers sequences for malignant forward primer HPVpU-1M, benign forward primer HPVpU-31B, and common reverse primer HPVpU-2R are described in Table 1. PCR was conducted as follows (as recommended by the fabricant).

Reaction mixtures were prepared in 50µl strip tubes as presented in Table 2 and Table 3. PCR was performed in a 96 well PTC-200 Peltier Thermal Cycler (MJ Research).

Mineral oil was not needed as this thermal cycler has a heated lid. The 30 cycles of the PCR reaction were as follows: 30 seconds at 94°C; 2 minutes at 55°C; and, 2 minutes at 72°C. When PCR was complete, 10µl of reactant was mixed with xylene cyanol loading dye and applied to a 2% agarose, 1% ethidium gel. Visualization of the band was perfomed using an ultra-violet table and bands were compared to a 100bp ladder (Invitrogen). Positive amplification should yield products between 228 bp and 268 bp in length. TAK 6603 HPV typing set included both a malignant and benign control template for verification of the PCR reaction. 1µl (1ng/µl) of the supplied control template was used in a 50µl reaction (the volume of water was adjusted accordingly) whenever a PCR run was done, and 10µl of the control was put on the gel with the samples. The malignant control size was 63 bp and the benign was 61 bp. A negative control was also run which contained water instead of DNA.

| **Reagents** | **Volumes used** |
|---|---|
| 10X PCR Buffer | 5 µl |
| $MgCl_2$ 50mM | 1.5µl |
| DNTP mixture (ea. 2.5mM) | 4 µl |
| HPVpU-1M (25 pmol/µl) | 1 µl |
| HPVpU-2R (25 pmol/µl) | 1 µl |
| TaKaRa Taq (5 units/µl) | 0.5 µl |
| Sample DNA (0.1µg/µl) | 10µl |
| Distilled sterilize water | 27µl |

Table 2: Amplification of malignant HPV types reaction mixture

| Reagents | Volumes used |
|---|---|
| 10X PCR Buffer | 5 µl |
| $MgCl_2$ 50mM | 1.5µl |
| DNTP mixture (ea. 2.5mM) | 4 µl |
| HPVpU-31B (25 pmol/µl) | 1 µl |
| HPVpU-2R (25 pmol/µl) | 1 µl |
| TaKaRa Taq (5 units/µl) | 0.5 µl |
| Sample DNA (0.1µg/µl) | 10µl |
| Distilled sterilize water | 27µl |

Table 3: Amplification of benign HPV types reaction mixture

**2.5 PCR reaction for house keeping gene detection**

Due to reduced efficiency of amplification of DNA templates from formalin-fixed specimens and inhibitory substances present in paraffin preparations, productive amplification was confirmed by β-globin DNA PCR for each specimen. Sequences for the primers used were 5'-CATGGTGCATCTGACTCCTG-3' and 5'-GAGCCAGGCCA TCACTAAAG-3'. The primers were designed with Primer3 program. Reaction mixtures were prepared in 0.5 ml strip tubes as presented in Table 4.

| Reagents | Volumes used |
|---|---|
| 10X PCR Buffer | 5 µl |
| $MgCl_2$ 50mM | 2.5µl |
| DNTP mixture (ea. 2.5mM) | 4 µl |
| β-globin forward primer (25 pmol/µl) | 1 µl |
| β-globin reverse primer (25 pmol/µl) | 1 µl |
| TaKaRa Taq (5 units/µl) | 0.5 µl |
| Sample DNA (0.1µg/µl) | 10µl |
| Distilled sterilize water | 26µl |

Table 4: Amplification of β-globin reaction mixture

PCR was performed in a 96 well PTC-200 Peltier Thermal Cycler (MJ Research). Mineral oil was not needed as this thermal cycler has a heated lid. Reaction conditions were as follows: 2 minutes at 94°C; and 30 cycles of 30 seconds at 94°C, 1 minute at 55°C and 1 minute at 72°C. 10 µl of the samples was put on a 1% agarose gel and stained with 1% ethidium bromide. A positive control was run in each experiment consisting of a plasmid with the PCR product inserted into the polylinker region (described below in section 2.6).

**2.6 PCR product purification and cloning**

Given a positive amplification, DNA products were gel purified by using the QIAquick gel purification kit (Qiagen) following the manufacturer's directions. Gel extracted bands were cloned using pGEM®-T Vector System I (Promega). Thermostable DNA polymerases, during PCR, add a single deoxyadenosine, in a template-independent fashion, to the 3′-ends of amplified fragments. pGEM®-T is a linearized plasmid containing deoxythymidine, the compatible overhang for ligation of PCR products. Ligation was performed with T4 DNA ligase, overnight at 4°C, using a 3:1 ratio of gel extraction product of PCR product to pGEM®-T vector. 5µl of the ligation product was used to transform 25µl of Max Efficiency DH5α *Escherichia coli* (Invitrogen). Cells were kept on ice with the plasmid for 30 minutes followed by a 50 second heat shock, and ice again for 5 minutes. One hundred µl of Luria-Bertani (LB) broth was added and the bacteria were shaken at 225 rpm at 37°C in an incubator-shaker. After one hour the bacteria were plated on agar plates containing ampicillin (100µg/ml), X-gal (20mg/ml) and IPTG (200mg/ml). Bacteria were grown overnight and blue and white selection was

performed, where blue colonies were bacteria that contained the empty relegated pGEM®-T vector and white colonies were those that contained the PCR insert. White colonies were picked with a bacteria culture loop and put in 3 mls of LB broth with 100μg/ml of ampicillin, shaken overnight at 300rpm at 37°C in an incubator-shaker. One ml of the bacterial culture was spun down at 14000 rpm in a centrifuge and plasmid DNA was extracted using QIAprep Spin Miniprep Kit following the manufacturer's directions. Insertion of the PCR product into pGEM-®-T insert was determined by performing a digestion using SstII and PstI restriction enzymes and running the project on a gel.

### 2.7 HPV DNA sequencing

Positive HPV samples were sent for sequencing at the McGill University and Genome Québec Innovation Centre. Positive HPV DNA was gel extracted and cloned into pGEM®-T (described in section 2-6) and sequenced using T7 and SP6 RNA polymerases. Sequences obtained were compared to the National Center for Biotechnology Information database by nucleotide-nucleotide BLAST alignment (blastn). Reference sequences used for typing and mutation analysis were obtained from the GenBank126 database under the accession numbers presented in Table5.

| HPV type GenBank | Accession number |
|---|---|
| HPV-6 | NC 000904 |
| HPV-11 | NC 001525 |
| HPV-16 | NC 001526 |
| HPV-18 | NC 001357 |
| HPV-31 | NC 001527 |
| HPV-33 | 001528 |
| HPV-35 | 001529 |
| HPV-52b | NC 001592 |
| HPV-58 | NC 001443 |

Table 5: GenBank database accession numbers for the complete genome of each HPV type

### 2.7.1 Sanger dideoxy sequencing

Sanger dideoxy sequencing was performed following the protocol described in the T7 Sequenase v2.0 PCR product sequencing kit® manual (Amersham BIOSCIENCE)[90] using 33Plabelled dATP. Since the desired PCR product has been purified, enzymatic pre-treatment is omitted. The only modification to the protocol involved the annealing step where the DNA template is snap cooled in the presence of the primer. This is achieved by briefly placing the tube of denatured template and primer into a container of 100% ethanol that has been cooled to – 80 °C. Samples were then transferred to an ice/water bath until sequencing.

### 2.7.2 Electrophoresis and Autoradiography

Samples were loaded on a pre-heated "Long Ranger" acrylamide gel (FMC Bioproducts) using a sharks-tooth comb125 The gel was run on a Gibco BRL S2 manual sequencing apparatus. Short runs of an hour and a half (resulting in data ranging from the primer up to approximately 300-bp) were performed. All runs were performed under the

same conditions: 1200 Volts, 10 mA and 40 W. Upon completion of the desired run(s), gels were transferred to a filter paper and dried for 2 hours at 80 °C using a 583 Gel Dryer® and HydroTech vacuum pump from BioRad. Gels were then placed in a film cassette with Kodak® autoradiography film for a period of time between 2 to 10 days depending on the age and strength of the isotope. Finally, the films were developed manually using Agfa® X-ray film developer and fixer.

### 2.8 Data analysis

Overall survival (OS) was calculated from the date of diagnosis to the date of last follow-up or death of any cause. Disease specific survival (DSS) was calculated from the date of diagnosis to the date of last follow up or the date of death from tongue cancer – patients who died from other causes are not included. Relapse-free survival (RFS) was calculated from the date of treatment completion to the date of documentation of clinical recurrence. Patients lost to follow up were included in all calculations and were censored from the date of last follow up. Survival curves and recurrence rates were plotted with the Meyer-Kaplan method and compared with the log-rank test. Univariate analysis was used to determine prognostic factors. Cox forward regression was used for multivariate analysis. Values are considered significant when $p<0.05$. All p values are two tailed.

# Chapter 3: Results

# 3. Results

## 3.1 Chart Review

A total of 199 patients were recruited. Only, 146 cases were reviewed. Of these, 121 had cancers of the oral cavity and 25 had cancers of the oropharynx; 62% were males, and 38% were females. Smoking is considered a strong risk factor for cancer of the oral cavity and cancer of the oropharynx, with a clear dose–response relationship between cancer risk and increasing number of cigarettes smoked per day. A clear dose–response relationship between risk for cancer and the number of years of smoking was also found. Similarly, the number of alcoholic drinks per day is considered a strong risk factor for cancer of the oral cavity and cancer of the oropharynx.

In the sample population in our study 42 % were smokers, 41% consumers of alcohol, and 30% had both risk factors. Also, we found HPV, from certain subtypes 6, 11, 16, 18, 31, 33, 35, 52b, 58 , was positive only in 0.5 % of the subjects (Figure 3). Carcinomas were distributed as follows: 40% had oral tongue cancer; 20% had floor of mouth; 10% had buccal cancer; 2% had lip cancer; 2% had hard palate; 7% had retromolar trigone cancer; 6% had base of tongue cancer; 3% had soft palate cancer; and, 2% had cancer in multiple sites. Cervical lymph node metastasis was noted in 30% of the patients (Figure 4).

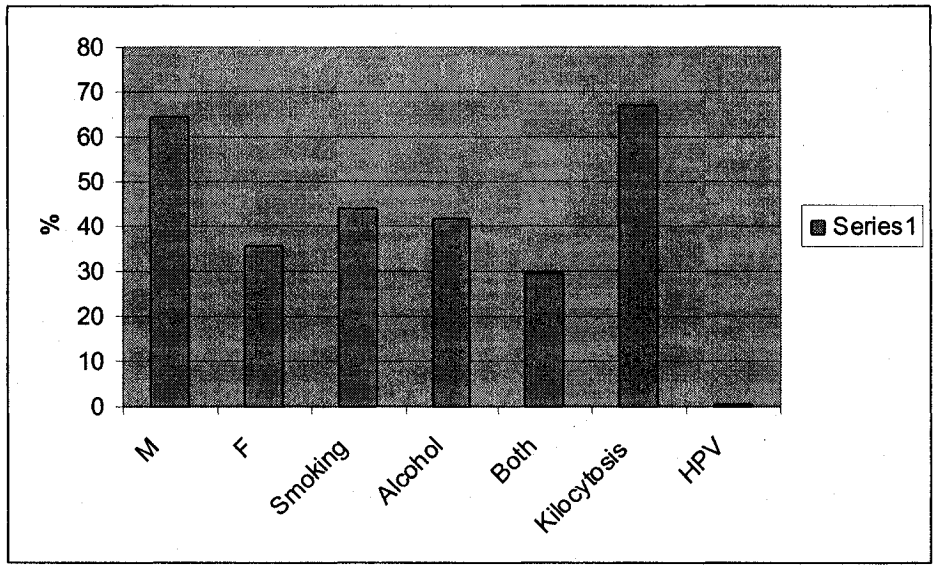


Figure 3: Distribution of a total of 146 patients

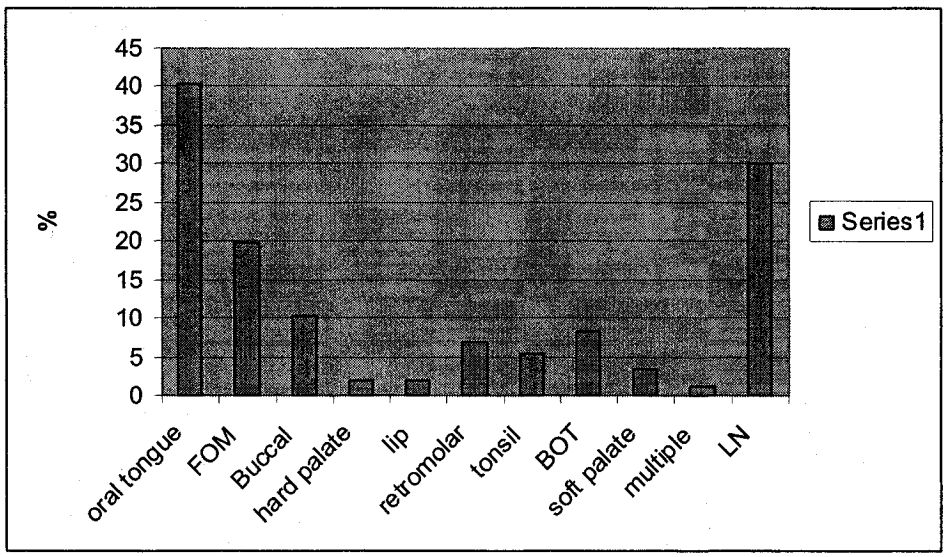


Figure 4: Tumor sites distribution

### 3.2 Koilocytosis and histological differentiation

Koilocytosis was noted in 67% of patients (Figure 3). Histological differentiation of the cancers in the sample population was as follows : 40% well differentiated; 36% moderately differentiated; and 24% poorly differentiated. (Figure 5). More specifically, koilocytosis was present in 67.2% of well differentiated lesions, 65.2% of moderately differentiated lesions, and 68.4% of poorly differentiated lesions (Figure 6).

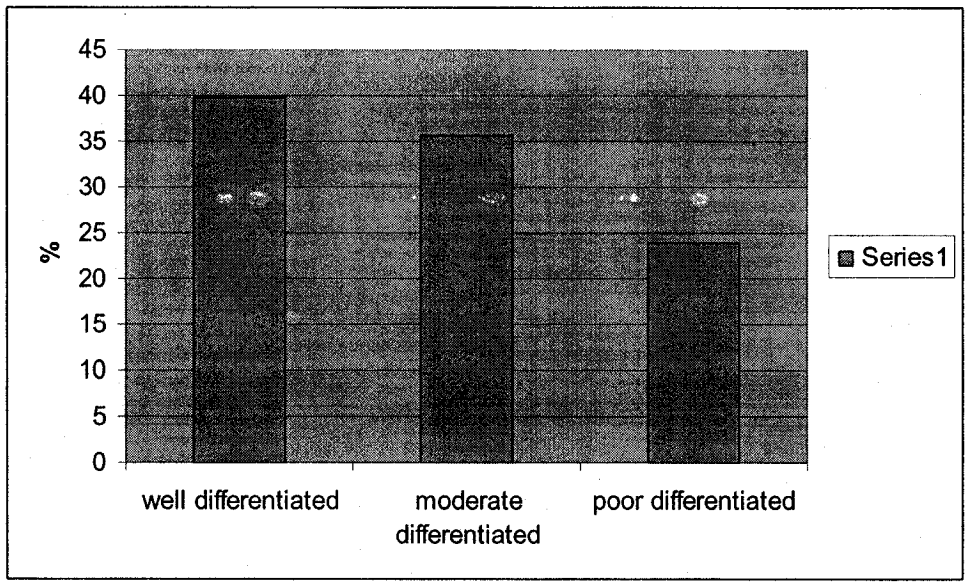


Figure 5: Histological differentiation of the cancers

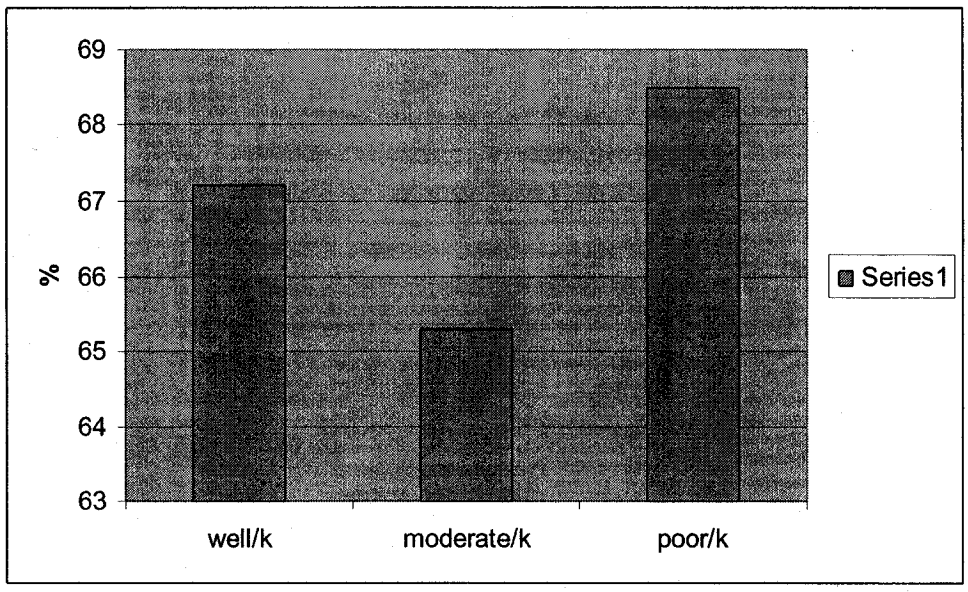


Figure 6: Association of koilcytosis with different histological differentiation

### 3.3 Koilocytosis and tumor grading

Cancer stage was ascertained for 146 (100%) of participating patients and was distributed as follows: 21% stage I, 23% stage II, 18% stage III, and 38% stage IV (Figure 7). Koilocytosis was identified in 73% of stage I, 68% of stage II, 68% of stage III, and 69% of stage IV patients (Figure 8).

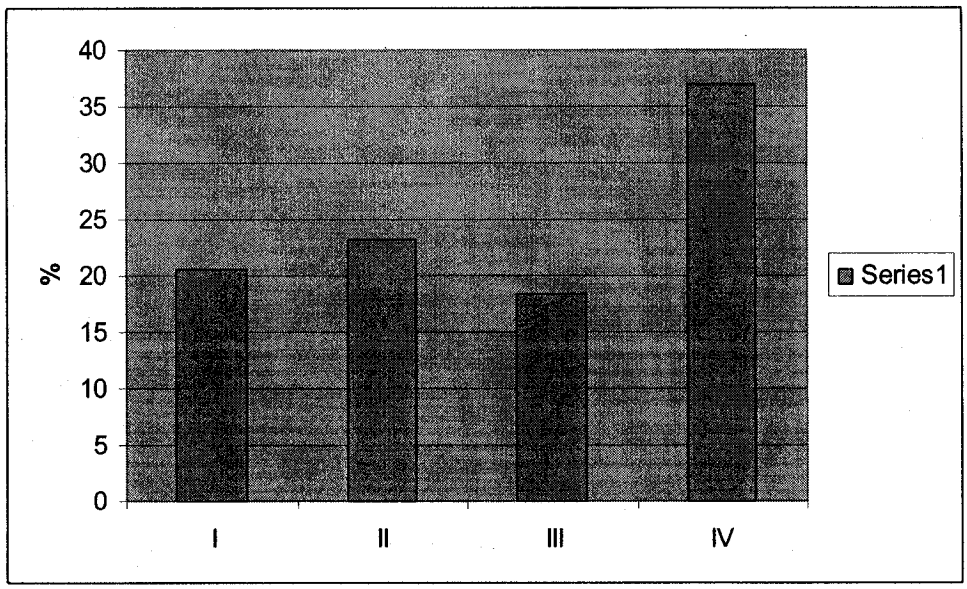


Figure 7: Tumor grading in 146 patients

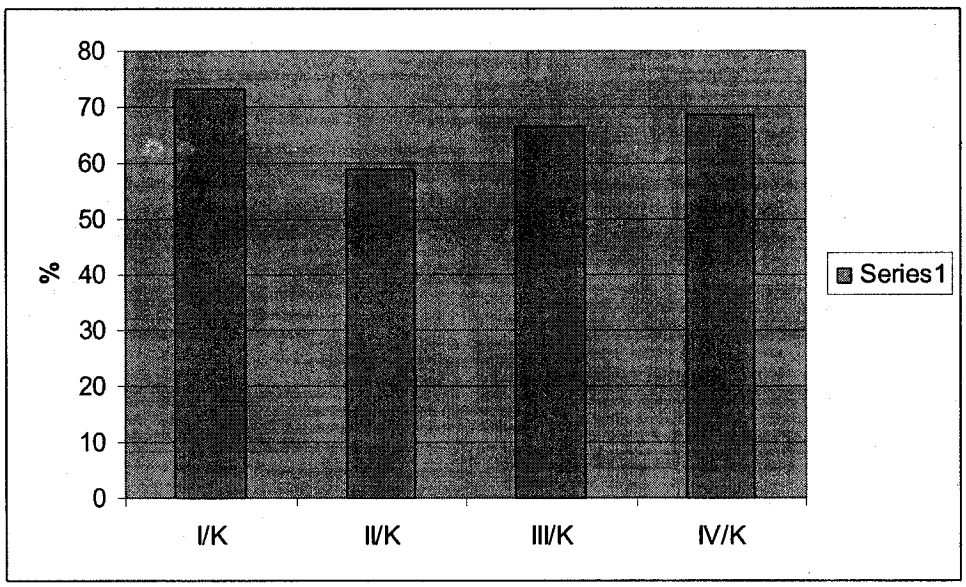


Figure 8: Association of koilocytosis with tumor grading

### 3.4 β-globin PCR

PCR of the β-globin gene was used to determine the quality of the DNA extracted from the paraffin preparations (Figure 9).

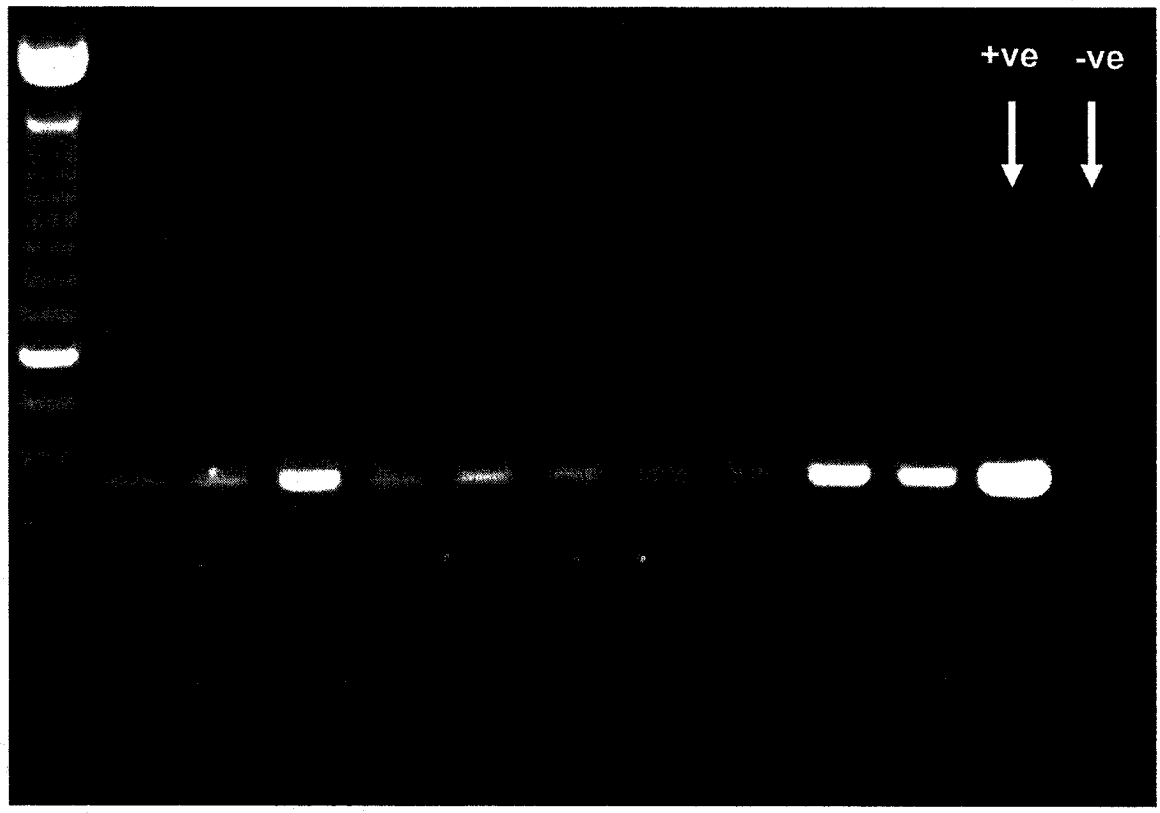


Figure 9: Representative example of β-globin amplification. PCR was performed using primers for β-globin, to determine the quality of the DNA of the samples. The figure is a representative example of 10 positive samples including a positive control and a negative control (indicated by arrows). The primers amplified a 363bp fragment

The primers were constructed so that they would amplify a fragment of genomic DNA bigger than the HPV samples. Of the pool of 191 samples, 36 samples were PCR negative for β-globin amplification and were eliminated from the pool of patients analyzed for HPV.

### 3.5 Malignant HPV PCR

Consensus primers were used for the PCR reactions to detect HPV so-called "high risk" subtypes (see methods Section 2.4). The primers were designed to detect HPV-16, -18, -33, -52b and -58 subtypes. Of the 155 samples that were β-globin positive, only one sample (<0.01%) was PCR positive using the malignant consensus primers (Figure 10). The band for the positive sample was purified from the gel and cloned into pGEM®-T vector by blunt end ligation with T4 DNA ligase. The DNA was sent for sequencing and when compared to the NCBI database, the sequence was 94% identical for the sequence of HPV-35 (NC_001529).

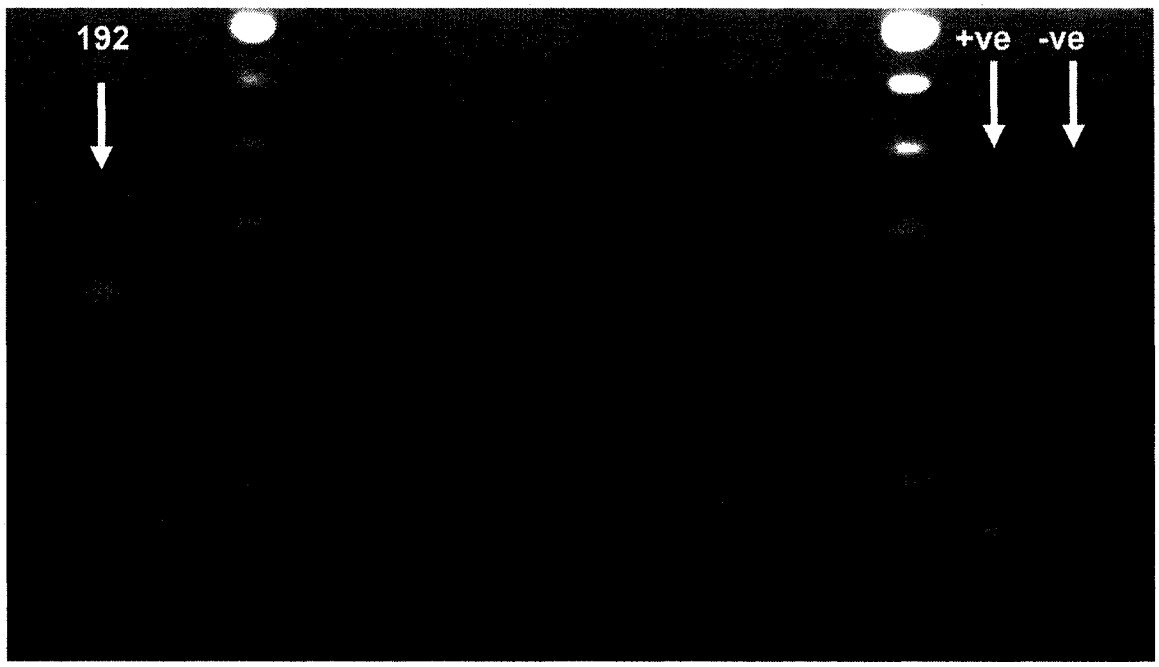


Figure 10: Representative example of so-called "high risk" HPV subtypes amplification. PCR was performed using consensus primers for the malignant subtypes. Including a positive control and a negative control indicated by arrows on the right hand side of picture. Positive sample shown by arrow on the left hand side of this picture.

### 3.6 Benign HPV PCR

Consensus primers were used for the PCR reactions to detect HPV so-called "low risk" subtypes (see methods section 2.4). The primers were designed to detect HPV-6, and -11 subtypes. Of the 155 samples that were β-globin positive, no PCR positive samples were detected using the so-called "low risk" consensus primers (Figure 11).


Figure 11: Representative example of so-called "low risk" HPV subtypes amplification. PCR was performed using consensus primers for the benign subtypes including a positive control and a negative control shown by arrows. Positive samples should lie between 228 bp and 268 bp. No samples were positive for HPV. A 61 bp positive control was used in each experiment

## 3.7 Statistical Analysis

### 3.7.1 Survival

#### 3.7.1.1 Overall survival (OS)

Overall survival (OS) was calculated from the date of diagnosis to the date of last follow-up or death of any cause. The overall survival was calculated for 5 years, and was found to be 71.5 % (Table 6).

| Time (number of years) | No Risk | No Event | Survival | std. err | lower 95% CI | upper 95% CI |
|---|---|---|---|---|---|---|
| 0 | 146 | 4 | 0.873 | 0.01351 | 0.946 | 0.999 |
| 1 | 121 | 11 | 0.884 | 0.02823 | 0.831 | 0.941 |
| 2 | 71 | 3 | 0.847 | 0.0343 | 0.782 | 0.917 |
| 3 | 47 | 2 | 0.811 | 0.04123 | 0.734 | 0.896 |
| 4 | 26 | 1 | 0.78 | 0.05007 | 0.687 | 0.884 |
| 5 | 12 | 1 | 0.715 | 0.0773 | 0.578 | 0.883 |

Table 6: Overall survival (OS) is calculated from the date of diagnosis to the date of last follow-up or death of any cause.

#### 3.7.1.2 Disease specific survival (DSS)

Disease specific survival (DSS) was calculated from the date of diagnosis to the date of last follow up or the date of death from tongue cancer – patients who died from other causes are censored at death. The disease specific survival was found to be 84.5% (Table 7).

| Time ( number of years) | No Risk | No Event | Survival | std. err | lower 95% CI | upper 95% CI |
|---|---|---|---|---|---|---|
| 0 | 145 | 2 | 0.986 | 0.00969 | 0.967 | 1 |
| 1 | 121 | 8 | 0.921 | 0.02404 | 0.875 | 0.969 |
| 2 | 71 | 3 | 0.882 | 0.03184 | 0.822 | 0.947 |
| 3 | 47 | 2 | 0.845 | 0.04005 | 0.77 | 0.927 |

Table 7: Disease specific survival (DSS) is calculated from the date of diagnosis to the date of last follow up or the date of death from tongue cancer – patients who died from other causes are censored at death.

#### 3.7.1.3 Relapse-free survival (RFS)

Relapse-free survival (RFS) was calculated from the date of treatment completion to the date of documentation of clinical recurrence. Patients lost to follow up are included in all calculations and were censored from the date of last follow up. The Relapse-free survival at 3 years was found to be 86.9% (Table 8).

| Time ( number of years) | No Risk | No Event | Survival | std. err | lower 95% CI | upper 95% CI |
|---|---|---|---|---|---|---|
| 0 | 139 | 5 | 0.964 | 0.0158 | 0.934 | 0.995 |
| 1 | 113 | 8 | 0.896 | 0.0275 | 0.843 | 0.951 |
| 2 | 66 | 2 | 0.869 | 0.0327 | 0.807 | 0.935 |

Table 8: Relapse-free survival (RFS) is calculated from the date of treatment completion to the date of documentation of clinical recurrence. Patients lost to follow up are included in all calculations and are censored from the date of last follow up.

### 3.7.2 Univariate analysis:

The Kaplan-Meyer method of analyzing survival curves was used (Table 9) to determine the association between survival and smoking, stage, koilocytosis, family history of cancer, immune status, site, exposure to radiotherapy, exposure to chemotherapy, pathological reports, and presence of radiotherapy complication. Survival was found to be significantly associated only with exposure to radiotherapy ($p=0.000107$) (Table 10), and presence of radiotherapy complications ($p=0.000405$) (Table 11).

For those patients who were treated with radiotherapy (Table 10), 71 patients had radiotherapy alone. The observed number of deaths was 14 patients, while the expected number of deaths was 10.4, yielding the Chi-2 = 2.4. This was statistically significant.

Similar results were found in patients who had been treated with radiotherapy and brachytherapy (6 patients had radiotherapy and brachytherapy). The observed number of deaths was 3 patients, while the expected number of deaths was 0.4, which means the Chi-2=16 (Table 10) is significant.

The association between survival and complications from radiotherapy was significant (Table 11). We found 14 patients had radiotherapy related complications, in which the expected number of patients suppose to have radiotherapy complication was 6.6, which means the Chi-2 was 12.5,. This was statistically significant.

| Survival and different risks | p value |
|---|---|
| Survival ( duration, outcome) and gender | 0.233 |
| Survival ( duration, outcome) and smoking | 0.433 |
| Survival ( duration, outcome) and stage | 0.198 |
| Survival ( duration, outcome) and koilocytosis | 0.206 |
| Survival ( duration, outcome) and family history of cancer | 0.428 |
| Survival ( duration, outcome) and alcohol | 0.373 |
| Survival ( duration, outcome) and immune | 0.113 |
| Survival ( duration, outcome) and site | 0.84 |
| Survival ( duration, outcome) and radiotherapy | 0.000107 |
| Survival ( duration, outcome) and chemotherapy | 0.324 |
| Survival ( duration, outcome) and pathological report | 0.378 |
| Survival ( duration, outcome) and XRT complication | 0.000405 |

Table 9: p value of survival with different risks, Kaplan-Meyer- method

| | N | Observed | Expected | (O-E) 2/E | Chi-2 |
|---|---|---|---|---|---|
| No post op Radiotherapy | 60 | 4 | 9.367 | 3.075 | 5.67 |
| Brachy alone | 9 | 1 | 1.727 | 0.306 | 0.35 |
| Radiotherapy alone | 71 | 14 | 10.468 | 1.192 | 2.41 |
| Radiotherapy + Brachy | 6 | 3 | 0.438 | 14.988 | 16.39 |

Table 10: Survival (duration, outcome) and radiotherapy

| | N | Observed | Expected | (O-E) 2/E | Chi-2 |
|---|---|---|---|---|---|
| Complication without XRT | 100 | 8 | 15.4 | 3.56 | 12.5 |
| Complication with XRT | 46 | 14 | 6.6 | 8.31 | 12.5 |

Table 11: Survival (duration, outcome) and XRT complication

### 3.7.3 Cancer stage and koilcytosis:

The association of the cancer stage and koilocytosis was estimated: the Chi-2 value was not significant (p=0.59).

# Chapter 4: Discussion and Conclusions

# 4. Discussion and Conclusions

## 4.1 Discussion of Results

This study evaluated the association between nine subtypes of HPV infection and cancers of the oral cavity and the oropharynx (HPV DNA in biopsy specimens, HPV subtypes 6, 11, 16, 18, 31, 33, 35, 52b, 58). We recruited more than 199 patients and of these, 146 patients met the selection criteria for this study. Of the 146 patients, 45 patients had positive cervical lymph nodes, yielding a total of 191 samples. Our results indicate that koilocytosis was present in 67% of all cases.

Furthermore, koilocytosis was equally distributed among different stages and histological grades. According to the literature[91-100] the presence of koilocytosis may provide a clue to the presence of HPV infection. In our results we found only one positive sample by PCR, which was HPV subtype 35. Therefore, two possible hypothesis are (1) other HPV subtypes not tested, which may be present, or, (2) koilocytosis may not have the same significance as in the uterine cervix.

Our results indicate that some HPV subtypes (HPV 6, 11, 16, 18, 31, 33, 35, 52b, 58 subtypes) does not appear to play a definite etiologic role in a substantial fraction of cancers of the oropharynx and the oral cavity. As only one case was positive for the HPV subtypes usually purified, this suggests other HPV subtypes should be tested. Cigarette smoking and alcoholic consumption seemed to be greater determinants for the development of cancer.

Lack of HPV detection may depend on the type of primers used in the study. Due to the broad range of human papillomavirus (HPV) types, use of specific primers may limit the sensitivity of the study.[101] To compensate for this problem, consensus primers

were used, where conserved bases between HPV types were used in the primer sequence and mismatches were kept at a minimum.[102] The primers amplified the E6 and E7 regions of HPV, a region of high homology between subtypes of the virus. While this decreases the specificity of the assay, these primers allow for the amplification of many HPV types to occur at the same time, thus increasing the sensitivity of the assay.

On the other hand, increasing sensitivity with the use of consensus primers decreases the specificity of detecting specific subtypes. The efficiency of the PCR reaction may be affected as the binding of the primer to its target may not be optimal. As the number of mismatches was kept low in the primer sequence, amplification of the most frequently studied subtypes of HPV is still possible with high sensitivity.[102]

Samples must be carefully prepared to avoid contamination and sample loss. PCR reactions are subject to inhibitory substances, such as phenolics, carbohydrates and humic acids, which may be introduced in the sample during the purification of DNA from paraffin preparations. These may generate false positives as they would inhibit DNA polymerase activity. After the extraction process we ensured that the samples were sufficient for PCR amplification by measuring the optical density values with a spectrophotometer. Additionally, before proceeding to HPV PCR, we performed a PCR amplification for the β-globin gene. At the same time this enabled us to conclude the quality of the DNA sample and if the quantity was sufficient for the PCR reaction.

The product of the β-globin amplification was 363 base pairs, and any positive samples should be capable of a PCR amplification of products of this size and below. Products of the HPV PCR reactions should yield products that are 228-268 base pairs, well below the size of the β-globin amplicon. After screening of the samples, we

eliminated the β-globin negative (PCR negative) prior to HPV PCR reactions. We used the same amount of DNA for the β-globin PCR reactions as those used for the HPV assays. A positive control was always used alongside the samples, and was positive in every experiment performed. Performing the PCR reactions in this manner allowed us to conclude that negative results were truly PCR negative using the consensus malignant and benign primers.

HPV DNA was detected in tumor biopsy specimens from 0.68% of patients with cancer. This is not entirely unexpected. There is a wide variance of HPV DNA presence in cancer reported in the literature.[90,103-108] Reported prevalence rates for HPV in primary oropharyngeal cancers range between 40% and 60%.[58,59,68,109,110] The prevalence rate may even be higher in lymph node metastases, as HPV-related tumors may have an enhanced propensity for metastatic spread.[68,106]

The vast majority (95%) of HPV-positive in the latter studies contained HPV16, the most common type found in genital cancers. Recent progress in uncovering the molecular genetic mechanisms underlying human cancers is translating into more effective strategies for patient care including refinements in tumor diagnosis. For patients presenting with metastatic squamous cell carcinoma of the head and neck, a practical and reliable method for pinpointing the site of tumor origin could obliviate the need for sophisticated imaging studies, define anatomical sites for directed biopsy and surgical excision, and better demarcate radiation fields.

To date, the overall impact of novel approaches for tumor localization has been limited. Using a microsatellite-based genetic approach, Califano *et al.*[111] were able to match a distinctive pattern of genetic alterations in the metastatic implant to a specific

region of the upper aerodigestive tract and, thus, infer the origin of otherwise occult primary tumors. This matching of genetic fingerprints, however, does not preclude the need for extensive tissue sampling of the upper aerodigestive tract, and it necessitates sophisticated methodologies unavailable to most diagnostic laboratories.

Other approaches have taken advantage of the fact that some HNSCCs are caused by certain oncogenic viruses that target specific regions of the upper aerodigestive tract. In effect, detection of a specific virus in the metastasis implicates the site of tumor origin. Most notably, detection of EBV in a neck metastasis reliably points to tumor origin from the nasopharynx.[112-116] Although this approach is readily feasible for diagnostic laboratories with ISH capabilities, application to this point has been limited, as only a small subset of HNSCCs has been linked to a tumorigenic virus. The potential role of viral probes as a tool to localize tumor origin has been expanded recently with the recognition that the HPV is an important causative factor in the development of a subset of HNSCCs.[1]

Compared to PCR-based techniques, the ISH method is inexpensive and readily feasible for routine use by many diagnostic laboratories. Direct visualization of viral tissue distribution better substantiates HPV as a causal agent. In contrast, the highly sensitive PCR technique may detect HPV DNA present in the sample that is not specific to tumor cell nuclei and, therefore, not of any pathophysiological significance.[108] Expansion of the hybridization armamentarium to provide broader coverage of high-risk HPV subtypes could improve sensitivity, but only at the expense of expediency and cost containment. IHC has not been a suitable alternative. The unreliability of viral oncoprotein detection in formalin-fixed tissues has prompted a search for surrogate

markers of HPV infection that are more feasible for routine IHC. Patients with HPV-induced HNSCC may benefit from HPV-targeted treatment strategies (e.g., therapeutic HPV vaccines[117]).

The majority of literature on oral cavity lesions and HPV has focused on squamous cell carcinomas. Many studies have been performed with a wide array of molecular assays described earlier. Once again, the data range from 0%[118-120] to 100%.[121] Many other reviews have looked at these trends to "tease out" factors that account for the differences between, and among, studies. In a large review of the literature examining the role of HPV in oral lesions, HPV was detected in 13.5% of normal mucosa and 26.2% of squamous carcinoma.[122,123] The authors noted that DNA was more likely to be detected in fresh-frozen than in paraffin-embedded samples, and that the mode of detection was a significant factor in the prevalence reported in various studies.[122] Another large review of head and neck samples noted that the HPV prevalence in HNSC, as detected by PCR, was 34.5%, by ISH 15.8%, and by Southern blot 24.5%.[124] Thus, it is no surprise that, overall, PCR exhibited a higher sensitivity and ability to detect the presence of HPV. However, PCR-positive lesions may have been a result of minute contamination or subclonal infection that does not necessarily indicate a real contribution to carcinogenesis.

The larger studies used PCR detected HPV infection rates of approximately 10 to 15%. Even when the most sensitive of techniques was used, there is still a low rate of detection of HPV in oral cavity malignancies. The significance of HPV DNA presence in the progression to malignancy is still unclear. It *is* clear, however, that oral carcinoma is different from cervical cancer, where HPV infection is necessary for disease

development. This current review highlights the same challenges identified in previous review articles: diverse patient populations with different likely rates of endemic infection, different molecular assays used by a variety of authors, a lack of understanding of the link between HPV and carcinogenesis in the integrated *vs.* the non-integrated state, and an unknown link between HPV DNA presence and activity. While many new studies have proposed epidemiologic, serologic, molecular, and mechanistic roles of HPV and its contribution to oral cancer, there continues to be debate and a wide range of reported prevalence in normal individuals and those with pre-malignant and malignant lesions. In addition to the technical aspects of HPV detection, the simple nomenclature regarding anatomic locations of oral cavity *vs.* oropharyngeal lesions is often unclear. The literature clearly supports the idea that oropharyngeal cancers are more likely to have HPV than other head and neck tumors. Anatomically, the oral cavity and oropharyngeal border is the posterior 1/3 of the oral tongue, which is clinically difficult to delineate in many cases. Thus, there may be a significant portion of oropharyngeal tumors that are included in the oral cavity group, falsely elevating the number of HPV-positive samples, or *vice versa*.

HPV 35 a new human papillomavirus (HPV) type, was detected by low-stringency Southern blot hybridization analysis of DNA from an endocervical adenocarcinoma.[125] The genomic DNA of the virus, which was obtained as two BamHI fragments of 3.75 and 4.1 kb, was molecularly cloned into lambda L47 and subsequently subcloned into pBR322 for further characterization. Hybridization studies demonstrated that these viral DNA isolates were only distantly related to other HPV and thus represented a new type of HPV, called HPV 35. A restriction enzyme map was prepared

which allowed a comparison of the genetic organization of this HPV with that of HPV 6b; the results demonstrated collinearity of the HPV 35 genome with that of HPV 6b. Prevalence studies revealed that HPV 35 was present in 2 of 158 (1%) anogenital intraepithelial neoplasia and in 3 of 69 (4%) anogenital cancers. Thus HPV 35 is a low-prevalence human papillomavirus associated with anogenital intraepithelial neoplasia and cancer.

The overall survival at 3 years was 81.1 % (Table 6); the disease specific survival after 3 years was 84.5 % (Table 7); the relapse-free survival after 3 years was 86.9 % (Table 8). The overall survival, disease specific survival, and relapse-free survival, after 3 years were similar. We did not find smoking, and tumor grading effect significantly on survival (Table 9). However, we found exposure to radiotherapy and radiotherapy complications showed decrease survival (Table 10, 11). We found the association of the cancer stage and koilocytosis was not significant ($p=0.59$).

**4.2 Summary**

HPV has been shown to be a significant carcinogen in cervical cancer, but the significance of human papilloma virus' contribution to oral squamous cell carcinoma has been studied for several decades and remains debated. Putative molecular mechanisms have been identified that clearly demonstrate its ability to disrupt key cellular elements responsible for the regulation of cell division and apoptosis. However, while epidemiologic and molecular data provide some evidence of high-risk HPV presence in oral pre-malignant and malignant lesions, it may exist in only a small minority of cases. Thus, HPV may be a contributing factor in a subset of oral malignancies but is not a necessity in all cases, as it is in cervical cancer. Alternatively, different subtypes (which

this study did not examine) may be involved. Further studies using newer molecular techniques will shed light on this controversial topic and clarify the prevalence of HPV DNA in these samples and, more importantly, elucidate the significance of HPV infection in the oral cavity.

Koilocytosis was present in 67% of all cases; it was equally distributed among different stages, and histological grades. According to the literature,[91-100] the presence of koilocytosis may give a clue to the presence of HPV infection. In our results only one sample was positive by PCR. Therefore, either koilocytosis is an indicator for other subtypes of HPV. According to the results of the present study, HPV does not appear to play a definite etiologic role in a substantial fraction of cancers of the oropharynx and the oral cavity.

HPV DNA was detected in tumor biopsy specimens from 0.68% of patients with cancer. Because koilocytosis is equally distributed among the histological grades, and stages, we could not assess whether the presence of koilocytosis plays a role in prognosis and, because HPV is negative, we do not know if koilocytosis is a marker of HPV. We could not establish the relation between HPV and prognosis.

The overall survival at 3 years was 81.1 % (Table 6), the disease specific survival after 3 years was 84.5 % (Table 7), the relapse-free survival after 3 years was 86.9 % (Table 8). The overall survival, disease specific survival, and relapse-free survival, after 3 years are similar. We did not find smoking, and tumor grading to have a significant effect on survival (Table 9). However, we found exposure to radiotherapy and radiotherapy complications showed decreased survival (Table 10, 11). We found that the association of the cancer stage and koilocytosis was not significant ($p=0.59$).

## 4.3 Conclusions

1- In our results we found only one positive sample by PCR. Therefore, two possible explanations are (1) other HPV subtypes not tested, which may be present, or, (2) koilocytosis may not have the same significance as in the uterine cervix.

2- Human papillomavirus (HPV 6, 11, 16, 18, 31, 33, 35, 52b, 58 subtypes) unlikely to cause oral & oropharyngeal cancers.

3- Smoking & alcohol are the most frequent causes of such cancers.

4- The overall survival, disease specific survival, and relapse-free survival, after 3 years are similar(+80%).

5- Exposure to radiotherapy and radiotherapy complications were associated with decrease survival.

6- The association of the cancer stage and koilocytosis was not significant (p=0.59).

## 4.4 Future Directions

The relation between HPV and head & neck cancers is subject to ongoing investigations and is slowly being understood. Our results indicate that some HPV subtypes(HPV 6, 11, 16, 18, 31, 33, 35, 52b, 58 subtypes) do not appear to play a definite etiologic role in a substantial fraction of cancers of the oropharynx and the oral cavity. This is not entirely unexpected. There is a wide variance of HPV DNA presence in cancer reported in the literature. As only one case was positive for the HPV subtypes usually purified , this suggests other HPV subtypes should be tested.

This study has introduced the notion that the oropharynx and the oral cavity were not associated with some HPV subtypes. However, by performing a PCR amplification for the β-globin gene. At the same time this enable to assess the quality of the DNA

sample and if the quantity is sufficient for the PCR reaction. Also, using PCR primers that detect very wide numbers of HPV subtypes may help to the association of head & neck cancers and a wide number of HPV subtypes.

Further studies using newer molecular techniques will shed light on this controversial topic and clarify the prevalence of HPV DNA in these samples and, more importantly, elucidate the significance of HPV infection in the oral cavity.

The possible integration of the HPV with head & neck cancers concepts will have major clinical implications on the diagnosis and management of head & neck cancers. The potential role of viral probes as a tool to localize tumor origin has been expanded recently with the recognition that the HPV is an important causative factor in the development of a subset of HNSCCs. Patients with HPV-induced HNSCC may benefit from HPV-targeted treatment strategies (e.g., therapeutic HPV vaccines[117]).

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## Appendix 1: Consent Form (English)

# Informed Consent Form

## Detection of Human Papilloma Virus in primary site of oral/ oropharyngeal cancer and in cervical lymph nodes: Correlation with clinico-pathological parameters and prognostic significance

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McGill Department of Otolaryngology, Head and Neck Surgery

### Purpose of the Study

There is some new research that links diseases of the head and neck to a virus called the Human Papilloma virus (also called HPV). It is a virus that is everywhere, on your hands and on the ground. It is the same virus that gives warts and that is involved in cervical cancer. There are more than a hundred types of HPV and those that cause warts are different than those that cause cervical cancer.

We think that some cases oral/oropharyngeal cancer may also be caused by HPV. The goal of our study is to see if HPV was involved in your disease and to see if has any link to how your disease behaved. This information may help us find new ways to prevent and treat the diseases in the future.

We can detect HPV by looking at your DNA, the molecule that contains all your genetic information and controls the activities of your cells. DNA can also provide information that determine your hereditary characteristics like your eye color, but we will not be looking at that part of the DNA in this study. We are only interested in the virus DNA that is incorporated in your DNA.

You are being asked to participate in this study because you had oral/oropharyngeal cancer. Oral/oropharyngeal cancer may be linked to a viral infection and we would like to test if this virus was present in the tissue that was removed.

Your participation in this study means that you will permit the use of the DNA from the tissue we removed during your biopsy or your surgery. We will be looking for the presence of the virus DNA. We will review your hospital chart to see if you had any factors that increased your risk to develop the disease and how the disease behaved in your particular case. This should help us understand how the disease behaves and how it is related to the virus in oral/oropharyngeal cancer.

## Progression of the research project

### Procedures

Since the material we will be using was removed at the time of your surgery, there are no additional procedures involved in this research.

**Medical visits**

You will not be required to have any additional visit to your physician or other health specialist as part or as a result of this study.

**Duration of the research project**

We expect the current research project to last a year.

**Scope of the research project**

This research involved patients from the Montreal General Hospital, the Royal Victoria Hospital and the Jewish General Hospital. The laboratory where the research will be based is at the Meakins-Christie Laboratories.

**Access to your medical record**

The research team will consult your medical record to obtain information, which is pertinent to the research project. This information will not be shared with anyone else.

**Combination with other information**

Your name will not be forwarded to any other organization.

## Collection, research and storage of genetic material

### Identification of the sample

We will protect the confidentiality of the samples by assigning them a specific code. Your DNA sample will not be specifically identified but a code will link you to the sample. Decoding can only be performed by the principal researcher or an individual authorized by the principal researcher.

### Length of storage

Samples of your DNA will be kept at at the Meakins-Christie Laboratories under the responsibility of Dr. Hamid for 5 years after the end of the research project. After this time, all the DNA samples will be destroyed using an enzyme and then discarded appropriately.

### Other research

The samples will be used only for the purposes of this research project.

## Benefits

You will receive no personal benefit from your participation in this research project. We hope, however, that the results obtained will permit us to further our knowledge in the area of oral/oropharynx by allowing us to determine if there is a link between HPV and oral/oropharyngeal cancer and eventually, benefit society as a whole.

## Risks

One of the risks associated with this research project relates to the disclosure of the results or the disclosure of your participation to third parties. Mere participation in genetic research projects could compromise or diminish your chances and the chances of your family obtaining insurance (life insurance, disability, mortgage or health) or certain types of employment.

## Confidentiality

### Safety/security of the data

All of the information obtained about you and the results of the research will be treated confidentially. This information will be coded and the code list will be locked in a filing cabinet in the investigator's office with limited access. Electronic files will be kept strictly confidential by all investigators and collaborators. Your participation and the results of the research will not appear in your medical record. The results of this study may be published or communicated in other ways, but it will be impossible to identify you.

**Third-party access to results**
Unless you have provided specific authorization or where the law permits or a court order has been obtained, your personal results will not be made available to third parties such as employers, governmental organizations, insurance companies or educational institutions. This also applies to your spouse, other members of your family and your physician.

However, for the purposes of ensuring the proper management of the research, it is possible that a member of an ethics committee, a Health Canada representative or the reviewers of a journal where the results will be published may consult your research data as well as your medical record.

## Communication of results

You can communicate with the research team to obtain information on the status of the work or the general results of the research project. Project updates will be posted on our website and will communicate with you by mail at the end of the project.

## Commercialization

The analysis of your DNA sample may contribute to the creation of commercial products from which you will receive no financial benefit.

## Voluntary participation

Your participation is completely free and voluntary. The quality of medical services available to you will not be affected by your decision. You may take the time necessary to reflect on your decision and discuss your participation in the project with persons close to you before giving us your answer.

You are free to withdraw from the study at any time without affecting your present or future medical care. If you withdraw, your DNA sample will be retraced and destroyed.

## Civil liability

If you suffer any injury as a result of your participation in this project, emergency care will be available.

## Resource persons

If you would like additional information regarding the research project or would like to communicate any change of address to us, you can contact :
Ombudsman (MUHC/MGH) ,tel 514-934-1934,ext 48306

Laurie Berlin (JGH ) ,tel 514-340-8222,ext 5833

## Questions

Dr. Karen Kost, the principal investigator or Dr. Khalid AL-Qahtani , researcher explained the nature and the progression of the research project. I can reach Dr. Kost at 514 934 1934 ext. 42273 or Dr. Khalid AL-Qahtani 514 249-9942 at any time regarding this study. I have familiarized myself with the consent form and have received a copy. I have had the opportunity to ask questions that have been answered. Upon reflection, I agree to participate in this research project.

## Consent statement

| | | |
|---|---|---|
| **Participant** | **(Print Name)** | **Date** |

## Verbal translation

I was present during the meeting between the research team member and the participant. I translated, for the participant, the consent form and all information conveyed/presented regarding the research project.

| | | |
|---|---|---|
| **Translator** | **(Print Name)** | **Date** |

## Agreement of the researcher

The research project, as well as the conditions of participation, were described to the participant. A member of the research team answered any questions and explained that participation was free and voluntary.

| | | |
|---|---|---|
| **Investigator** | **(Print Name)** | **Date** |

## Appendix 2: Consent Form (French)

# Informed Consent Form

## Detection of Human Papilloma Virus in primary site of oral/ oropharyngeal cancer and in cervical lymph nodes: Correlation with clinico-pathological parameters and prognostic significance

### Chercheurs

Dr. Karen Kost, Oto-rhino-laryngologiste, Chirurgienne de la Tête et du Cou, CHUSM
Dr. Martin Black, Oto-rhino-laryngologiste, Chirurgien de la Tête et du Cou, Hôpital Général Juif
Dr. Michael Hier, Oto-rhino-laryngologiste, Chirurgien de la Tête et du Cou, Hôpital Général Juif
Dr. Gerald Domanowski, Pathologiste, CHUSM
Dr. Qutayba Hamid, MD,PhD,FRCP, Laboratoires Meakins Christie
Dr. Khalid AL-Qahtani, Reisdent en Otolaryngologie, Université McGill

### Institutions

Centre Universitaire de Santé McGill (Hôpital Général de Montréal et Hôpital Royal Victoria), Hôpital Général Juif

### Funding

Recherche sur le cancer de la Tête et du Cou, Département d'Otorhinolaryngologie, Université McGill

### But de l'étude

Des recherches récentes semblent indiquer un lien entre les maladies de la tête et du cou et le virus du papillome humain (VPH). Ce virus est partout, sur vos mains, sur le sol. C'est le même type de virus qui cause les verrues et le cancer du cervix. Il y a plus de 100 variétés de ce virus et ceux qui causent les verrues sont différents de ceux qui causent le cancer.

Nous croyons que certains cas de cancer de la cavité buccale sont causés par ce virus. Le but de notre étude est de tester le tissue que nous avons enlevé lors de votre chirurgie

pour voir si le tissu était infecté par le virus. Cette information devrait nous aider à prévenir et traiter les maladies de langue

Nous pouvons sétecter la presence du virus en analysant votre ADN, cette molécule qui contient votre infomation génétique. L'ADN contient aussi de l'information qui détermine vos caractéristiques héréditaires comme la couleur de vos yeux. La seule analyse d'ADN que nous ferons vise le virus VPH. Nous ne ferons pas d'autre analyse génétique.

Nous vous demandons votre participation dans cette recherche parce que vous avez été atteint(e) d'un cancer de la cavité buccale. Nous croyons que ce cancer peut être lié à une infection virale et nous aimerions vérifier si ce virus est présent dans le tissu de que nous avons enlevé de votre langue lors de votre chirurgie.

En acceptant de participer à cette étude, vous nous permettez d'analyser l'ADN provenant des tissus que nous avons enlevés lors de votre chirurgie. Vous nous permettez aussi de réviser votre dossier médical pour tenter de déterminer si vous avez des facteurs de risque pour ce type de cancer et évaluer comment le cancer s'est comporté. Ceci nous permettra de mieux comprendre les causes du cancer de la cavité buccale.

## Processus de recherche

### Procédures

Puis que le tissue que nous allons utiliser provient de la chirurgie que vous avez déjà eue, vous n'aurez pas à avoir aucune autre procédure médicale ou chirurgicale.

**Visites Médicales**

Pour participer à cette étude, vous n'aurez pas à faire aucune visite médicale supplémentaire ni a rencontrer aucun autre professionnel de la santé.

**Durée du projet de recherche**

Ce projet de recherche devrait durer un an.

**Portée du projet de recherche**

Ce projet rassemble les patients des hôpitaux suivants : Montréal Général, Royal Victoria et Général Juif. Le laboratoire de recherche sera basé au centre Meakins-Christie.

**Accès à votre dossier médical**

L'équipe de recherche consultera votre dossier médical pour obtenir seulement l'information pertinente à cette recherche. Aucune information ne sera révélée à qui que ce soit hors du groupe de recherche.

**Autres informations personnelles**

Votre nom ne sera pas donné à aucune autre organisation.

## Collecte, recherche et protection du matériel génétieque

### Identification du spécimen

Nous protégerons la confidentialité des spécimens en utilisant un système de codes. Votre ADN ne sera pas identifié de manière personnelle, mais un code sera utilisé pour lier votre nom à votre éprouvette. Le système de code sera accessible seulement aux personnes autorisées par le chercheur principal.

**Durée de rangement**

Vos spécimen d'ADN seront conservés au laboratoire Meakins-Christie sous la responsabilité du Dr. Hamid pour une durée totale de 5 ans après complétion de ce projet de recherche. À cette date, vos spécimens seront détruits en utilisant une enzyme puis ils seront jetés de manière appropriée.

**Autre recherche**

Les spécimens ne seront utilisés seulement que dans le cadre de ce projet de recherche.

## Bénéfices

Votre participation dans notre étude ne vous apportera pas de bénéfice personnel direct. Cependant, votre participation nous aide à mieux comprendre votre maladie et pourrait éventuellement nous aider à développer de nouvelles techniques pour prévenir et traiter votre maladie et éventuellement la société en général.

## Risques

Un des risques associés à votre participation à notre étude tient à la divulgation des résultats de cette étude ou de votre participation à cette étude. Le seul fait de participer à une étude génétique pourrait compromettre ou diminuer vos chances et / ou celle des membres de votre famille d'obtenir de l'assurance (vie, incapacité, emprunt ou santé) ou certains types d'emploi.

## Confidentialité

**Accès et sécurité de l'information**

Toutes les informations collectées à votre sujet, de même que les résultats de cette recherche seront traité de manière strictement confidentielle. Toutes les informations seront codées et la liste de déchiffrage des codes sera gardée dans une filière sous clé au bureau du chercheur principal. Toute information électronique sera traitée de manière strictement confidentielle par tous les chercheurs et collaborateurs. Votre participation et les résultats de la recherche n'apparaîtront pas dans votre dossier médical. Les résultats de cette étude pourront êtres publiés ou communiqués au public, mais ce sans qu'il soit possible de vous identifier.

**Accès aux résultats**
À moins d'avoir obtenu votre autorisation expresse ou que la loi ne le permette ou que votre dossier de recherche soit sommé d'un ordre de la cour, vos résultats personnels ne seront pas accessibles à qui que ce soit hors du groupe de recherche (donc pas accessible à un employeur, organisation gouvernementale, compagnie d'assurance, ou institution d'enseignement). Ceci s'applique aussi à votre partenaire, aux membres de votre famille et à votre médecin.

Cependant, il est possible que pour assurer la qualité de ce projet de recherche, un membre du comité d'éthique, un représentant de Santé Canada ou que les éditeur d'un journal scientifique où les résultats de cette recherche seront publiés, consulte les données de recherche obtenues et votre dossier médical.

## Communication des résultats

Vous pouvez communiquer avec les membres de l'équipe de recherche pour obtenir de l'information sur le statut de ce projet de recherche. Des nouvelles regardant le progrès de notre recherche seront publiés sur notre site internet et nous communiqueront avec vous par courrier lors de la complétion de notre projet.

## Commercialisation

L'analyse de votre ADN pourrait contribuer à la création de produits commerciaux dont vous ne recevrez aucun bénéfice.

## Participation Volontaire et Retrait de Votre Participation

Votre participation dans cette recherche est strictement volontaire. Vous pouvez refuser de participer ou pouvez retirer votre participation en tout temps, et ceci sans aucune explication nécessaire, pénalité ou perte de bénéfice. Si vous décidez de ne pas participer ou si vous voulez retirer votre participation, vous n'aurez aucun préjudice par rapport à vos soins médicaux ou à votre participation dans d'autres projets de recherche. Les chercheurs peuvent annuler votre participation en tout temps si ceci semble être dans votre meilleur intérêt.

## Responsabilité civile

Si vous souffrez de quelque dommage physique résultant de cette étude, des soins d'urgence seront disponibles.

## Personnes ressources

Si vous désirez des information supplémentaires en lien avec ce projet de recherche, ou voudriez communiquer un changement d'addresse, veuillez contacter :
Ombudsman (MUHC/MGH) ,tel 514-934-1934,ext 48306
Laurie Berlin (JGH ) ,tel 514-340-8222,ext 5833

## Questions

La docteure Karen Kost, la responsable de ce projet de recherche, ou Dr. Khalid Al-Qahtani, chercheur, sont disponibles pour discuter toutes procédures, la nature et le progrès de ce projet de recherche. Vous pouvez contacter Dr. Kost au 514 934 1934 ext. 42273 ou Dr. Khalid Al-Qahtani au 514 249 9942 en tout temps.

J'admets m'être familiarisé(e) avec cette formule de consentement et j'en ai reçu une copie. J'ai eu l'opportunité de poser des questions auxquelles ont m'a répondu de manière satisfaisante. Après considération, j'accepte de participer à ce projet de recherche.

## Déclaration de Consentement

J'ai lu ce document de consentement, et désire participer à l'étude proposée. On m'a donné l'opportunité de poser des questions et on a répondu à toutes mes questions. J'ai eu un temps suffisant pour considérer cette offre et pour obtenir conseil si je le désire. On me donnera une copie signée de ce document de consentement. En signant ce document, je maintiens tous mes droits légaux.

_____ _____

**Participant** (Nom) **Date**

## Traduction verbale

J'étais présent(e) durant la rencontre entre le membre de l'équipe de recherche et le participant. J'ai traduit la formule de consentement pour le participant, de même que toute information apportée par le chercheur sur le projet de recherche

_____ _____

**Traducteur** (Nom) **Date**

## Déclaration du chercheur

Le projet de recherche, de même que les conditions de participation on été discutées avec le participant. Un membre de l'équipe de recherche a répondu à toutes les questions posées par le participant et expliqué que sa participation était libre et volontaire.

_____ _____

**Chercheur** (Nom) **Date**

## Appendix 3: Ethics Approval

May 31, 2004

Dr. Karen Kost
Department of Otolaryngology
Montreal General Hospital
1650 Cedar Avenue
Montreal, Quebec H3G 1A4

Dear Dr. Kost,
We have received your request for review by the Institutional Review Board, Faculty of Medicine, of the research proposal entitled "Detection of Human Papilloma Virus in Primary Site of Oral/Oropharyngeal Cancer and in Cervical Lymph Nodes: Correlation with Clinico-Pathological Parameters and Prognostic Significance", submitted by your Otolaryngology resident, Dr. K. Al-Qahtani

As this study involves no more than minimal risk and in accordance with Article 1.6 of the Canadian Tri-Council Policy Statement of Ethical Conduct for Research Involving Humans and U.S. Title 45 CFR 46, Section 110 (b), paragraph (1), we are pleased to inform you that approval for the study (May 2004) and consent form (May 2004) was provided via an expedited review by the Chair on May 31, 2004, valid until **May 2005.** The study proposal will be presented for corroborative approval at the next meeting of the Committee on August 23, 2004, and a certification document will be issued to you at that time.

A review of all research involving human subjects is required on an annual basis in accord with the date of initial review and approval. The annual review should be submitted at least one month before **May 2005**. Should any modification to the study occur over the next twelve months, please advise IRB appropriately.

We ask you to take note that it is the responsibility of the investigator to deposit a copy of the approved research protocol and consent form with the Research Ethics Board of each hospital where study data is collected. **Failure to do so may result in the invalidity of data collected and possible freezing of research funds.**

Yours sincerely,
Serge Gauthier, M.D.
Chair
Institutional Review Board

cc: Dr. K. Al-Qahtani
Ms. F. Cantini – JGH
Ms. E. Boyle – MUHC/MGH
Ms. L. Fateen – MUHC/RVH
AØ5-M59-04B