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Human papillomavirus infection of the oesphagus and its association with squamous cell carcinoma : a restropective study of cases seen at Chris Hani Baragwanath hospital in 2009 and 2010

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HUMAN PAPILLOMAVIRUS INFECTION OF THE OESOPHAGUS AND ITS ASSOCIATION WITH SQUAMOUS CELL CARCINOMA: A RETROSPECTIVE STUDY OF CASES SEEN AT CHRIS HANI BARAGWANATH HOSPITAL IN 2009 AND 2010.

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0102003D

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of

Master of Medicine in the branch of Anatomical Pathology

October, 2014

DECLARATION

I, Dawn-Lee van der Byl, declare that this research report is my own work. It is being submitted for the degree of Master of Medicine in the branch of Anatomical Pathology to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



30 October 2014.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

CONFERENCE PRESENTATION

Van der Byl D, Hale MJ, Naidoo S.

Human papillomavirus infection of the oesophagus and its association with squamous cell carcinoma: A retrospective study of cases seen at Chris Hani Baragwanath Hospital in 2009 and 2010.

Presented as a poster presentation at IAP 2012.

ABSTRACT

Background

There are two principal types of oesophageal carcinoma with squamous cell carcinoma being the most common globally. There are several different etiological factors in the development of oesophageal squamous cell cancer (OSCC). The significance of these risk factors appears to vary by region. The human papillomavirus has been implicated in the aetiology of OSCC. Furthermore, it has been postulated that the differences in the prevalence of HPV in OSCC is related to the geographical region in which the study was conducted with HPV playing a more significant role in high risk areas.

Aim

The aim of this study was to establish the presence or absence of HPV infection in the oesophagus and any association it may have with oesophageal squamous cell carcinoma.

Materials and methods

All cases of oesophageal squamous cell carcinoma that were diagnosed between 01 January 2009 and 31 December 2010 at Chris Hani Baragwanath Hospital were included in the study. All the cases were assessed for the presence or absence of condylomatous atypia. PCR was performed on all the cases as well as on 10

cases of normal control oesophageal biopsies. PCR was performed using the GP5+ and GP6+ primers.

Results

The database search revealed 96 eligible cases of which 95 showed amplification of the β-globin gene and underwent PCR. Of these, two cases showed histological features of condylomatous atypia. All 95 cases failed to demonstrate the presence of HPV DNA by PCR.

Conclusion

HPV is a common infection in the South African population and is known to be carcinogenic in other sites. Its role in OSCC has remained controversial, however, this study does not support the theory that HPV has a role to play in the pathogenesis of OSCC.

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ABBREVIATIONS

CI: Confidence interval

DNA: Deoxyribonucleic acid

H&E: Haematoxylin and eosin

HPV: Human papillomavirus

ISH: In situ hybridisation

OSCC: Oesophageal squamous cell cancer

Pap: Papanicolaou

PCR: Polymerase chain reaction

CHAPTER 1

1.0 INTRODUCTION

There are two principal types of oesophageal carcinoma, namely adenocarcinoma of the oesophagus and squamous cell carcinoma of the oesophagus, with squamous cell carcinoma being more common than adenocarcinoma globally. Several other epithelial and non-epithelial tumours have also been described, such as carcinoid tumours, sarcomas and melanomas [(1)].

Adenocarcinoma of the oesophagus most commonly arises in the background of Barrett oesophagus which develops in the setting of chronic gastro-oesophageal reflux disease [(2)].

There are several different etiological factors in the development of oesophageal squamous cell cancer (OSCC). The significance of these different risk factors appears to vary by geographical location [(3)].

Alcohol and tobacco are considered the primary aetiology of most cases of OSCC that are diagnosed in Western countries, accounting for more than 90% of the cases. Although both are significant in the aetiology of OSCC individually, together the effects of their toxins are multiplied, increasing the chances of developing OSCC [(4)]. Another substance of abuse that is implicated as a cause is opium [(4, 5)], which when smoked, exposes the individual to carcinogens that may be involved in

the aetiology of OSCC [6].

Nutritional factors as a cause for OSCC include temperature factors, nutritional deficiencies and contamination. In parts of South America where a herbal beverage is consumed very hot, the almost burning temperature of the drink is considered to be a risk factor for the development of OSCC possibly due to the chronic oesophagitis caused by the excessively high temperatures. This has been mirrored in studies from other countries assessing the intake of other hot drinks [4]. In some regions, deficiencies of vitamins and trace elements are considered critical to the development of OSCC [5].

Contamination of food by fungi is known to potentially produce nitrosamines or the precursors of nitrosamines. These are thought to be important in the development of OSCC [5]. In South Africa it is the consumption of Fusarium fungal contaminated home-brewed beer that has been postulated as the aetiological agent owing to its nitrate reducing capabilities and its subsequent production of carcinogenic nitrosamines [7].

Human papillomavirus (HPV) has been implicated in the aetiology of OSCC but this has not been proven definitively [4]. This association, however, seems to be most significant in high risk areas only [1].

In view of South Africa's high risk rate for oesophageal carcinoma, I decided to investigate the presence of HPV DNA in OSCC in a large series of patients attending Chris Hani Baragwanath Hospital.

CHAPTER 2

2.0 AIMS AND OBJECTIVES

The aim of this study was to determine the presence or absence of HPV DNA in oesophageal squamous cell carcinoma. If HPV DNA is detected in a statistically significant number of OSCC cases and not in normal oesophageal biopsies a causal relationship can be supported.

The objectives were to:

1. Determine the presence of HPV-DNA using PCR in a large series of cases diagnosed over a two year period.
2. Determine the age, gender and race of the patients diagnosed with this form of carcinoma.
3. Assess for the presence or absence of condylomatous atypia in cases of OSCC and compare this to the PCR findings.

CHAPTER 3

3.0 LITERATURE REVIEW

3.1 Epidemiology and demographics of squamous cell carcinoma of the oesophagus

In 2002, cancer of the oesophagus was the sixth most diagnosed cancer in men and the ninth most diagnosed cancer in women globally as reported by the International Agency for Research on Cancer. It has a dismal 5-year survival rate of less than 10% worldwide [3]. It has a high variation in incidence worldwide, with more than a fifty- fold variation in incidence between high and low risk areas [8].

The countries with the highest documented risk for oesophageal cancer are parts of Iran, China, Kazakhstan [4] as well as East and South Africa [8].The areas with the lowest recorded levels of oesophageal cancer are Western Africa and South Eastern parts of Asia [8].

Oesophageal squamous cell carcinoma is diagnosed more frequently in men than women with a 2-10 times higher incidence [4]. It is most common in adults over the age of 45 years [1].

Racial and ethnic differences have been noted in the incidence of OSCC. It is more prevalent amongst African-Americans as compared to Caucasian Americans [4] with a nearly six times higher incidence in African Americans [1]. In central Asia,

Caucasians are at a lower risk as compared to individuals of Turkish or Mongolian descent [4].

3.2 HPV oncological potential

HPV has been investigated with relation to cervical cancer for many years [9]. Through this well established association, it has earned the reputation of having oncogenic potential [5]. It has further been linked to cancer of the vulva, vagina, penis, anus and oropharynx [9]. Its role in the causation of OSCC remains inconclusive in the literature [10].

3.3 HPV subtypes

To date, over 140 types of HPV have been described [11]. The L1 gene is the most conserved gene in the HPV genome and therefore, this gene is used when classifying new HPV types. In 1995, at the International Papillomavirus Workshop, a new definition was decided upon using the L1 gene as a reference. A new HPV type is only classified as such when, following genome cloning and subsequent analysis of the L1 gene, it is shown that this new type differs by more than 10% from the closest known HPV type. If this difference is in the range of 2-10%, it is classified as a subtype and if less than 2%, it is classified as a variant [12].

The oncogenic potential of the HPV types are classified according to their prevalence in cervical cancer. These classifications are high-risk, low-risk or a

probably high-risk type [9].

The HPV types with an undisputable link to cervical cancer have been classified as high-risk types. These high-risk types include HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 [9]. Worldwide, HPV types 16, 18, 31 and 45 have been attributed with causing over 90% of all cervical squamous cell carcinomas [13]. Of these four, HPV 16 deserves a special mention as it single-handedly can be linked to over 50% of these cancers [14].

Low-risk types are HPV types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108 [9]. These types are attributed with causing benign warts and are not considered to be oncogenic [13].

The third category that exists is termed 'probably high-risk'. The types that fall into the probably high-risk category are 26, 53, 66, 68, 73 and 82. They are classified as such as they have been detected in cases of cervical cancer but there is insufficient literature to assess their oncogenic potential [9].

3.4 HPV genome

HPV is a circular, double-stranded DNA virus that invades the mucous membranes and basal cutaneous cells where it makes use of the host cells' DNA replication mechanisms to reproduce itself [9]. It usually exists as a nuclear episome but it is also capable of integrating its genome into the host's genome. This integration has been found to lead to an unstable genome, allowing for malignant transformation to

occur [15].

The HPV genome consists of 8 genes. There are 6 non-structural genes (E1, E2 and E4-7) and 2 genes coding for capsid proteins (L1 and L2) [15].

Each gene has a different function. The L1 and L2 genes code for a major and a minor capsid protein respectively. L2 also aids in the assembly of intact virions. The E1 and E2 genes are highly conserved and are involved in DNA replication and genome encapsidation. E2 has the added role of regulating the expression of the E6 and E7 genes. Gene E4 facilitates viral assembly and E5 is involved in protein upregulation of growth factor receptors [15]. E6 and E7 genes encode for proteins that disrupt the cell cycle checkpoints ensuring the cells never leave the cell cycle. This is done by binding to and degrading proteins that would normally cause either apoptosis or would regulate terminal differentiation. For E6, this protein is p53 and for E7 it is the Retinoblastoma family of proteins [13]. When a HPV genome is integrated into the host's genome there is usually disruption of the E2 gene. This leads to a loss in the regulation of E6 and E7 leading to genome instability and cancer promotion [15].

3.5 HPV life cycle

HPV is an epitheliotropic virus. Its replication is closely related to the replication and differentiation of the epithelial cells that it infects [13]. Under normal conditions, basal epithelial cells divide and the daughter cell that is produced will migrate to

the suprabasal layers. They differentiate and thus lose the ability to divide. This process is interrupted by HPV infection [15].

Initially, infectious viral particles gain access to the basal layers of the epithelium. A plausible mode of access is through micro-wounds of the epithelium [13]. In these basal cells the viral particles enter the nucleus and viral replication occurs. The virus is maintained in the basal layers at low levels with each infected cell containing approximately 50-100 genomes. With cell division, the virus too divides and each daughter cell contains an even number of genomes [15].

This division is followed by migration of one of the daughter cells into the suprabasal layers. In these upper layers the E6 and E7 genes come into effect. They ensure the infected cell does not leave the cell cycle and that HPV replication can continue with the use of the host epithelial cells' replication machinery. In the upper layers, there is gene amplification. E1 and E2 are responsible for this replication as well as for DNA packaging into capsids. Finally there is release of the newly-formed virions from the cells [15].

3.6 HPV in oesophageal squamous cell carcinoma

The role of HPV in oesophageal squamous cell carcinoma was first proposed by Syrjänen in 1982 [16]. Since then the pages of medical journals are testament to over 60 articles on the subject of the role that HPV has to play in the aetiology of oesophageal squamous cell cancer.

The data in the current literature shows vastly divergent results. A review of the literature undertaken by Poljak *et al.* up until 1997 showed HPV prevalence rates of between 0 and 66% of cases of OSCC. Poljak *et al*. found in their series no evidence of HPV in 120 cases of OSCC [10]. Several factors have been considered in the literature in an attempt to elucidate the impact they may have on HPV prevalence in OSCC.

It has been postulated that the differences in the prevalence of HPV in OSCC are related to the geographical region in which the study was conducted with HPV having a more significant role in areas which are high risk for OSCC [5]. Herbster *et al*. conducted their study in Brazil comparing the HPV prevalence rates among two different population groups within Brazil, the one group was from southern Brazil, a high incidence area and the second was from south eastern Brazil, a medium incidence area. They demonstrated HPV in 23% of cases from the high risk areas as compared to only 8% of the cases from the medium risk area. This supports the theory of the causal relationship of HPV in OSCC in geographical regions which are high risk for OSCC [17]. Two studies from Iran, a high risk area, that have been recently published show conflicting results on this theory. The study published by Yahyapour *et al*. found HPV in 27,7% of their cases [11] which further supports this theory, however in a study by Abdirad *et al*. HPV was only detected in 8,6% of their cases [18] which is not a significant correlation.

Biron *et al*. [19] found an increase incidence of cervical cancer in female patients with oropharyngeal squamous cell carcinoma as compared to a control group and

postulated frequent HPV co-infection of the two sites in these patients. Unfortunately, none of the studies looking at OSCC and HPV commented on the incidence of cervical cancer, cervical dysplasia or the prevalence of genital warts in the population. This would have given an estimation as to the overall prevalence of HPV in the study population, thus helping with the interpretation of results. In countries with a low or decreasing incidence of HPV related cervical disease, one would not expect a significant HPV burden and a negative HPV result would not be significant. The converse would be true in that a positive HPV result in these countries would have been a significant result.

Polymerase chain reaction (PCR) is commonly used and is considered to be the most sensitive method of HPV detection [5]. Technical factors with the PCR procedure have been cited as a possible reason for the discordant results in the literature, especially with respect to the selection of primer sets [10]. The use of the L1 region which is highly conserved across many HPV genotypes is most frequently used to detect HPV by PCR [10-11, 17-18, 20]. The E6 region has also been employed by some [10, 20]. Matsha *et al*. found that of their 52 HPV positive cases, all were positive using the L1 region, however, only 32 were positive with E6 and they postulated poor primer design for the discordant results when comparing L1 and E6 primers [20]. Herbster *et al*. performed PCR for the L1 gene on all their samples in two rounds, the first round used the MY09/MY11 primer set and the second round used the GP5+/GP6+ primer pair. Subsequently, in situ hybridisation (ISH) was performed on the samples which were PCR positive. Of

these PCR positive cases, 89% were also positive with ISH. An equal number of PCR negative samples were also tested with ISH and none of them were positive [17].

Several studies have subtyped the HPV that has been detected in their cases of OSCC. In Iran high risk HPV 16 and low risk HPV 6 were detected at an equal frequency in the HPV positive cases tested by Abdirad *et al*. [18]. The study by Yahyapour *et al*. which was also conducted in Iran also found an equal combination of low risk and high types, namely low risk HPV11 and high risk HPV45 [11]. Vaiphei *et al*. found more than one viral type in most of their cases with high risk HPV 52 being the most frequent [21]. There does not appear to be a correlation between HPV subtype and the development of OSCC suggesting that there may be another factor which may play a role in the development of OSCC. Of particular interest is the high prevalence of low risk HPV subtypes in the OSCC cases.

The role of HPV in oesophageal cancer can be proven by establishing its role in the malignant transformation of oesophageal mucosa. One could extrapolate that HPV infection is responsible for oesophageal cancer if one could provide evidence of a step-wise progression from oesophageal mucosa with HPV infection, to dysplasia, to carcinoma in situ and then invasive carcinoma of the oesophagus.

Vaiphei *et al*. assessed the presence of HPV in OSCC as well as in the adjacent mucosa of the same patient. They detected HPV in 16 of their 23 tumour samples

(70%) and in 15 of the 23 adjacent mucosa samples (65%). Of interest is that high risk HPV types were detected in two of the tumour cases but not in their adjacent mucosa and the converse was also found with two cases showing high risk HPV in the mucosa but not the tumour [21].

In South Africa, the last few decades have seen a change in the face of medicine, a change in disease progression and disease profiles. HIV is the underlying cause that this change stems from. With the HIV pandemic in many countries, the progression to invasive carcinoma of the cervix has been noted to happen at an increased frequency in a younger age group among HIV positive individuals as compared to their HIV negative age-matched controls [9]. Unfortunately, there are no studies that have looked at the HIV status of the patients with OSCC to see if there was a trend that mirrors that of cervical cancer and HPV.

Alcohol and smoking are well recognised risk factors for the development of OSCC [4]. A retrospective study was performed by Qi *et al*. and the cases of OSCC were compared to matched controls. Smoking and alcohol consumption were assessed as independent risk factors. They found HPV in 38,7% of tumour cases and 29,0% of control cases. In the patients who were non-smoking and non-alcohol consuming there was no significant difference in HPV prevalence rates amongst cases of OSCC. In patients who were seropositive for HPV and who were both smokers and alcohol consuming, there was a significantly higher risk for the development of OSCC [22]. The increased prevalence of HPV in OSCC amongst smokers was mirrored by Vaiphei *et al*. [21]. This association however was not

confirmed by Herbster *et al*. who found no significant differences in HPV prevalence amongst smokers as compared to non-smokers or amongst people who consumed alcohol and those who did not. They did not assess if there was a difference in HPV prevalence in patients who both smoked and drank alcohol [(17)].

South Africa is a known high risk area for OSCC and since the 1960's the incidence rate has been increasing to almost epidemic proportions [(7)]. A study conducted in the Transkei region of South Africa detected HPV DNA by PCR in 52 of 114 (46%) of their OSCC cases as well as in 7,3% of their normal control cases. The predominant subtype was HPV 11 found in 43% of cases followed by HPV 39, HPV 16 and HPV52. Their data supports the aetiological association of HPV and OSCC [(20)].

CHAPTER 4

4.0 MATERIALS AND METHODS

4.1 Clinical setting

This study was carried out in the Anatomical Pathology Department of Chris Hani Baragwanath Hospital which is located in Soweto, Gauteng, South Africa. This is a large hospital with approximately 3200 beds which provides health care primarily to residents of greater Soweto. However, it is also a tertiary care academic hospital and functions as a major referral centre from other areas of South Africa.

4.2 Sample

The National Health Laboratory Services database at Chris Hani Baragwanath Hospital, DISA, was searched for all archived cases of OSCC that were diagnosed between 01 January 2009 and 31 December 2010. The search was undertaken using the SNOMED codes for “esophagus” and “squamous cell carcinoma, NOS”. The pathological reports, slides and paraffin wax embedded blocks were retrieved. All of the cases were allocated a consecutive number and the names of the patients were not revealed to anyone except the principal investigator.

4.3 Inclusion and exclusion criteria

- Patients younger than 18 years of age were excluded.
- Duplications were excluded (biopsy, surgical specimens or recurrences for the same patient).
- Cases in which the material in the paraffin wax block was cut through or inadequate (insufficient tissue within the tissue block to cut the 4 x 10µm sections for the PCR) were excluded.
- Only cases of primary squamous cell carcinoma were included.

4.4 Histological assessment

The archived slides for each case were examined by the student in order to confirm the diagnosis and to ensure that enough representative material was available in the block. Enough material was defined as the presence of invasive carcinoma which was more than focal single cell invasion. In addition, if multiple ribbons or levels had previously been examined, each ribbon or level was examined to ensure that the invasive component had not been cut away and was still present on the last ribbon or level in such cases. Each case was histologically assessed for the presence of squamous cell carcinoma. In addition, the surface epithelium of each case was assessed for the presence of condylomatous atypia which is a morphologic indication of HPV infection, the features of which include parakeratosis, papillomatosis, nuclear atypia, multinucleation, perinuclear

vacuolation and basal cell hyperplasia.

4.5 Gathering and analysis of data

The patients' age, gender and race were taken from the biopsy request forms and hospital sticker. This data together with the number identifying the case were captured on the data collection form (Appendix 1) and when complete, they were transferred onto an Excel Spreadsheet for analysis.

4.6 Polymerase chain reaction

- **Sample collection**

The archived paraffin-embedded formalin-fixed tissue blocks of these cases were retrieved. From each block, 4 x 10µm sections were cut into Eppendorf tubes. Between each case the work surface was sterilised, the technologist changed gloves and the microtome blade was changed to avoid cross contamination between cases. For each step and with each sample, a new pipette tip and tube was used to avoid cross contamination. Each new tube was clearly labelled with the study number prior to being used.

PCR was performed as per departmental protocol as described below.

- **Removal of paraffin from the paraffin-embedded tissue blocks**

Prior to starting, the work surface was decontaminated by wiping down the surface

using a 3% Virkon solution followed by exposure with UV light for ten minutes. To remove the paraffin, 1000µL of Xylene was added to each labelled Eppendorf tube. Each sample underwent vortex vigorously for 15 seconds to mix the paraffin and Xylene. The samples were then placed in a centrifuge at 13000rpm for five minutes. The supernatant was then removed by pipetting. The above steps were then repeated. To remove any residual Xylene, each sample was centrifuged at 13000rpm for a further five seconds. The supernatant was then removed with a pipette. To remove any residual Xylene, 1200µL of ethanol (96-100%) was added to each Eppendorf tube. Each sample underwent gentle vortex for 15 seconds to mix the alcohol with the tissue. The samples were then placed in a centrifuge at 13000rpm for five minutes. The supernatant was then removed by pipetting. The above steps were then repeated. To remove any residual alcohol, each sample was centrifuged at 13000rpm for a further five seconds. The supernatant was then removed with a pipette. Each tube was then opened and placed in a Dry Bath at 37˚C for 15 minutes allowing any remaining ethanol to evaporate.

- **DNA extraction**

A proteinase buffer solution was then made by combining 1800µL of Elution buffer and 200µL of proteinase K (10%) and mixing by vortexing for a few seconds. Into each Eppendorf tube, 200µL of the proteinase buffer was added to the tissue sections and mixed by vortexing for a few seconds. The tubes were then placed in an incubator overnight. The incubator lysed the tissue and released the DNA by agitating the tissue at 56˚C. The following day the tubes were removed from the

incubator and 200µL of Buffer AL was added to each tube. Each sample was then vortexed for 15 seconds and incubated at 70˚C for 10 minutes to inactivate the proteinase K. The samples were then removed from the incubator and briefly centrifuged to remove any drops from the inside of the lid. The supernatant was then removed and added to a new labelled tube. To the supernatant, 200µL of ethanol (96-100%) was added and mixed by vortexing for 15 seconds. For the next few steps of the DNA isolation process, a QIAGEN DNA Micro Kit (Qiagen, Germany) was used. The supernatant mixture was carefully added to the Qiagen tube ensuring the QIAamp Spin Column was not touched or damaged. The tubes were then closed and centrifuged at 8000rpm for one minute. The QIAamp Spin column was then removed from the tube with the filtrate and placed in a new labelled 2ml collection tube. The tube with the filtrate was discarded. To each tube, 500µL of Buffer AW1 was added and then the tubes were centrifuged at 8000rpm for 1 minute. The QIAamp Spin column was then removed from the tube and placed in a new labelled 2ml collection tube. The old collection tube was discarded. To each tube, 500µL of Buffer AW2 was added. The tubes were centrifuged at 8000rpm for one minute and then at 13000rpm for three minutes to dry the membrane. The QIAamp Spin column was then removed from each tube and placed in a new labelled 2ml collection tube. The old collection tube was discarded. Into each new tube, 30µL of Buffer AE was added to the centre of the membrane and allowed to stand for one minute at room temperature. The tubes were then centrifuged at 8000rpm for one minute. The centrifuge removed the DNA from the QIAamp Spin Column membrane into the collecting tube. New

collection tubes were taken and labelled. From the old collection tubes, the QIAamp Spin Columns were removed and discarded and the fluid transferred to the new collection tubes with pipettes. These tubes containing the DNA solution were then refrigerated at 2-10C until used.

- **DNA quantification check**

To assess the quantity of the DNA obtained, a Thermo Scientific NanoDrop 1000 spectrophotometer was used. First the spectrophotometer was calibrated with 2µl of Buffer AE. For each sample, 2µl was added to the spectrophotometer and a reading obtained. The results of the DNA extraction and quantification are provided in Appendix 2.

- **DNA quality check using the beta-globin gene**

A number of components may cause inhibition of PCR by interfering with one of the enzymatic reactions of PCR. An internal control in the form of β-globin is used to monitor the DNA extraction procedure and check for possible inhibition. β-globin is used as it is a house-keeping gene that is found in all nucleated host cells. Template DNA of the β-globin gene is co-purified when DNA extraction is performed thus monitoring the DNA extraction procedure. Detection of β-globin by real-time PCR confirms that inhibitors are not present.

In this study, the β-globin gene was amplified using the primer pair PCO4 (forward) and GH20 (reverse) (Table 1). The β-globin master mix was made in a final

reaction volume of 18 µl and contained 10.0 µl of 2X SensiMix™ (Bioline, United Kingdom), 2.0 µl of Primer Mix (PCO4 [10mM] and GH20 [10mM]) (Whitehead Scientific, United Kingdom) and 6.0 µl of sterile PCR grade ddH_2O (Adcock Ingram, South Africa). (Appendix 3). The PCR master mix was made by adding the various components in order and mixed by gentle pipetting

Table 1: Sequence of the oligonucleotides used as β -globin PCR primers

Gene	Primers	Sequences	Size (bp)
β-globin	PCO4 (forward) GH20 (reverse)	5'-CAACTTCATCCACGTTCACC-3' 5'-GAAGAGCCAAGGACAGGTAC-3'	268

The specimen was prepared for PCR in a labelled 0,2ml tube by adding 18µl from the β-globin PCR master mix and 2.0µl of the DNA isolate. The tubes were placed in the sample tray which was in turn placed in the Rotor-Gene 6000 real-time system. The thermal profile is indicated in Appendix 4. The average melting point of the β-globin amplicon should be 85,5°C +/- 1°C. This amplicon can be revealed as a 268bp on an agarose gel.

- **PCR procedure**

The HPV L1 gene was amplified using the primer pair GP5+ (forward) and GP6+ (reverse) (Table 2). The PCR master mix was made for all reactions in a final reaction volume of 50 µL and contained, 5.0 µl of 10X Reaction Buffer (with $MgCl_2$, 15mM) (Roche, Germany), 4.0 µl of $MgCl_2$ (25 mM) (Roche, Germany), 1.0 µl of

dNTPs (10 mM each) (Roche, Germany), 1.0 µl of GP5+ (10 µM) (Whitehead Scientific, United Kingdom), 1.0 µl of GP6+ (10 µM) (Whitehead Scientific, United Kingdom) and 0.4 µl of Taq DNA polymerase (5 U/µl) (Roche, Germany). (Appendix 5). The master mix reaction contained 5 µl of template DNA. PCR master mix was made by adding the various components in order and mixed by gentle pipetting. This amplicon can be revealed as a 150bp on an agarose gel.

Table 2: Sequence of the oligonucleotides used as HPV PCR primers

Gene	Primers	Sequences	Size (bp)
HPV L1	GP5+ (forward) GP6+ (reverse)	5'-TTTGTTACTGTGGTAGATACTAC-3' 5'-GAAAATAAACTGTAAATCATATTC-3'	150

The DNA concentration of the amplified product varied between the samples with an average DNA concentration of 166.1 Ng/µL. The tubes were placed in the sample tray which was in turn placed in the GeneAmp 9700 (Applied Biosystems) PCR System for PCR cycling. The thermal profile is indicated in Appendix 6 and was as follows: initial denaturation at 94°C for 4 minutes, 40 cycles with the cycling profile of step 1 at 95°C for 1 minute, step 2 at 55°C for 1 minute and step 3 at 72°C for 1 minute and the final extension at 72°C for 5 minutes.

Each step of the procedure was controlled with positive and negative controls. Positive controls were obtained from previously proven HPV positive cases. For negative controls, a 'no template' control was used in which nuclease-free water was substituted as a template. Every HPV PCR run contained at least two positive

controls and one negative control.

- **Electrophoresis**

A 3% ethidium bromide stained agarose gel was used to visualise the PCR product. The 3% agarose gel was prepared using 3g of Agarose (Bioline, United Kingdom) and 100ml of TAE buffer (Invitrogen, United Kingdom) which contains ethidium bromide. On a parafilm 1µl of loading dye was mixed with 20µl of each amplified PCR product and loaded into the wells. The first well contained the DNA ladder (GeneRuler 50bp DNA Ladder) (Fermentas, Canada) which was prepared by mixing 2µl of the DNA ladder with 2µl of loading dye and 12µl of Sabax water. The subsequent wells contained the controls and then the samples. The samples were separated for 45 minutes at 100V and then photographed.

4.7 Control and reliability of the data

Ten cases of normal oesophagus were identified and tested for the presence of HPV DNA in exactly the same way using PCR. These cases were included to assess for the presence of HPV in the oesophagus in a population without OSCC.

The diagnosis of squamous cell carcinoma is straight forward and the scientists in the molecular biology laboratory are very experienced at reading electrophoresis gels. Thus it was not considered necessary to perform intraexaminer or interexaminer reliability tests. Results of the data were regarded as both reliable and reproducible.

4.8 Data analysis

Data analysis was performed using appropriate techniques and software. Microsoft Excel 2010 ® was used to store data as a spread sheet and statistical calculations were performed in STATISTICA version 11 by StatSoft, Inc. (2012) [www.statsoft.com].

Descriptive statistics in the form of mean, median and standard deviation were used to describe the ages; and frequency analysis was used to describe the gender and race of the patients. The PCR results reflected whether there was a presence or absence of HPV DNA.

4.9 Statistical significance

In order to compare the categorical data HPV positivity and negativity was statistically compared in the OSCC and the normal control cases. As the data was to be categorical the Chi-square test (Yates correction) was used unless one of the categories was less than 10 in which case a Fisher's exact was used. A p value of <0.05 indicated statistical significance.

4.10 Ethical consideration

The Department of Anatomical Pathology has been granted blanket clearance by the Committee for Research on Human Subjects (medical) of the University of the Witwatersrand for the use of archived slides and tissue blocks for investigative

procedures and medical biology procedures. Nevertheless, an ethics application has also been submitted and clearance for this investigation has been received under clearance number M110404. (Appendix 7).

CHAPTER 5

5.0 RESULTS

5.1 Sample size

A total of 96 cases were identified from the NHLS database search and met the inclusion criteria for the study. In one of these cases, DNA was not amplified with the β-globin gene and was subsequently excluded from the study. Ten biopsies showing normal oesophageal mucosa were included as controls.

5.2 Demographics

The study population comprised OSCC from 58 males and 38 females with a male to female ratio of 1,53:1.

The age of the study population ranged from 26 to 94 years with a mean of 62.3 years (SD=12,37) and a median of 61 years (Figure 1). The mean ages of males was 60,6 years and in females the mean age was 64,8 years (Figure 2). This difference was not statistically significance (p=0.1). Age was not available for two patients, one male and one female.

Race was only recorded in 37 of the cases (38,5%). In all these cases the race was recorded as “Black”.

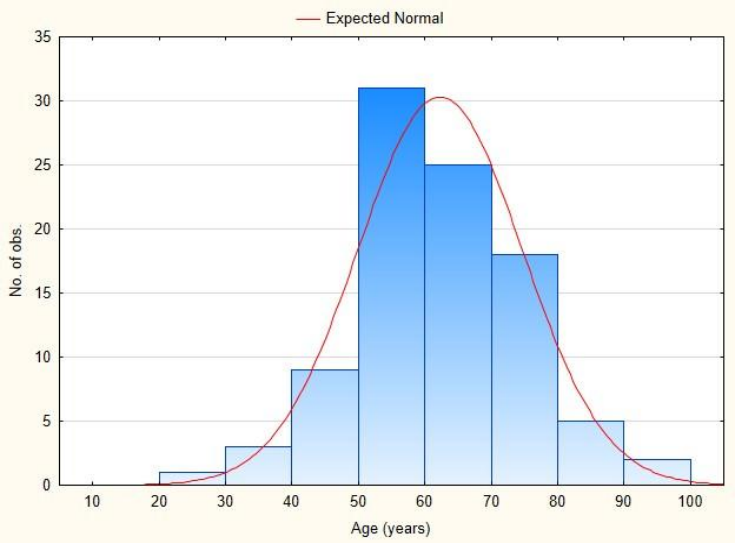


Figure 1: Histogram of the ages measured in years in the study population of patients with OSCC with the expected normal distribution of cases with this age range (n=94)

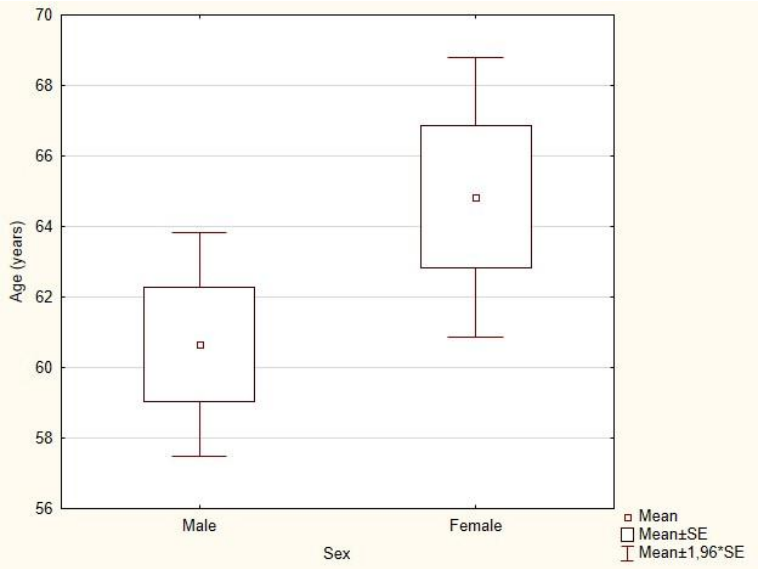


Figure 2: Boxplot of age in years by gender as found in the study population of patients with OSCC

5.3 Histological features

Histological assessment confirmed oesophageal squamous cell carcinoma in all the cases (Figure 3).

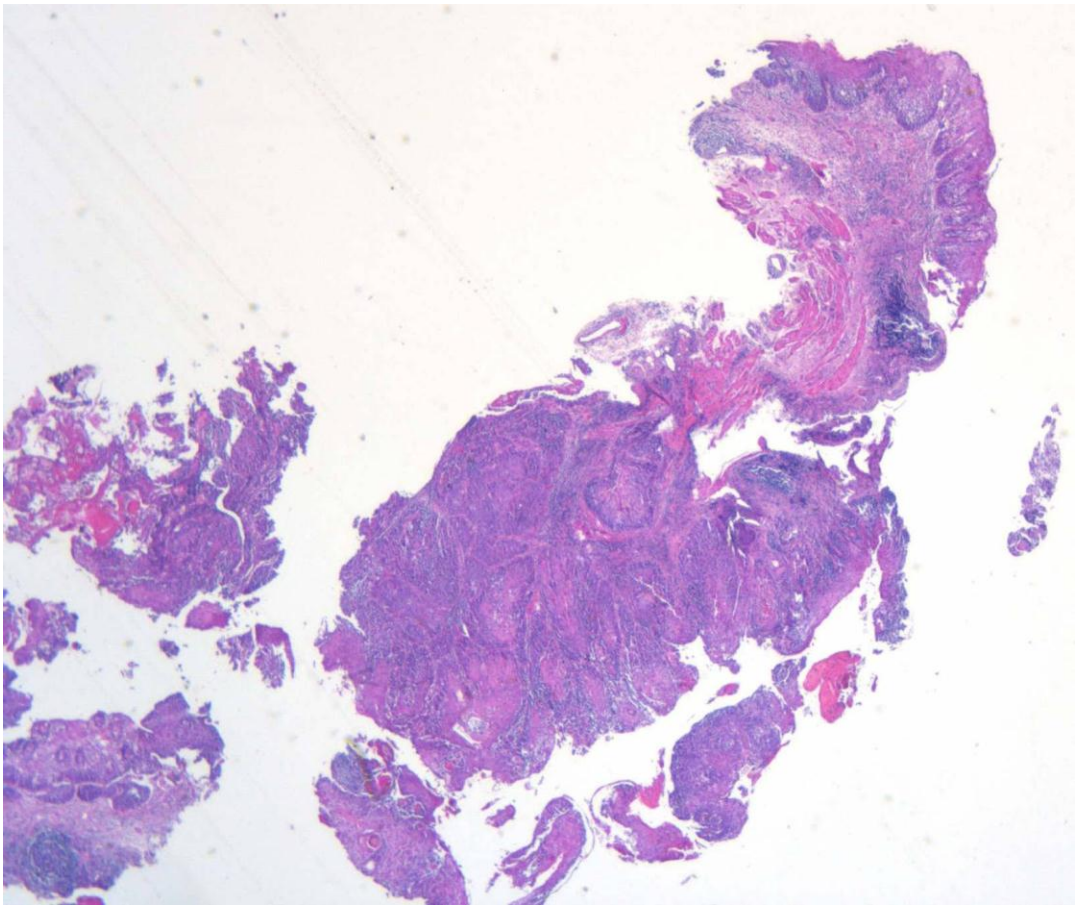


Figure 3: Invasive squamous cell carcinoma of the oesophagus (H&E, x20)

Histological assessment of the oesophageal squmaous cell carcinoma cases showed evidence of condylomatous atypia in two of the 96 (2,1%) cases (Table 3, Figure 4 and Figure 5). Both of these were biopsy specimens.

Table 3: Presence of condylomatous atypia in cases of oesophageal squamous cell carcinoma and normal control specimens

	Condylomatous atypia	
	Present	**Absent**
OSCC	2/96	94/96
Normal biopsy	0/10	10/10

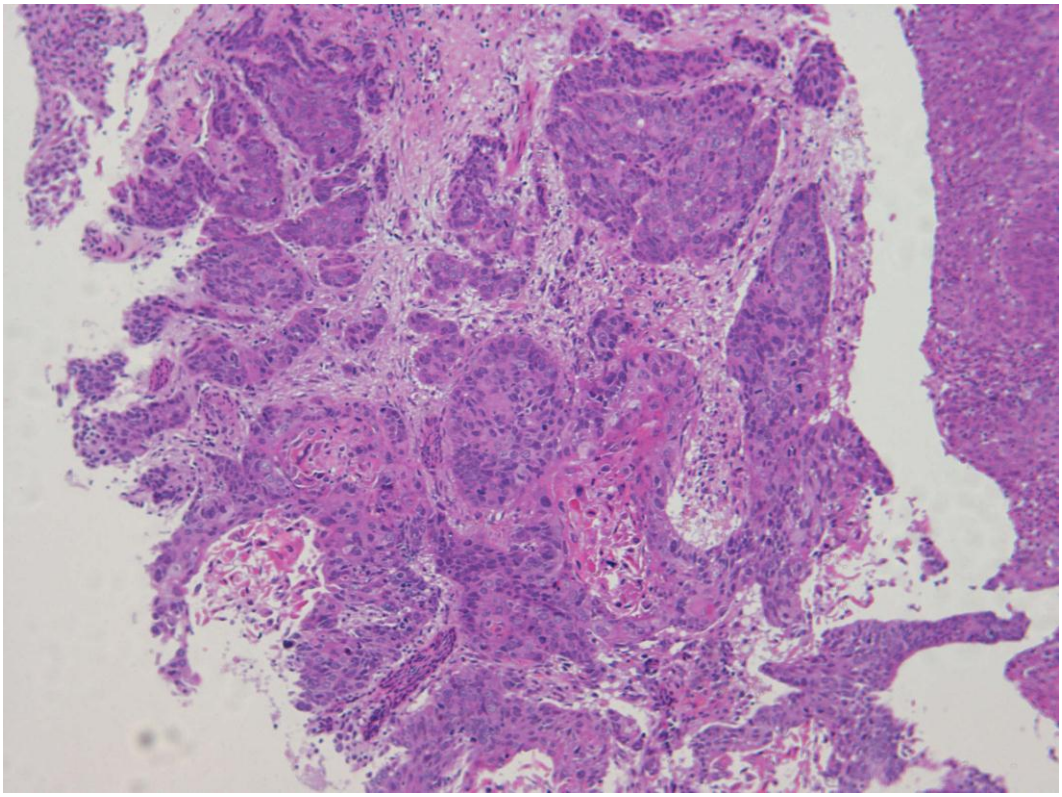


Figure 4: Invasive squamous cell carcinoma of the oesophagus (H&E, x100)

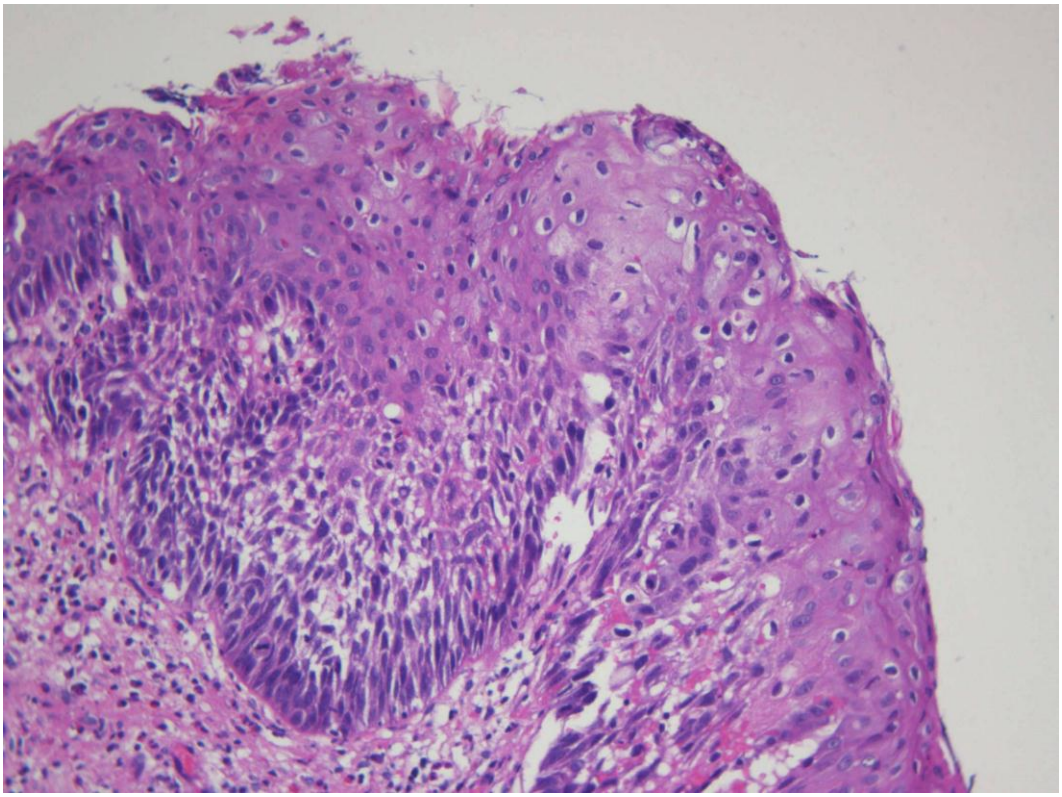


Figure 5: High power view of Figure 4 showing condylomatous atypia of the mucosa (H&E, x200)

There was no condylomatous atypia or invasive squamous cell carcinoma in the normal control cases.

5.4 PCR results

PCR was performed on 95 cases of oesophageal squamous cell carcinoma using the GP5+ and GP6+ primers. All 95 cases failed to demonstrate the presence of HPV DNA (Figure 6 and Table 4). The β-globin gene would not amplify in one case of OSCC and it was deemed unsuitable for the HPV PCR reaction.

All ten cases of the normal mucosa tested negative for the presence of HPV DNA.

In every PCR run the two positive HPV controls were positive producing the expected DNA fragment of150bp on electrophoresis. The negative control was negative in every PCR run.

As all of the cases in both groups were negative, the Chi-square test could not be performed.

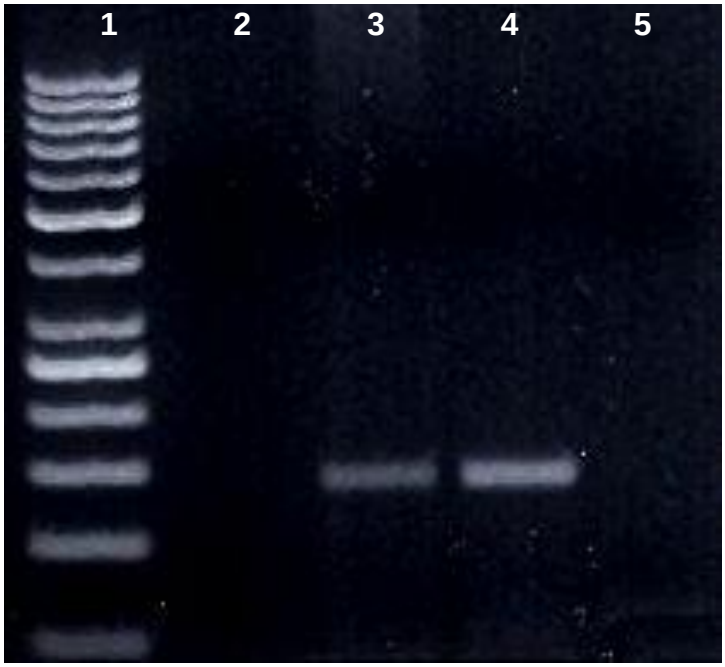


Figure 6: A 3% ethidium bromide stained agarose gel run at 100V for 45 minutes showing DNA fragments produced by PCR amplification of the L1 gene from HPV using GP5+/GP6+ primers

Lane 1: 50bp DNA ladder; Lane 2: OSCC case; Lanes 3-4: Positive controls; Lane 5: Negative control

Table 4: Detection of HPV DNA in cases of oesophageal squamous cell carcinoma and normal control specimens

	HPV PCR	
	Negative	**Positive**
OSCC	95/95	0/95
Normal biopsy	10/10	0/10

CHAPTER 6

6.0 DISCUSSION

6.1 Major findings of the study

In this study HPV could not be demonstrated in any cases of OSCC that were diagnosed at Chris Hani Baragwanath Hospital in 2009 and 2010. This proves that there is no role for HPV in the aetiology of OSCC in this study population. This is a significant finding given the HPV burden in South Africa.

The incidence rate of OSCC in this study population was higher in men as compared to women which correlates with that reported globally. [4]

The mean age of the study population was 62,3 years. This is comparable to the mean age that was reported in other studies such as 59,6 years by Poljak *et al*. [10] and 58.84 years by Abdirad *et al*. [18].

Although the race of the patients was only recorded in 38,5% of the cases, all the patients were of the same racial demographic. This study was conducted at Chris Hani Baragwanath Hospital in Soweto which although providing care to patients of all races, is predominantly frequented by black patients. This may explain the finding of only a single racial profile in this study.

6.2 The meaning of the study and why it is important

HPV has been proven to be oncogenic and successful screening programmes have been instituted and a HPV vaccine has been developed to further assist in reducing the burden of HPV-related disease. Should HPV have been detected this may have impacted on management protocols with respect to future screening for OSCC and HPV as well as guidelines for implementing the HPV vaccine. However, given the complete absence of HPV in these cases it is evident that other aetiological agents are at work in this study population that are unrelated to HPV. At the present time there is no need for HPV screening programmes or vaccine schedules in respect to OSCC.

6.3 Similar studies

The results shown in this study are comparable to those found previously by other authors such as Poljak *et al*. in Poland [10]. Although Abdirad *et al*. found HPV in isolated cases they found the prevalence of HPV in OSCC too low to be significant [18]. Both of these studies made use of the L1 region for HPV detection which was the same primer used in this study. In conflict to this, Herbster *et al*. [17] and Yahyapour *et al*. [11] found statistically significant HPV levels in their cases of OSCC also using the L1 region primers for PCR. The reason for the discordance of results cannot be explained by the risk for OSCC prevalence in a country as previously suggested [5] or by method of detection [10] although technical issues

such as cross contamination should be considered.

In a previous study from South Africa by Matsha *et al*., a significant HPV prevalence was found in cases of OSCC and HPV was also detected in normal oesophageal biopsies. The study was conducted in a different region, namely the Transkei region in South Africa which is a high risk area for OSCC. It is also considered a rural area as compared to the urbanised area of Soweto where this study was conducted. They predominantly found HPV 11 which is a low risk oncogenic subtype of HPV [(20)]. This raises the possibility that this may have been a co-incidental finding as opposed to a significant association. Traditional risk factors such as smoking and alcohol consumption in this population was not specifically addressed in this study. In particular the consumption of home-brewed beer was not considered and this may have influenced the HPV results.

6.4 Alternative explanations of the findings

The findings which have been previously published show HPV prevalence ranging from 0-66% [(10)]. It is possible that there is no correlation between OSCC and HPV with only incidental HPV DNA being detected. The role of cross contamination has not been specifically addressed and standard protocols to avoid cross contamination during PCR were used in all studies, however, cross-contamination at the time of biopsy has not been considered. Many studies used the same GP5+/GP6+ primer pair to the L1 gene as was used in this study which eliminates significant variables with respect to the study design.

The two cases demonstrating condylomatous atypia subsequently proved to be negative for HPV by PCR. This may be as a result of an artefact that was introduced during the processing of the tissue or as a result of koilocytic mimics such as glycogenation of the cytoplasm.

6.5 Clinical relevance

These findings are clinically relevant in that immediate management protocols need not be altered at this point in time. HPV has not been shown to be an important factor in this study population and is not a factor that needs to be addressed by clinicians.

6.6 Study limitations

Only a single HPV DNA primer set was used in this study namely primers to the L1 region. Once the first round were proven negative, a different primer set or method of HPV detection could have been used on all the cases or a random subset to confirm the negative results.

This study was based on all cases occurring within a specified time frame that fulfilled eligibility criteria and not a specific sample size. A post-hoc power analysis was not possible as the incidence rates between the comparative groups was the same. An attempt was made to calculate sample size *a priori*. It was also not possible to calculate sample size as the expected incidence of HPV in the oesophagus is zero and if the incidence is based on previous literature the range of

0-66% quoted in the literature [10] is too wide to estimate sample size. Poljak *et al*. performed a literature review of all 35 studies occurring prior to 1997, with only two of these studies having more cases than the 95 OSCC cases tested here [10]. Of the most recent studies published on this subject, Yahyapour *et al*. had 177 cases of which 27,7% detected HPV [11], Qi *et al*. had 225 cases of which 38,7% detected HPV [22] and Vaiphei *et al*. had only 23 OSCC samples in which HPV detected in 87% of their cases [21]. The sample size differed between these studies although HPV positivity was detected in all of them. A sample size of 95 cases in this study is not out of keeping with that published in the literature although a longer time frame could have been considered in order to increase the sample size.

6.7 Further suggestions for research

Correlation with HPV screening programmes could be considered in future studies. Once the predominant HPV type in the given population was identified from Pap smear screening programmes, additional more specific primer sets could be considered.

It would also be worthwhile correlating Pap smear results in patients with proven OSCC to determine if there is any correlation between HPV prevalence on Pap smear and HPV prevalence in OSCC cases. Balamurugan *et al*. [23] found a statistically significant increase in OSCC among survivors of cervical squamous cell carcinoma although they attributed this finding to tobacco-use and not HPV infection.

CHAPTER 7

7.0 CONCLUSION

HPV is a common infection in the South African population and is known to be carcinogenic in other sites. Its role in OSCC has remained controversial, however, this study does not support the theory that HPV has a role to play in OSCC.

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Appendix 1: Data Collection Sheet

Research number:		
Case number:		
Clinical details:	Age:	
	Gender:	
	Race:	
Histology:	Diagnosis:	
	Condylomatous atypia:	
HPV PCR (Positive or negative):		

Appendix 2: DNA Extraction and Quantification Log

DNA/RNA Isolation log : QIA amp DNA Micro kit

Assay: DNA Extraction and Quantification Log

Name: DAWN VAN DER BIJL Date: 01/12/12

REAGENT	LOT NO.	EXP DATE	DATE REC	DATE IN USE
Qiagen Micro kit	139302602	31-10-12	31-10-11	2-12-11
Xylene	1037954	05-2012	21-7-11	27-7-11
Ethanol	1036254	01-2016	01-07-11	12-07-11

Spectrophotometer: Nanodrop 1000 Asset: 060204

NO.	TUBE NO.	REHYDRATION	DNA QUANTIFICATION				
			260/280	260/230	Ng/ul		
1.	HPV1	35µl	2.05	4.19	103.5		
2.	HPV2	35µl	3.86	-0.13	3.2		
3.	HPV3	35µl	2.01	-2.35	29.2		
4.	HPV4	35µl	2.07	3.98	112.7		
5.	HPV5	35µl	1.92	-1.77	17.5		
6	HPV6	35µl	2.01	2.08	481.1		
7.	HPV7	35µl	2.06	3.48	153.7		
8.	HPV8	35µl	2.02	1.76	34.5		
9.	HPV9	45µl	2.07	-10.82	69.0		
10.	HPV10	35µl	2.07	2.63	467.0		
11.	HPV11	35µl	2.21	-1.09	13.5		
12.	HPV12	35µl	0.53	-0.02	0.5		
13.	HPV13	35µl	1.79	-0.06	1.3		
14.	HPV14	35µl	1.99	3.48	150.7		
15.	HPV15	35µl	2.05	2.88	156.8		
16.	HPV16	35µl	2.05	3.92	52.9		
17.	HPV17	35µl	1.68	1.15	53.1		
18.	HPV18	35µl	2.08	-366.05	46.9		
19.	HPV19	35µl	2.02	2.50	480.7		
20.	HPV20	35µl	2.05	2.25	387.1		
21.	HPV21	35µl	2.21	1.98	151.2		
22.	BLANK	50 µl	-1.78	-0.01	0.2		
23.							
24.							
25.							
26.							

DNA/RNA Isolation log : QIA amp DNA Micro kit

Assay: DNA Extraction and Quantification Log

Name: DAWN DER BIJL Date: 02/01/12

REAGENT	LOT NO.	EXP DATE	DATE REC	DATE IN USE
Qiagen Micro kit	139302602	31-10-12	31-10-11	2-12-11
Xylene	1037954	05-2012	21-7-11	27-7-11
Ethanol	1036254	01-2016	01-07-11	12-07-11

Spectrophotometer: Nanodrop 1000 Asset: 060204

NO.	TUBE NO.	REHYDRATION	DNA QUANTIFICATION				
			260/280	260/230	Ng/ul		
1.	HPV22	35µl	1.54	1.40	127.6		
2.	HPV23	35µl	2.07	1.71	158.8		
3.	HPV24	35µl	2.02	2.08	495.1		
4.	HPV25	35µl	2.00	1.41	53.0		
5.	HPV26	35µl	2.10	2.02	203.0		
6	HPV27	35µl	1.96	1.89	67.2		
7.	HPV28	35µl	2.20	1.87	110.8		
8.	HPV29	35µl	2.17	1.84	152.5		
9.	HPV30	45µl	2.10	1.89	335.0		
10.	HPV31	35µl	2.12	1.92	227.6		
11.	HPV32	35µl	2.11	1.75	240.6		
12.	HPV33	35µl	2.14	1.98	248.6		
13.	HPV34	35µl	2.19	1.68	151.0		
14.	HPV35	35µl	2.06	2.02	515.7		
15.	HPV36	35µl	2.09	1.84	748.8		
16.	HPV37	35µl	2.06	2.01	453.5		
17.	HPV38	35µl	2.10	2.09	381.6		
18.	HPV39	35µl	2.18	1.21	133.3		
19.	HPV40	35µl	2.21	1.23	79.2		
20.	HPV 41	50µl	2.15	1.89	225.8		
21.	BLANK	50µl	-0.71	2.16	-0.8		
22.							
23.							
24.							
25.							
26.							

DNA/RNA Isolation log : QIA amp DNA Micro kit

Assay: DNA Extraction and Quantification Log

Name: DAWN VAN DER BIJL Date: 02/12/11

REAGENT	LOT NO.	EXP DATE	DATE REC	DATE IN USE
Qiagen Micro kit	139302602	31-10-12	31-10-11	2-12-11
Xylene	1037954	05-2012	21-7-11	27-7-11
Ethanol	1036254	01-2016	01-07-11	12-07-11

Spectrophotometer: Nanodrop 1000 Asset: 060204

NO.	TUBE NO.	REHYDRATION	DNA QUANTIFICATION				
			260/280	260/230	Ng/ul		
1.	HPV41	35µl	1.63	1.92	121.6		
2.	HPV42	35µl	1.71	1.99	158.2		
3.	HPV43	35µl	2.08	1.88	335.0		
4.	HPV44	35µl	1.41	1.52	24.6		
5.	HPV45	35µl	2.08	2.16	151.0		
6	HPV46	35µl	1.98	2.01	515.7		
7.	HPV47	35µl	1.92	1.99	67.2		
8.	HPV48	35µl	1.69	1.87	79.2		
9.	HPV49	45µl	1.88	1.81	225.8		
10.	HPV50	35µl	1.67.	1.84	53.0		
11.	HPV51	35µl	1.71	1.92	158.8		
12.	HPV52	35µl	1.62	1.75	110.8		
13.	HPV53	35µl	1.88	1.98	151.6		
14.	HPV54	35µl	1.92	1.68	133.3		
15.	HPV55	35µl	1.31	1.63	52.3		
16.	HPV56	35µl	1.81	1.94	67.8		
17.	HPV57	35µl	2.01	2.09	225.8		
18.	HPV58	35µl	1.88	1.99	141.3		
19.	HPV59	35µl	1.85	2.01	121.3		
20.	HPV61	35µl	1.71	1.87	44.6		
21.	HPV62	35µl	1.69	1.88	101.2		
22.	HPV63	35µl	1.41	1.67	55.2		
23.	HPV64	35µl	1.56	1.74	87.3		
24.	HPV65	35µl	1.62	1.88	27.2		
25.	BLANK	50µl	-5.6	0.16	-2.3		
26.							

DNA/RNA Isolation log : QIA amp DNA Micro kit

Assay: DNA Extraction and Quantification Log

Name: DAWN VAN DER BIJL Date: 05/12/11

REAGENT	LOT NO.	EXP DATE	DATE REC	DATE IN USE
Qiagen Micro kit	139302602	31-10-12	31-10-11	2-12-11
Xylene	1037954	05-2012	21-7-11	27-7-11
Ethanol	1036254	01-2016	01-07-11	12-07-11

Spectrophotometer: Nanodrop 1000 Asset: 060204

NO.	TUBE NO.	REHYDRATION	DNA QUANTIFICATION				
			260/280	260/230	Ng/ul		
1.	HPV66	35µl	1.84	1.92	99.6		
2.	HPV67	35µl	1.62	1.99	15.2		
3.	HPV68	35µl	1.97	1.88	181.6		
4.	HPV69	35µl	1.41	1.62	24.6		
5.	HPV70	35µl	2.08	2.01	203.5		
6	HPV71	35µl	1.96	1.92	137.5		
7.	HPV72	35µl	1.91	1.87	44.8		
8.	HPV73	35µl	1.72	1.91	79.6		
9.	HPV74	45µl	1.88	1.81	225.8		
10.	HPV75	35µl	1.62	1.84	141.2		
11.	HPV76	35µl	1.68	1.92	162.3		
12.	HPV77	35µl	1.71	1.75	101.8		
13.	HPV78	35µl	1.92	2.01	151.6		
14.	HPV79	35µl	1.94	1.68	131.2		
15.	HPV80	35µl	1.95	1.72	52.3		
16.	HPV81	35µl	1.81	1.94	17.8		
17.	HPV82	35µl	2.16	2.09	225.8		
18.	HPV83	35µl	1.88	1.99	141.3		
19.	HPV84	35µl	1.85	1.92	121.3		
20.	HPV85	35µl	1.54	1.87	84.6		
21.	HPV86	35µl	1.99	1.88	1112		
22.	HPV87	35µl	1.84	1.67	55.2		
23.	HPV88	35µl	1.78	1.74	87.3		
24.	HPV89	35µl	1.89	1.88	127.2		
25.	BLANK	50µl	-2.01	-1.3	-4.1		
26.							

DNA/RNA Isolation log : QIA amp DNA Micro kit

Assay: DNA Extraction and Quantification Log

Name: DAWN VAN DER BIJL Date: 05/12/11

REAGENT	LOT NO.	EXP DATE	DATE REC	DATE IN USE
Qiagen Micro kit	139302602	31-10-12	31-10-11	2-12-11
Xylene	1037954	05-2012	21-7-11	27-7-11
Ethanol	1036254	01-2016	01-07-11	12-07-11

Spectrophotometer: Nanodrop 1000 Asset: 060204

NO.	TUBE NO.	REHYDRATION	DNA QUANTIFICATION				
			260/280	260/230	Ng/ul		
1.	HPV90	35µl	1.08	1.62	28.7		
2.	HPV91	35µl	2.01	2.04	151.6		
3.	HPV92	35µl	1.95	1.99	121.5		
4.	HPV93	35µl	1.88	1.92	56.2		
5.	HPV94	35µl	1.89	1.91	104.8		
6	HPV95	35µl	1.96	2.01	91.2		
7.	HPV96	35µl	1.93	1.99	88.9		
8.	HPV98	35µl	2.08	2.01	215.3		
9.	HPV99	45µl	1.98	2.16	89.5		
10.	HPV100	35µl	1.93	2.04	112.9		
11.	HPV101	35µl	2.16	2.11	153.8		
12.	HPV102	35µl	2.01	2.06	150.7		
13.	HPV103	35µl	1.88	1.99	44.2		
14.	HPV104	35µl	1.78	1.94	480.7		
15.	HPV105	35µl	1.94	2.08	387.2		
16.	HPV106	35µl	1.92	1.98	151.2		
17.	BLANK	50µl	-7.2	-0.08	-0.17		
18.							
19.							
20.							
21.							
22.							
23.							
24.							
25.							
26.							

Appendix 3: β-globin Master Mix for PCR

PCR Reaction	**1X**	**X36**
2X SensiMix™	10.0 µl	360 µl
Primer Mix (10mM)	2.0 µl	72 µl
Sterile PCR grade ddH_2O	6.0 µl	216 µl
Total	**18.0 µl**	
Sample	**2.0 µl**	

Appendix 4: β-globin PCR Profile

Cycle	Cycle point
Hold @ 95°C, 10 minutes	
Cycling (50 repeats)	Step 1 @ 95°C, hold 10 seconds
	Step 2 @ 60°C, hold 10 seconds
	Step 3 @ 72°C, hold 15 seconds
Final extension @ 85,5°C for 5 minutes	

Appendix 5: HPV Master Mix for PCR

PCR Reaction	**1X**	**X28**
Sterile PCR grade ddH_2O	32.6 µl	912.8 µl
10X Reaction Buffer (with $MgCl_2$, 15mM),	5.0 µl	140 µl
$MgCl_2$ (25 mM)	4.0 µl	112 µl
dNTPs (10mM each)	1.0 µl	28 µl
GP5+ (10 µM)	1.0 µl	28 µl
GP6+ (10 µM)	1.0 µl	28 µl
Taq DNA polymerase (5 U/µl).	0.4 µl	11.2 µl
Total	**45.0 µl**	
Sample	**5.0 µl**	

Appendix 6: HPV PCR Profile

Cycle	Cycle point
Hold @ 94°C, 4 minutes	
Cycling (40 repeats)	Step 1 @ 95°C, hold 1 minute
	Step 2 @ 55°C, hold 1 minute
	Step 3 @ 72°C, hold 1 minute
Final extension @ 72°C for 5 minutes	
Hold @ 4°C ∞	

Appendix 7: Ethics Clearance Certificate

M110404

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Dawn-Lee van der Byl

CLEARANCE CERTIFICATE	**M110404**
PROJECT	Human Papillomavirus Infection of the Oesophagus and its Association with Squamous Cell Carcinoma: A Restrospective Study of Cases seen at Chris hani baragwanath Hospital in 2009 and 2010
INVESTIGATORS	Dr Dawn-Lee van der Byl.
DEPARTMENT	School of Pathology/Anatomical Pathology
DATE CONSIDERED	2006/05/11
DECISION OF THE COMMITTEE*	Approved unconditionally
	06/05/2011

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 2006/05/11 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof M Hale

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Appendix 8: Approval of Title

Faculty of Health Sciences
Medical School, 7 York Road, Parktown, 2193
Fax: (011) 717-2119
Tel: (011)717-2075/6

Reference: Ms Salamina Segole
E-mail: Salamina.segole@wits.ac.za
16 November 2011
Person No: 0102003D
PAG

Dr D Van der Byl
P O Box 1634
Glenvista
2058
South Africa

Dear Dr Van der Byl

Master of Medicine in the specialty of Anatomical Pathology: Approval of Title

We have pleasure in advising that your proposal entitled "*Human papillomavirus infection of the oesophagus and its association with squamous cell carcinoma: A retrospective study of cases seen at Chris Hani Baragwanath Hospital in 2009 and 2010*" has been approved. Please note that any amendments to this title have to be endorsed by the Faculty's higher degrees committee and formally approved.

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences