HUMAN PAPILLOMAVIRUSES IN OESOPHAGEAL CANCER

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CONTENTS

DEDICATION

To my late brother **Bob Matsha,** my niece **Unathi**, **Rajiv**, my better half and my two daughters **Ondela Thato (Ondi)** and **Tamar Ilona (Bubu)**.

Thank you for your patience, unwavering support, love and encouragement.

ABSTRACT

Oesophageal cancer is a major health problem in South Africa. It is the most common cancer among black males and third most common among females. The Transkei region of the Eastern Cape, South Africa has one of the highest incidences of oesophageal cancer in the world. The incidence and mortality of this disease are nearly equal due to late diagnosis. There is substantial evidence that oesophageal cancer result from a combination of environmental and genetic factors. Infection by human papillomaviruses has been implicated in squamous cell carcinoma of the oesophagus, but the field remains controversial. Despite the high incidence of oesophageal cancer in Transkei, South Africa, no study has been examined the association between HPV and oesophageal cancer.

The first phase of the project was to determine the presence of HPV DNA by PCR using HPV L1 and E6 consensus primers in tumours obtained from patients with squamous cell carcinoma of the oesophagus. The types of HPV were determined by sequence analysis and identified by searching the GenBank database using BLAST software. Immunohistochemistry using anti-HPV was used to identify HPV antigens from the same tumour biopsies. HPV DNA was detected in 44% of the squamous cell tumours of the oesophagus with HPV types 11 followed by HPV type 39 as the most predominant. HPV type 39 has previously not been reported to be associated with squamous cell carcinoma of the oesophagus.

The second phase of the study was to establish if HPV DNA is present in precancerous lesions of the oesophagus. HPV DNA screening of cells obtained by brush cytology was carried out on volunteers participating in an early screening programme for oesophageal cancer. The objective was to use the data to increase the accuracy in the interpretation of results obtained by brush cytology in identifying high-risk individuals as is commonly done with Pap test for cancer of the cervix. HPV DNA was present in 50% of asymptomatic individuals with malignant cells and 17% of those with dysplasia compared to 2% of individuals with no abnormal morphological

changes. In this 2% HPV DNA positive benign subjects other factors such as fungal infection and chronic inflammation were observed. More than 80% of the volunteers indicated that they induced vomiting on a regular basis while 100% of the HPV DNA positive individuals engaged in this practice. It is possible that this cultural practice has been overlooked and may be playing an important role in the development of oesophageal cancer.

The transforming activity of HPV has been localised to the HPV E6 and HPV E7 gene products. HPV E6 protein interferes with the p53 tumour suppressor proteins, and has also been suggested to inactivate the recently identified p73 gene product. Thus, the final phase of the project was to investigate the expression of p53 and p73 proteins in tumours containing HPV DNA by immunohistochemical methods. Furthermore, the effect of HPV E6 on p53 and p73 protein levels was investigated by transient transfection of normal human fibroblasts with E6 expressing plasmids. No significant correlation was observed between p53 and HPV status. Conversely, a statistically significant positive correlation between p73 protein expression and HPV status was observed. The p73 protein expression levels were significantly higher in cells containing any of the HPV subtypes indicating that both low-and high-risk HPV subtypes had common effects on p73.

Transfection and flowcytometry analysis have shown that both HPV11 (low risk) *and HPV16* (high-risk) E6 proteins inhibited apoptosis. Consistent with known HPV E6 interactions with p53, HPV16 E6 expressing fibroblasts had reduced endogenous p53 levels. The levels of endogenous p73 were increased in cells transfected by both HPV 11 and HPV 16 E6 constructs further indicating that both low- and high- risk HPV types have a similar effect on p73. The importance of these results suggest that the classification of HPV subtypes in oesophageal cancer may have to be revised and that HPV DNA in oesophageal cancer is not a bystander, but may be using different mechanisms in the transformation of cells. These results have formed a basis for further investigations as to how the HPV types that are found in oesophageal cancer (some of which are considered low oncogenic in cervical cancer) can potentially contribute to tumorigenesis.

CHAPTER 1
LITERATURE REVIEW

1 INTRODUCTION

Carcinoma of the oesophagus poses a significant health problem because the development of the disease is largely asymptomatic, resulting in late diagnosis with a poor prognosis. In the majority of cases, by the time patients begin to suffer from progressive dysphagia and seek medical help, the disease is already in a late stage and only palliative treatment can be offered. Consequently, the incidence and mortality for oesophageal cancer (OC) are nearly equal.

1.1 CLASSIFICATION OF TUMOURS OF THE OESOPHAGUS

There are striking differences in the histologic types of oesophageal cancer. The two major types of oesophageal cancer are adenocarcinoma and squamous cell carcinoma (SCC). Adenocarcinomas are typically located in the distal oesophagus and occur predominantly in white males of industrialised countries (Day et al., 1994). Squamous cell carcinoma of the oesophagus (OSCC) is more prevalent and accounts for 50 to 60% of all oesophageal tumours. The developing world accounts for 85% of the total global incidence of oesophageal cancer, primarily oesophageal squamous cell carcinoma.

Oesophageal SCC is located predominantly in the middle and the lower third of the oesophagus, only 10 to 15% being located in the upper third (Figure 1.1) (Hamilton et al., 2000). The other types of oesophageal tumours occur rarely, these include verrucous squamous carcinoma, basaloid squamous cell carcinoma, spindle cell carcinoma, adenosquamous carcinoma, small cell carcinoma, mucoepidermoid carcinoma, adenoid cystic carcinoma, and undifferentiated carcinoma. This thesis focuses on molecular analysis of oesophageal SCC in Transkei, South Africa, where the incidence of oesophageal cancer is reportedly one of the highest in the world.

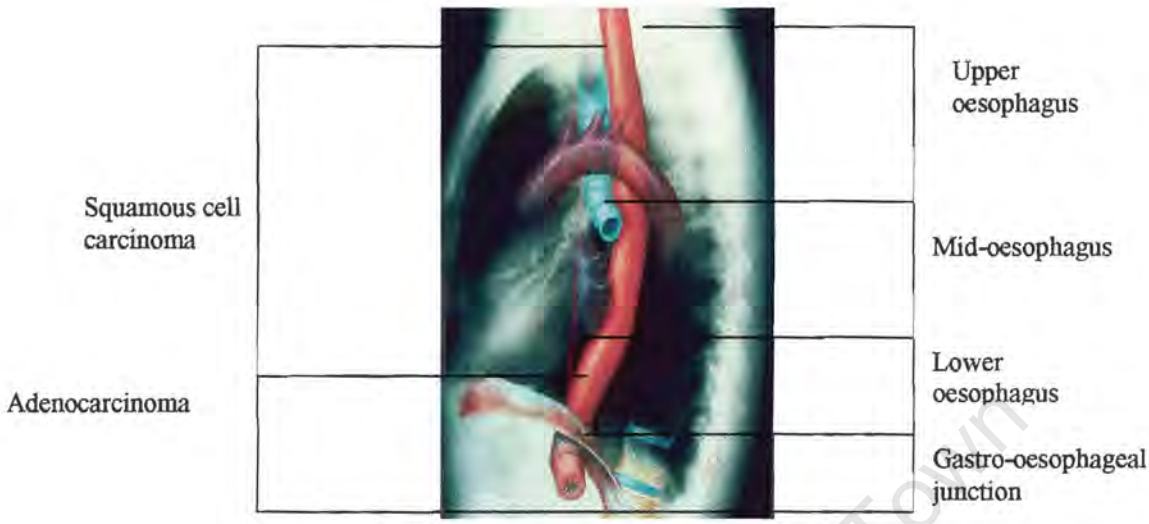


Figure 1.1

Anatomy of the oesophagus. Regional distributions of squamous cell carcinoma and adenocarcinoma in the oesophagus.

1.2 EPIDEMIOLOGY

Oesophageal cancer is the eighth most common cancer in the world with an uneven ethnic and geographical distribution (Canon et al., 1997). The incidence of OSCC in different countries is summarised in table 1.1. There are several well-defined high- risk areas (China, Iran and South Africa) and low-risk areas (Europe and North America) of oesophageal cancer. In both high- and low-risk areas, oesophageal cancer is exceedingly rare in individuals under the age of 30 and the median age is around 65 in both males and females (WHO, 2000).

Table 1.1

Oesophageal incidences in different countries. The table below is a summary of worldwide incidence (per 100 000) of oesophageal SCC in males (WHO, 2000).

Region	Regional average per 100 000
North America	5.2
South America	11.0
Southern Africa	11.9
Iran	51.6
China	19.3
Russia	7.8
Australia	4.6

1.2.1 High-risk areas

1.2.1.1 China

Oesophageal SCC accounts for more than 90% of all resected tumours in China (Li et al., 1997). There are six areas within China that have been identified as having a high incidence of oesophageal cancer. These include the Linxian County in Henan Province, Northern Sichuan, and the Da-Bie mountainous region at the border of Hebei Province, Southern Fukien, northern Jiangsu, and northern Xingiang. Each of these areas has reported different incidence rates. The highest incidence of OC in China, 199 cases per 100 000, is reported in the area south of the Taihang Mountains and is one of the highest in the world (Hou et al., 1998).

1.2.1.2 Iran

In Iran, majority of patients is between 35 and 54 years, and nearly all come from the poorest stratum of the rural society. In northeastern Iran, specifically the provinces of Gonbad, Maquandaran and Gorgon, up to 180 new cases per 100 000 have been recorded each year (Dowlatshahi et al., 1985). In the southern Fars province, the risk for males and females is nearly equal, but older females have a higher incidence than older males (Sadeghi et al., 1977).

1.2.1.3 South America

The countries with a high incidence of oesophageal cancer in South America include Brazil, Uruguay and Argentina. In Southern Brazil the city of Porto Alegre has a high incidence of oesophageal cancer and the age standardized incidence rate for males has been estimated to be 18.9 per 100 000.

1.2.1.4 France and Italy

The high incidence areas in France are Normandy and Calvados, and in Italy it is the northern part of the country. The incidence of oesophageal cancer in these two countries could be as high as 30 per 100 000 population in males. In 1975 the male to female ratio of oesophageal cancer in Brittany, France, was reported to be 23 to 9 (Tuyns et al., 1975). However, recent changes in distribution pattern in France indicate that the rate of OSCC has increased steadily in low-risk areas, particularly among females (Hamilton et al., 2000).

1.2.1.5 Africa

In Africa, the country that has been thoroughly screened for the true incidence of oesophageal cancer is South Africa. The other countries that have been reported to have a high incidence of oesophageal cancer in Africa include Zimbabwe, Malawi and Mozambique, with Zimbabwe having the highest incidence of OC where the age standardized incidence rate had been estimated to be 196 per 100 000 in black males.

The Transkei region of the Eastern Cape has been identified as a region with a high incidence of oesophageal cancer in South Africa worldwide. A high incidence of oesophageal cancer amongst the black Xhosa speaking people of the Transkei region was first noticed and reported by Burrell in 1957. Subsequent research confirmed this as the most frequently observed cancer in the Transkei. It further established marked differences in oesophageal cancer incidence rates in different districts of Transkei.

The four districts that have been studied extensively are in northeastern Transkei (Lusikisiki and Bizana) and the other two in the south -western part (Butterworth and Kentani) (Figure 1.2). The northeastern region has been classified as a low risk area and the south western as a high risk one. The population of the northeastern districts is relatively stable, predominantly rural and comparatively more affluent than that of rural areas in the southwestern part of Transkei. Butterworth district is an industrial area with a growing urban population, whereas Kentani is a typical rural district with a conservative traditional population.

The age standardised rates (ASIR) for confirmed cases of oesophageal cancer range from 2.6 and 1.8 in males and females respectively, in the northeastern district of Bizana to 103.1 to 25.6 in males and females respectively in the southwestern district of Butterworth (Rose et al., 1973). These results were further corroborated by subsequent surveys carried out between the periods 1981 to 1984 (Jaskiewicz et al., 1987), 1985 to 1990 (Makaula et al., 1996), and 1991 to 1995 (Somdyala et al., 2002). The results from these surveys have shown consistently high rates in the southwestern districts of Kentani, a high but decreasing rate in Butterworth and progressively increasing rates in the northeastern districts of Bizana and Lusikisiki, which were previously considered as low incidence areas.

Recently, oesophageal cancer rates in Transkei have been reported to be declining, whether this is an apparent or real decrease is currently being

investigated (Sitas et al., 2002). There is now mounting evidence that oesophageal cancer may not be confined to rural Transkei, but is also commonly seen in other urban Africans (Van Rensburg et al., 1985; Kneebone et al., 1985; Jaskiewicz et al., 1992). The age standardised incidence rate in Soweto, South Africa's largest urban black community was reported to be 125 per 100 000 in males and 37 per 100 000 in females (Kneebone et al., 1985).

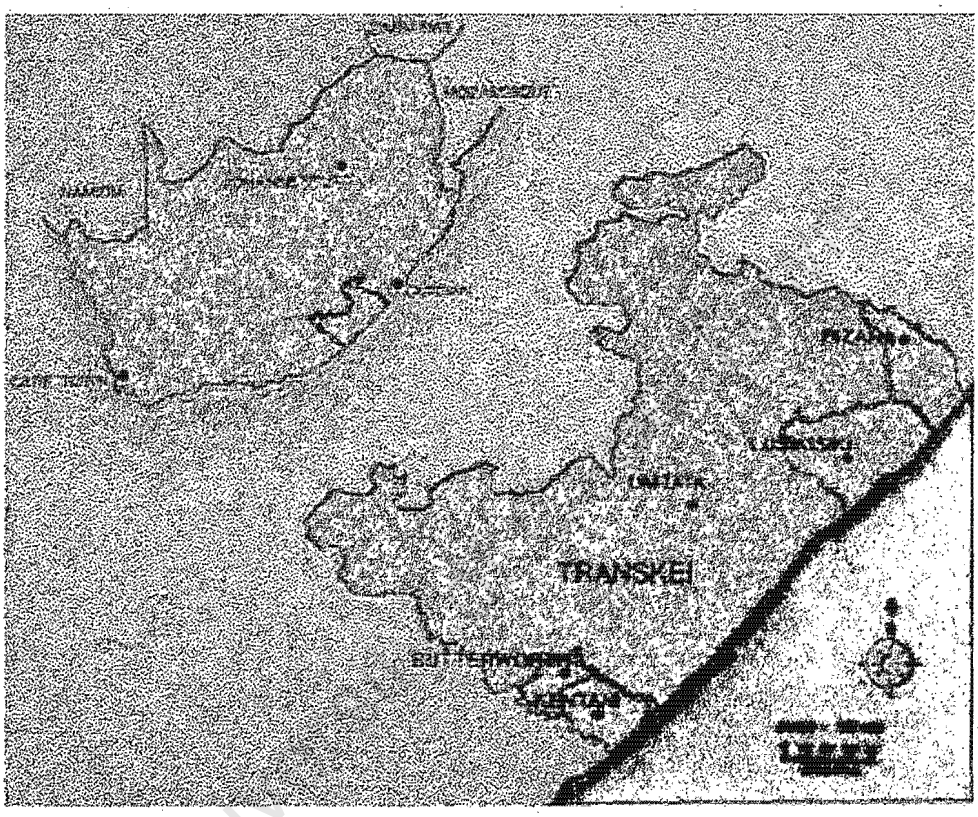


Figure 1.2

Transkei, South Africa. Location of Transkei (green) in South Africa and the location of the four districts in which the incidence rates of OC have been determined (bottom right).

1.2.2 Low-risk areas of oesophageal cancer

In low risk areas such as North America, Australia and Europe, the age standardised annual incidence does not exceed 5 per 100 000 population in males and 1 per 100 000 in females. However, demographic studies have shown ethnic variations within the populations of North America. The incidence of

oesophageal squamous cell carcinoma is more than five times among African Americans (16.8 per 100 0000) than among Caucasian men (1.0 per 100 000) (Pera et al., 2001). In African American males and females mortality as a result of OC has been estimated to be 12.4 per 100 000 and 2.6 per 100 000 respectively, while for white females it is 1.0 per 100 000. In the Caucasian populations the most predominant oesophageal tumours are adenocarcinomas.

1.3 AETIOLOGY OF OESOPHAGEAL CANCER

The aetiology of oesophageal SCC is known to be complex and composed of multiple factors, those caused by the environment being of greatest importance. The disease preferentially occurs in groups with a low socio-economic status or those afflicted with poverty (Tollefson et al., 1985). This seems to be a common denominator not only in Transkei, but also in other high incidence areas of the world such as Iran (Cook-Mozaffari et al., 1979). However, presently no definite aetiological factors are known.

There exists enormous disparity in the aetiology of the disease among various countries. In industrialised countries, consumption of alcohol and tobacco, especially in combination are the major risk factors. In other high incidence areas, nutritional deficiencies, ingestion of hard foods and /or hot liquids and infection with fungi and human papillomaviruses are also risk factors.

1.3.1 Nutritional deficiencies

The role of nutritional deficiency in the development of oesophageal cancer was made more than three decades ago by Larson et al (1975), after observing that women in North Sweden with a condition characterised by iron deficiency and low levels of several micronutrients were likely to have hypopharyngeal and upper oesophageal cancer. This was further strengthened by the observations that a poor diet, comprising mainly of carbohydrates with a low intake of animal protein, fruits, and green vegetables to be associated with an increased risk of

oesophageal cancer in both high- and low-risk incidence areas (Li et al., 1980; Yang et al., 1980; Sons et al., 1987; Mahboubi et al., 1980; Schottenfeld et al., 1984; Jaskiewicz et al., 1987; Pottern et al., 1981).

Countries with high oesophageal cancer rates such as China, Iran, northern Italy, and South Africa, have mostly maize and wheat as staple foods, and micro-nutrients such as ß-carotene, several B vitamins, vitamin C, magnesium, zinc and certain minerals have been reported to be at low levels (van Rensburg., 1981).

Studies carried out in the seventies and eighties have found nutritional deficiencies in people living in the high-risk areas in South Africa. Significant deficiencies in vitamin A, E, and B12 levels in addition to folate that was generally associated with lower intakes of green vegetables, fruits and animal proteins have been reported. Intakes of less than two-thirds the Recommended Daily Allowance (RDA) was frequently observed with inadequate intakes of calcium (90%), nicotinic acid (79%), riboflavin (55%), and ascorbic acid (50%), (Groenewald et al., 1981). Riboflavin for example, has been shown to increase the mitotic rate of oesophageal mucosal cells of baboons (Warwick et al., 1973).

Mineral deficiencies such as selenium have also been reported. Subjects of households with previously reported oesophageal cancer cases had significantly lower whole blood selenium levels. Similarly, mean Se levels of subjects with premalignant or malignant oesophageal cytological changes were significantly lower than in those of subjects without such lesions (Van Helden et al., 1987; Jaskiewicz et al., 1987; & 1988).

Furthermore, nutritional deficiencies can develop by chronic alcohol abuse, and the subsequent exposure to carcinogenic substances, for example those present in smoke may result in transformation. Alcohol has been suggested to aggravate the nutritional deficiency induced in maize-rich diets, or that lesions caused by

niacin and/or riboflavin deficiency may enhance the topical action of alcohol, possibly favoring the penetration of carcinogenic compounds (Correa et al., 1982).

1.3.2 Alcohol and tobacco

Several studies have shown that alcohol and tobacco intake to be major risk factors for oesophageal cancer. Emphasis has been placed on the synergistic relationship between these when the same individual uses both, with the major role being played by tobacco especially in pipe smoke (McGlashan et al., 1982).

Tobacco smoke is known to contain a wide variety of chemicals that have been shown to be highly mutagenic and carcinogenic and oxidants that deplete the body's antioxidants (Schectman et al., 1991; Duthie et al., 1991). Opium and tobacco pyrolysis products for example, commonly sucked or chewed in Iran and Transkei respectively, displayed mutagenic activity in Salmonella typhimurium strains TA98 and TA100 in the presence of rat liver microsomes (Hewer et al., 1978).

In contrast to what has been observed for lung and laryngeal cancer, pipe and hand rolled cigarettes entail a risk that is probably greater than commercial cigarette smoking (Tuyns et al., 1983). A study in France found that strong and dark tobacco as well as hand rolled cigarettes were major risk factors, with the former having a predilection for the upper third of the oesophagus and the latter for the lower third (Launoy et al., 2000).

It has been repeatedly shown that alcohol use is significantly associated with an increased risk of oesophageal cancer. It is reported that alcohol is not carcinogenic *per se*, but acts as a solvent, interacting and increasing the penetration of other carcinogens into the basal cell layers in the oesophagus. In addition chronic use of alcohol interferes with absorption and metabolism of

presence of Fusarium monoliforme has been correlated with oesophageal cancer in China (Yang et al., 1980; Zhen et al., 1984), Italy (Franceschi et al., 1990) and South Africa (Marasas et al., 1982; 1988; 1981).

In the Transkei, harvested maize is normally separated into healthy and mouldy batches that are primarily intended for human consumption and beer brewing respectively (Sydenham et al., 1990). Fumonisin levels have been measured in both healthy and mouldy maize over two seasons, very high levels were detected in mouldy maize (0.1 to 140 mg/kg) compared to healthy maize (< 0.05 to 10.2 mg/kg). Levels were also generally higher from the high oesophageal cancer incidence areas than those obtained from the low incidence areas (Rheeder et al., 1992; Sydenham et al., 1990).

The structural similarity of fumonisins with sphingolipids has led to the hypothesis that fumonisins may inhibit the synthesis of sphingosine by inhibiting the enzyme sphinganine N-acyltransferase. Sphingosines are important components of cell membranes and have been shown to regulate growth factor receptors and act as second messengers for a wide variety of factors, including the tumour necrosis factor, and interleukin -1. Accumulation of sphingosines leads to cell toxicity and altered cell behavior.

Extracts of fumonisin cultures have also been shown to induce the formation of novel DNA adducts. In Mexico, where corn based products in the form of tortillas are consumed, mycotoxins have been linked to birth defects (Mobio et al., 2000). Whilst contamination of maize by fusarium moniliforme is a worldwide phenomenon (Sydenham et al., 1993), the diet of the majority of Transkei population is mainly maize based, thereby exposing them to relatively high levels of mycotoxins on a daily and ongoing basis. However, it is still unclear whether this fungus plays a significant role in the development of oesophageal cancer.

1.3.4 Cultural practices

Although alcohol and smoking have been found to be important risk factors, in other high incidence areas such as Iran, they play a negligible role in the development of oesophageal cancer. In figure 1.3 the age standardised incidence rates per 100 000 in different countries and proportions in percentage due to alcohol and tobacco are summarised (WHO, 2000). Thus it is likely that each high-risk area has its own regional practices that are probably contributing to the pathogenesis of oesophageal cancer.

Oesophageal cancer seems to be promoted by physical damage to the oesophageal mucosa followed by specific carcinogenic induction the form of which may differ from country to country (Albert et al., 1991). The oesophageal mucosa is located at the route of entry for foreign, often harmful agents including chemical substances from lifestyle habits.

In South Africa, the consumption of home brewed beer (umqombothi) has been strongly associated with oesophageal cancer. Mouldy maize, which has been shown to be contaminated with mycotoxins, is usually used for flavour when the home brewed beer is prepared. In addition, the ingestion of pipe stem extracts (Injonga), a substance known to be mutagenic and contain high levels of nitrosamines has been associated with oesophageal cancer (Hewer et al., 1978). Other factors that may enhance the formation of N-nitroso compounds include microorganism contamination of food and water, low molybdenum content in soil and food as well as zinc deficiency.

The association between the practice of regular self-induced vomiting and oesophageal cancer in South Africa has been hypothesised, but never explored. This practice may pose a major insult to the oesophageal mucosa, bathing it in gastric and duodenal juices, which may result in oesophagitis. Chronic oesophagitis is strongly associated with OSCC in high-risk areas (Oettle et al., 1986; Munoz et al., 1982).

In other high-risk areas like China and Iran, ingestion of hard foods (pickled vegetables) and hot beverages at temperatures of 60 to 88^{0}C are other habitual practices associated with oesophageal cancer (Ghadirian et al., 1987; Nakachi et al., 1988). The hard foods and hot beverages are suggested to be physical irritants to the oesophagus, and via the continuous injury of the oesophageal epithelium, carcinogenesis in the oesophagus may be facilitated (Yang et al., 1980).

In parts of India, the risk of oesophageal cancer is believed to be due to tobacco chewing alone or with betel nut, betel leaf, slaked lime or catechu, a resin from acacia (Ranadive et al., 1979). Similarly, in Sri Lanka, the disease is common in females and is related to the habit of chewing tobacco and betal quid. Oesophageal cancer is found in high proportions among immigrants from India in Pakistan

In industrialised countries such as France, North America and Australia alcohol and tobacco consumption is associated with high risk of developing oesophageal cancer. Interestingly, in North America, an inverse relationship between income level and oesophageal cancer has been observed (Ernster et al., 1982). In Alaska, oesophageal cancer is seen mostly in females and is associated with hide chewing.

1.3.5 Human papillomaviruses

Papillomaviruses are small, nonenveloped DNA viruses. Human papillomaviruses (HPV's) are widespread infectious agents of humans and animals. Many specific types of human papillomaviruses involved in cancer have been identified and characterised. To date more than 70 distinct types of HPV's have been described on the basis of DNA sequence and consist of both cutaneous and mucosal types (Villa et al., 1997). The involvement of HPV's in cervical cancer is well established, and this has led to the classification of these viruses as either low or high oncogenic risk types depending on the type of tissue

in which they are found, that is, normal tissue or benign, or tumour tissue, respectively.

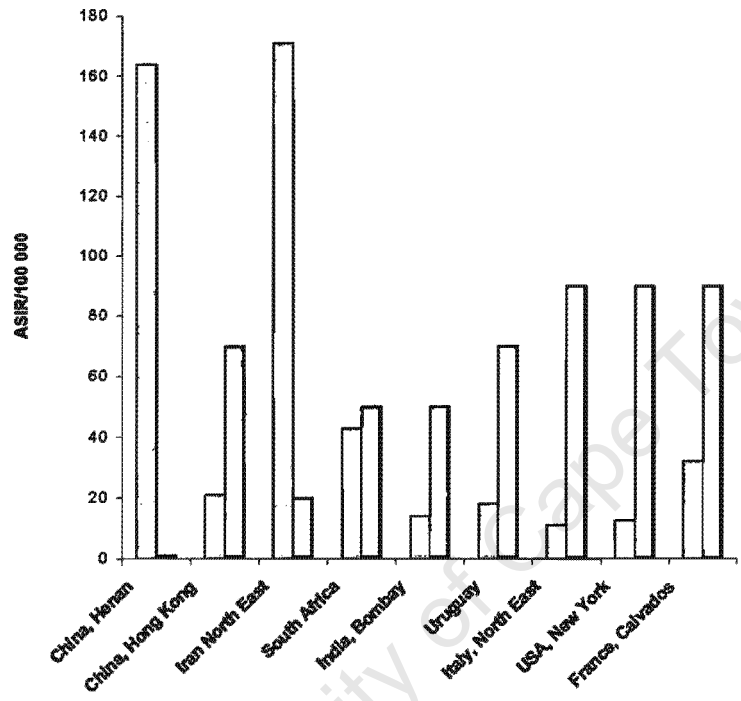


Figure 1.3

Squamous cell carcinoma of the oesophagus. Age-standardised incidence rates (ASIR) per 100 000 (blue) and proportions (%) due to alcohol and tobacco (pink).

1.3.5.1 Molecular Biology of HPV

1.3.5.1.1 Genome structure

The genomic structure of all papillomaviruses appears to have a similar general structure. The HPV genome consists of a circular double-stranded DNA of about 8000 nucleotides. Amongst the types whose complete DNA sequence has been determined, a series of conserved open reading frames (ORF's) have been found

on one strand of DNA while the other strand is apparently noncoding (Chen et al., 1982; Schwartz et al., 1983; Danos et al., 1984). These ORF's are divided into three segments, a late region that encodes two structural viral capsid proteins, an early region compromising of six genes, namely, E1, E2, E4, E5, E6, and E7, and a non-coding region known as the long control region (LCR), (Figure 1.4).

The late genes, L1 and L2 the sequences of which are highly conserved among papillomaviruses, encode the capsid proteins. Following their synthesis, these proteins are directed to the cell nucleus, where viral particle assembly takes place. The non-coding region (LCR) of the viral genome has been shown to contain sequences involved in viral origin of replication and the transcriptional responsive elements that regulate HPV gene expression.

The full length E2 gene product in conjuction with E1 protein have been identified and shown to be required for genital HPV replication in vivo. The product of the E4 gene, which is expressed as a late gene despite its localisation in the early region seems to be involved in the maturation and release of papillomavirus particles. The remaining early genes (E5, E6, and E7) are responsible for the transforming activities of human papillomaviruses (Villa et al., 1997).

1.3.5.1.2 Transformation by HPV

Genital high-risk HPV DNA's were found to immortalise the life span of human genital keratinocytes in vitro. Dissection of the genes responsible for this immortalisation capacity revealed that the HPV E6 and E7 genes were required. These two genes are selectively retained and expressed in cervical carcinomas (Wilcynski et al., 1988). The E7 proteins form complexes with the retinoblastoma susceptibility gene product (Rb) and related proteins (zur Hausen et al., 1996).

Rb is a tumour suppressor protein involved in the progression control of the cell cycle. It controls the cell progression by interacting with and inhibiting

transcription factors such as E2F. The cell cycle controls association and dissociation of the E2F/Rb complex is regulated by phosphorylation and dephosphorylation of Rb, consequently the E2F is released to activate transcription of genes required in the G1/S transition. The E7 protein of HPV binds hypophosphorylated Rb, consequently the E2F is released to activate transcription of genes required in the G1/S phase transition. This process eventually leads to proliferation of abnormal cells that otherwise would have been halted and repaired by cell cycle check mechanisms.

The HPV E6 protein of high risk types complexes with cellular protein E6-AP and the product of p53 gene, and facilitates TP53 degradation via the ubiquitin - dependent proteolytic system. HPV E6 of both low- and high-risk HPV's binds to p53, but only E6 of oncogenic HPV types target p53 for degradation. The p53 gene is the best-characterised tumour suppressor gene involved in the negative regulation of cell proliferation by regulating gene transcription, monitoring and halting the cell cycle in the presence of DNA damage, and in apoptosis. p53 gene mutations are present in almost 50% of all human cancers, and the interaction of HPV E6 with p53 is thought to have the same effect as a p53 mutation.

HPV E6 proteins have also been shown to interact with the newly identified gene p73, however at this stage of research this is not clear. Functional inactivation of p73 via direct DNA binding of HPV E6 of both low- and high-risk types has been reported (Park et al., 2001). There are two schools of thought regarding the function of p73, one suggest that p73 is a tumour suppressor gene, because p73 is structurally and functionally similar to p53. Like p53, p73 has been shown to transactivate p21 and induce apoptosis (Figure 1.5). However the lack of p73 mutations in cancers studied thus far, and the overexpression of p73 gene products in cancer tissue versus normal tissue suggest that the expression of this gene product is oncogenic.

HPV E5 gene is usually lost or disrupted following viral integration into the host chromosome, hence its transformation functions in human cancers has not been thoroughly studied. The E5 gene product is a highly hydrophobic protein anchored in the cell membrane that can interact with other membrane associated proteins, the epidermal growth factor receptor in the stimulation of epithelial cell proliferation.

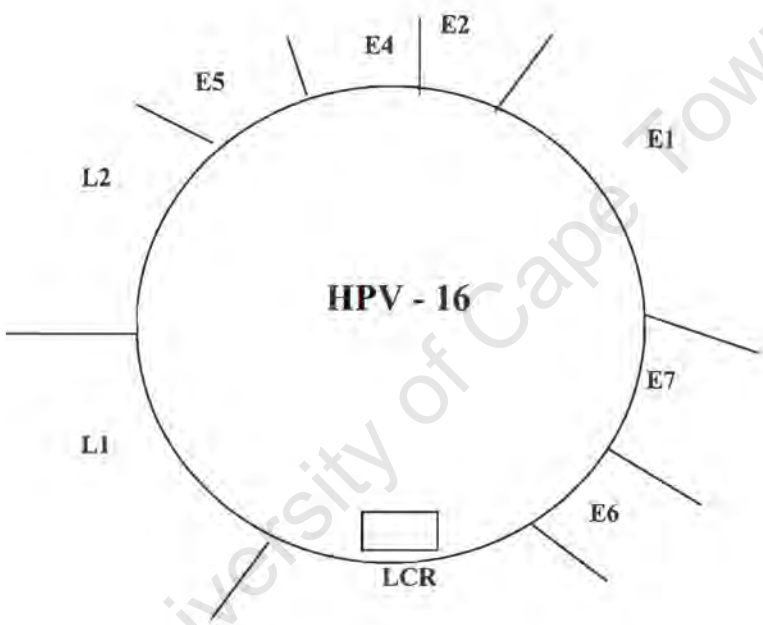


Figure1.4

HPV 16. Schematic representation of the genomic organization of HPV16. The early (E) and late (L) genes, and the long control region (LCR) are indicated.

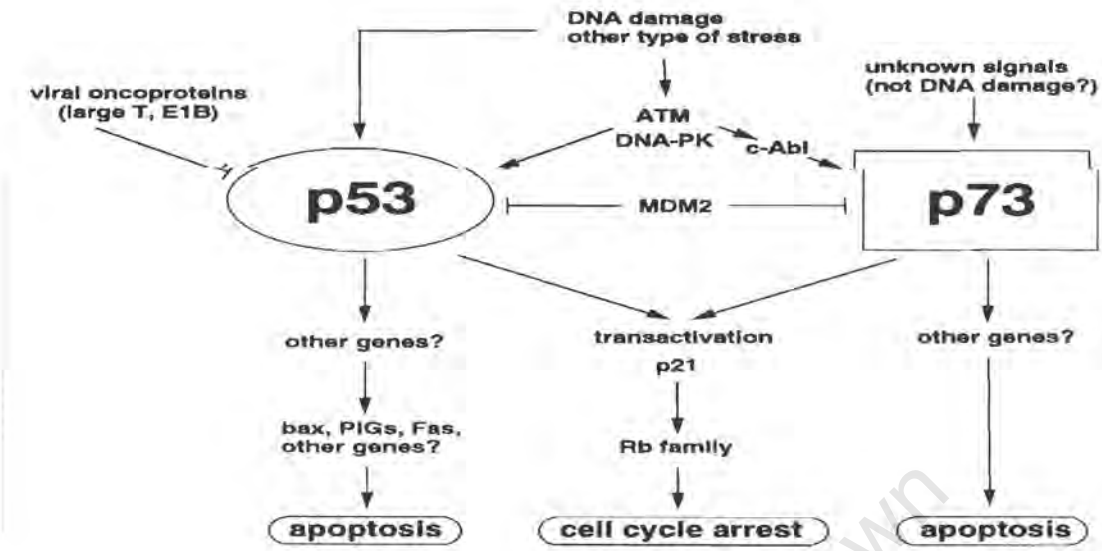


Figure 1.5
p53 and p73 pathways. Diagrammatic pathways by which p53 and p73 induce cell cycle arrest and apoptosis in response to various stimuli, including genotoxin. Both proteins share a common pathway via transactivation of p21 (Ichimiya et al., 2000).

1.3.5.2 HPV in oesophageal cancer

The basis for investigation into the aetiological role of HPV in the development of oesophageal carcinoma was initiated by Syrjanen in 1982. The authour examined 60 patients with squamous cell carcinoma of the oesophagus and discovered that 40% had morphological features of HPV infection. Since then, contradictory observations with regards to the role of HPV in OC have been reported (Table 1.2). It has been speculated that the contradictory results observed in different HPV detection studies may be due to variations in sensitivity and specificity of the techniques used (Sur et al., 1998). To date, HPV DNA has been investigated in 1485 and 2020 oesophageal squamous cell carcinomas by in situ hybridisation (ISH) and polymerase chain reaction (PCR), respectively. In situ hybridisation detected HPV DNA in 22.9% of cases, whilst PCR could identify HPV DNA in 15.2% cases (Syrjanen et al., 2002).

HPV DNA has been described in a high proportion of oesophageal cancer from high-risk regions worldwide (Table 1.2). However, variations within regions also exists in the frequency with which HPV is detected. Chang et al (1993), evaluated 776 biopsy specimens from 363 patients using low-stringency *in situ* hybridisation and confirmed the presence of HPV DNA in 85 (23.4%) patients. Forty percent of the 85 HPV positive tumours were shown to contain at least one type of (HPV, HPV6, 11, 16, 18, or 30) DNA sequences. In contrast, another study performed in the same province, but where tissue samples were analysed for the presence of HPV DNA by Southern blot analysis using HPV 16 and HPV 18 probes, and by PCR with HPV consensus primers showed no evidence of HPV 16, HPV 18 or any other subtypes (Lu et al., 1995).

In Beijing, China, the incidence of oesophageal cancer is significantly lower than that reported from high risk areas of China. Suzuki et al (1996), examined 83 OSCC from Beijing for HPV DNA by ISH for HPV types 6/11; HPV 16/18; and HPV 31/33/35, and PCR using consensus primers for the HPV L1 gene, and type specific primers for the HPV 6, HPV 16, and HPV 18. In *situ* hybridisation and PCR using consensus primers failed to demonstrate the presence of HPV DNA, but the type specific primers were able to demonstrate HPV DNA in 3 of the specimens (4.3%). The HPV types identified were HPV 6 in one sample and HPV 16 in the other two. Interestingly, in comparison with another PCR study involving Chinese patients from Fuzhou, Chen et al (1994) demonstrated HPV infection in 60% of the 40 formalin fixed paraffin embedded sections of oesophageal carcinoma specimens.

Similar or even higher prevalence of HPV DNA among OSCC patients from South Africa has been found in other PCR, hybridisation and immunohistochemical studies. HPV DNA in patients with OSCC from South Africa has also been confirmed in an international study. Togawa et al (1994), investigated the presence of HPV DNA from OSCC samples collected from different regions of the world (Italy, France, Japan, Iran, USA, and South Africa),

and found the presence of HPV DNA in 17% of the 18 OSCC cases from South Africa by PCR. The region with a high incidence of OC in South Africa, Transkei, has never been systematically investigated for the involvement of HPV infection. Recently, Matsha reported the presence of HPV DNA in 46% of the OSCC cases from Transkei analysed by PCR using consensus primers (Matsha et al., 2002). The HPV types identified in that study were HPV types 11, 16, 52, and 39. In cervical lesions HPV types 16 and 39 are classified as high oncogenic HPV types.

The pooled data of high-risk areas show a range in the prevalence of HPV DNA in oesophageal cancer from 13% to 63%, with the overall incidence of about 22% (Lam et al., 2000). Therefore, these results support the hypothesis that HPV may play a significant role in oesophageal carcinogenesis in those areas with a high prevalence of the disease. However, whether the low-risk HPV types found in oesophageal cancer are oncogenic in this particular human organ as well as molecular mechanisms in which HPV is involved in the malignant transformation in the oesophagus has not been investigated.

The presence of HPV DNA in low incidence geographic regions such as North America, Europe and Australia remains controversial. Kulski et al (1986), reported the incidence of HPV positivity (50%) in Australian patients with OSCC using hybridisation techniques. In 1990, the same author repeated these experiments in another set of OSCC patients and HPV DNA was present in 23% of OSCC cases from Australia. These results suggest that HPV infection is present in some OSCC from low risk areas. However, in North America, reports have been very consistent reporting low or absence of HPV prevalence in patients with OSCC (Kiyabu et al.,1989; Paz et al., 1997; Suzuk et al., 1996). The conclusions drawn from these studies was that HPV infection occurs infrequently in association with OSCC in America.

1.3.6 Genetic predisposition in oesophageal cancer

Familial predisposition of oesophageal cancer has been poorly studied. One study that seem to suggest a strong association between family history of cancer, particularly oesophageal cancer and the risk of developing OSCC has been conducted by Dr Ballo (data unpublished). In that study, 22% of oesophageal cancer patients had a first degree relative with another cancer and in 62% of these cases the relatives had oesophageal cancer. In other studies the evidence is weak and inconclusive (Hu et al., 1992; Carter et al., 1992).

Nevertheless, familial predisposition to oesophageal cancer has been associated with focal non-epidermolytic palmoplantar keratoderma (NEPPK or tylosis). This autosmal, dominantly inherited disorder of the palmar and plantar surfaces of the skin has been designated the tylosis oesophageal cancer (TOC) gene and maps to 17q25. Marger et al (1993)have shown that 90% to 95% of patients with tylosis are at risk of developing SCC of the oesophagus by the age 65. Loss of TOC gene may alter oesophageal inegrity thereby making it more susceptible to enviromental mutagens. In South African oesophageal cancer patients, the association of TOC gene abnormality and oesophageal cancer has not been studied yet.

Table 1.2 Detection of human papillomavirus (HPV) in oesophageal squamous cell carcinomas

Method used	Country	HPV type	No. of samples	%positive	Reference
IHC	China	Ag	31	23%	Mori, 1989
HB	China	-	51	49%	Chang, 1990
ISH	China	6, 11, 16, 18	51	43%	Chang, 1990
FISH	China	11, 16, 18	80	4%	Chang, 1990
SB	China	16	24	50%	Li, 1991
PCR	China	6, 11, 18, 18	51	49%	Chang, 1992
SB	China	11, 16, 18, 30	20	40%	Chang, 1992
ISH	China	6, 11, 16, 18, 30	363	23%	Chang, 1993
PCR	China	GP	40	60%	Chen, 1994
SB, PCR	China	16, 18	35	0%	Lu, 1995
SB, PCR	China	16, 18	103	36%	He, 1996
PCR	China	GP	70	4%	Suzuk, 1996
PCR	China	16, 18	152	21%	He, 1997
ISH	China	wide spectrum	36	8%	Chang, 1997
PCR	China	CP	117	17%	de Villiers, 1999
PCR	China	CP	101	17%	Chang, 2000
ISH	China	6, 11, 16, 18, 30, 53	700	17%	Chang, 2000
PCR	China	CP	32	6%	Pexito, 2001
PCR	China/SA	CP	63	30%	Lavergne, 1999
HB	South Africa	-	70	33%	Hille, 1986
IHC	South Africa	Ag	70	10%	Hille, 1986
HB	South Africa	-	20	65%	Hale, 1989
PCR	South Africa	various	14	43%	Williamson, 1991
ISH	South Africa	6, 7, 16, 18, 30	10	30%	van Rensburg, 1993
PCR	South Africa	CP, 16, 18	18	17%	Togawa, 1994
PCR	South Africa	E6, GP	9	67%	Cooper, 1995
ISH	South Africa	6, 11, 18, 31, 33	48	52%	Cooper, 1995
PCR	South Africa	CP	50	46%	Matsha, 2002
Dot blot	France	6/11, 16/18	12	42%	Benamouzig, 1992
ISH	France	6, 11, 16, 18, 31, 33	12	8%	Benamouzig, 1992
PCR	France	6, 11, 16, 18, 31, 33	75	0%	Benamouzig, 1995
PCR	Italy	-	18	0%	Rugge, 1997
PCR	Italy	CP, 16, 18	45	0%	Talamani, 2000
PCR	Italy	CP, RFLP	17	47%	Astori, 2001
IHC	Japan	Ag	15	13%	Mori, 1989
PCR	Japan	CP, 16, 18	45	7%	Toh, 1992
ISH	Japan	6, 11, 16, 18, 31, 33	71	34%	Furihata, 1993
ISH	Japan	16, 18	42	31%	Ono, 1994
PCR	Japan	CP	31	0%	Akutsu, 1995
IHC	Japan	Ag	61	8%	Nakamura, 1995
PCR	Japan	CP	72	21%	Shibagaki, 1995
PCR	Japan	CP, 16, 18	27	63%	Khurshid, 1998
PCR	Japan	CP	45	7%	Sugimachi, 1998
ISH	Japan	6, 11, 16, 18	123	30%	Takahashi, 1998
PCR	Japan	CP	75	16%	Kawaguchi, 2000
IHC	Japan	Ag	4	0%	Kuwano, 2001
FISH	Australia	11, 13, 16, 18	10	50%	Kulski, 1986
FISH	Australia	6, 11, 16, 18	39	23%	Kulski, 1990
PCR	USA	16/18	13	0%	Kiyabu, 1989
PCR	USA	6, 16, 18	23	4%	Suzuk, 1996
PCR	USA	73	1	100%	West, 1996
PCR	USA	-	11	0%	Paz, 1997
PCR	USA	CP, RFLP, 16	51	2%	Turner, 1997
ISH	UK	6, 11, 16, 18, 31, 33	4	0%	Ashworth, 1993
PCR	UK	-	22	0%	Morgan, 1997

Ag, HPV antigens; CP, consensus primers; FISH, filter in situ hybridisation;, GP, general primers; HP, histological biopsy; IHC, immunohistochemistry; ISH, in situ hybridisation; PCR, polymerase chain reaction; RFLP, restriction fragment length polumorphism, SB, Southern blot hybridisation

1.4 MOLECULAR ALTERATIONS IN OESOPHAGEAL CANCER

Molecular basis of cellular transformation in human cancers has been extensively studied. The key features of a malignant cell are, dysregulated cell division and differentiation, genomic instability, loss of normal senescence and invasion into adjacent tissue (Bishop et al., 1987). Oesophageal cancer is no exception, alteration in genes especially those that encode regulators of the G1 to S phase transition of the cell cycles are common in squamous cell carcinoma of the oesophagus. The genes that have been selectively studied in OSCC are summarised in table 1.4. The respective proteins of these genes interact with each other in complex networks, therefore accumulated effects of multiple gene alterations in tumour cells may lead to aggressive tumour behaviour and poor prognosis in patients with oesophageal squamous cell carcinoma.

1.4.1. p16/CDKN2

Cell cycle progression from G1 to S phase is strictly regulated by several cell cycle -dependent serine kinases (Cdks) inhibitors, including the p16/CDKN2 gene product. The Cdks are regulated and activated by a set of proteins, cyclins, whose expression or activity varies in a cell cycle-dependent manner (Rosen et al., 1994). Inactivation of the p16/CDKN2 gene through various mechanisms, such as homozygous deletions, loss of expression due to aberrant DNA methylation or missense mutations, may result in enhanced tumour progression.

Gamieldien et al (1998) reported p16/CDKN2 somatic mutation in 28% of patients with oesophageal cancer. In other studies involving OSCC patients, the incidence of p16/CDKN2 mutations ranges between 0% to 86% (Busatto et al., 1998; Esteve et al., 1996; Chan et al., 1997; Muzeau et al., 1997; Xing et al., 1999; Yang et al., 1997; Shamma et al., 1998). Exons one and two were mainly analysed since these two exons cover almost 97.5% of the coding region for p16/CDKN2 (Sun et al., 1995).

Table 1.4 Genetic alterations in squamous cell carcinoma of the oesophagus.

Gene	Location	Tumour abnormality	Function	Reference
p16/CDKN2	9p22	Homozygous loss Promoter methylation	CDK inhibitor (cell cycle control)	Sun et al., 1995
Cyclin D1	11q13	Amplification	Cell cycle control	Hinds et al., 1992
Rb	13q14	LOH loss of expression	Cell cycle control	Friend et al, 1986
p53	17p13	LOH, mutations	G1 arrest, apoptosis genetic stability	Levine et al, 1991
p73	1p36.3	overexpression	apoptosis	Kaghad et al., 1997
EGFR	17p13	amplification, overexpression	Signal transduction	Aaronson et al, 1991
TOC	17q25	LOH	Tumour suppressor	Marger et al., 1993
c-myc	8q24.1	Amplification	Transcription factor	Evan et al., 2001
FEZ1	8p22	Transcription shutdown	Transcription factor	Hamilton et al., 2000
DLC1	3p21.3	Transcription shutdown	Growth inhibition	Hamilton et al., 2000

1.4.2 Cyclin D1

Cyclin D1 is a cell regulatory protein that is expressed at high levels during the G1 phase of cell cycle (Matsushime et al., 1991). The gene product of cyclin D1

controls cell cycle progression by activating their cyclin-dependent kinases (CDK4 and CDK2), which leads to the phosphorylation of the retinoblastoma protein, and in turn causes cells to enter the S phase of the cell cycle. (Sherr et al., 1999). Therefore, inhibition of cyclin D1 will arrest the cells before the S-phase and overexpression will contribute to the uncontrolled cell proliferation eventually resulting in malignant cell accumulation. Tendency to develop breast cancers has been observed in transgenic mice engineered to overexpress cyclin D1 (Wang et al., 1994).

Overexpression of cyclin D1 has been commonly observed in oesophageal cancer tissues (20% to 40%), and is frequently detected in cancers that retain expression of the Rb protein, in agreement with the notion that these factors co-operate within the same signalling cascade (Lu et al., 2000). Amplification of cyclin D1 has been suggested to be a marker for poor prognosis (Shimada et al., 1999; Nagasawa et la., 2001). In South Africa, 29% of patients with OC displayed overexpression of cyclin D1, with 75% of patients having lymph node metastaisis (Chetty et al., 1999).

1.4.3 Retinoblastoma (Rb)

The cell cycle is controlled at several set points, whether a cell will synthesise DNA and traverse the cycle, arrest, differentiate or undergo programmed cell death is controlled at two main restriction points, G1 and G2/M (Hartwell et la., 1989). Rb seems to be a fundamental component of the cell cycle clock. Loss of Rb or binding of Rb to viral oncoproteins, for example HPV E7 proteins, that prevent its binding to transcription factors (for example E2F) causes unregulated cell division (Kato et al., 1993).

In oesophageal cancer, loss of heterzygosity (LOH) and mutations of the Rb gene have been observed (Monden et al., 1996; Li et al., 1993). In one such study, mutation and expression of Rb in human oesophageal cancer biopsies from Linxian, China, were investigated by PCR-direct sequencing and Northern

blot hybridisation. Rb gene deletions and mutaions in exons 17 and 21 were observed (Li et al., 1993).

1.4.4 p53

The ability of wild type p53 to induce cell cycle arrest and allow repair of damaged DNA or lead to apoptosis has been well characterised. p53 achieves cellular proliferation control by activating or repressing cellular genes involved in cell cycle control. For example, wild type p53 is associated with induction of a gene p21/WAF1 and Bcl-2 family members (Bax, Bak, Puma, and Noxa) (Mantessano et al., 1996; Johnstone et al., 2002). The p21/WAF1 gene product binds to and inhibits kinase activity of members of the cyclin-dependent kinase family and Bcl-2 antagonise Bax and or Bak and inhibits drug-induce apoptosis (Reed et al., 1999). Loss of p53 protein, or mutations in the gene is the central mechanism through which cells escape normal controls on their proliferation.

Mutated p53 tends to have a longer half-life than wild type p53 and tend to be overexpressed (Levine et al., 1991).The p53 gene has been reported to be mutated in 35% to 80% of oesophageal squamous cell carcinoma (Hamilton et al., 2000). Immunohistochemical studies have indicated altered p53 overexpression in 42% to 51% in OSCC patients from South Africa (Hendricks et al., 2002).

1.4.5 p73

p73 has been recently identified and is located on chromosome 1p36.3 that is frequently deleted in neuroblastoma and other cancers (Kaghad et al., 1997). It has been shown that ectopic overexpression of p73 activates p21/WAF1 and induce apoptosis (Jost et al., 1997). However, animal model systems have demonstrated that transgenic mice lacking the p73 gene are not susceptible to tumours, but instead show developmental abnormalities (Yang et al., 2000). To date, no p73 gene mutations have been identified in human cancers including

oesophageal cancer (Benard et al., 2003). Nonetheless, it is strongly suggested that p73 may be involved in acquisition and maintainance of malignancy.

Mutations, LOH and expression of the p73 gene have been investigated in a liimited number of oesophageal cancer cases. The absence of mutations has been in concordance with published reports (Cai et al., 2000; Nimura et al., 1998). Similarly, altered mRNA p73 expression in the tumour tissue versus the normal has been observed. However, differences with regards to LOH (8% and 64%) exist between the two reports. The conclusions drawn from these reports was that perhaps in oesophageal cancer, p73 plays a partial compensatory role for defective p53, because the association between elevated p73 expression and p53 defects was statistically significant.

1.4.6 Other Genetic Alterations

Other potentially important genetic alterations include alterations of growth signal receptor and transduction genes. The epidermal growth factor receptor (EFGR) protein encodes transmembrane receptor tyrosine kinases (Zhou et al., 1994). Alterations in EGFR have been found in many other tumours such as breast, ovarian, gastric and brain tumours (Montesano et al., 1996). Amplification and overexpression of EGFR gene status have been found in oesophageal cancer (15% to 30%).

The c-myc gene is located on chromosome 8q24.1 and is thought to induce cell proliferation in the presence of survival factors, such as Bcl-2, and apoptosis in the absence of survival factors. In addition, it can induce cells to drug -induced apotosis (Evan et al., 2001). Amplificantion of c-myc has been shown in 14% to 25% of oesophageal cancers and is suggested to contribute to tumour progression, however this is still speculative (Montesano et al., 1996; Hamilton et al., 2000).

Understanding the molecular events that contribute to oesophageal and other cancers can provide paradigm to explain the relationship between cancer genetics and treatment sensitivity and should enable a more rational approach to anticancer drug design and therapy.

1.5 DIAGNOSIS OF OESOPHAGEAL CANCER

1.5.1 Clinical manifestation

The majority of patients with oesophageal cancer present with dysphagia which unfortunately is a late symptom in the natural history of the disease. In high incidence areas such as China, early screening programmes have been able to detect individuals with early oesophageal cancer. In those individuals with early oesophageal cancer symptoms, if present, are mild. The symptoms reported are gastric pain, burning sensation on swallowing, slight dysphagia, substernal discomfort and heartburn (Liu et al., 1995).

1.5.2 Oesophagography

This is usually the initial invasive diagnostic approach in symptomatic patients. This method relies on radiological contrast observation of abnormalities such as luminal masses, ulcerations or strictures. Oesophagrams can also be used as an aid to tumour staging by determing the oesophageal axis. However, this method of investigation is unreliable and it has been proposed as an alternate for early screening in populations considered to be at high risk (Levine et al., 1997; Ohno et al., 1997).

1.5.3 Endoscopy

Presently, endoscopy is the most accurate method for detection of oesophageal cancer. It provides biopsy material for further investigations. Endoscopy with in vivo staining (Chromoendoscopy), utilises either toulidine blue or Lugol solution. Lugol solution reacts specifically with glycogen in the normal squamous epithelium, whereas precancerous lesions and cancerous lesions are not

stained. Endoscopy with Lugol stain has been shown to improve the rate of detection of superficial esophageal cancers (Sugimachi et al., 1991).

Combination of endoscopy and ultrasonography can assist to investigate the depth of tumour infiltration and para-oesopahgeal lymph node involvement in early and advanced stage of oesophageal cancer. Tumour growth is characterised as swelling of the oesophageal wall with or without direct invasion to surrounding organs. Computed tomography (CT) and magnetic resonance imaging (MRI) can give information on local and systemic spread of OSCC.

1.5.4 Brush Cytology

Brush cytology of the oesophagus is both reliable and cost-effective for early detection of OC in screening programmes. Two methods (Balloon, and Nabeya capsule) are used for the retrieval of oesophageal cells for cytology. In a study that compared brush cytology biopsies with tumour biopsies obtained by endoscopic examination, squamous cell dysplasia as displayed by cytology and squamous cell cancers in the same site and patient was observed (Dawsey et al., 1993). Thus, it was suggested that brush cytology and endoscopy are complementary diagnostic methods for detection of oesophageal cancer. However, the expertise required for endoscopic examination makes this method impractical and costly for mass screening programmes.

1.5.5 Pathology

Once all the clinical investigations have been performed and the patient has been classified as having oesophageal cancer, histopathologic examination of the biopsy is of paramount importance to ascertain whether the tumour biopsy taken is truly cancerous. This is important not just for the patients, but for other studies that may be done on that particular biopsy specimen. Tumour type, stage and grade of tumour are obtained by histopathologic examination. These parameteres are essential in planning treatment and in the prediction of prognosis of these patients.

1.6 TREATMENT OF OESOPHAGEAL CANCER

The treatment offered to most patients with OSCC is palliative and as mentioned earlier, majority of these patients are already at a late stage of disease on presentation.The mean survival of patients not considered for treatment is usually measured in months or a few weeks in those with metastatic disease.

Surgery (complete tumour resection or reconstruction), chemotherapy and radiation and brancytherapy are the treatments available for oesophageal cancer. In a few cases where the cancer is diagnosed early, tumour resection is the treatment of choice. The survival of patients who had received sugical treatment is 68% to 85% of those in stage 1 and II, and 15% to 28% in stage III (Roth et al., 1994).

Cistaplatin and 5 Fluorouracil in combination is considered to be the standard therapy for OSCC patients (Ajani et al., 1994). Radiotherapy together with brancytherapy are used as palliative treatment measures (Smalley et al., 1994). Despite improvements in treatment regimes, the five year survival of patients with oesophageal cancer still remains at less than 10%. In South Africa, Mannell et al (1989), investigated the one year survival of oesophageal cancer patients that had been treated; 37% by oesophageal intubation, 35% by radiation, 22% by chemotherapy and 17% by surgery. In those patients with local spread of the disease the one year survival was 11% and 1% in those with advanced disease.

1.5 IMPORTANCE OF THIS STUDY

Despite nearly 50 years of research, there are still no clear answers to the high incidence of oesophageal cancer in the Transkei region. Residence in the Eastern Cape, South Africa of more than 5 years duration and poverty seem to be a risk factor as evidenced by the increasing incidence of oesophageal cancer in the resident population of Soweto, many of whom are migrants from the Eastern Cape (Sitas et al., 1998). It is likely that the answers lie in the Eastern

Cape and are probably linked to dietary and cultural practices. The population of Transkei is estimated at 3.2 million and more than 80% of which is rural and poor.

Currently much emphasis in cancer research is placed on early detection and biomarkers, and preventive measures. However, application of preventive measures becomes meaningless in the absence of known aetiologic factors. Early detection of this disease seems to be the only factor that would change the mortality and morbidity rates due to oesophageal cancer. Molecular biology techniques have provided improvements in early detection and prognosis of other cancers. For example, HPV DNA detection in cervical Pap smears. To date, however, no molecular biomarker has been identified for oesophageal cancer.

HPV DNA detection by various methods allows accurate interpretation of Pap smear test results in cervical cancer screening programmes followed by appropriate action that may prevent the development of cancer. Mass screening programmes using brush cytology techniques may be a feasible means for early detection of oesophagel cancer in high-risk areas like China and South Africa.

In this project the first aim was to determine if HPV infection is present in patients with squamous cell carcinoma of the oesophagus from the Transkei and if so, correlate the presence of HPV infection with precursor lesions identified by brush cytology. In addition to this, other habitual factors known to contribute to the development of oesophageal cancer were studied in order to be able to establish a set of criteria that would identify an individual being at high-risk for oesophageal cancer. The establishment of such a criteria would enable high risk individuals to undergo perhaps regular screening procedures for the disease in which early lesions, if detected, could be treated. The other objective of this study was to investigate the molecular mechanism by which HPV may contribute to the

development of oesophageal cancer by analysing some of the genes known to interact with HPV in the transformation of cells.

CHAPTER TWO

HUMAN PAPILLOMAVIRUS TYPES ASSOCIATED WITH OESOPHAGEAL CANCER IN TRANSKEI.

2.1 INTRODUCTION

The Transkei region of the Eastern Cape has been identified as a region with a high incidence of squamous cell carcinoma of the oesophagus in South Africa worldwide. The disease occurs more frequently in men, where the lifetime risk of developing this cancer is one in 39 (Chalasani et al., 1998). There appears to be a distinct racial variation in the types of oesophageal cancer (OC), with predominance of squamous cell cancer among blacks, whereas adenocarcinoma is seen more frequently in whites (Sitas et al., 1998). Presently the aetiology of oesophageal cancer is unknown, however many factors including the presence of human papillomavirus (HPV) DNA has been associated with the development of oesophageal squamous cell carcinoma.

Human papillomavirus has been implicated as a causative agent in a variety of human squamous cell carcinomas (SCC), including those of the oral cavity (de Villiers et al., 1985), larynx (Scheurlen et al., 1986) cervix (Williamson et al., 1994), and anogenital region (Neil et al., 1990). Human papillomaviruses are small non-enveloped DNA viruses belonging to the Papovaridae family, and more than 70 papillomavirus types have been identified on the basis of sequence divergence (Villa et al., 1997; de Roda Husman et al., 1995). The association of certain types of HPV primarily with normal tissue or benign lesions, as opposed to the cancer associated types, has led to the concept of low (e.g. types 6, 11 and 33) and high (e.g. types 16 and 18) risk HPV's.

The first compelling evidence that HPV might be involved in oesophageal cancer dates back to the observations made in 1982 by Syrjanen et al. The author examined 60 patients with squamous cell carcinoma of the

oesophagus and discovered that 40% had morphological features of HPV infection. Since then contradictory observations with regards to the presence of HPV in oesophageal cancer have been reported with reported prevalence rates ranging from 0% to 71% (Table 2.1).

HPV DNA has been described in a high proportion of oesophageal cancers from high-risk regions. However, variations within regions also exist in the frequency with which HPV is detected. Chang et al, (1993), evaluated biopsy specimens using low-stringency *in-situ* hybridisation and confirmed the presence of HPV DNA in 85 out of 363 patients with oesophageal squamous cell carcinoma (OSCC). In contrast, another study also performed in the same province, where tissue samples were analysed for the presence of HPV DNA by Southern blot hybridisation using HPV 16 and 18 probes and by PCR with HPV consensus primers showed no evidence of HPV DNA (Lu et al., 1995). In South Africa the presence of HPV DNA among OSCC patients has been previously reported (Togawa et al., 1994; Poljak et al., 1993; Williamson et al., 1991; Cooper et al., 1995), but none of these studies targeted Transkei, a region with a high incidence of OC in South Africa.

The differences in HPV detection rates are probably a result of variations in the specificity and sensitivity of the analytical techniques used. Compared with other techniques, the polymerase chain reaction (PCR) is a simple, rapid and sensitive method for the detection of HPV DNA in tissue samples. Furthermore, the use of degenerate consensus primers is an advantage in PCR based studies because these primers can detect a wide spectrum of HPV types. However, the E1 and L1 genes, which are suitable targets for consensus primers may be lost or disrupted after viral DNA integration, whereas the E6 and E7 genes which are thought to be retained intact (Wilczynski et al., 1988) in all carcinomas are too variable to be targeted with consensus primers.

This chapter determined the incidence and types of HPV in tumours from patients with squamous cell carcinoma of the oesophagus and individuals without cancer by PCR. Since differences in HPV detection rates in different PCR based studies have been previously observed (Chang et al., 1992; Talamani et al., 2000; Astori et al., 2001; Williamson et al., 1991), PCR was performed using consensus primers to both the E6 and L1 genes. Immunohistochemistry using an antibody that targets HPV L1 proteins was also used to confirm the presence of HPV antigens. HPV DNA positive samples with the L1 primer set were further subjected to sequence analysis to determine the HPV type.

Table 2.1

Detection of human papillomavirus (HPV) in oesophageal squamous cell carcinomas

Method used	Country	HPV type	No. of samples	%positive	Reference
IHC	China	Ag	31	23%	Mori, 1989
HB	China	-	51	49%	Chang, 1990
ISH	China	6, 11, 16, 18	51	43%	Chang, 1990
FISH	China	11, 16, 18	80	4%	Chang, 1990
SB	China	16	24	50%	Li, 1991
PCR	China	6, 11, 18, 18	51	49%	Chang, 1992
SB	China	11, 16, 18, 30	20	40%	Chang, 1992
ISH	China	6, 11, 16, 18, 30	363	23%	Chang, 1993
PCR	China	GP	40	60%	Chen, 1994
SB, PCR	China	16, 18	35	0%	Lu, 1995
SB, PCR	China	16, 18	103	36%	He, 1996
PCR	China	GP	70	4%	Suzuk, 1996
PCR	China	16, 18	152	21%	He, 1997
ISH	China	wide spectrum	36	8%	Chang, 1997
PCR	China	CP	117	17%	de Villiers, 1999
PCR	China	CP	101	17%	Chang, 2000
ISH	China	6, 11, 16, 18, 30, 53	700	17%	Chang, 2000
PCR	China	CP	32	6%	Pexito, 2001
PCR	China/SA	CP	63	30%	Lavergne, 1999
HB	South Africa	-	70	33%	Hille, 1986
IHC	South Africa	Ag	70	10%	Hille, 1986
HB	South Africa	-	20	65%	Hale, 1989
PCR	South Africa	various	14	43%	Williamson, 1991
ISH	South Africa	6, 7, 16, 18, 30	10	30%	van Rensburg, 1993
PCR	South Africa	CP, 16, 18	18	17%	Togawa, 1994
PCR	South Africa	E6, GP	9	67%	Cooper, 1995
ISH	South Africa	6, 11, 18, 31, 33	48	52%	Cooper, 1995
PCR	South Africa	CP	50	46%	Matsha, 2002
Dot blot	France	6/11, 16/18	12	42%	Benamouzig, 1992
ISH	France	6, 11, 16, 18, 31, 33	12	8%	Benamouzig, 1992
PCR	France	6, 11, 16, 18, 31, 33	75	0%	Benamouzig, 1995
PCR	Italy	-	18	0%	Rugge, 1997
PCR	Italy	CP, 16, 18	45	0%	Talamani, 2000
PCR	Italy	CP, RFLP	17	47%	Astori, 2001
IHC	Japan	Ag	15	13%	Mori, 1989
PCR	Japan	CP, 16, 18	45	7%	Toh, 1992
ISH	Japan	6, 11, 16, 18, 31, 33	71	34%	Furihata, 1993
ISH	Japan	16, 18	42	31%	Ono, 1994
PCR	Japan	CP	31	0%	Akutsu, 1995
IHC	Japan	Ag	61	8%	Nakamura, 1995
PCR	Japan	CP	72	21%	Shibagaki, 1995
PCR	Japan	CP, 16, 18	27	63%	Khurshid, 1998
PCR	Japan	CP	45	7%	Sugimachi, 1998
ISH	Japan	6, 11, 16, 18	123	30%	Takahashi, 1998
PCR	Japan	CP	75	16%	Kawaguchi, 2000
IHC	Japan	Ag	4	0%	Kuwano, 2001
FISH	Australia	11, 13, 16, 18	10	50%	Kulski, 1986
FISH	Australia	6, 11, 16, 18	39	23%	Kulski, 1990
PCR	USA	16/18	13	0%	Kiyabu, 1989
PCR	USA	6, 16, 18	23	4%	Suzuk, 1996
PCR	USA	73	1	100%	West, 1996
PCR	USA	-	11	0%	Paz, 1997
PCR	USA	CP, RFLP, 16	51	2%	Turner, 1997
ISH	UK	6, 11, 16, 18, 31, 33	4	0%	Ashworth, 1993
PCR	UK	-	22	0%	Morgan, 1997

Ag, HPV antigens; CP, consensus primers; FISH, filter in situ hybridisation;, GP, general primers; HP, histological biopsy; IHC, immunohistochemistry; ISH, in situ hybridisation; PCR, polymerase chain reaction; RFLP, restriction fragment length polumorphism, SB, Southern blot hybridisation

2.2 RESULTS

2.2.1 OESOPHAGEAL BIOPSIES

The Umtata General Hospital is a 1000 bed facility and the major tertiary centre for the Transkei, to which all cases of oesophageal cancer are referred. Two hundred paraffin embedded biopsies of histological confirmed SCC of the oesophagus were obtained from archival material in the department of anatomical pathology of the Umtata General Hospital during the period 1995 to 1998. One of the limiting factors for HPV incidence studies is the difficulty in obtaining normal tissue biopsies of the oesophagus. For this reason forty-one paraffin embedded biopsies of the oesophagus taken from individuals with no histopathologically confirmed OSCC were used as normal controls in this study. Ten fresh biopsies were collected from patients undergoing endoscopy in 1998. Briefly, the biopsies excised from the patient during endoscopy were snap frozen in liquid nitrogen and thereafter stored at –70°C until required.

A pathologist confirmed histopathology classification using the World Health Organization (WHO) system. More than 70% of the patients had SCC keratinising type (Table 2.2). Clinical diagnosis of each of the normal subjects was obtained from their respective hospital records. Just over 50% of the "normal" subjects had oesophagitis (Table 2.3).

The demographic data of each of the cancer patients and normal controls were retrieved from their respective hospital records (Table 2.4). All the cancer biopsies used in this study were taken from black patients who presented clinically with varying degrees of dysphagia.

Table 2.2

Histopathology of the paraffin wax embedded tumour biopsies. Summary of the histopathological characteristics of the tumour biopsies used in this study.

Histopathological findings	No. of biopsies
SCC Keratinising type	152 **(76%)**
SCC non keratinising type	40 **(20%)**
SCC papillary type	3 **(1.5%)**
Carcinoma in situ	4 **(2%)**
SCC basaloid	1 **(0.5%)**

Table 2.3

Clinical diagnosis of the normal patients. Summary of the clinical characteristics of the patients whose biopsies were shown to be non-cancerous.

Clinical findings	No. of patients
Cancer phobia	1**(2.4%)**
Pain	1 **(2.4%)**
Oesophagitis	23 **(56.1%)**
Dysphagia	12 **(29.3%)**
Unknown	4 **(9.8%)**

Table 2.4

Demographic data. Summary of the ages and sex of patients whose biopsies were used in this study.

	Males	Females	Mean age	Total
SCC biopsies				
a) Paraffin embedded	151	49	58 ± 18	200
b) Fresh biopsies	8	2	56 ± 11	10
Total	159	51		210
Sex ratio	3 :	1		
Normal biopsies				
Paraffin embedded	27	14	45 ± 16	41
Sex ratio	1.5 :	1		

2.2.2 DNA EXTRACTION

DNA was isolated from biopsies using the standard DNA isolation procedures as described in materials and methods (section 6.1.1 and 6.1.2). Agarose gel electrophoresis and spectrophotometric absorbance measurement at 260 and 280nm were done to assess the quality and quantity of the DNA. Generally there was a 2-fold yield in DNA from fresh compared to paraffin embedded biopsies. For DNA quality, a ratio (A_{260}/A_{280}) of 1.8 to 2.0 is considered ideal. DNA obtained from paraffin embedded biopsies was of poor quality. This variation could be attributed to DNA degradation commonly observed in paraffin embedded biopsies.

2.2.3 PCR AMPLIFICATION OF HPV SEQUENCES

The polymerase chain reaction (PCR) was used to amplify the L1 and E6 regions of the HPV DNA and human ß-actin gene as described in materials and methods (section 6.2) and table 2.5. Human ß- actin was used as an internal control to monitor amplification of the DNA in HPV negative samples (Figure 2.1). The failure to amplify ß- actin in any given DNA sample was reason enough to exclude the particular DNA sample from the study. Amongst the paraffin embedded biopsies 78 DNA samples obtained from SCC biopsies and 3 DNA samples from normal biopsies were excluded based on this criterion.

HPV sequences from the E6 region were amplified using degenerate consensus primers to produce a product of 240 bp (Figure 2.2). The E6 amplification system is highly specific but has a narrower HPV – type spectrum and is known to amplify HPV types 6, 11, 16, 18, 31, 33, 39, 42, 45, and 52 (Cooper et al., 1995). Instead of relying on one pair of degenerate primers, it uses a mixture of positive and negative strand primers that target the same region of the different HPV types. The advantage of the E6 amplification system is that this early region is most likely to be retained if viral DNA integration has occurred in the host chromosome (Wilczynski et al., 1988).

For the L1 region of HPV, the degenerate MYO9/MY11 primer set is capable of amplifying a wide spectrum of HPV types (HPV5, 6, 8, 11, 16, 18, 26, 27, 30, 31, 33, 35, 39, 40, 41, 42, 43, 45, 47, 48, 51, 52, 53, 54, 55, 58, and 59) (Schiffman et al., 1991) to produce a PCR product of 450 bp. The GP5+/GP6+ primer set is a non-degenerate primer set that detects a wide range of HPV types (HPV6, 11, 13, 16, 18, 31, 32, 33, 35, 39, 40, 42, 43, 44, 45,51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 63, 65, 66, and some yet unidentified types) (De Roda Husman et al., 1995), using a lower annealing temperature and produces a PCR product of approximately 150 bp.

The nested polymerase chain reaction (PCR) was first performed using the degenerate MY09/MY11 primer set, followed by nested PCR with the GP5+/GP6+ set as described in materials and methods (section 6.2.2.2). PCR amplification using either MYO9/MY11 or GP5+/GP6+ separately failed to detect HPV DNA especially in paraffin embedded biopsies (figure 2.3A and 2.3B). Using the nested PCR approach HPV DNA could be detected in some of the samples (figure2.3C). Of the 122 ß-actin positive paraffin embedded biopsies 48 were positive for HPV DNA using the L1 amplification system and 36 were positive with the E6 amplification system (Table 2.6).

2.2.4 SEQUENCING OF PCR PRODUCTS

To identify the HPV types, all the positive PCR products obtained with the nested L1 amplification system were subjected to direct DNA sequence analysis using the T7 Sequenase version 2.0 DNA PCR product sequencing kit (Amersham) as described in materials and methods (section 6.3). The nucleotide sequences obtained were subsequently subjected to Basic Local Alignment Search (BLAST ncbi. nih.gov), which is a set of similarity search programmes designed to explore all of the available sequence databases. The biopsies were shown to contain DNA to HPV types 11, 16, 39 and 52. The low risk HPV-11 was the most common subtype (52%) in all the SCC biopsies (Figure 2.4). In the biopsies from normal individuals none of the high-risk HPV subtypes were detected (Table 2.7).

2.2.5 IMMUNOHISTOCHEMICAL STAINING WITH HPV ANTIBODY

Paraffin embedded sections that were shown to be positive for HPV by the polymerase chain reaction were selected for the study. Evidence of papillomavirus antigen was sought in these sections by means of commercially available antihuman papillomavirus antibody (Clone K1H8, DAKO). This antibody reacts with a nonconformational internal linear epitope of the major capsid protein of HPV1, which is broadly expressed among, the different HPV subtypes.

The anti-HPV antibody immunoreacts with formalin fixed tissues containing HPV types 6, 11, 16, 18, 31, 33, 42, 51, 52, 56, and 58.

The tissue sections were stained using the automated DAKO universal stainer and IHC software as described in materials and methods (section 6.9). After counter staining in haematoxylin, the slides were observed under an Olympus BHS microscope (Zeiss). A cervical precancer lesion was used as a positive control for the detection of viral product (Figure 2.5 A). In SCC of the oesophagus biopsy sections that were positive for HPV DNA by PCR, presence of viral papilloma was not detected using this method (Figure 2.5 B).

Table 2.5

PCR primer sets. Primers used in PCR amplification of HPV DNA and beta actin showing the corresponding annealing temperatures and PCR product sizes

Primers	region	annealing temperature	PCR product
MYO9: 5'-CGTCCMARRGGAWACTGATC-3'	L1	55°C	450
MY11: 5'-GCMCAGGGWCATAAYAATGG-3'	L1	55°C	450
GP5+: 5'-TTTGTTACTGTGGTAGATAC TAC-3'	L1	40°C	150
GP6+: 3'-CTTAT ACTAAATGTCAAATAAAAAG-5'	L1	40°C	150
WD 72: 5'-CGGTCGGGACCGAAAACGG-3'	E6	55°C	240
WD 76: 5'-CGGTTSAACCGAAAMCGG-3'	E6	55°C	240
WD 154: 5'-TCCGTGTGGTGTGTCGTCC-3'	E6	55°C	240
WD 67: 5'-WGCAWATGGAWWGCYGTCTC-3'	E6	55°C	240
WD: 66: 5'-AGCATGCGGTATACTGTCTC-3'	E6	55°C	240
ß-actin F: 5'-TGACGGGGTGACCCACACTGTGCCCATCTA-3'	ß-actin	57°C	650
ß-actin R:5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG3'	ß-actin	57°C	650

M, A+C; R, A+G; W, A+T; Y, C+T, S, G+C

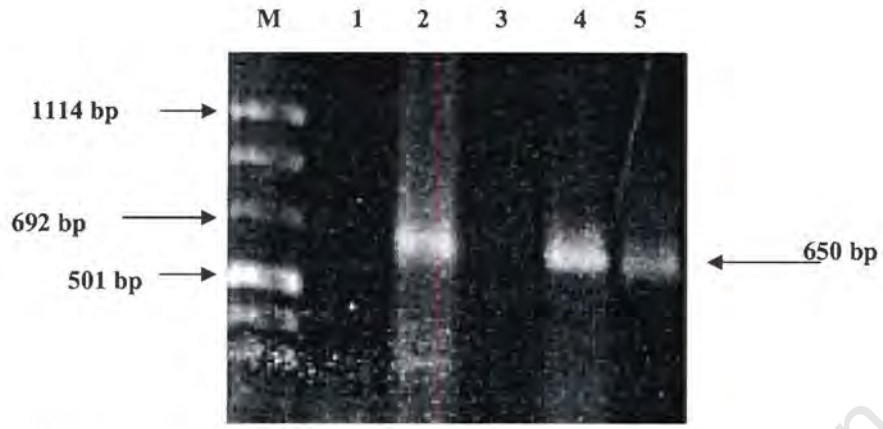
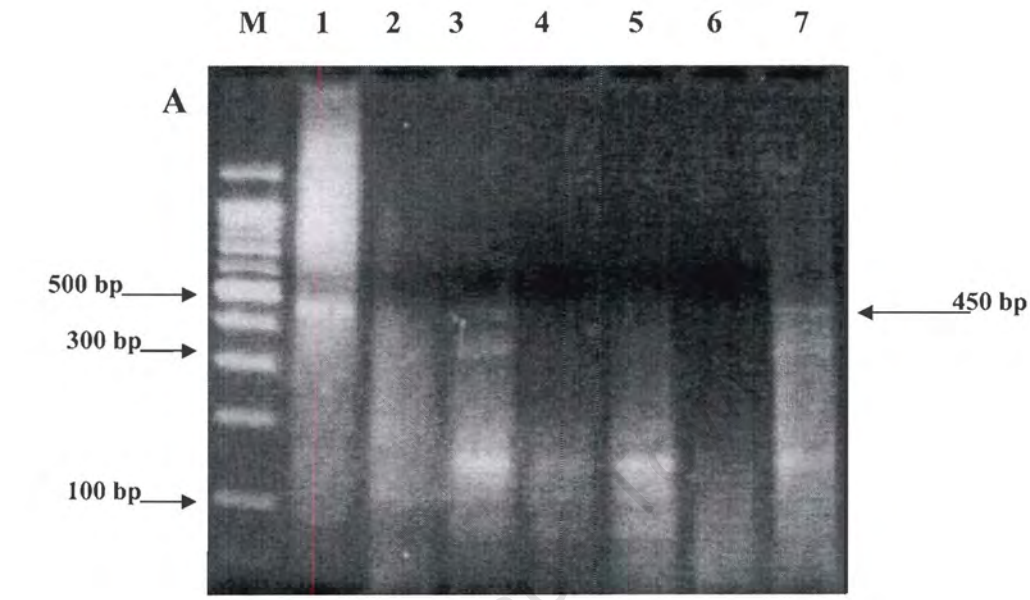


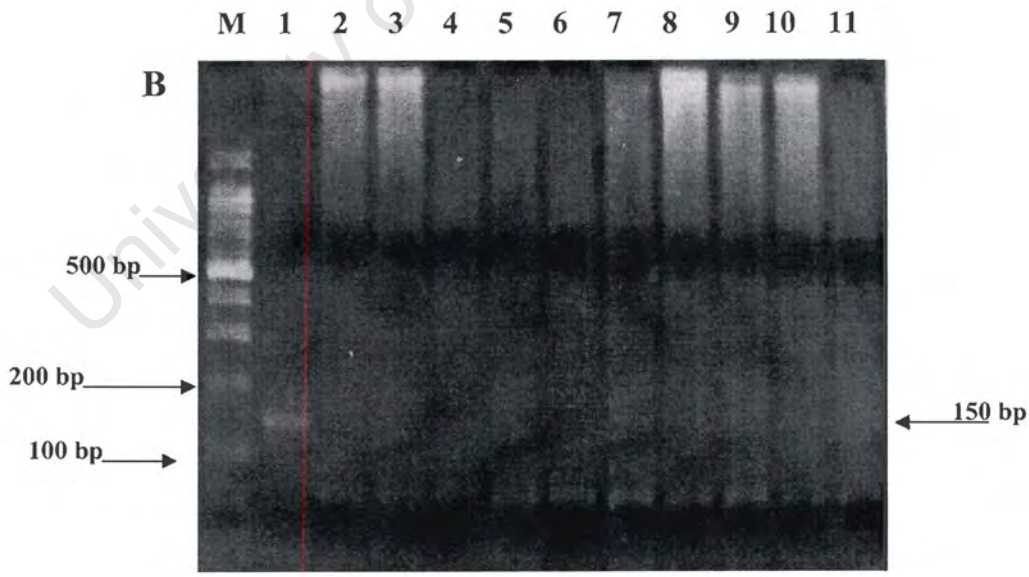
Figure 2.1

Beta actin PCR amplification. DNA was isolated from biopsies and subjected to PCR amplification using conditions shown in table 2.6. Lane 1 is a negative control and lane 2 is a positive control derived from HeLa cells (cervical cell line). The samples in lanes 4 and 5 were positive and the sample in lane 3 was negative for beta actin. The ß-actin PCR product is 650 bp.

Figure 2.2

PCR amplification using E6 consensus primers. DNA was isolated from biopsies and subjected to PCR amplification using conditions shown in table 2.6. Lane 1 is a negative control and lane 2 is a positive control. The samples in lanes 3, 4, 8, and 9 were positive for HPV, whereas the samples in lanes 5, 6, 7, and 10 were negative. The E6 PCR product is 240 bp.





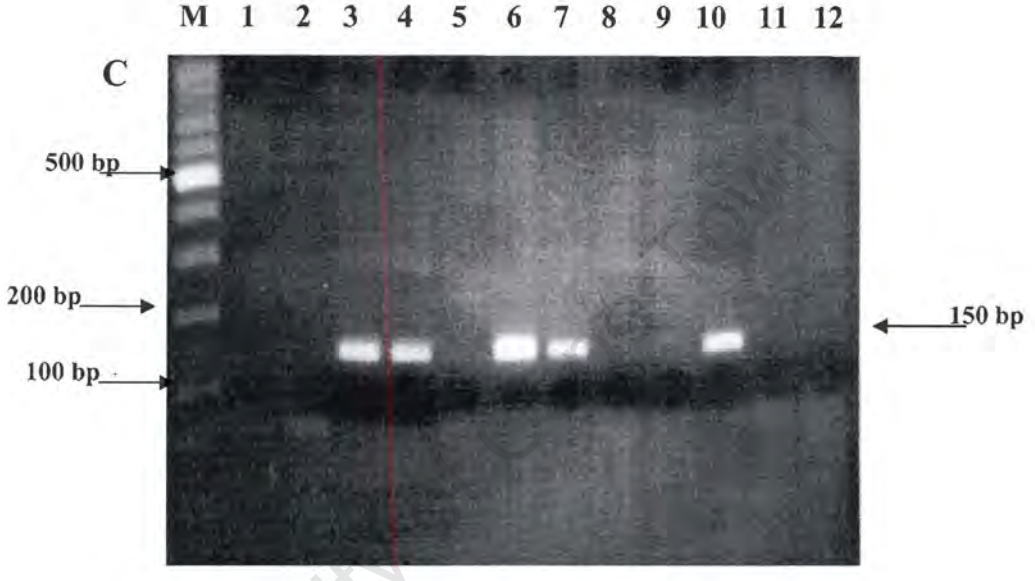


Figure 2.3

HPV L1 PCR amplification. A; PCR amplification using the HPV L1 MY09/MY11 primer set. lane 1 is the positive control and lane 2 is the negative control. The other lanes had very low HPV DNA amplification. The size of the PCR product is 450 bp. B; PCR in which the internal primers GP5+/GP6+ were used. Lane 1 is the positive control and lane 12 is the negative control. The DNA from the tumour biopsies also had very low amplification as shown by the rest of the lanes. C; is the nested PCR amplification. Lane 1 and 2 are the negative controls and lane 3 is a positive control derived from a patient with cervical carcinoma known to be HPV positive. The samples in lanes 4, 6, 7, and 10 were positive for HPV, whereas the samples in lanes 5, 8, 9, 11, and 12 were negative for HPV. The size of the PCR product is 150 bp.

Table 2.6

PCR results. Summary of the results of PCR using the HPV L1 and E6 consensus primers.

HPV region	OSCC paraffin biopsies	OSCC fresh biopsies	Normal
L1	48/122 **(39%)**	4/10 **(40%)**	3/38 **(7.9%)**
E6	36/122 **(30%)**	6/10 **(60%)**	**0%**
L1 & E6	30/122 **(24.5%)**	4/10 **(40%)**	**0%**
L1 but E6 negative	18/122 **(14.8%)**	4/10 **(40%)**	3/38 **(7.9%)**
E6 but L1 negative	6/122 **(4.9%)**	2/10 **(20%)**	**0%**
Total prevalence	54/122 **(44.2%)**	6/10 **(60%)**	3/38 **(7.9%)**

(L1 & E6) + (L1 but E6 negative) + (E6 but L1 negative) = Total prevalence

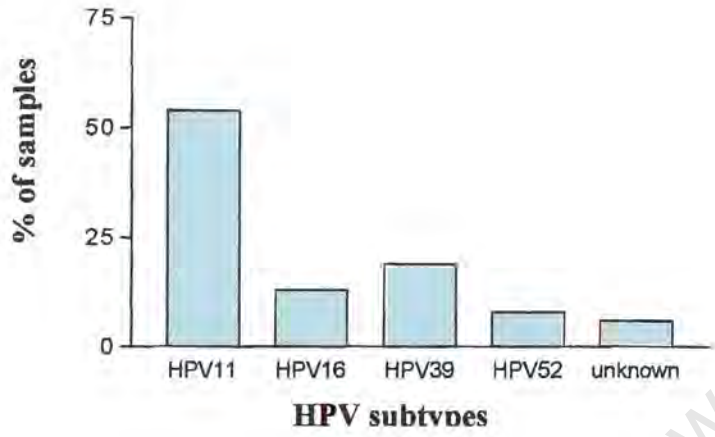


Figure 2.4

Frequency of HPV types in oesophageal cancer biopsies. PCR products positive for HPV DNA using the L1 amplification system were subjected to DNA sequence analysis as described in materials and methods (section 6.3) and used to identify the HPV subtypes.

Table 2.7

Frequency of HPV types in normal paraffin embedded biopsies. Paraffin embedded biopsies from patients without squamous cell carcinoma of the oesophagus were also subjected to nested PCR with L1 primer sets. PCR products obtained were further subjected to sequence analysis as described in materials and methods (section 6.3).

HPV types	No. of biopsies
HPV 11	1
HPV 32	1
Unknown	1

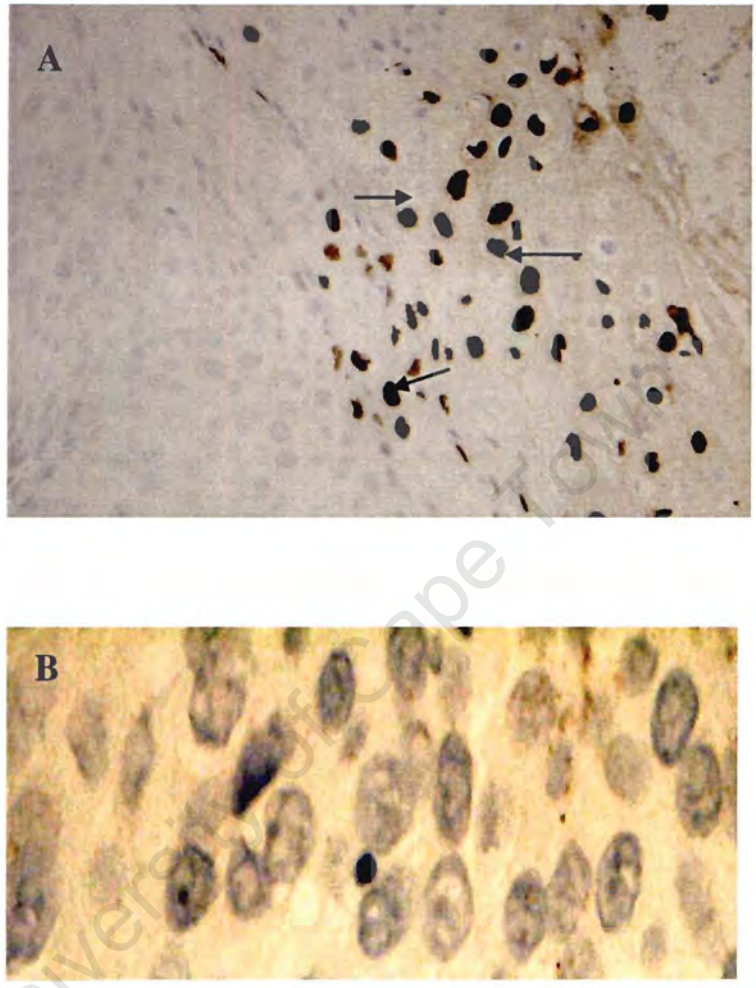


Figure 2.5

A; photomicrograph of the cervical precancer lesion in which the details of the HPV – staining reaction are shown (arrows). Positive staining, brown precipitate is solely localised to the nuclei of the cells.B; paraffin section of SCC of the oesophagus that was positive for HPV DNA by PCR showing no viral papilloma staining.

2.3 QUALITY CONTROL

In order to confirm that there was no mixing of tissue blocks used, the paraffin embedded tissue blocks together with the haematoxylin/eosin stained slides were retrieved and a histopathologist confirmed the pathologic diagnosis of each tissue block. A second histopathologist blindly histopathologically scored randomly selected slides.

A new microtome blade was used to section each tissue block to avoid cross contamination. Sectioning of tissue blocks and DNA extraction was done in small batches of not more than 10 blocks at a time.

PCR studies are prone to false positive results, hence the negative control used in the first round of PCR (MY09/MY11) was included in the second round when nested PCR was performed. In order to confirm accuracy and precision of the PCR results, HPVDNA positive and negative samples were randomly selected and included in subsequent PCR batches. At least two weeks apart, the sequencing gels were read and blast alignment analysis done each time.

2.4 DISCUSSION

Several studies during the past 2 decades have shown the presence of HPV DNA in biopsies of patients with oesophageal cancer (summarised in table 2.1). Studies have generated conflicting and often contradictory data, which may be attributed to the techniques used for HPV DNA analysis. The prevalence of HPV DNA in low incidence geographic regions such as North America, Europe and Australia has been reported low or absent. In high incidence areas high HPV prevalence rates have been reported, but remain controversial. In addition, variations in the prevalence rates of HPV from the same geographical areas have also been reported. For example, the presence of HPV DNA has been confirmed in 23.4% of patients with oesophageal cancer in China (Chang et al., 1993), whereas another study carried out in the same area (Lu et al., 1995) found no evidence of HPV DNA. Similarly, prevalence rates of 50% and 23% have been reported in patients with oesophageal cancer from Australia; in both instances the same technique was used (Kuslki et al., 1986 and 1990).

In this study, the prevalence of HPV DNA in patients with SCC of the oesophagus was found to be high in that 44% of paraffin embedded biopsies and 60% of fresh biopsies contained HPV DNA compared to 7.9% in biopsies from individuals with no evidence of oesophageal squamous cell carcinoma. Similar or even higher prevalence of HPV infection among OSCC patients from South Africa has also been found in other PCR, hybridisation and immunohistochemical studies (Togawa et al., 1994; Poljak et al., 1993; Williamson et al, 1991; de Villiers et al., 1999). Williamson et al (1991), detected HPV DNA in 71% of patients with OSCC using HPV L1 consensus primers and Hille et al (1986) detected HPV antigen in 30% of OSCC by immunohistochemistry. These findings support the notion that HPV may play a role in the development of oesophageal cancer in South Africa.

The failure of HPV detection in PCR positive samples by immunohistochemistry, in this study clearly demonstrates the discrepancies between different techniques

that are used for the detection of HPV. The antibody targeted the group specific structural protein and is highly specific, but detects only cells expressing the late viral gene. Similarly the use of primer pairs targeting different regions of the HPV genome showed different detection rates in PCR amplification of HPV DNA. A prevalence of 30% was obtained using the HPV E6 consensus primers compared to that of 39% when using the HPV L1 consensus primers (nested PCR). This is because the E6 consensus primers have a narrower HPV type spectrum compared to that of the L1.

False positive PCR results are of major concern in most laboratories, false negative results get less attention. Sampling errors and inhibition of PCR might miss PCR positive cases. PCR by HPV L1 outer primers (MY09/MY11) or inner primers (GP5+/Gp6+) failed to detect HPV DNA in paraffin embedded biopsies. Detection of HPV DNA was possible only when nested PCR was performed using these primer pairs. Zehbe et al (1996) compared the sensitivity of nested PCR with GP5+/GP6+ and MY09/MY11 and with the primer pairs alone and concluded that biopsies proved negative with nested PCR were either truly negative or contain HPVs not detectable with either primer system. Furthermore the sensitivity of nested PCR has been reported to be 10- to 100-fold higher compared with using MY09/MY11 alone (Evander et al., 1992). In addition the quality of DNA from paraffin sections was poor compared to that of fresh biopsies, suggesting that this may have inhibited the efficiency of amplification and sensitivity. PCR sensitivity on paraffin sections requires about 400 copies for detection (Shibata et la., 1988), and PCR amplifying shorter fragments are favorable in conditions of less efficient amplification.

The earlier studies discussed above have shown that HPV types 16 and 18 were commonly detected in oesophageal cancer. In this study HPV type 11 was the predominant type accounting for more than 50% of HPV types detected. Chang and colleagues (Chang et al., 1993) also detected HPV-6 and HPV-11 in oesophageal cancer biopsies by in situ hybridisation using a mixed HPV6/11

probe. Furthermore, HPV type 11 was reported in a fatal case of oesophageal and bronchial papillomatosis (Hording et al., 1989). HPV-6 and HPV-11 are generally found in benign genital condylomata. Although found exclusively in benign lesions, these HPV subtypes have occasionally been detected in malignant lesions. HPV type 11 has been implicated in the malignant progression of laryngopharyngeal lesions, cancer of the anus (Zemstov et al., 1992), and penile carcinoma (Dianzani et al., 1998).

In this study HPV type 39 was detected, which has never been shown to be present in oesophageal cancer. According to sequence comparison data, HPV39 most closely resembles HPV 18 and is phylogenetically classified together with HPV 45 in the mucogenital high-risk group. These HPV's have been detected in a disproportionately high percentage of rapidly progressive invasive cervical carcinomas. HPV 39 has been detected in erythroplasia of Queyrat (Wieland et al., 2000), a carcinoma in situ that mainly occurs on the glands, the prepuce, or the urethral meatus of elderly men.

In summary, this chapter has shown that HPV DNA's are definitely present in oesophageal cancer patients from the Transkei, South Africa, however clear evidence for a role in tumoregenesis is still lacking.

CHAPTER THREE

HUMAN PAPILLOMAVIRUS DETECTION BY BRUSH CYTOLOGY IN ASYMPTOMATIC INDIVIDUALS

3.1 INTRODUCTION

The conventional diagnostic methods for oesophageal cancer (OC) are barium oesophagography and endoscopy combined with biopsy and/or cytological brushing. Despite improvements in detection methods, mortality from oesophageal cancer has not declined (Boring et al., 1994) partly because few patients qualify for treatment as most of the patients are already in late stage of the disease at the time of diagnosis. Surgery remains the main therapy, but early diagnosis may allow complete tumour resection and improve the chance for cure (Streitz et al., 1993).

The three screening methods for early detection of oesophageal cancer are occult blood detection, endoscopy with the help of iodine staining to detect abnormal mucosa, and abrasive brush cytology. Endoscopy is by far the most accurate, but impractical and expensive for screening programmes. In South Africa abrasive brush cytology has been the technique of choice for early detection of oesophageal cancer in mass screening programmes (Lazarus et al., 1994; 1992; Jaskiewicz et al., 1987; Sumeruk et al., 1987).

Though abrasive brush cytology screening of cells has primarily achieved the identification of precancerous lesions (Roth et al., 1997; Lazarus et al., 1992; 1994), a portion of patients with cellular abnormalities may be missed or may not be sufficiently distinct. For example Jacob et al (1990) carried out a prospective endoscopic surveillance with biopsy and brush cytology in 255 asymptomatic high-risk US veterans. The authors experienced difficulties in differentiating inflammatory from dysplastic changes in both cytological and histological examination.

In Pap smear screening for cervical cancer, a method similar to brush cytology of the oesophagus, high frequencies of false negative results occur because of errors in both sampling and the interpretation of smears (Villa et al., 1997). However, the establishment of infection with high-risk genital HPV types as aetiological agents in cervical cancer is indisputable such that HPV DNA detection has been introduced as an adjunct to cytology and this has improved the accuracy of Pap test results.

Whilst mass screening programmes for oesophageal cancer have been carried out in South Africa, none of these studies had included HPV DNA detection in the brush biopsies obtained. In other areas with high incidence of OC, there are limited reports that have investigated the involvement of HPV in biopsies obtained by cytological brushing (Li et al., 2001; Trottier et al., 1997; Chang et al., 1990). The detection of HPV DNA in either normal or abnormal cells obtained by brushing may assist in identifying high-risk individuals as well as quantifying the total risk of developing oesophageal cancer. Furthermore if HPV DNA is detected in morphologically abnormal cells as well as in the corresponding biopsy specimens that are histologically proven to be cancerous, it may provide additional evidence in its possible aetiological role in oesophageal cancer.

In chapter 2, biopsies from patients with oesophageal squamous cell carcinoma were shown to harbour HPV DNA, suggesting that it may be a factor in OC development. This chapter aims to investigate the presence of HPV DNA in cells obtained by brush cytology from asymptomatic subjects in Transkei, South Africa and to correlate the presence of HPV with the cytological morphology as well as other habitual practices. Cells were collected from subjects undergoing early screening using an encapsulated brush, the Nabeya capsule (Nabeya et al., 1983) and a questionnaire was administered for each individual.

3.2 RESULTS

3.2.1 Brush Biopsies used in this study

Volunteers for brushing were recruited from 3 different health centres within a radius of 50km from Umtata, Transkei, South Africa. A consent form as well as a questionnaire was administered to each individual (appendix 1).

Thirty four percent of the volunteers had previously suffered from swallowing difficulties, but at the time of screening they were asymptomatic. Two hundred brush biopsies were obtained during the period of 2000 to 2001 using the Nabeya capsule (Figure 3.1) and sample composition is shown in table 3.1. Briefly, the capsule together with a bundled portion of the attached string was swallowed with a glass of water while the subject held the other end of the string. After 10 minutes the brush was slowly withdrawn, inducing the exfoliation of the oesophageal mucosal cells. Cytological slides were prepared and fixed immediately. Following the preparation of the slides, the brush with the remaining cells was placed in a container that had been half filled with ice cold normal saline and transported on ice to the laboratory the same day. The brush was vortexed vigorously to remove the cells and removed from the saline. The saline suspension was centrifuged and cells stored at –70°C until required.

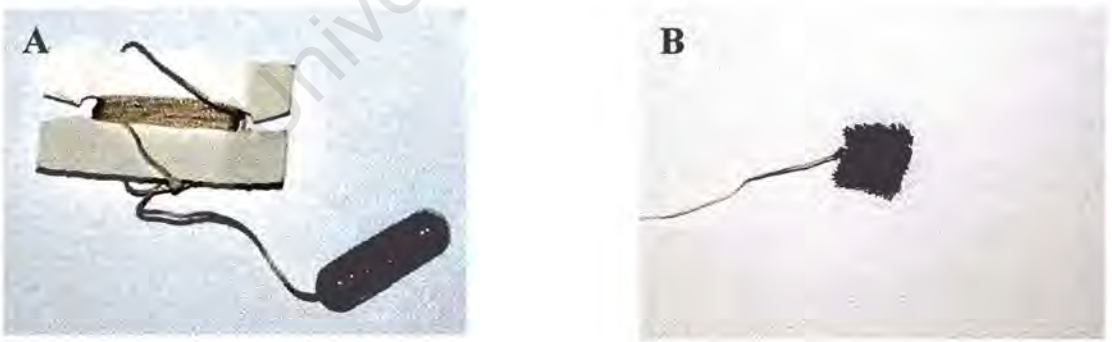


Figure 3.1

Nabeya Capsule. A; the sponge sampler compressed in a dissolvable capsule. B; the sponge sampler after dissolution of the gelatine capsule.

Table 3.1

Composition of volunteers used in this study. Summary of the age and sex of the volunteers whose cytology brush biopsies were used in this study is shown.

Sex	No. of subjects	Mean age
Male	49 **(24.5%)**	52 ± 20
Female	151 **(75.5%)**	45 ± 28
Total	**200**	

3.2.2 CYTOPATHOLOGY

The slides were stained and examined as described in materials and methods (section 6.1.3). The cytological results were primarily graded as either suspicious of malignancy (presence of malignant cells), dysplasia (mild, moderate, or severe) as atypical, or with no pathologic changes as benign. Other changes (secondary diagnosis) such as fungal elements and/or chronic inflammation and/or keratinisation were recorded for each case (Figure 3.2). All the slides and results were confirmed by a cytopathologist. Malignancy was suspected in two cases whilst dysplasia was observed in 14 other cases (Table 3.2). Fungal elements were present in 19 of the 184 benign subjects whilst chronic inflammation was observed in eight.

3.2.3 HPV DNA DETECTION AND TYPING

This was a blind study, i.e. cytological findings were not decoded before HPV DNA detection. DNA was isolated from the cells that had been stored at –70°C as described in materials and method (section 6.1.3). The polymerase chain

reaction PCR and sequencing of the HPV DNA positive samples were carried out as described in materials and methods (section 6.2 and 6.3, respectively).

Forty-eight (24%) of the two hundred brush biopsies were not amplifiable as shown by the results of the housekeeping gene, ß-actin gene. In total 6 (3.9%) subjects were positive for HPV DNA. PCR using the HPV L1 consensus primers could only detect 3 of the subjects whilst the HPV E6 primers detected 5, and 2 could be detected by both HPV L1 and E6 primer pairs. Three (21.4%) of the subjects with abnormal cytological morphology (malignant cells and dysplasia) were shown to harbour HPV DNA. The three (2%) HPV DNA positive but benign samples had either chronic inflammation or fungal infection. Sequence analysis revealed that 2 (33.3%) had HPV type 11, 1(16.6%) HPV type 32 and 3 (50.1%) unknown HPV infections (Table 3.3).

3.2.4 EPIDEMIOLOGIC FACTORS

Information regarding habitual practices was obtained from the volunteers by means of a questionnaire (Appendix 1). Of the 152 subjects whose DNA samples were of amplifiable quality epidemiologic factors such as smoking cigarettes, chewing tobacco, drinking beer or spirits, induced vomiting, and family history were correlated with HPV status using the statistical package STATISTICA.

There was a statistically significant difference in the frequency of males who used tobacco compared to females (72% versus 7%, $P = 0.01$). Besides consumption of alcohol, more than 80% of the subjects induced vomiting at least once a month. The number of subjects with HPV infection was too small to make meaningful statistical calculations. All the HPV positive subjects practised vomiting and 83% of them used alcohol. Smoking may be biased in HPV positive subjects because 114 (75%) of females compared to 38 (25%) of males volunteered for the study (Table 3.4).

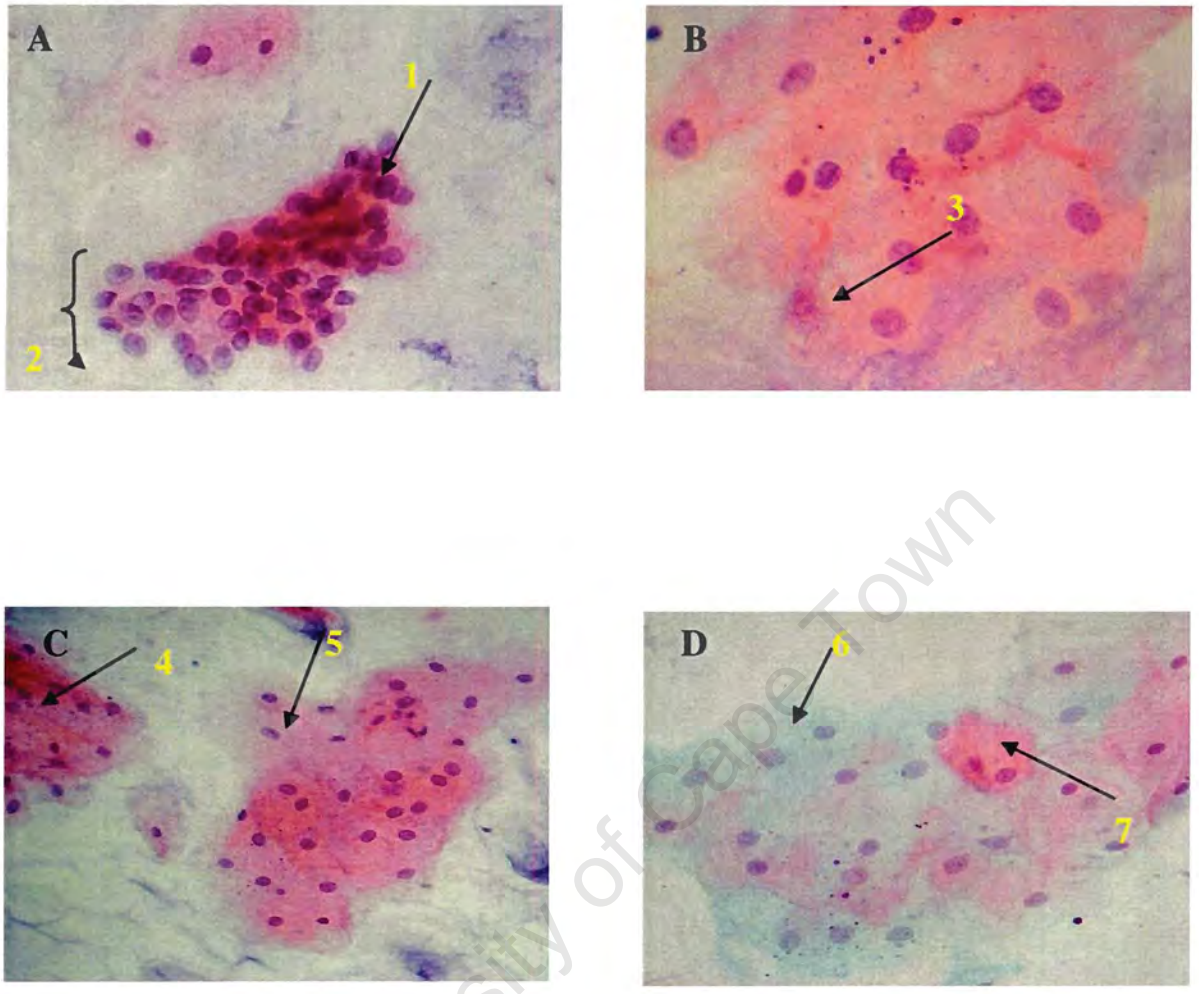


Figure 3.2

Cytotopathology. A (X200); dysplastic changes with malignant cells (arrow 1), bracket labelled 2 represents cells in a rosette form which is consistent with malignancy, B (X400); dysplastic cells with possible HPV infection (arrow 3), C (X200); benign cell changes with fungal infection and inflammation (arrow 4 and arrow 5 respectively), D (X200);normal non-keratinising and keratinising cells (arrows 6 and 7, respectively).

Table 3.2

Cytological findings. Summary of the morphology of cells obtained from the volunteer individuals participating in the study.

Cytopathology	No. of subjects
(a) Atypical	14 **(7%)**
(b) Suspicious of malignancy	2 **(1%)**
(c) Benign	184 **(92%)**
(c-i) Benign with chronic inflammation	8 **(4.3)**
(c-ii) Benign with fungal infection	19 **(10.3%)**

Table 3.3

HPV status in the brush biopsy samples. Summary of the results obtained by PCR and sequencing of DNA obtained from the brush biopsies of apparently normal individuals.

	Suspicious	Atypical	Benign
No. of subjects	2 **(1%)**	12 **(8%)**	138 **(91%)**
HPV L1	1 **(50%)**	2 **(17%)**	**0%**
HPV E6	1 **(50%)**	1 **(8%)**	3 **(2%)**
HPV type	HPV 11	HPV 11&32	
HPV with fungal infection	**0%**	**0%**	1 **(5%)**
HPV with chronic inflammation	**0%**	**0%**	2 **(25%)**

Total HPV prevalence = 3.9% (HPV L1 +HPV E6)

Table 3.4
Habitual factors. Summary of the habitual practices of the subjects used in this study.

	Males	Females	HPV positive
Total	38 **(25%)**	114 **(75%)**	6 **(3.9%)**
Alcohol consumption	31 **(86%)**	87 **(79%)**	5 **(83%)**
Tobacco consumption	26 **(72%)**	8 **(7%)**	1 **(16.6%)**
Induce vomit (100%)	34 **(94%)**	95 **(86%)**	6
Family history	3 **(8%)**	13 **(12%)**	1 **(16.6%)**

3.3 DISCUSSION

High mortality rates due to oesophageal cancer have been reported world-wide due to late diagnosis and poor prognosis and77.4% of patients in South Africa are already at an advanced stage when they seek medical help Alberts et al (1991). The reasons for the delay in seeking medical help could be attributed to the absence of early symptoms in affected individuals, this ruling out detection and improved survival. In China, significant improvements in survival rates are seen when oesophageal cancers are resected at an early stage. Approximately 90% survival in intramucosal cancers has been observed, whereas in situ carcinomas were associated with a 95 -100% five-year survival. (Li H et al., 1997).

Epidemiologic studies have reported OC to be more common in males than females and the mortality rate is 4 to 6 times higher in males than females (Blott et al., 1994). In this regard, the present study has been biased by the fact that more than 75% of the subjects screened were females. We had previously encountered a similar phenomenon in other epidemiological studies conducted in this population (Erasmus et al., 2001), generally females volunteered in larger numbers than males, suggesting that females tend to take health issues much more seriously than their male counterparts.

Abrasive brush cytology for early detection of oesophageal cancer has been performed using two types of samplers. One is the balloon sampler, which is a catheter with a terminal inflatable balloon covered with cotton or nylon mesh. The other is a sponge ball compressed in a dissolvable capsule, the Nabeya capsule. The sensitivity and specificity of these two samplers have been studied by various research groups, in 1992 Lazarus assessed the reliability of the Nabeya capsule and reported high sensitivity (90%) and specificity (99.9%) in the diagnosis of early cancer of the oesophagus among asymptomatic South African individuals. Similarly Roth et al (1997) screened asymptomatic individuals from a

high-risk population in Linxian, China, and reported specificity of 81% and 92% when using the balloon and sponge samplers, respectively. The sponge sampler is now locally manufactured and inexpensive, hence we used it to obtain cytological material from the 200 subjects screened.

Cytological findings from the smears prepared from the brush biopsies were classified as benign (no pathologic changes), atypical (dysplasia mild, moderate or severe), and suspicious of malignancy (presence of malignant cells). Dysplastic changes were detected in 7% and malignant cells were present in 1% of the subjects screened similar to previous reports from South Africa and other high-risk areas (Jaskiewicz et al., 1987). Similarly in Iran, the incidence of dysplasia was reported to be 3.7% (Crespi et al., 1979). In South Africa, brush biopsy screening for oesophageal cancer is infrequent and irregular and limited to research projects. It is thus not available as a routine investigation tool in medical facilities. The presence of malignant cells or dysplastic cell changes in asymptomatic subjects strongly demonstrates the need for a regular, accurate and non-invasive method for the identification of high-risk individuals.

Human papillomavirus DNA detection in cells obtained by the non-invasive abrasive brush biopsy technique could be similar to that in Pap smears of the cervix. In Pap smears the clearest role for HPV DNA testing is to improve diagnostic accuracy and limit patients with benign or mildly abnormal cytological test results from undergoing unnecessary invasive, colposcopy. When positive brush cytology (dysplasia) of the oesophagus was obtained, endoscopic examination with biopsy was recommended. Three patients agreed to undergo endoscopic examination with biopsy and in one case malignancy was confirmed. In the presence of an accurate screening method, the two subjects for whom negative results were found, could have been spared the invasiveness of this procedure.

During the last three decades, data have been accumulated regarding a possible aetiological role of HPV in the development of oesophageal cancer. The majority of these studies have used sample material obtained from tissue biopsies of patients with squamous cell carcinoma of the oesophagus. Studies using material from cytological brush biopsies of asymptomatic individuals are limited. HPV DNA was detected in 50% (1) of subjects with malignant cells (suspicious of malignancy) and 17% of those with mild to moderate dysplasia (atypical) compared to 2% of those with no abnormal morphological changes (benign). These results suggests that HPV infection might be an early event and HPV DNA detection might be useful in further quantifying the total risk of developing oesophageal cancer.

The total prevalence of HPV DNA was found to be 3.9% and this could be attributed to the region surveyed. The subjects studied were from a Transkein region that had previously been described as having a relatively lower incidence of oesophageal cancer (Burrell et al., 1957, Rose et al., 1981). In China, Li (2001), investigated the presence of HPV DNA from a high and low incidence area of Anyang and reported 1.9-fold higher prevalence of HPV DNA by PCR and 2.2-fold higher prevalence by in situ hybridisation from the high incidence area.

One of the healthy volunteers in this study was shown to harbour HPV type 32, whilst two others had HPV type 11 which was the most predominant HPV type that was found in the tissue biopsies of squamous cell carcinoma of the oesophagus (Chapter 2). HPV type 32 is classified as benign, however the E7 protein of this virus has been shown to have a high binding affinity for the Rb protein, but is incapable of inducing its degradation (Caldeira et al., 2002).

It has been reported that HPV favours areas of chronic mucosal irritation, metaplasia, or iatrogenic injury (Kashima et al., 1993). Of great interest was the fact that two of the three HPV positive, but benign subjects showed chronic

inflammation abnormalities on cytology. More than 80% of the volunteers in this study indicated that they induced vomiting on regular basis with 100% of the HPV DNA positive engaging in this practice. The volunteers reported to be either using salt water, herbal concoctions or "holy" water usually obtained from Christian cult groups to induce vomiting. This process is believed to cleanse the individual of evil drinks or food that one may have intentionally been given by foes. It is possible that this cultural practice has been overlooked and may be playing an important role in the development of oesophageal cancer in the Transkei population. Self-induced vomiting may be a regular and major assault of the oesophageal mucosa, bathing it in gastric and possibly duodenal juices and cause oesophagitis, a condition that is believed to be a precursor of oesophageal cancer (Sammon et al., 1992). Furthermore, it has been reported that in the oesophagus, gastroesophageal reflux can cause a chronic mucosal injury leading to metaplasia and susceptibility to HPV (Odze et al., 1993; Parnell et al., 1978).

Consumption of alcohol and tobacco particularly in combination has been strongly associated with oesophageal cancer. More than 80% of the volunteers studied consume alcohol and 72% of males smoked. Amongst the HPV positive subjects in this study only one subject indicated that he smoked, whilst 80% consumed alcohol. It is thought that mutagens present in smoke constituents may cooperate with papillomavirus in the induction of cervical malignancies in different ways (Jackson et al., 1993). Similarly, in the upper aerodigestive tract, it has been suggested that cancer development as a consequence of alcohol and tobacco consumption may be intensified by HPV infection (Pillai et al., 2000).

Of the three subjects with benign lesions who had HPV DNA, one had fungal elements that was not identified, but it can be speculated that this could have been the result of a complication from immunosupression, for example human immunodeficiency virus (HIV) which is highly prevalent in South Africa. In a study conducted on immunosuppressed individuals, 17% oesophageal brush biopsies

were shown to contain HPV DNA by PCR using HPV L1 consensus primers (Trottier et al., 1997). It is thought that in certain cases HPV infection and HPV associated carcinoma are late complications of chronic immunosuppression (Rudlinger et al., 1986; Beutner et al., 1991)

Familial history has received minimum attention amongst the possible risk factors for oesophageal cancer. Overall 17 (11%) of the volunteers studied had a family history of OC, males and females reporting 8% and 12% respectively for the first degree relative. Only one patient that had malignant cells and HPV DNA reported a first-degree family relative with oesophageal cancer. One study that attempted to investigate the association of family history and oesophageal cancer, found autosomal recessive mendelian inheritance pattern was found in 4% of the population examined (Carter et al., 1992). In another study conducted by Dr R Ballo using a hospital based cancer registry from Groote Schuur hospital in Cape Town, 28 of 129 (22%) OC patients had a first degree relative with cancer with 62% of these having had oesophageal cancer (data unpublished). Therefore, these results suggest a possible role for family history in the development of OC.

The data in this chapter suggest that HPV might be an important role player in the pathogenesis of oesophageal cancer. However, the overall HPV DNA prevalence in subjects with dysplastic changes (21.4%) suggests that other risk factors, rather than HPV alone may have synergistic actions with HPV in the pathogenesis of oesophageal cancer. It is likely that HPV infection in oesophageal cancer is an early event as demonstrated by the detection of HPV DNA in subjects with dysplastic cells. Screening programmes for HPV DNA detection could thus be used as an additional tool in identifying high-risk individuals.

CHAPTER FOUR

ANALYSIS OF A ROLE FOR HPV DNA IN THE DEVELOPMENT OF OESOPHAGEAL MALIGNANCIES.

4.1 INTRODUCTION

The development of oesophageal cancer (OC), as with all other cancers, is thought to arise through a complex combination of environmental factors and the accumulation of numerous genetic alterations, such as p53 and p16 gene mutations (Montesano et al., 1996). Amongst the environmental factors, the presence of HPV DNA has been implicated in the pathogenesis of oesophageal cancer. However, the role of HPV DNA as well as the mechanisms underlying the interaction between HPV DNA and genetic factors remains ill defined in oesophageal cancer. Numerous attempts to clarify the role of HPV in oesophageal cancer are well documented, but frequently these studies do not go beyond HPV DNA detection (Matsha et al., 2002; de Villiers et al., 1999; Lavergne et al., 1999 Suzuki et al., 1996) inspite of the available information regarding the function of these viral proteins.

Cloned DNAs of the high risk HPVs were found to be very efficient at immortalising genital tract keratinocytes, the normal host cell for these viruses. This immortalisation capacity is a characteristic not shared by the cloned DNAs of the low risk viruses. Dissection of the HPV genes revealed that E6 and E7 were necessary for efficient immortalisation (Wood-worth et al., 1989; Pecoraro et al., 1989). E6 and E7 from the high risk HPV types have the ability to alter pathways involved in the cell cycle control, interacting with and neutralising the regulatory function of two tumour suppressor proteins, p53 and Rb, respectively.

Wild type p53 expression suppresses transformation by several oncogenes and results in a block of the cell cycle before DNA replication, i.e. in G1. To overcome this obstacle, certain viruses encode proteins that functionally inactivate p53, for example the SV40 large T antigen prevents transactivation of p53 target genes through association with the p53 DNA binding domain

(Rupper et al., 1993). E6 of high-risk HPV types induce wild type p53 degradation through the ubiquitin proteasome pathway (Scheffner et al., 1990), thus abolishing induced growth arrest and apoptosis in response to DNA damage. (Kessis et al., 1993; Foster et al., 1997).

An exclusive relationship between HPV infection and p53 mutations has been reported in human anogenital cancers (Crook et al., 1991; Yaginuma et al., 1991; Iwasaka et al., 1993; Denk et al., 2001). In oesophageal squamous cell carcinoma (OSCC) coexistence of p53 mutations and HPV DNA or p53 over expression and HPV DNA has been observed (Cooper et al., 1995; Chang et al., 1994; Lam et al., 1997; Hasegawa et al., 2002). Mutant p53 proteins are more stable than their wild type counterparts, and their accumulation in tumour cells can be detected immunohistochemically.

Recently two structural homologues of the p53 gene, p63 and p73 have been identified (Kaghad et al., 1997; Yang et al., 1998; Osada et al., 1998). Presently, the functional role of p73 in cancers is still under investigation, but there is evidence indicating that p73 is a tumour suppresser gene that is targeted during tumorigenesis and undergoes loss of expression. The findings that p73 gene is structurally and functionally similar to p53 and that ectopic p73 overexpression activates p21/WAF-1 and induces apoptosis are some of the reasons behind the tumour suppressor gene role for p73 (Jost et al., 1997, Ichimiya et al., 2000). Furthermore, Flores et al (2002), has shown that p73 together with p63 cooperate with p53 during DNA damage induced apoptosis.

However, the lack of p73 mutations in tumours thus far studied questions this tumour suppressor gene role (Kovalev et al., 1998; Yokomizo et al., 1999; Nimura et al., 1998; Sunhara et al., 1998; Zaika et al., 1999). In addition, wild type (wt) p73 over expression associated with malignant tissue, but not with its matched normal counterpart, has been found in breast cancer (Zaika et al., 1999), lung cancer (Mai et al., 1998), prostate cancer (Takahashi et al., 1998), colorectal cancer (Sunahara et al., 1998) and oesophageal cancer (Cai et al., 2000).

The mechanisms leading to p73 inactivation are not well characterised, but various types of mutant p53 and HPV E6 protein interactions with p73 have been suggested to inactivate p73 (Strano et al., 2002; Park et al., 2001). Park et al (2001) have demonstrated inactivation of p73 by E6 proteins of both low- and high-risk HPV types. This suggests that E6 of low risk HPVs may at least in part, contribute to the E6 mediated transformation and hyper proliferation of cells.

No published information is available on the relationship between HPV infection and p73 status in oesophageal squamous cell carcinoma. If the HPV E6 protein plays a causative role in the development of OSCC either via p53 degradation or p73 inactivation, it should be possible to identify impaired p53 and /or p73 expression by immunohistochemical means. The demonstration of such a relationship should help in determining a functional role for HPV infection in the pathogenesis of oesophageal cancer.

In the previous two chapters HPV DNA was shown to be present in 44% OSCC biopsies as well as in 21.4% of cells that display dysplasia. In this chapter p53 and p73 protein expression levels were measured immunohistochemically in all the OSCC biopsies used in chapter 2, and correlated to the HPV status. To test the effect of HPV E6 on endogenous p53 and p73 protein expression, normal human fibroblasts were transiently transfected with HPV E6 from a low- (HPV type 11) and a high- (HPV type 16) risk group. Cell cycle progression was monitored by flow cytometry and p53 and p73 protein levels were measured by western blot analysis.

4.2 RESULTS

4.2.1. Oesophageal Squamous Cell Carcinoma Biopsies

The 122 paraffin embedded OSCC biopsies used in Chapter 2 were cut into consecurtive sections of 3µm thick as described in materials and methods (section 6.9). In order to confirm that the correct blocks were retrieved, haematoxylin/eosin staining was performed. The hematoxylin/eosin stain was ambiguous in 8 tissue blocks and these were not included in the study.

Using the World Health Organisation (WHO) TNM classification (WHO, 1999) a pathologist confirmed histopathologic classification. Grading of OSCC is based on the parameters of mitotic activity, anisonucleosis and degree of differentiation. Well-differentiated tumours are characterised by abundant keratinisation and intercellular bridges whilst poorly differentiated tumours may lack these features. Moderately differentiated tumours exhibit features between the well and poorly differentiated tumours (Figure 4.1).

Equivocally poorly differentiated tumours were further confirmed by immunohistochemical staining using a monoclonal antikeratin antibody, (Monoclonal Mouse Anti-Human Cytokeratin, Clone MNF 116, DAKO) (Figure 4.2). Of the 114 OSCC tumour biopsies, moderately differentiated tumours were the most common accounting for more than 60% of OSCC cases (Figure 4.3).

4.2.2 p53 Immunohistochemical Staining of the OSCC Biopsies

Monoclonal Mouse anti-Human p53 Protein, Clone DO-7 (DAKO) was used as the primary antibody. The tissue sections were stained using the automated DAKO universal stainer and IHC software (DAKO) as described in materials and methods (section 6.9.5). Sections from a colon biopsy were included in each batch as positive control, while the primary antibody incubation was omitted for negative control (Figure 4.4).

The immunohistochemically stained OSCC sections were assessed and scored independently by two histopathologists. In all cases but one, there was

complete agreement concerning the staining pattern. Consensus agreement was achieved on discussion and review of the one case.

p53 expression was considered to be present if more than 10% of nuclei showed uniform staining and was graded into 1+, 2+ or 3+ corresponding to 10 - 25%, 25 – 50% or > 50% of cells staining positive, respectively (Figure 4.5). Five fields were randomly selected and scored under low magnification. Seventy percent of the 114 tumour biopsies showed p53 accumulation with intense staining in 58.7% of OSCC cases, (2+ = 10.5%, and 3+ = 48.2%) (Figure 4.6).

In all the samples containing squamous cell carcinoma and neighbouring normal epithelium, less than 10% of the cells showed a positive signal for p53 in the normal cells (Figure 4.7).

4.2.3 Relationship between tumour grade and p53 expression

The prognostic value of tumour differentiation status is debatable, but generally poorly differentiated tumours have been shown to be associated with poor survival (Tores et al., 1999). The association between p53 expression and tumour grade may serve as an additional tool in determining the prognosis of patients. The p53 scores in section 4.2.2 were carried out blind with regards to the grade of the tumour. The data in section 4.2.1 and 4.2.2 were analysed using the statistical package, STATISTICA. p53 accumulation was evident in 68%, 81% and 71% of moderately, well, and poorly differentiated tumours, respectively (Figure 4.8) Differences in p53 expression between the different tumour grades were assessed by means of Chi square test and no statistically significant differences were observed (P = 0.49).

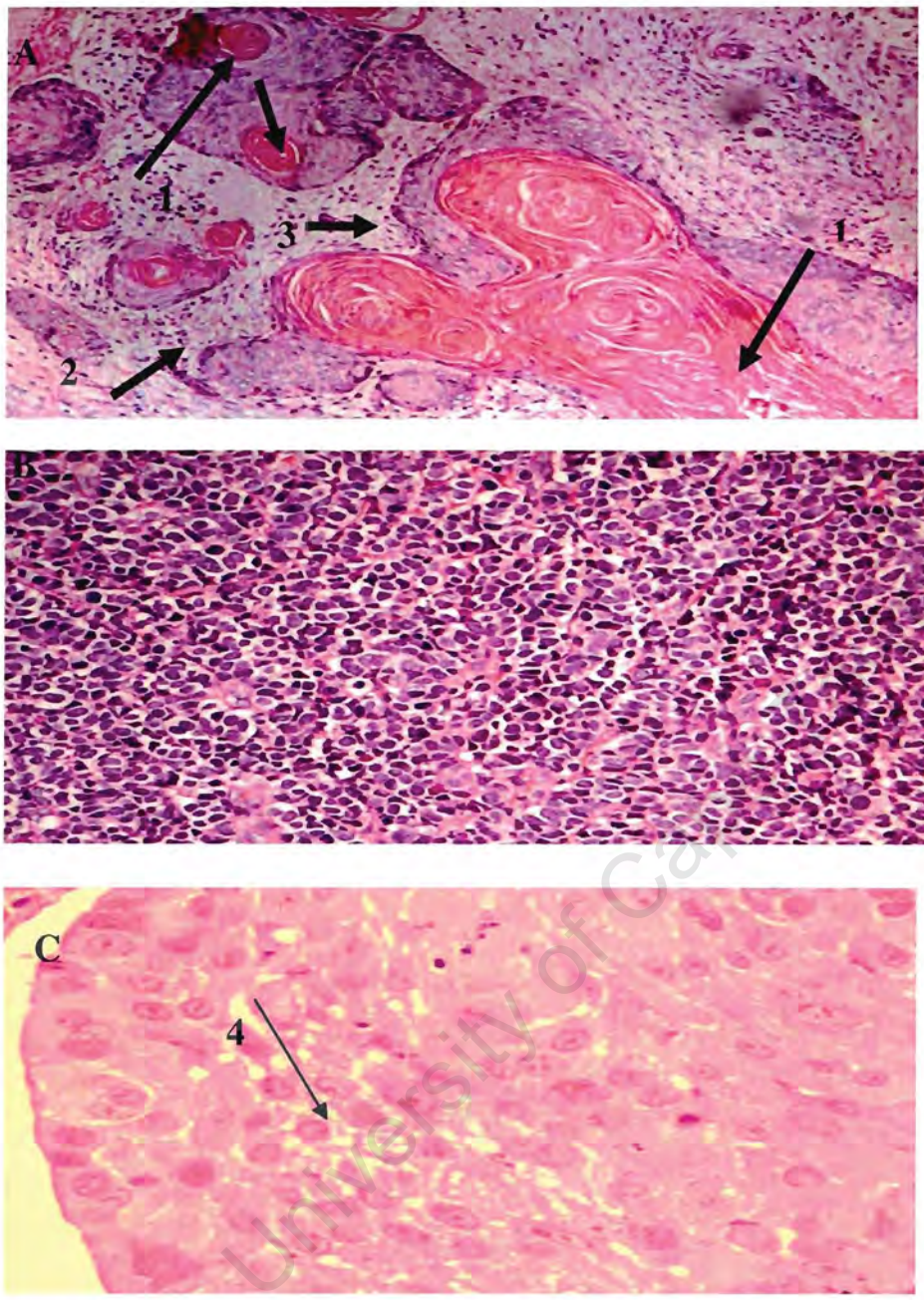


Figure 4.1 Haematoxylin/Eosin staining of Oesophageal Squamous Cell Carcinoma. A; Well-differentiated tumour (100X magnification) depicted by large differentiated keratinising squamous cells (arrow 1) with lymphoid infiltration (arrow 2). The small basal cell type cells are in the periphery of the cancer nests (arrow 3). B; Poorly differentiated tumour (200X magnification) consisting of small squamous cell type with small amount of eosonophilic cytoplasm. C; Moderately differentiated tumour (400X magnification) featuring keratinising squamous cells and intercellular bridges (arrow 4).

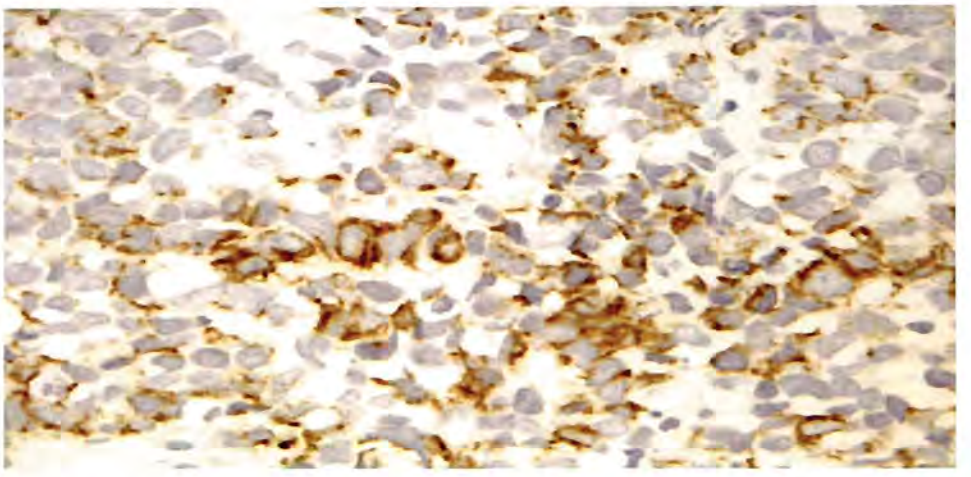


Figure 4.2 Cytokeratin Stainining. Equivocal poorly differentiated OSCC tumour sections were immunostained using Anti-human Cytokeratin antibody as described in materials and methods (section 6.9.4) Positive immunoreactivity (400X magnification) of poorly differentiated OSCC with the Anti-Human Cytokeratin antibody.

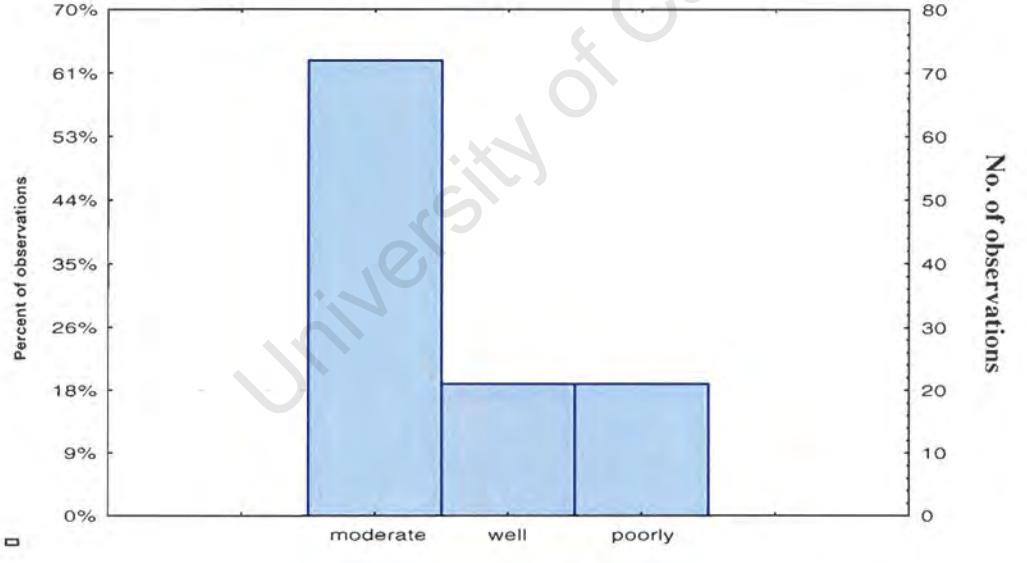


Figure 4.3 Pathology Grade. The 114 OSCC tumour biopsies used in this study were stained with haematoxylin/eosin and graded by a pathologist using the WHO criteria. Well; well differentiated, moderate; moderately differentiated, and poorly; poorly differentiated. The frequency distribution of the well, moderately and poorly differentiated tumours are indicated (No. of observations).

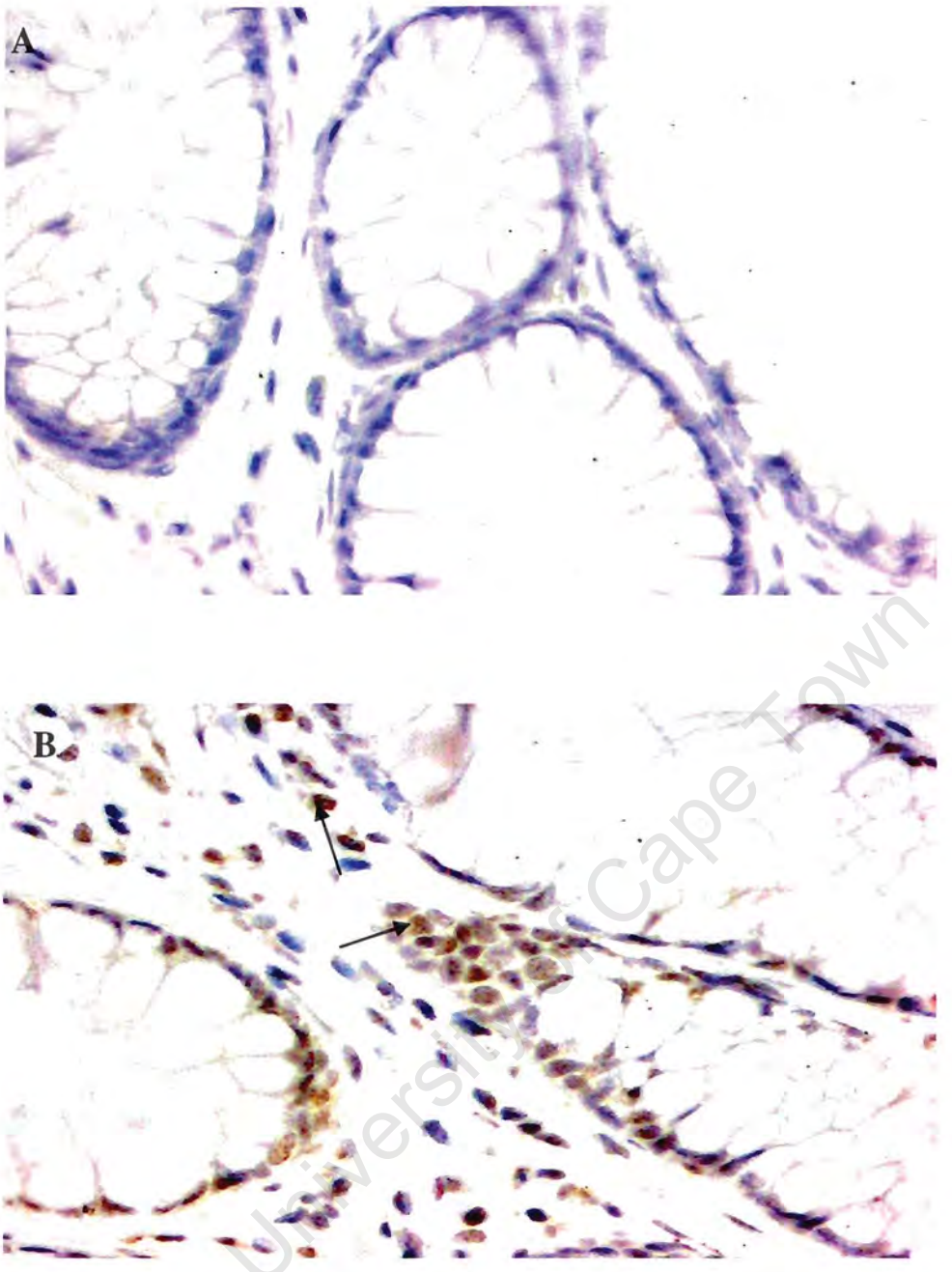


Figure 4.4 p53 Expression in Positive Control Colon Sections. Sections from a colon biopsy that had previously been shown to react with anti-p53 clone DO-7 (DAKO) were immunostained using anti-p53 as the primary antibody as described in materials and methods (Section 6.9.5). A; negative control in which the primary antibody incubation was omitted, B; positive control showing positive p53 immunoreactivity in the nuclei of cells (arrows).

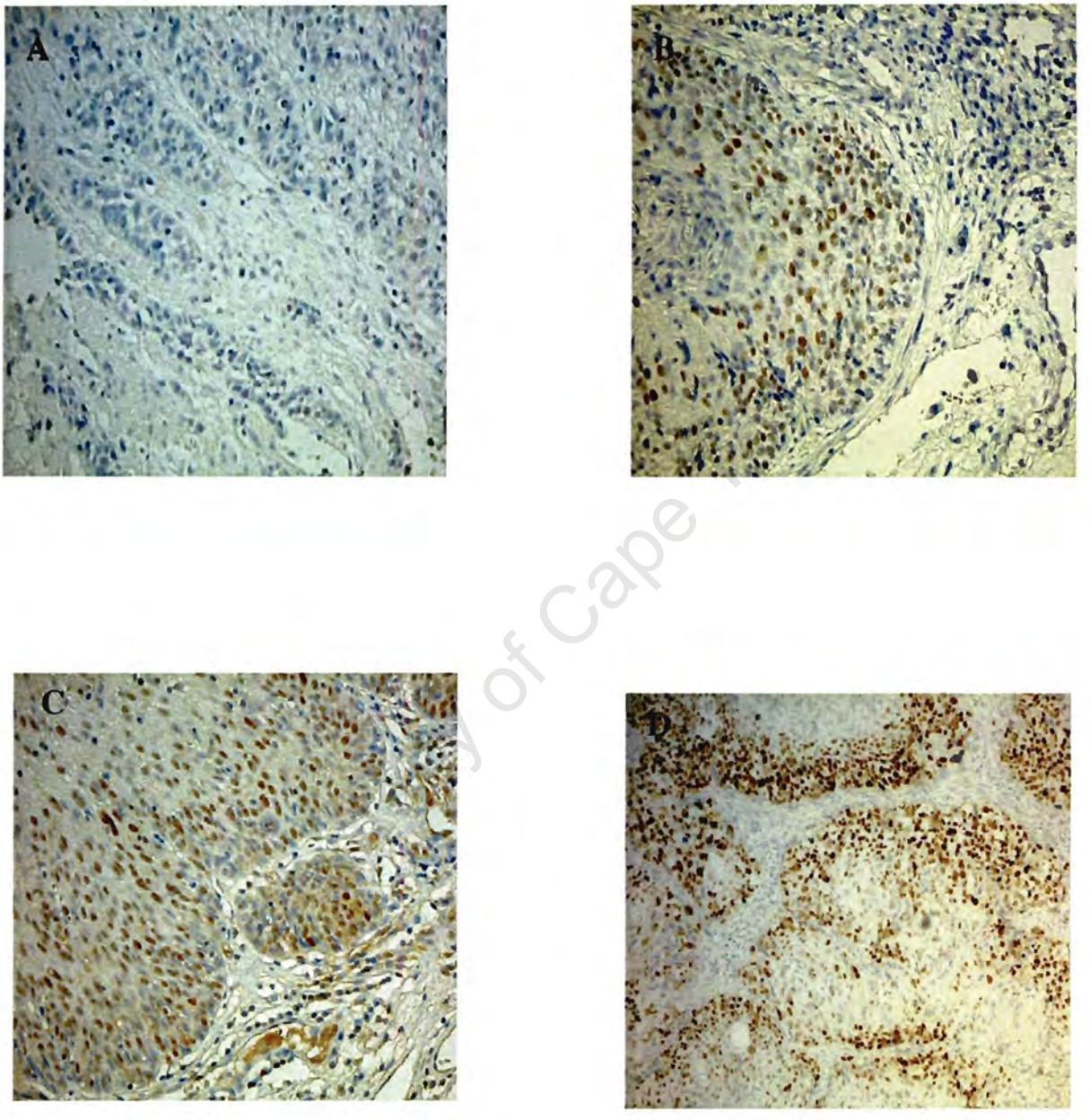


Fig. 4.5 p53 Expression in Oesophageal Squamous Cell Carcinoma. p53 was detected immunohistochemically using anti-p53 antibody as described in materials and methods (Section 6.9.5). A; (200X magnification) p53 negative tumour tissue, B; (200X magnification) 1+ p53 expression, C; (200X magnification) 2+ p53 expression, D; (100X magnification) 3+ p53 expression.

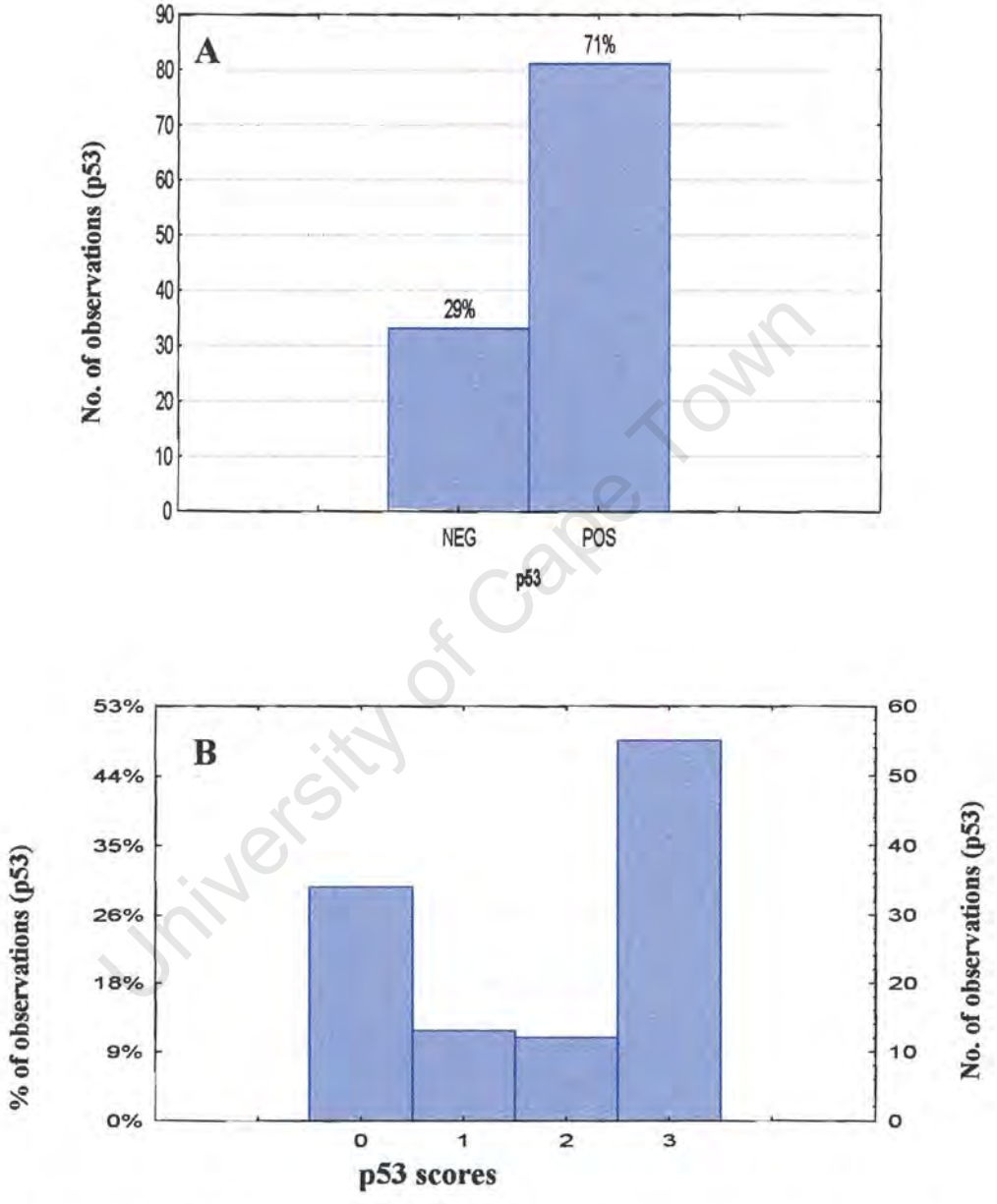


Figure 4.6 Overall p53 Immunostaining of OSCC Biopsies Used in this Study. The OSCC tumour biopsies were immunostained using anti-p53 antibody. After immunostaining two independent histopathologists examined the tumour sections and each tumour section was scored using the criteria mentioned in section 4.2.3. A; p53 results when p53 expression is divided into p53-negative (p53 = 0) and p53-positive (p53 = 1+, 2+ or 3+), the percentage of p53 positive or negative are indicated on top of the appropriate bar on the graph, B; detailed breakdown of p53 expression in OSCC. The numbers of cases are indicated (No of observations).

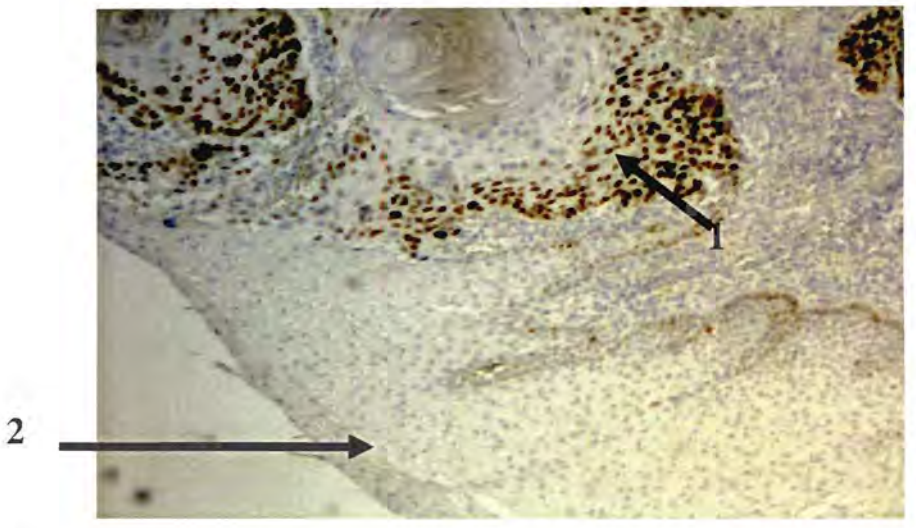


Fig. 4.7

p53 Immunostaing in Surrounding Normal Epithelium (100X magnification). In those tumour samples containing surrounding normal epithelium p53 expression was shown to be negative. Arrow 1 shows tumour nest scoring 3+ for p53 expression surrounded by p53 negative normal epithelium (arrow 2)

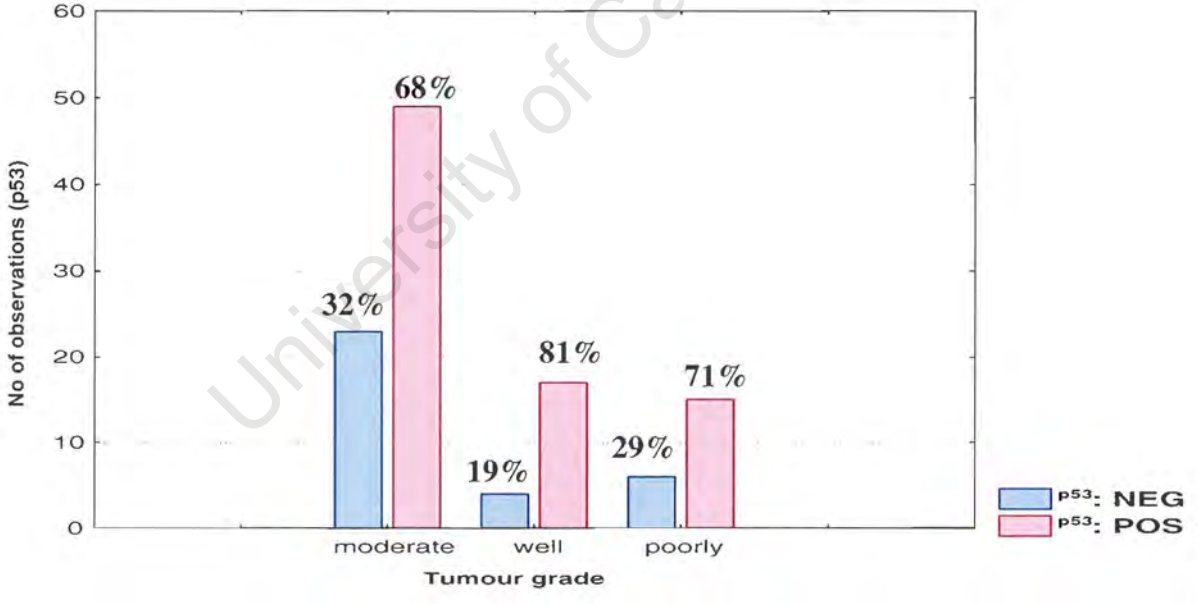


Figure 4.8 Relationship between Tumour grade and p53 levels. p53 expression between the different tumour grades was analysed using the statistical package STATISTICA. The percentage of p53 negative or positive biopsies for each differentiation status is indicated on top of the bar graph and the number of cases is indicated on the Y-axis.

4.2.4 Immunochemical Staining of OSCC Biopsies for p73

Expression of p73 was assessed using a rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.) as described in materials and methos (Section 6.9.6). This antibody is capable of immunoreacting with p73 isoforms α and β. It was determined that pressure cooking in EDTA (pH 8.0) for antigen retrieval and overnight incubation of the primary antibody at 4^0C yielded the best results. After immunostaining two independent histopathologists assessed the slides.

Immunoreactivity was found to be diffuse and nuclear and in particular in the nuclei of epithelial cells. When observed in the surrounding normal epithelia, immunoreactivity was restricted to the to nuclei of the cells close to the basal layer and within the basal layer (Figure 4.9). In tumour cells, the staining remained generally nuclear but demonstrated an increased intensity. p73 nuclear accumulation was scored using the same criteria used for scoring p53 in section 4.2.2 (Figure 4.10).

The extent of nuclear p73 expression was significant in tumour cells, thus in normal neighbouring epithelia p73 expression was observed in 2 of the 26 (7.7%) cases and in 70 (61.4%) of tumours. Intense p73 immunoreactivity (15% were 2+ and 32.4% were 3+) was observed in 47.4% of the 114 OSCC tumour biopsies (Figure 4.11). The pattern of p73 immunostaining in the different tumour grades was not statistically significant (*P* value = 0.14). Eighty one percent, 57% and 60% of well, poorly and moderately differentiated tumours were positive for p73, respectively (Figure 4.12).

4.2.5 Relationship between HPV Infection, p53 and p73

The p53, p73 and HPV data were analysed using the statistical package, STATISTICA. Frequency distribution and correlations of all investigated factors were analysed by the Chi square test and Spearmans Rank correlation test, respectively. Co-expression of both p53 and p73 was statistically significant ($r = 0.48$, P-value < 0.05) in 54% of tumours studied (Figure 4.13).

No statistically significant relationship was observed between p53 protein expression and HPV status ($P = 0.16$). p53 expression was evident in 36% HPV DNA-positive and 35% HPV DNA-negative OSCC cases. Conversely, p73 protein expression showed differences between HPV DNA-positive and negative OSCC cases ($P = 0.01$). In order to examine whether there exist a relationship between the three variables; p53, p73 and HPV status, logarithm linear analysis of the three variables was performed. This statistical test did not identify other relationships between the three variables, but confirmed the significant co-expression of p53 and p73, which was increased in HPV DNA positive samples (30%). Absence of both p53 and p73 expression was observed in 5% of HPV DNA-positive biopsies and 14% of HPV DNA-negative samples (Figure 4.14).

The HPV subtype proportions were not sufficient for further meaningful statistical analysis with regards to p53 or p73 protein expression. However, distribution analysis of the various subtypes showed differences with regards to p53 protein expression. A decreased (4%) p53 protein expression was observed in HPV type 16 infected tumours, while p73 expression was generally increased among all the other HPV subtypes (Figure 4.15).

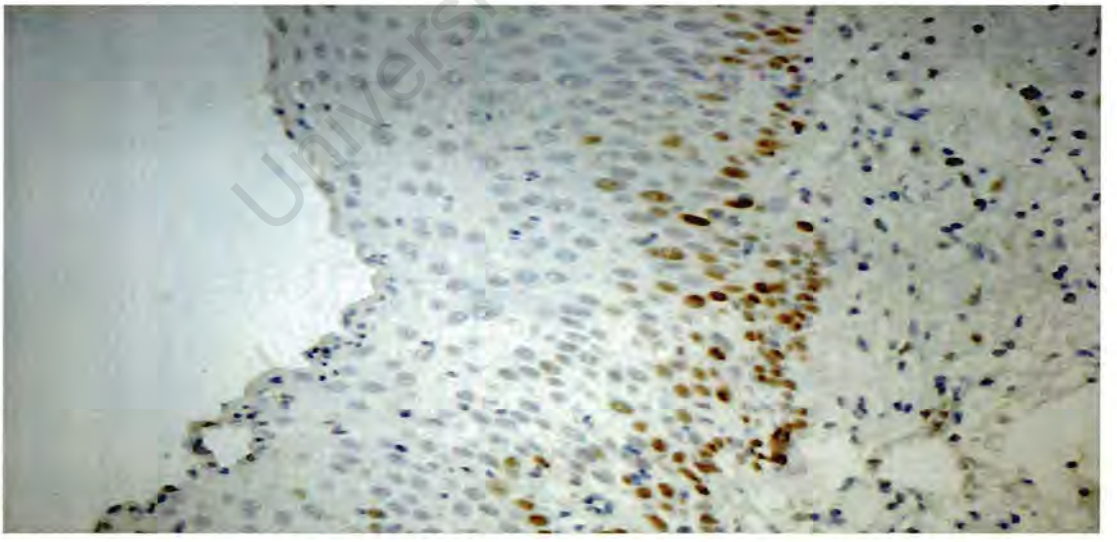


Figure 4.9 p73 Immunostaining in Surrounding Normal Epithelium.(X 400 magnification). p73 was detected immunohistochemically using rabbit polyclonal p73 H-79 antibody (Santa Cruz Biotechnology, Inc) as described in materials and methods. p73 immunoreactivity was observed in the basal layer.

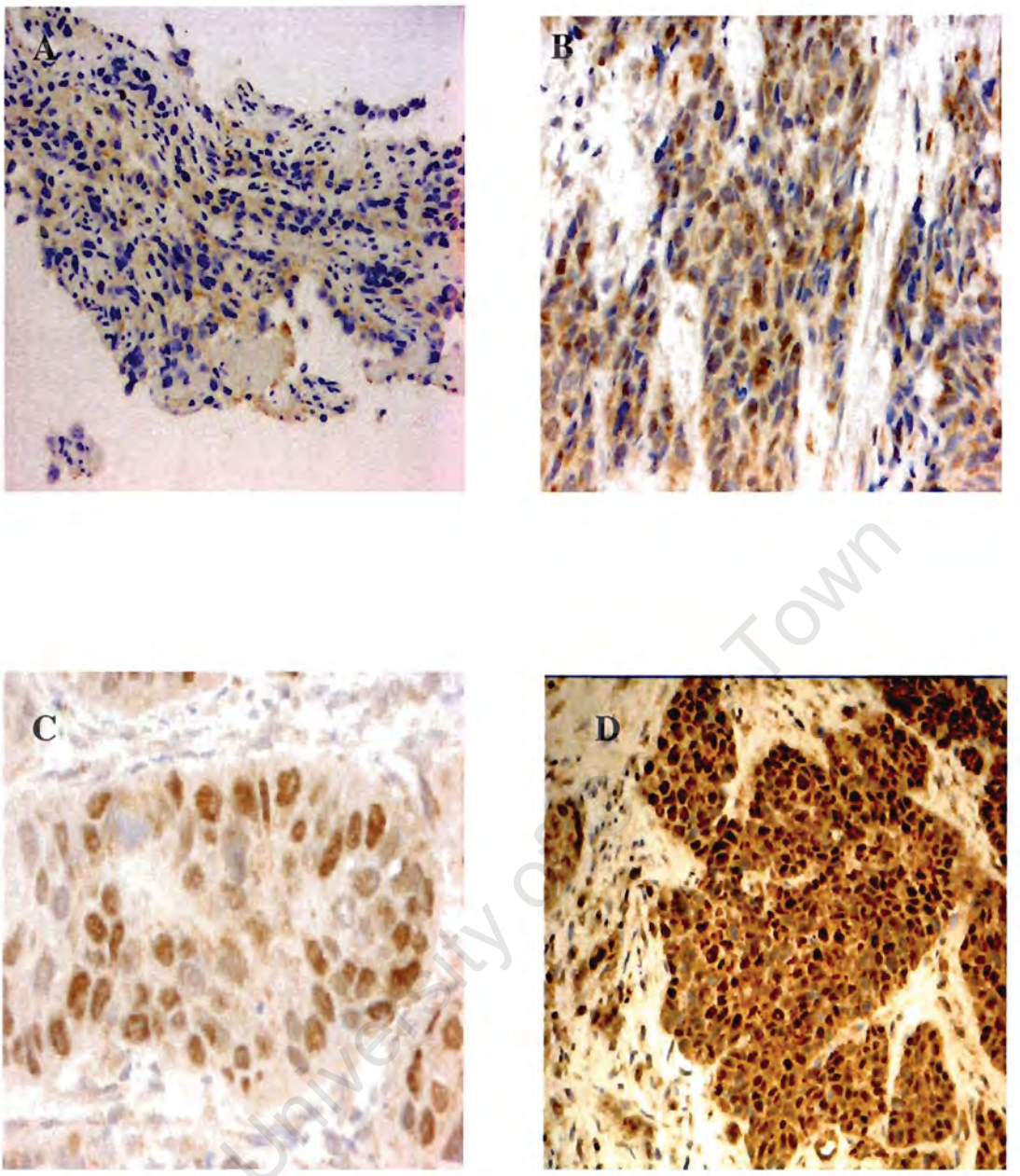


Figure 4.10 p73 expression in Oesophgeal Squamous Cell Carcinoma. Detection of p73 was performed immunohistochemically as described in materials and methods (Section 6.9.6). After immunostaining the tumour sections were scored using the criteria mentioned in section 4.2.3. A; (200X magnification) non specific (background) p73 negative cells, B; (200X magnification) one plus p73 expression, C; (400X magnification) two plus p73 expression, D; (200X magnification) three plus p73 expression.

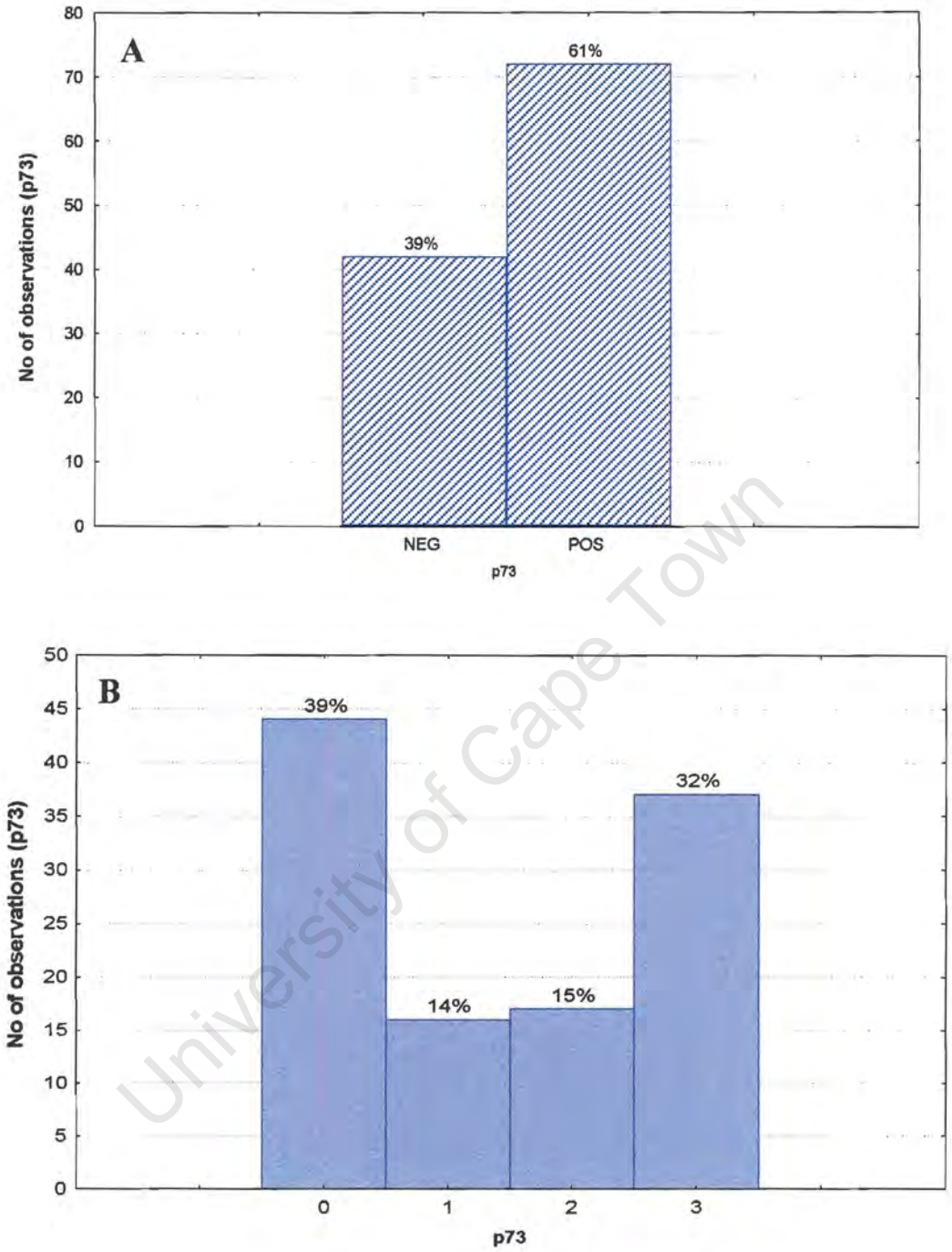


Figure 4.11 p73 Immunoreactivity in the Oesophageal Squamous Cell Carcinoma biopsies. p73 was detected by immunohistochemical means using anti p73 clone H-79 (Santa Cruz Biotechnology, Inc) as described in materials and methods (Section 6.9.6). After immunostaining the tissue sections were scored as described in section 4.2.3. A; p73 in all tumour biopsies when p73 is divided into p73-negative (p73 = 0) or p73-positive (p73 1+, 2+, or 3+), B; detailed breakdown of p73 expression in all the tumour biopsies.

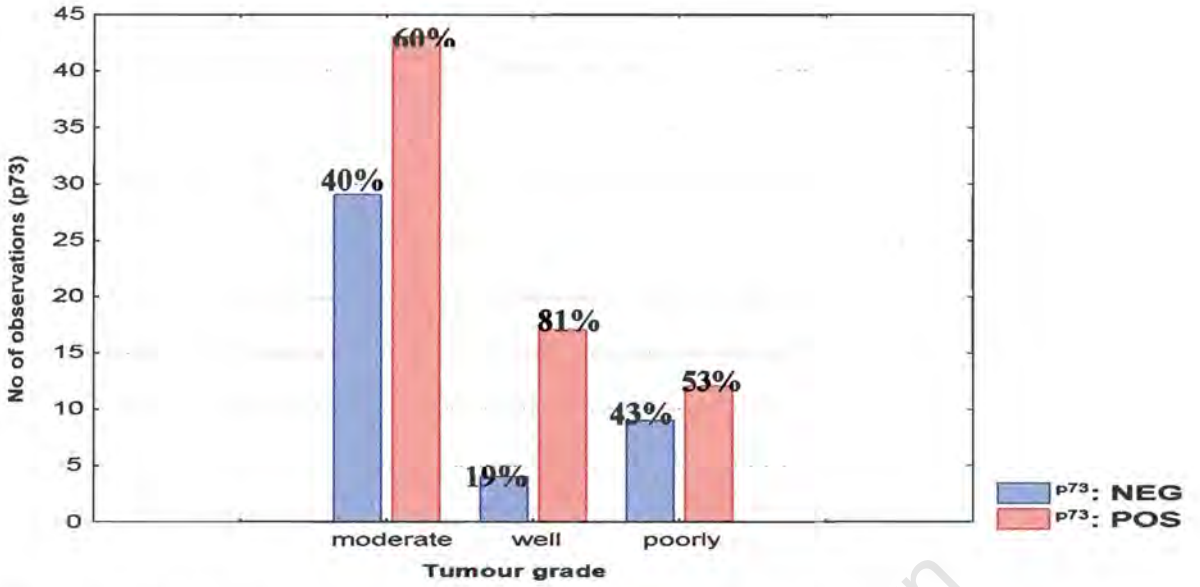


Figure 4.12 Relationship between Tumour grade and p73. p73 expression between the different tumour grades was analysed using the statistical package STATISTICA. The percentage of p73 negative of positive for each tumour differentiation category are indicated on top of the bar graph and the number of cases are indicated on the Y-axis.

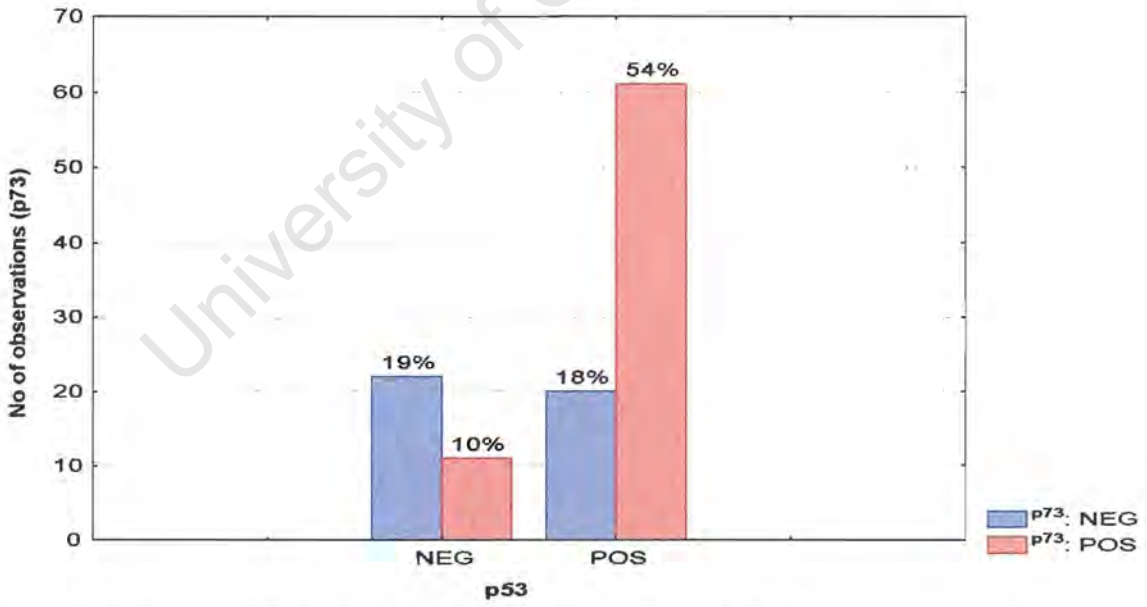
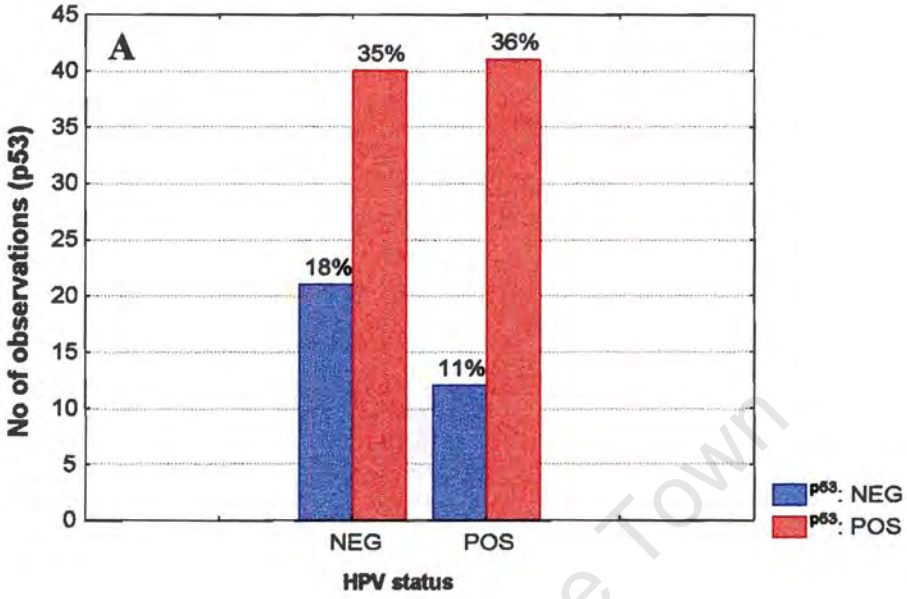
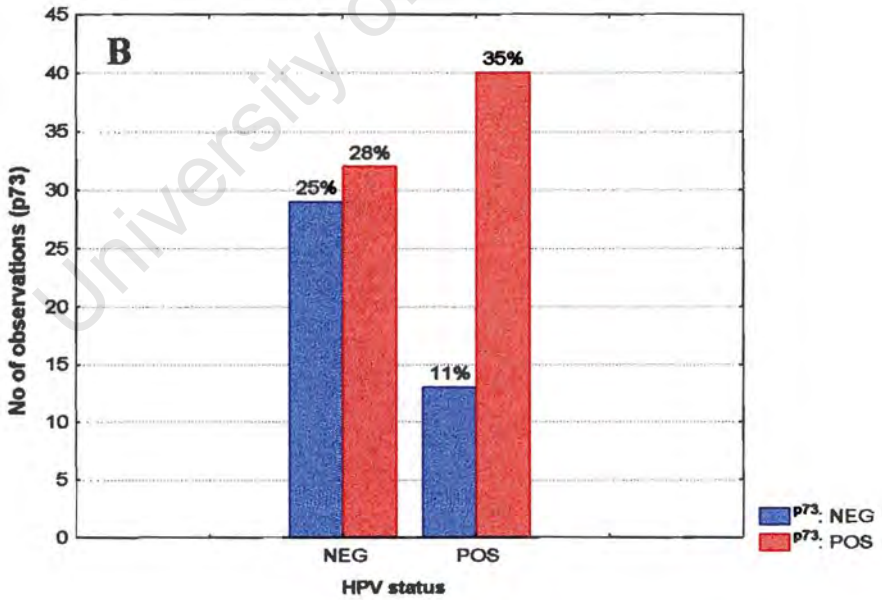


Figure 4.13 Relationship between p53 and p73. The OSCC biopsies were immunohistochemically stained for p53 and p73 protein expression as described in materials and methods (section 6.9.5 and 6.9.6, respectively). The data was captured on Excell and analysed using STATISTICA software. Co-expression of p53 and p73 was examined by observing the number of p73 positive samples in either p53 negative or positive tissue sections. The percentages of the differences in p73 expression in either p53 positive or negative samples are indicated on the bar graph.





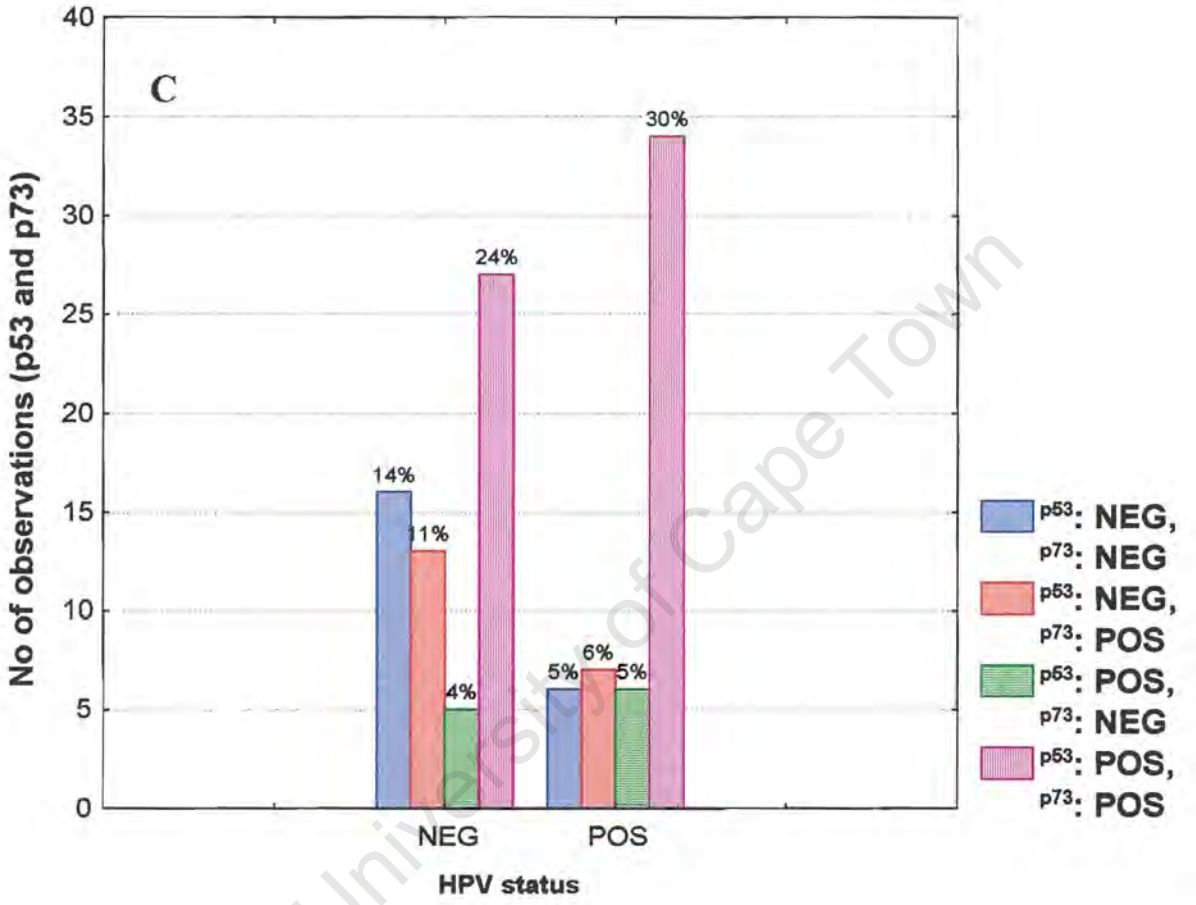


Figure 4.14 Relationship between HPV status, p53 and p73. The HPV DNA status and HPV subtypes were determined in chapter 2 while p53 and p73 status was analysed in section 4.2.4 and 4.2.6, respectively. The data was analysed using the statistical package STATISTICA. A; The number of observations for each variable is shown on the Y-axis and the percentage on the bar graph. A; p53 status on either HPV DNA-positive or –negative samples, B; p73 status on either HPV DNA-positive or –negative samples, C; logarithm linear analysis of p53, p73 and HPV status.

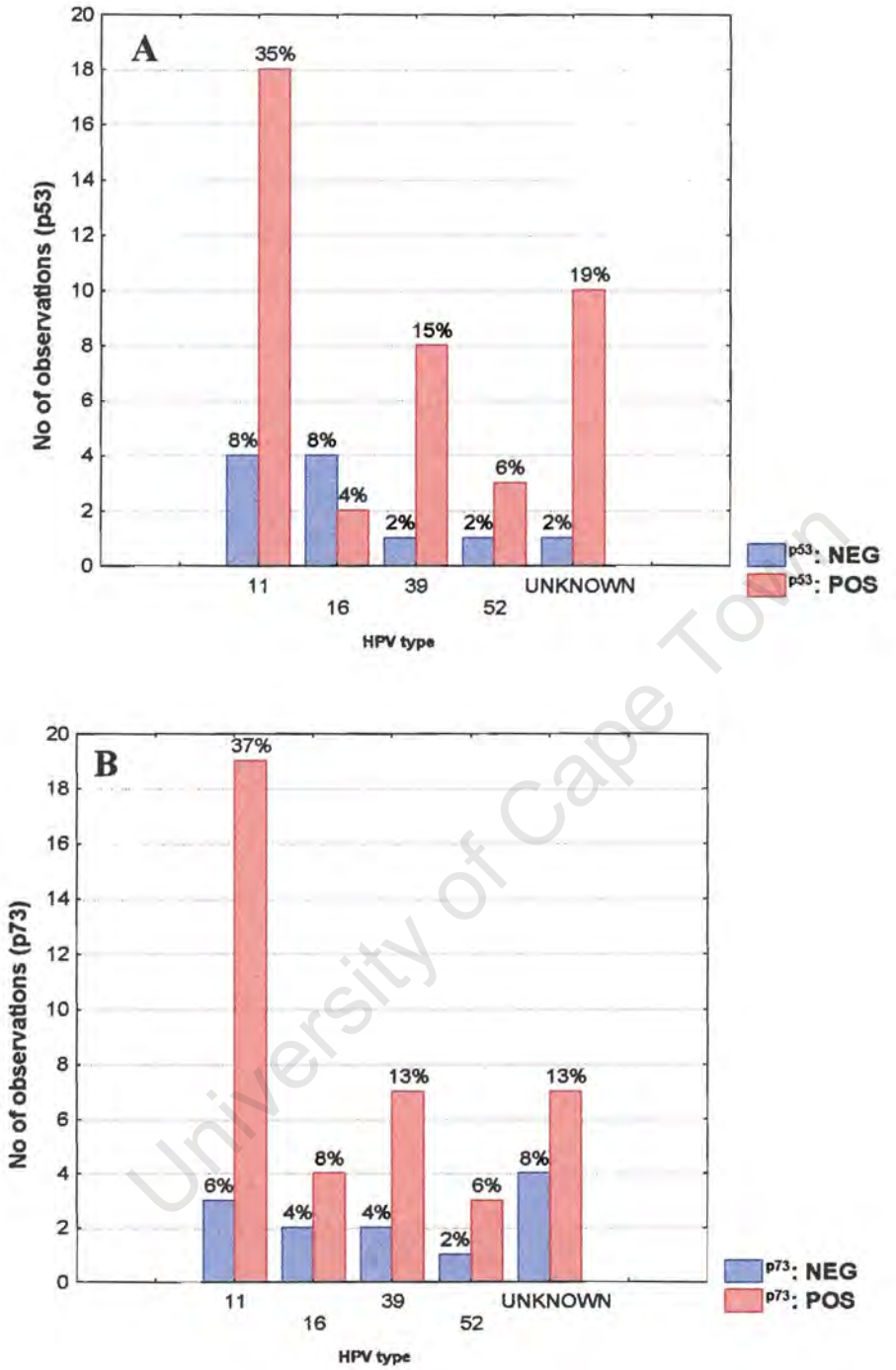


Figure 4.15 HPV subtypes, p53 and p73. Frequency distribution of p53 or 73 expression between the various HPV subtypes. The percentage of tumours expressing p53 or p73 within each HPV subtype is shown on the bar graph. A; p53 protein expression and HPV subtypes, B; p73 protein expression and HPV subtypes.

4.2.6 Functional analysis of HPV E6 Proteins

4.2.6.1 Plasmid Constructs Used In This Study

The constructs pCDNA3: HPV-11 E6 and pCDNA3: HPV-16 E6 constructs (a gift from Dr Lawrence Banks, International Centre For Genetic Engineering and Biotechnology, Trieste, Italy) contain the HPV-11 E6 and HPV-16 E6 open reading frames (ORF's), respectively in the mammalian expression vector Pcdna3. pCMV ß-gal containing the bacterial ß-galactosidase gene driven by the cytomegalovirus promoter construct was included as an internal control to monitor and standardise transfection efficiency. pCDNA3 (Empty vector) was used as background control. Plasmid DNA prepared by CsCl/ethidium bromide density gradient centrifugation were subjected to DNA sequence analysis to confirm that the constructs contained the correct inserts.

4.2.6.2 Cell Culture and Transfection of Fibroblasts

Normal WI38 human embryonic lung fibroblasts and their SV40 transformed counterparts SVWI-38 were maintained in Dubelccos modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum and antibiotics as described in section 6.5.1. Cells were plated in triplicate into 35 mm dishes and transiently transfected using FuGENE 6 transfection reagent (Roche). Briefly, cells were grown to approximately 60 – 80% confluency, cotransfected with 100ng of pCMV ß -gal and 2µg of plasmid DNA or empty vector. Reporter assays were performed 48 hours after transfection. Figure 4.16 summarises the process steps followed during the transfection procedure.

4.2.6.3 Cell Cycle progression of Transfected Fibroblasts

To monitor the effects of HPV E6 expression on cell cycle progression, the distribution of cells in G1, S, and G2/M phases of the cell cycle was analysed. Cells were treated with 034µM doxorubicin (SIGMA) 24 hours after transient transfection to induce DNA damage and harvested by trypsinisation at 12 hours and 24 hours after treatment as discussed in materials and method (section 6.5). In order to test if the doxorubicin concentration used was sufficient to induce DNA damage and arrest cell growth at the G2/M phase, non-transfected cells were treated with 0.34µm doxorubicin and the G2/M

phase monitored (Figure 4.17). Harvested cells were fixed, stained with propidium iodide (PI) immunoflorescence, and analysed by FACScan (Facs Calibre, Becton and Dickinson). The data were captured in Cell Quest (Becton and Dickinson) and analysed using Modfit Verity software (USA) (Figure 4.18).

The distribution of cells in the various phases was not significantly different between the two harvesting time points, i.e. at 12 and 24 hours. Apoptosis was significantly reduced in cells transfected with either pCDNA3: HPV-11 E6 or HPV-16 E6 plasmid constructs. The G2M phase of the cells transfected with HPV E6 constructs was not affected by the doxorubicin treatment and the distribution of the cells in G1 and S phases was not significantly different between the non-transfected and transfected cells (Figure 4.19).

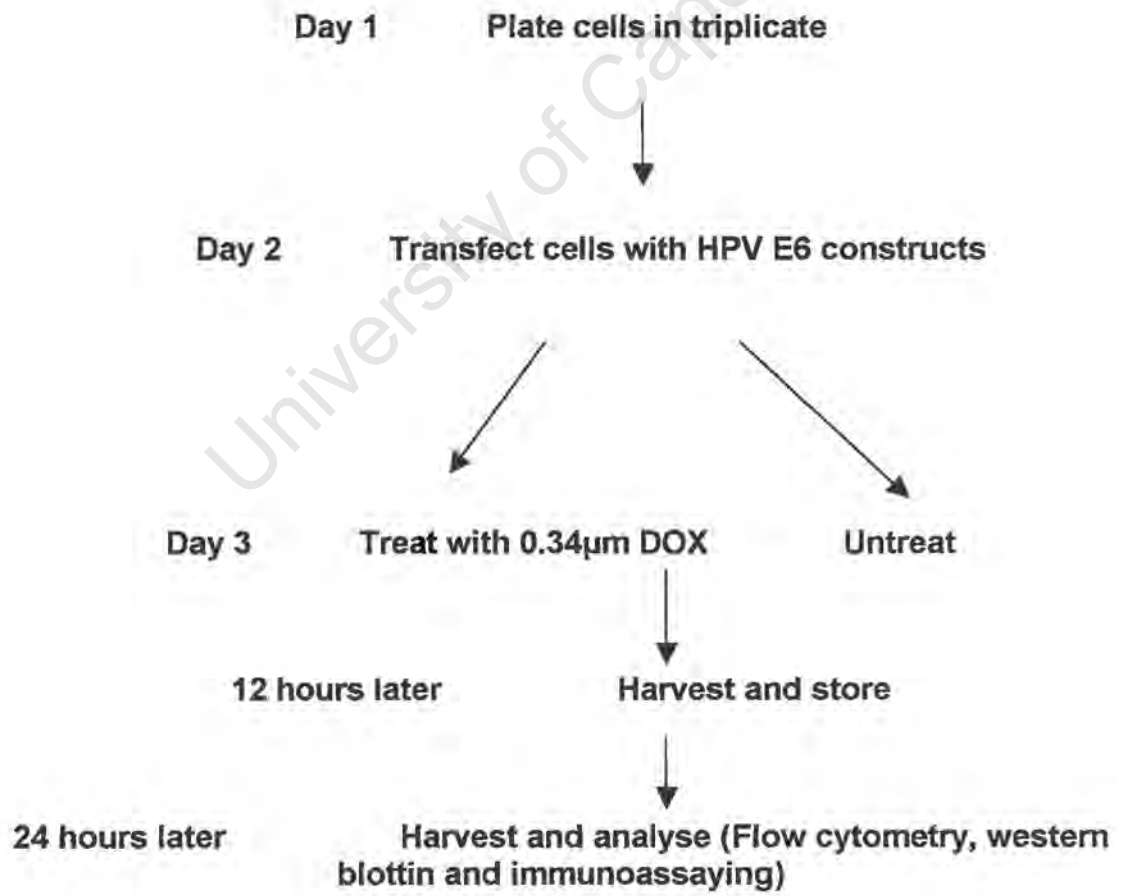


Figure 4.16 Schematic overview of transfection and doxorubicin treatment of normal human fibroblasts.

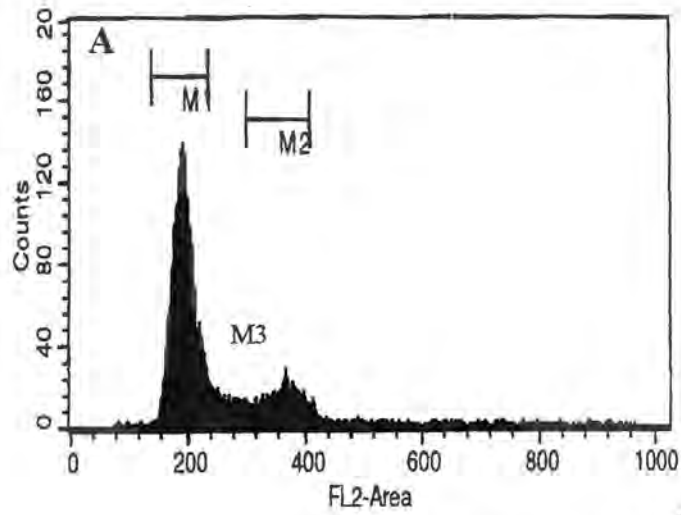
4.2.6.4 Effect of HPV E6 on p53 and p73, accumulation.

To delineate the mechanisms by which HPV E6 affect cell cycle control, cells were grown on overnight cover slips as described in section 6.5.4 and endogenous p53 and p73 protein levels were measured by immunohistochemistry as described in section 6.9. p53 accumulation was absent in cells that had not been treated with doxorubicin. After immunostaining, 5 fields were randomly selected and the numbers of cells positive for either p53 or p73 were counted.

In HPV16 E6 transfected cells, p53 immunoreactivity was significantly reduced while in HPV 11E6 transfected cells, p53 expression was similar to non-transfected cells (Figure 4.20 A to D and I). The expression of p73 protein was intense and increased in cells transfected with either HPV-11E6 or HPV-16 E6 plasmid constructs. p73 expression in mock transfected cells was normal indicating that the intense p73 expression observed in HPV E6 transfected cells was not due to the vector, but the HPV E6 proteins (Figure 4.20 E to H and J).

4.2.6.5 Western blot Analysis

The WI38 cells were transfected in 35mm dishes with 2µg plasmid DNA (HPV11 E6 or HPV16 E6), treated with doxorubicin after 24 hours and harvested at 48 hours after transfection as discussed in section 6.7. Briefly, to detect endogenous p53, cells growing in monolayer were removed form plastic dishes by scraping and collected along with floating cells by centrifugation. Protein from the total cell lysate preparations were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and probed with anti p53 and an anti tubulin as control. HPV16 E6 expressing cells exhibited low levels of p53 and almost undetectable levels after 24 hours of doxorubicin treatment (Figure 4.21). The anti p73 antibody was not suitable for western blot analysis.

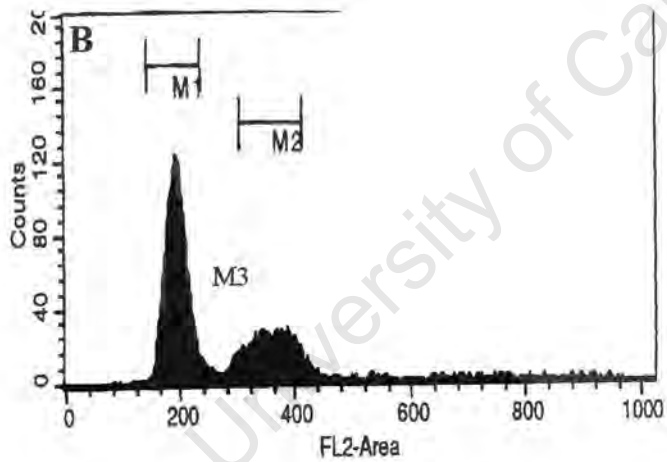


Area:

G1 = 68.55%

G2/M = 16.27%

S = 15.18%



Area:

G1 = 61.04%

G2/M = 28.95%

S = 10.01%

Figure 4.17 Effect of Doxorubicin on WI38. The fibroblasts were plated and grown overnight to 60 – 80% confluency. Semi-confluent cells were treated with 0.34µm doxorubicin and harvested after 24 hours. The cells were then incubated in the presence of RNAse A and propidium iodide as described in section 6.6. The numbers of events are shown on the Y-axis and fluorescence on the X-axis. Marker M; G1 phase cells, M2; G2/M and M3; S phase. The area under the curve is given as a percentage of the total area. A; untreated cells, B; treated cells.

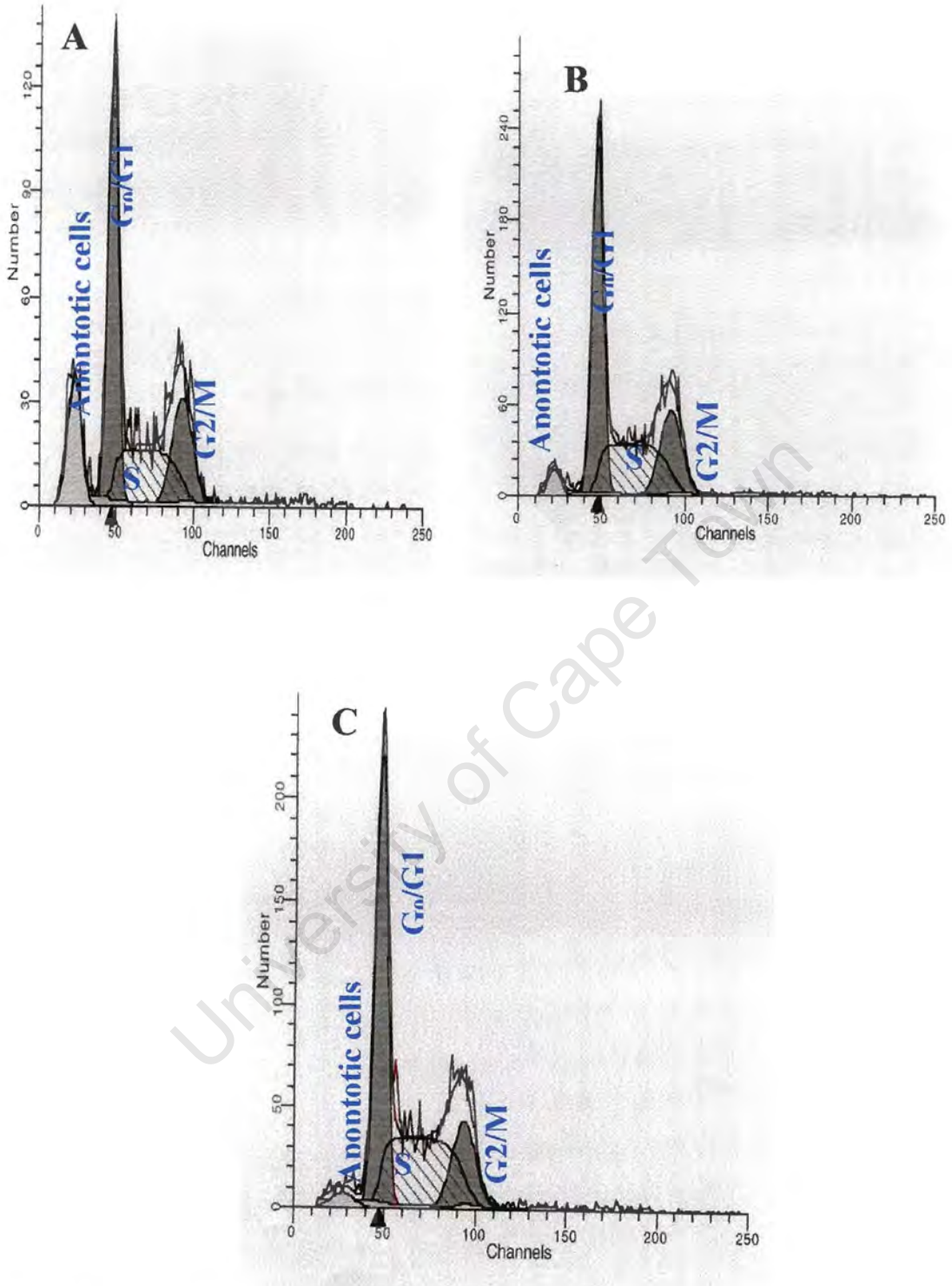
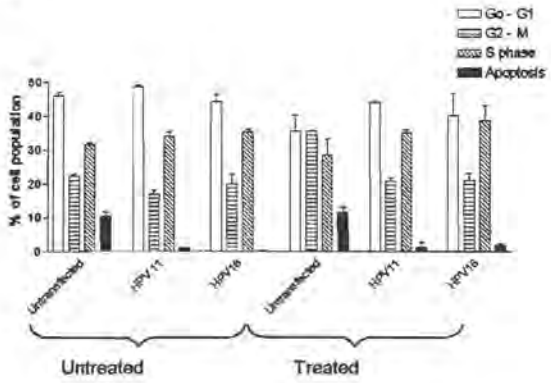


Figure 4.18 Modfit Verity software (USA) analysis of cell-cycle phases. WI38 cells were transfected with HPV E6 constructs, processed for propidium iodide immunoflourescence and analysed by FACScan analysis as described in section 4.2.6.3. The data captured in Cell Quest was further analysed using Modfit Verity software (USA). The number of events is shown on the Y-axis and channel cell cycle phases on the X-axis. A; non-transfected cells, B; pCDNA3: HPV-11 E6 transfected fibroblasts and C; pCDNA3: HPV-16 E6 transfected fibroblasts.

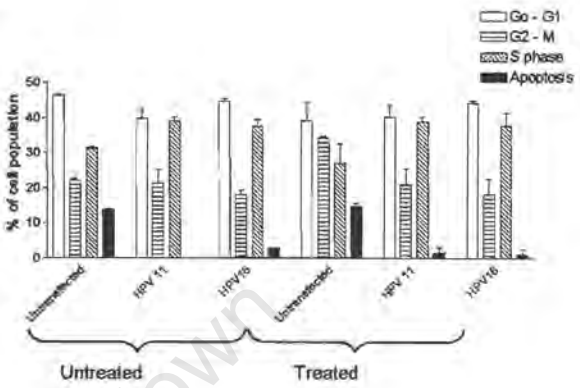
12 hours

24 hours

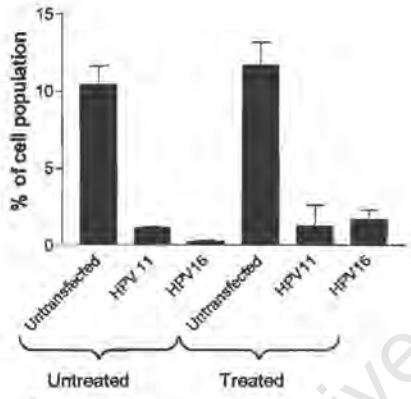
A



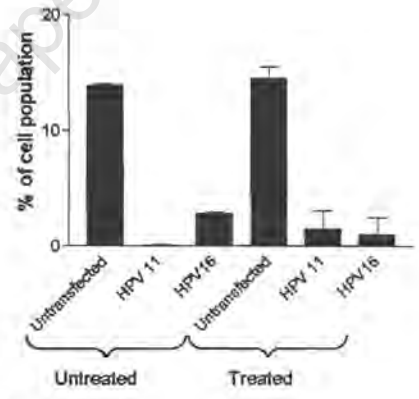
B



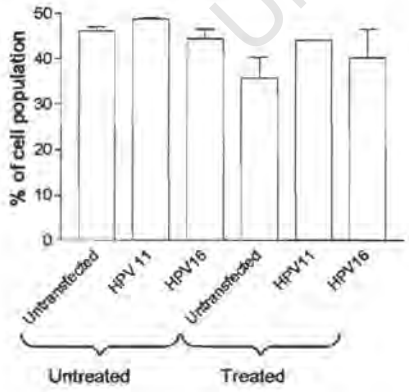
C



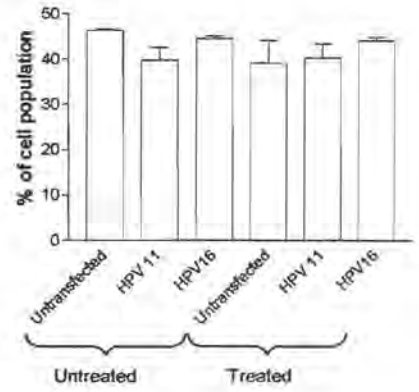
D



E



F



12 hours

24 hours

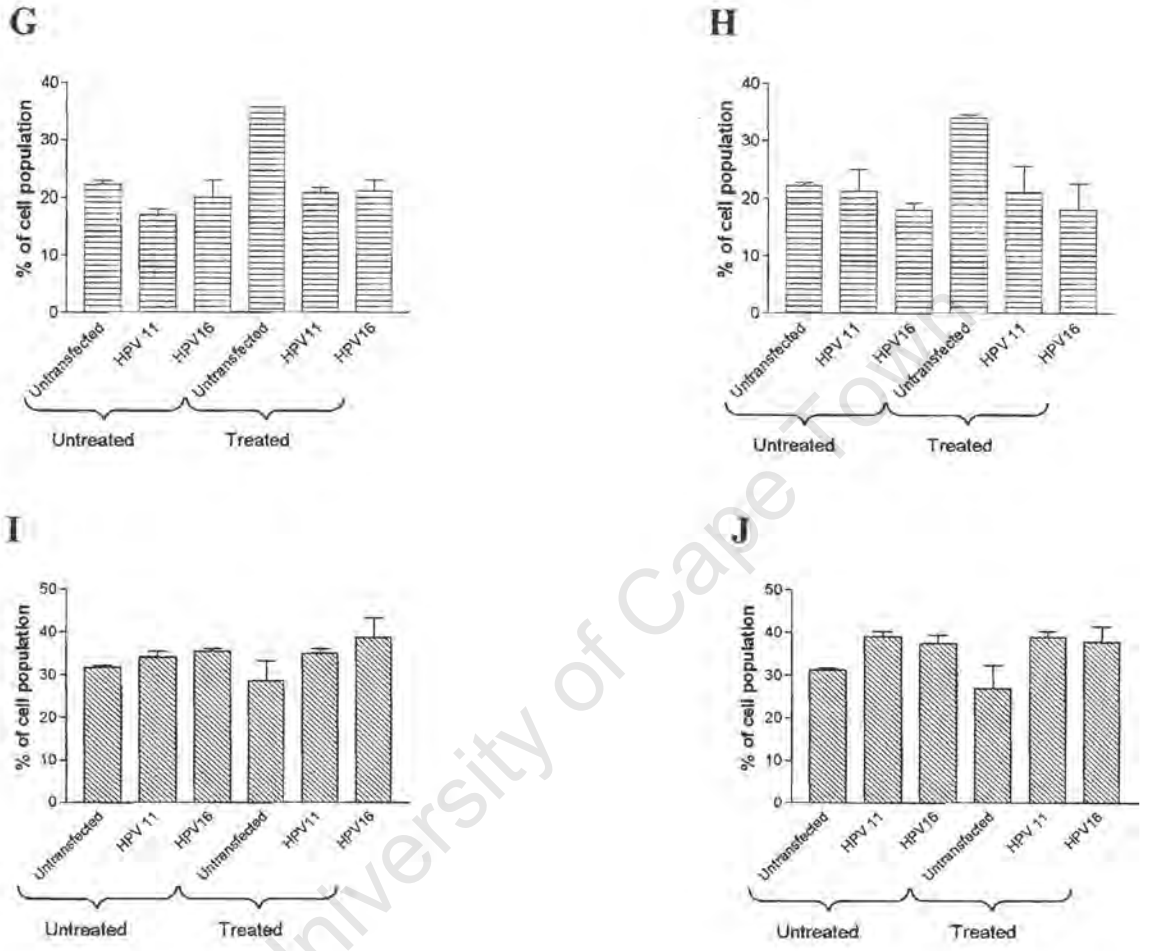
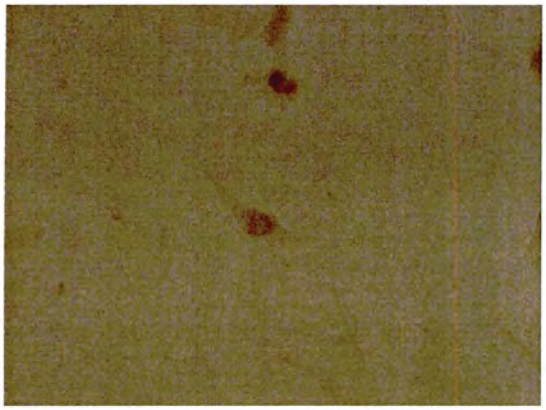


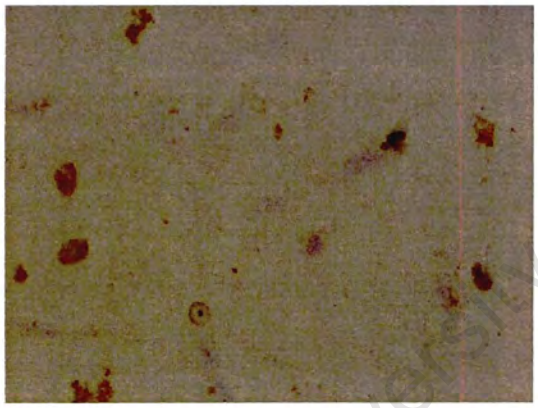
Figure 4.19 Distribution of cells in the different phases of the cell cycle. Confluent cells were transfected with HPV11 E6 or HPV16 E6 and treated with 0.34um doxorubicin. The distribution of cells in the various cell cycle phases was captured using Cell quest and analysed using Modfit Verity software (USA). The above graphs represent percentages of cells in apoptosis, Go/G1, S, and G2/M phase at different periods after harvesting. Overall overview of the entire cell cycle phases at different time points are shown in fig A and B, respectively. Breakdown of apoptosis, G0/G1, G2/M and S phases at 12-hour harvest time point are shown in fig C, E, G and I, respectively. Distribution of cells in apoptosis, G0/G1, G2/M and S phases at 24-hour harvest time point is shown in fig D, F, H and J, respectively.

p53

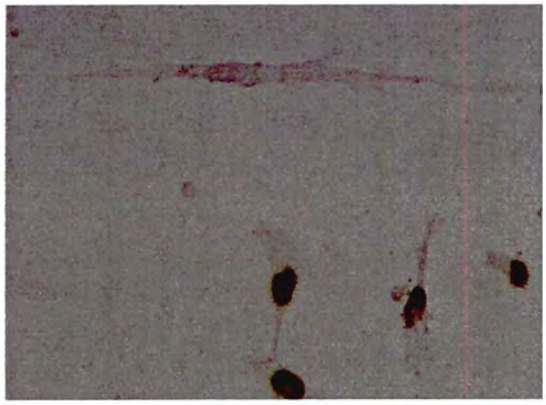
A Non transfected



B Mock

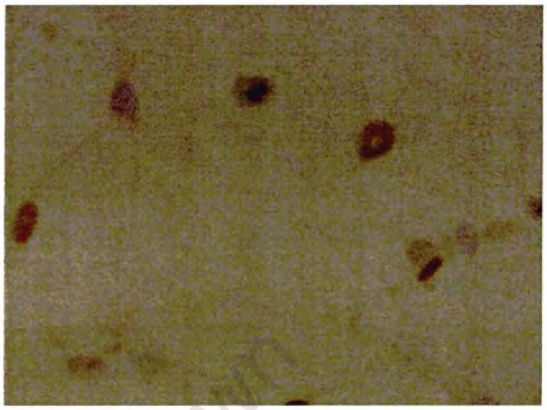


C HPV 11-E6

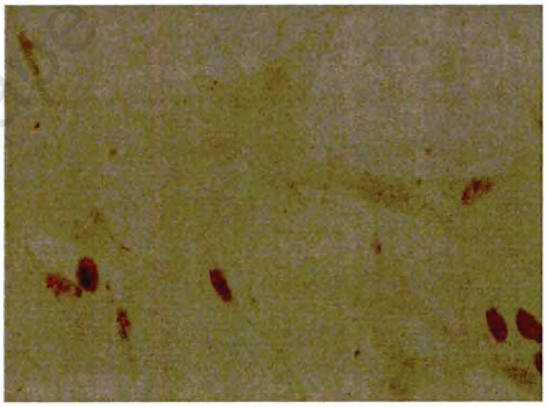


p73

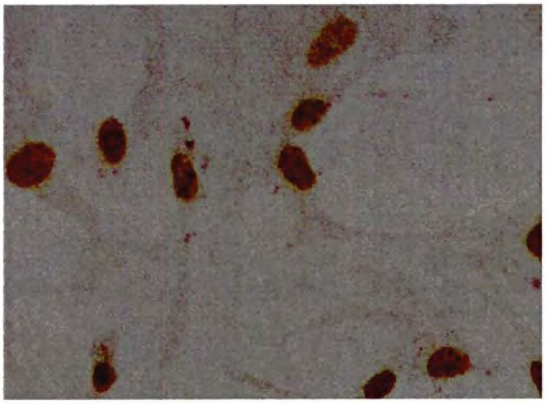
E Non transfected



F Mock



G HPV 11-E6



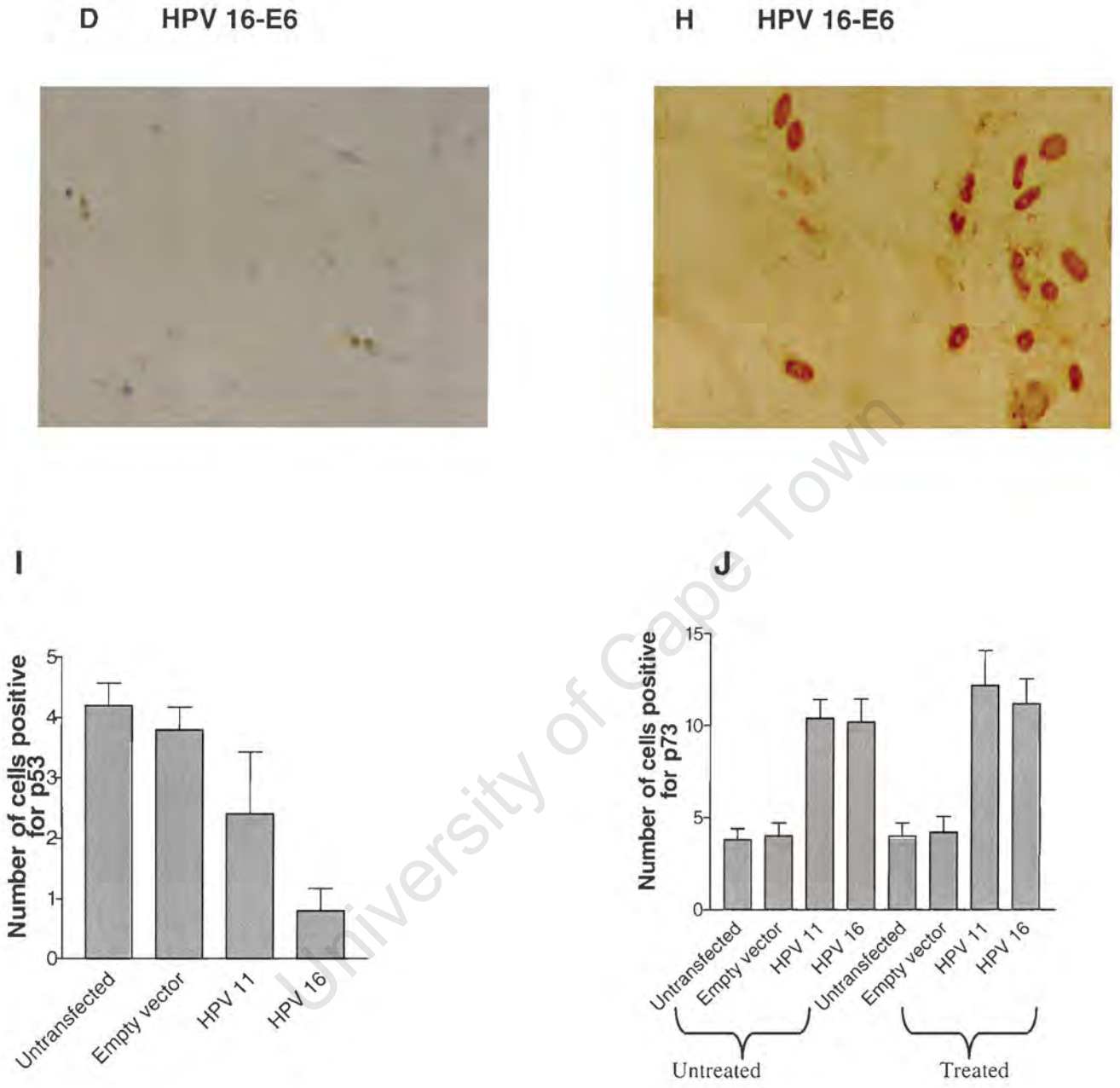


Figure 4.20. Immunostaining for p53 and p73 in transfected fibroblasts. Cells were plated on coverslips, transfected and treated with doxorubicin as described in section 4.2.6.4. Endogenous p53 and p73 were detected immunohistochemically. Expression of p53 in non-transfected, mock transfected, pCDNA3: HPV-11 E6 or pCDNA3: HPV-16 E6 is shown in figure A, B, C and D, respectively. Endogenous expression of p73 in non-transfected, mock, pCDNA3: HPV-11 E6 or pCDNA3: HPV-16 E6 transfected cells is shown in figure E, F, G and H, respectively. The number of cells positive for p53 or p73 are presented in fig. I, and J respectively.

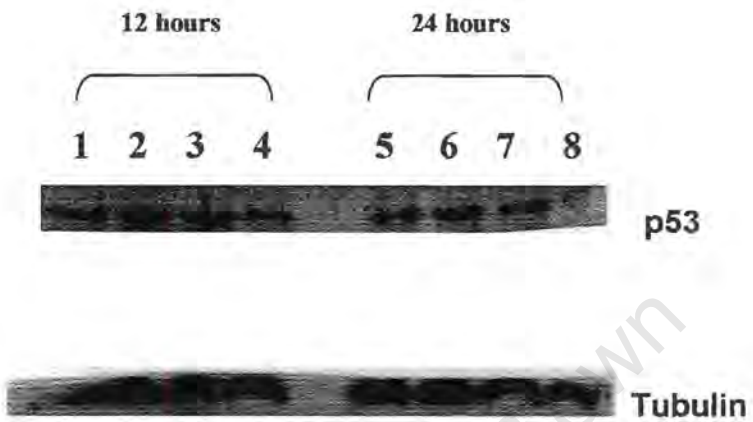


Figure 4.21.

Western blot analysis for p53 protein in transfected cells. Total protein lysate was prepared as described in section 6.7.1 Approximately 50µg of protein was electrophoresed on a 12% SDS polyacrylamide gel. The protein was transferred onto a nitrocellulose membrane and probed with anti-p53 antibody (DAKO). Anti-tubulin was used to monitor loading accuracy. Lanes 1 and 5; non-transfected, lanes 2 and 6; empty vector, lanes 3 and 7; HPV 11-E6, lanes 4 and 8; HPV 16-E6.

4.3 DISCUSSION

In this study, each tumour biopsy was classified into well, moderately and poorly differentiated types according to the histological classification of WHO. Sixty three percent (63.1%) were moderately differentiated, this is in agreement with WHO report which states that moderately differentiated carcinomas account for about two thirds of oesophageal squamous cell carcinomas (WHO, 2002). The prognostic impact of tumour differentiation is not clear, but poorly differentiated tumours have been associated with a poor prognosis (Tores et al., 1999). Other potential prognostic factors include tumour stage at diagnosis, treatment received and patient's general health status. Molecular alterations in certain genes such as p53, p16 and Rb may enhance tumour cell proliferation, invasiveness and metastatic potential and thus may have a bearing on survival.

p53 protein accumulation was observed in 70% of OSCC samples analysed but no significant differences were observed between well, moderate and poorly differentiated OSCC. These results confirm previous reports that showed p53 accumulation ranging between 42% and 85% in oesophageal squamous cell carcinoma (Sasano et al., 1992; Wagata et al., 1993; Wang et al., 1993; Ohashi et al., 1997; Chetty et al., 1999; van Heerden et al., 1998 Kanamoto et al., 1999; Sarbia et al., 1994; Bennet et al., 1992; Wang et al., 1993)). While the accumulation of p53 protein has been associated with poor prognosis in breast (Thor et al., 1992), colon (Sun et al., 1992), stomach (Martin et al., 1992 and lung (Quinlan et al., 1992) cancers, its prognostic significance in OSCC is uncertain. Shimiya (1993), reported on a series of 105 OSCC patients in which p53 accumulation was found to be a significant prognostic indicator. In contrast, Kanamoto (1999) evaluated the significance of p53 protein expression in 239 patients with OSCC and found that p53 protein expression did not correlate with prognosis in univariate and multivariate survival analysis.

Overexpression of p53 (2+ and 3+) was observed in more than 50% of OSCC samples analysed and is usually correlated with the presence of p53 gene mutations. In Lin-xian, China, a highly significant correlation between the presence of p53 gene mutations and nuclear p53 accumulation has been observed of 12 (75%) OSCC samples analysed (Liang et al., 1995). In another study, immunohistochemical and genetic analysis gave a concordant result in 84% of the OSCC cases studied (Seta et al., 1998). Accumulation of p53 protein has also been demonstrated in more than 70% of dysplasic (Shi et al., 1996). These studies suggest that abnormalities in p53 expression may be closely associated with the pathogenesis of OSCC and that immunoreactivity of p53 protein is a good indicator of the tumours with altered p53 function.

Immunohistological testing of the distribution of p53 positive cells showed that they were present in less than 10% in the normal epithelium surrounding the carcinoma as has also been observed in other studies (Gao et al., 1994; Bennet et al., 1992; Sarbia et al., 1994; Chen et al., 2003). It is possible that the presence of p53 protein detectable by IHC could represent accumulation of wild-type p53 protein capable of exerting tumour suppressor functions in response to DNA damage.

Inactivation of p53 protein by human papillomavirus E6 protein is another mechanism by which p53 function may be lost. No statistical differences were observed between p53 overexpression and HPV DNA in the OSCC cases studied since p53 protein accumulation was observed in both HPV-positive (36% of cases) and HPV-negative (35% of cases) biopsies. Previous reports have demonstrated both lack of correlation between p53 and HPV DNA in OSCC (Hirai et al., 1999; Takahashi et al., 1998; Lam et al., 1997; Hasegawa et al., 2002) and an inverse correlation between the presence of HPV DNA and the presence of p53 overexpression (Shibagaki et al., 1995; De He et al., 1997; Fuihata et al., 1993). De He (1997), for example, reported lower rates of p53 mutations in HPV DNA positive OSCC cases, while Lam (1997) detected p53 mutations in both HPV-positive and HPV negative cases studied. The presence of dysfunctional but immunohistochemically detectable p53 in HPV-

infected tumour cells could be the results of HPV E6 protein interactions with p53 C-terminus. It has been demonstrated that HPV E6 proteins are capable of binding to the p53 C-terminus and such interactions inhibit degradation of p53 (Li et al., 1996; Lechner et al., 1992). However, more direct histological evidence such as detection of the p53-E6 complex is required to clarify this interaction.

Meaningful statistical analysis could not be performed with regards to p53 accumulation and HPV subtypes. However, in tumours harbouring HPV 11 DNA , p53 expression was elevated compared to HPV 16 infected tumours and HPV 11 was the most predominant type accounting for more than 50% of the HPV types found. Unlike the HPV E6 of high-risk type (HPV16) the HPV E6 proteins of low risk type (HPV 11) binds to p53 but cannot induce its degradation.

HPV E6 proteins have also been linked to the inactivation of the recently identified p73 gene that is structurally and functionally similar to p53 (Ichimiya et al., 2000, Park et al., 2001, Kaghad et al., 1997)). Furthermore, p73 give rise to different mRNAs by alternate splicing and the by use of different promoters. At least seven different p73 isoforms (alpha to delta) are generated in normal cells (Kaghad et al., 1997; De Laurenzi et al., 1998; 1999; Ueda et al., 1999; Ishimoto et al., 2002), but the role of p73 in tumorigenesis has not been well characterised.

Using an antibody that can detect the alpha and beta isoforms of p73, accumulation of p73 was observed in 61.4% of OSCC tumours compared to 7.7% of cells in the surrounding normal epithelium. It is tempting to postulate that accumulation of p73 in the OSCC is due to p73 gene mutations. However, in all the tumours screened so far, only 0.5% of tumours harbored p73 mutations (Melino et al., 2002). The differences in p73 expression between normal and tumour cells, however, indicate that p73 may be a contributor to the process of tumorigenesis in oesophageal squamous cell carcinoma. Similarly, wild-type p73 overexpression associated with malignant tissue, but not with its matched normal counterpart has been observed in

breast cancer (Zaika et al., 1999), lung (Mai et al., 1998), prostate (Takahashi et al., 1998), colorectal (Sunhara et al., 1998) and oesophageal (Cai et al., 2000) cancers. In contrast, the major isoform p73 showed nuclear localisation in both normal squamous epithelium and carcinoma of the head and neck.carcinomas (Laurens et al., 2001). Similar to observations in this study, when expressed in the normal epithelium, p73 accumulation was confined to cells close to the basal layer and within the basal layer and in the tumour cells immunoreactivity was mainly localised in the nucleus and showed a diffuse pattern.

In contrast to p53 expression and HPV DNA, a direct correlation between p73 accumulation and presence of HPV DNA was observed ($r = 0.48$). The expression of p73 in tumours studied was increased in all the HPV subtypes including the unidentified types. These results are in agreement with the observations of Park et al(2001), in that HPV E6 of both low- and high-risk HPV types can functionally inactivate p73 by direct binding without inducing its degradation. In addition, these results may suggest that HPV 11, termed low-risk in the cervix may be oncogenic in oesophageal cancer via a different mechanism. Interestingly, in HPV DNA-positive but p53-negative tumours, the accumulation of p73 protein was greatly reduced (11%), while in HPV DNA-positive and p53- positive tumours, p73 expression was evident in 64% suggesting that dysfunctional p53 proteins together with HPV DNA may contribute synergistically to oesophageal carcinogenesis.

Statistically significant co-expression of p53 and p73 was observed in 53% of the tumours, further strengthening the notion that there exists a complex network of interactions between p53, and p73. Flores (2002) investigated the role of p63 and p73 in p53 DNA damage induced apoptosis using a series of mouse embryo fibroblasts lacking either p63 or p73 in single or double-knockout cells lacking both p63 and p73 but retaining p53. Treatment of these cells with doxorubicin revealed that cells lacking p63 or p73 alone showed an intermediate resistance to apoptosis as opposed to double knockouts that were equally resistant to apoptosis as p53 knockout cells, suggesting that p63 and p73 are required for p53 dependent DNA damage induced apotosis. In

addition, it has been shown that mutant p53 interacts with p73 or p63, but this is a characteristic not shared by wild type p53. Strano et al (2002) have demonstrated that p53 mutants can engage with different p73 isoforms in a physical association. This association impairs in vitro and in vivo p73 sequence specific DNA binding, consequently inhibiting their transcriptional activity. Moreover, the authors demonstrated that in cells carrying endogenous p53 mutations, p73 proteins are unable to recruit some of the target gene promoters. Recently, inactivation of p73 by dominant-negative proteins such as 72R p53 mutants has been shown to lead to resistance of cells to apoptosis induced by genotoxic agents (Bergamaschi et al., 2003; Irwin et al., 2003).

Consistent with known HPV E6/p53 interactions, in the present study, infection of fibroblasts with HPV16 E6 was shown to result in reduced levels of endogenous p53 as shown by Western blot analysis and immunostaining of fibroblasts transfected with HPV-16 E6. In HPV11 E6 expressing fibroblasts, endogenous p53 levels were not significantly reduced. It has been shown that HPV16 E6 complex with cellular protein E6-AP and facilitates p53 degradation via the ubiquitin-dependent proteolytic system (Scheffner et al., 1990). Activation of p53 by DNA damaging agents in HPV-positive cancer cells has previously been reported (Sakari et al., 2000) but, in this study, treatment of cells with doxorubicin after transfection with HPV E6 constructs did not increase the levels of p53. The failure of doxorubicin to activate p53 could be due to the concentration (0.34μm) used. Similarly, Kessis et al (1993) also failed to induce p53 in HeLa (cervical cancer cell line containg HPV E6 gene) cells using 0.5nM actinomycin D. These experiments were repeated by Sakari et al (2000), and p53 levels were activated when the authors used ten fold higher concentrations.

Apoptosis was markedly inhibited in HPV16 E6 and HPV11 E6 infected fibroblasts. This inhibition of apoptosis by also the low risk HPV type 11 supports the notion that the E6 proteins have p53-independent transforming activities. Indeed, HPV18 and HPV16 are the high-risk types most frequently found in malignant cervical lesions, yet the inability of HPV18 E6 proteins to

bind p53 has been demonstrated (Hengstermann et al., 2001). Furthermore, the E6 proteins have been reported to interact with several other proteins and that these interactions may contribute to HPV E6 cellular transformation (Montavani et al., 1999).

The effect of HPV E6 in normal fibroblasts resulted in increased levels of p73 as shown by immunostaining of cells transfected with either HPV 11 or HPV 16 E6 plasmid constructs. These results support the previous findings that HPV E6 of both low- and high-risk HPV types can functionally inactivate p73 by direct binding, but without inducing its degradation (Park et al., 2001).

In summary this study has demonstrated a relationship between HPV and p73 in oesophageal squamous cell carcinoma. In addition, infection of fibroblasts with low- or high-risk HPV subtypes greatly impaired apoptosis suggesting that there exist other mechanisms by which HPV E6 proteins contributes to hyperproliferation of cells, thereby leading transformation. The previous lack of evidence for a relationship between HPV and other genes, such as p53, that are known to interact with these viruses in transformation of cells has led other investigators to postulate that HPV DNA in oesophageal cancer is incidental. The results of this study have formed a basis for further investigation with respect to the classification and role of HPV subtypes in oesophageal cancer.

CHAPTER FIVE

CONCLUSION

5.1 CONCLUSION

Oesophageal cancer is one of the most fatal cancers worldwide with a five-year survival below 15%. The incidence, histology and aetiology of oesophageal cancer vary remarkably between and within countries as well as between ethnic groups. Western countries such as Europe and America have a very low incidence of oesophageal cancer, with a higher proportion of adenocarcinomas of the oesophagus and are associated with alcohol and tobacco consumption. In contrast, countries such as Iran, China and South Africa have a much higher incidence of squamous cell carcinoma and very little adenocarcinomas. Oesophageal cancer in these areas is partly attributed to low socio-economic status and cultural practices. In South Africa the disease affects mainly African males and the highest incidence of oesophageal cancer occurs in the Transkei region of the Eastern Cape.

Infection by human papillomaviruses was first implicated in the aetiology of oesophageal cancer in 1982. However, the aetiological role for human papillomaviruses (HPVs) in squamous cell carcinoma of the oesophagus remains controversial. In South Africa, several research groups have produced variable results with regards to HPV involvement in oesophageal cancer. These studies originated from geographical areas with low incidence of oesophageal cancer and the techniques used differ vastly in sensitivity.

In this study we initially determined the presence of HPV DNA in patients with squamous cell carcinoma of the oesophagus from Transkei, South Africa by polymerase chain reaction and immunohistochemistry. All the biopsies used were obtained from patients of African (Black) origin. The sample size comprised 122 paraffin embedded biopsies, 10 fresh biopsies from patients with squamous

cell carcinoma (SCC) of the oesophagus and 38 paraffin embedded biopsies of the oesophagus from normal individuals.

HPV DNA was detected in 44.2% of paraffin embedded tumour biopsies and in 60% of fresh tumour biopsies as opposed to 7.9% of control samples. These results are similar to other reports from high incidence areas of oesophageal cancer and suggest that HPV DNA may be a contributing factor in the development of oesophageal cancer. No HPV antigens were detected by the immunohistochemical method in samples that had been shown to harbour HPV DNA by PCR. The immunohistochemistry results clearly demonstrate that various HPV detection techniques have different specificity and sensitivity and this could be attributed to the conflicting HPV detection rates in oesophageal cancer.

In oesophageal cancer as with all cancers, there is a long interval between exposure to the causative factors and the development of the disease. Therefore if HPV contributes to the development of oesophageal cancer, then HPV DNA should be detected in precancerous lesions of the oesophagus. Invasive carcinoma of the oesophagus is usually preceded by a stage of dysplasia or carcinoma *in situ*. The second phase of this study therefore was to determine the presence of HPV DNA in cells obtained by brush cytology and correlates the HPV status with the morphology of the cells. Human papillomaviruses have a considerable malignant potential, particularly when chemical or physical carcinogens are involved as synergistic factors. Other habitual factors that are known to contribute to the development of oesophageal cancer were therefore covered by means of a questionnaire.

Of the subjects studied, 8% had dysplastic changes on cytological examination, similar to previous reports from South Africa. The total prevalence of HPV infection was 3.9% in the general population. In those subjects with dysplastic changes 21.4% were shown to harbour HPV DNA by PCR analysis whilst HPV

DNA was detected in only 2% of subjects without lesions. Interestingly subjects without lesions but positive for HPV DNA had other abnormal changes such as chronic inflammation and fungal infection. This is the first study to report on HPV involvement in precancerous lesions of the oesophagus in South Africa. A large sample size, particularly comprising of patients with dysplastic morphological changes is required to validate these results. Such a study would not only clarify the role of HPV in oesophageal cancer, but would also establish if HPV screening would be necessary to identify high-risk individuals.

A survey of habitual practices revealed that drinking was common between males and females, while tobacco consumption was more common in males. This could explain the high incidence of oesophageal cancer in males. Interestingly, self-induced vomiting, a practice which probably has been overlooked in the development of oesophageal cancer in South Africa was observed in more than 80% of subjects screened and in all HPV infected individuals. The continuous introduction of gastric juice to the oesophagus may introduce chronic physical damage of the oesophagus, which is favoured by human papillomaviruses. The implication of this cultural practice in the development of oesophageal cancer needs to be further investigated.

The E6 proteins of high-risk HPV contribute to tumorigenesis by interacting with wild type p53 and induce its degradation. However, in oesophageal cancer and in many other cancers, p53 is usually mutated. Indeed, immunohistochemical staining for p53, which has also been used as a tool to detect p53 mutations, revealed a total of 70% of patients with accumulation of p53 protein while no correlation was found between p53 expression and HPV 11 status. These results suggest that it is unlikely that HPV contributes to tumorigenesis via p53 degradation in oesophageal cancer. The presence of dysfunctional but immunohistochemically detectable p53 in HPV infected tumour cells could be the result of HPV E6, particularly HPV type 11 which is known to bind to the p53 - carboxyl terminus without inducing its degradation. Transfection of normal human

fibroblasts with HPV E6 constructs from both low- (HPV 11) and high-risk (HPV 16) HPV types resulted in decreased levels of endogenous p53 by the high-risk HPV16 E6.

It is possible that in oesophageal cancer, HPV E6 proteins contribute to transformation and hyperproliferation of cells via inactivation of other genes. HPV positivity was directly correlated ($p = 0.01$) with p73 protein expression and this was independent of HPV type. These results were further confirmed by the observation of increased endogenous p73 protein levels in cells transfected with either HPV 16 or HPV 11 E6 constructs. This is the first study that reports on the relationship between p73 protein expression and HPV status in oesophageal cancer. Other studies with more emphasis on direct interaction between HPV E6 proteins and p73 are required to validate these results.

In summary this study has enriched our understanding of HPV involvement in oesophageal cancer. It has clearly demonstrated that infection by human papillomaviruses may be a factor in the pathogenesis of oesophageal cancer. Furthermore, the data presented here demonstrate that the molecular mechanism by which HPV contributes to the development of oesophageal cancer is clearly different from that in cervical cancer. The data also shows that certain HPV types considered low-risk in cervical cancer might possibly be oncogenic in oesophageal cancer.

CHAPTER SIX
MATERIALS AND METHODS

6.1 DNA EXTRACTION

6.1.1 Extraction of DNA from Paraffin Wax Embedded Tissues

Two to three 10µm thick sections were cut from wax blocks, placed into 1.5 ml microcentrifuge tubes. Sections were dewaxed by sequential washes of xylene and dehydrated using 95% ethanol as follows: 1ml xylene was added to the sections in microcentrifuge tubes and vigorously mixed for 30 minutes at room temperature. The tissue was pelleted by centrifugation at full speed for 5 minutes, and the xylene removed. This was repeated 3 times with fresh 1ml xylene. After the last removal of xylene, the sections were dehydrated by the addition of 0.5 ml of 95% ethanol and mixed by several inversions. The tissue was pelleted and ethanol removed as described above. Dehydration of tissue sections was repeated twice. Following dehydration of tissue sections, the samples were dried under vacuum in a Savant SVC 100 Speedvac. The dried samples were digested overnight at 37^{0}C with 100µg/ml Proteinase K in a paraffin wax sections digestion buffer (section 6.11). The enzyme was inactivated at 95^{0}C for 10 minutes and the absorbency of an aliquot of each sample was measured at 260 nm and 280 nm (Beckman 650 DU spectrophotometer, USA) to assess the quantity and quality of DNA before storage at -20^{0}C.

6.1.2 Extraction of DNA from Fresh Tissue

Fresh tissue biopsies excised from the patients during endoscopy was snap frozen in liquid nitrogen and thereafter stored at -70^{0}C until required. Tissue samples were homogenised in 2 volumes of digestion buffer (section 6.11) using an Ultra-Turex homogeniser and incubated at 50^{0}C overnight. Following overnight incubation, 2µl of RNase A (10mg/ml) was added and the samples were incubated at 50^{0}C for 30 minutes. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and the samples shaken vigorously and centrifuged at 5000rpm for 10 minutes in a Beckman J-21C centrifuge. After

centrifugation, the aqueous phase was removed and placed in a new microcentrifuge tube. The aqueous phase was mixed by inversion with an equal volume of chloroform: isoamly alcohol (24:1) and centrifuged as above. Two volumes of ice cold 95% ethanol was added to the aqueous phase to precipitate the DNA by placing the samples at -20^{0}C for 30 minutes. Precipitated DNA was pelleted by centrifugation, and washed with 70% ethanol. DNA was dried under vacuum and resuspended in a suitable volume of 1X TE buffer (section 6.11) and stored at -20^{0}C until required.

6.1.3 Extraction of DNA from Cells obtained by Brush Biopsy Samples

The Nabeya capsule sponge containing the oesophageal cells was rinsed in normal saline in a 50ml centrifuge tube. The cells were pelleted by centrifugation at 5000 rpm for 10 minutes and stored at -70^{0}C. The stored cells were thawed, resuspended in 100µl digestion buffer (section 6.11), incubated at 55^{0}C overnight. Inactivation of enzyme was achieved by heating the samples at 95^{0}C for 10 minutes. Samples were centrifuged at full speed in a microcentrifuge tube for 5 minutes, supernatant was transferred to a clean tube and stored at -20^{0}C until required.

6.2 POLYMERASE CHAIN REACTION (PCR) PROTOCOLS

6.2.1 PCR reaction set-up for ß-actin

Reaction mixture

10X Amplification buffer	2µl
dNTP mix (2.5mM)	1.6µl
$MgCl_2$ (25mM)	1.2µl
Forward primer (50pmol)	0.7µl
Reverse primer (50pmol)	1.3µl
DNA	1µl
Taq DNA Polymerase (5U/µl)	0.2µl
Sterile distilled water	12µl
Total volume	**20µl**

Primer pair	PCR conditions	Product size
ß-actin F: 5'-TGACGGGGTGACC	94°C: 2min (initial denaturation)	
CACACTGTGCCCATCTA-**3'**	94 °C: 1min	
ß-actin R:5'-CTAGAAGCATTTG	57 °C: 1min	**650bp**
CGGTGGACGATGGAGGG-**3'**	72 °C: 1min	
	72 °C: 2min (extension)	

6.2.2 PCR reaction for HPV DNA

6.2.2.1 HPV L1 outer primers

Reaction mixture

10X Amplification buffer	5µl
$MgCl_2$ (25mM)	4µl
dNTP mix (2.5mM)	1µl
MY09 (100pmol final)	1µl
MY11 (100pmol final)	1µl
Taq DNA Polymerase (5U/µl)	0.5µl
DNA template (100 - 500 ng)	2µl
Sterile distilled water	35.5µl
TOTAL VOLUME	**50µl**

Primer pair	PCR conditions	Product size
MYO9: 5'-CGTCCMARRGGAWAC	94°C: 5min (initial denaturation)	
TGATC-**3'**	94 °C: 1min	
MY11: 5'-GCMCAGGGWCATAAY	55 °C: 1min } 40 cycles	**450bp**
AATGG-**3'**	72 °C: 1min	

72 °C: 5min (extension)

M, A+C; **R**, A+G; **W**, A+T; **Y**, C+T, **S**, G+C

6.2.2.2 HPV L1 - nested PCR (inner primers)

Reaction mixture

Amplification buffer (X10)	5µl
$MgCl_2$ (25mM)	4µl
dNTP mix (2.5mM)	1µl
GP5+ (50pmol final)	1µl
GP6+ (50pmol final)	1µl
Taq DNA polymerase (5U/µl)	0.5µl
DNA (PCR product, section 6.2.2.1)	2µl
Sterile distilled water	35.5µl
Total volume	**50µl**

Primer pair	PCR conditions		Product size
GP5+: 5'-TTTGTTACTGTG GTAGATAC TAC**-3'**	94: 5min (initial denaturation)		
	94: 1min 40: 2min 72: 1.5min	40 cycles	**150bp**
GP6+: 3'-CTTAT ACTAAA TGTCAAATAAAAAG**-5'**	72: 7min		

6.2.2.3 HPV E6 region

Reaction mixture

10X Amplification buffer	5µl

$MgCl_2$ (25mM)	4µl
dNTP mix (2.5mM)	4µl
WD 72 (10pmol final)	1µl
WD 66 (10pmol final)	1µl
WD 154 (10pmolfinal)	1µl
WD 76 (40pmol final)	1µl
WD 67 (40pmol final)	1µl
Taq DNA polymerase (5U/µl)	0.5µl
DNA template (100 -500ng)	2µl
Sterile distilled water	26.5µl
Total volume	**50µl**

Primer Pair	**PCR conditions**		**Product size**
WD 72: 5'-CGGTCGGGACC GAAAACGG**-3'**	94°C: 5min (initial denaturation)		
WD 76: 5'-CGGTTSAACCG AAAMCGG**-3'**	94 °C: 1min 55 °C: 1min 72 °C: 1min	40 cycles	**240bp**
WD 154: 5'-TCCGTGTGGTG TGTCGTCC**-3'**	72 °C: 5min (extension)		
WD 67: 5'-WGCAWATGGAW WGCYGTCTC**-3'**			
WD: 66: 5'-AGCATGCGGTAT ACTGTCTC**-3'**			

M, A+C; **R**, A+G; **W**, A+T; **Y,** C+T, **S,** G+C

6.3 DNA SEQUENCE ANALYSIS

6.3.1 Sequencing of PCR Products

Sequencing reactions were based on the Sanger dideoxy chain-termination method of sequencing (Sanger et al., 1977). PCR products were sequenced directly using the *T7* Sequanase PCR Product Sequencing Kit (Amersham) as per the manufacturer's instructions. Briefly, 5µl of the PCR product was treated with exonuclease I and shrimp alkaline phosphatase at 37^{0}C for 15 minutes and then subjected to sequence analysis. The resulting sequencing products were separated on a 6% polyacrylamide gel containing 7M urea in 1XTBE.

6.3.2 Denaturing Polyacrylamide Gel Electrophoresis

The 6% gel was constituted as follows: 15ml of 40% Acrylamide/Bisacrylamide
42g urea
10ml TBE (X10)
50ml distilled water

The above mixture was warmed to solubilise the urea, made up to 100ml and filtered under vacuum. Thereafter, 500ul of 10% ammonium persulphate and 50µl TEMED was added.

The gel was cast into 45cm long glass plates and allowed to set. Prior to sample loading the gel was pre-electrophoresed at 55W until it reached a temparature of 50^{O}C. 3.5µl PCR sequence reaction was mixed with 2ul formamide loading buffer (section 6.11) and heated to 80^{O}C for 3 minutes before loading. The gel was then electrophoresed at 55W until the xylene cyanol dye was about 5cm from the bottom.

After electrophoresis, the glass plates were separated and the gel transferred to a piece of Whatman 3MM filter paper. The gel was then covered with Saran (plastic) wrap and dried under vacuum at 60^{O}C for 30min to 1 hour. Dried gel was aligned with the x-ray (Agfa) film and exposed for 16 to 48 hours at room temperature or at -70^{O}C with screens using a spring-loaded metal cassette.

6.4 PLASMID DNA PREPARATION

6.4.1 Preparation of Competent Cells

5ml of Luria broth (LB) (section 6.11) was inoculated with 50µl of a glycerol stock of E. coli XL1-Blue cells and incubated at 37^{0}C with vigorous shaking for 16 hours. 300µl of the overnight culture was inoculated into 300ml fresh autoclaved Luria broth and incubated at 37^{0}C with shaking until the OD_{650} of the culture was between 0.2 and 0.4. The cells were pelleted by centrifugation at 5000rpm in a JA-10 rotor for 10 minutes at 4^{0}C. The cell pellet was gently resuspended in 40ml of cold 60mM $CaCl_2$, 10mM Pipes, pH 7.2 and incubated on ice for 20 minutes. The solution was transferred to a 30ml Corex ^{TM} tube and the cells were pelleted by centrifugation at 5000rpm in a JA20 rotor at 4^{0}C. The cells were then resuspended in 4ml of cold 60mM $CaCl_2$, 10mM Pipes, pH 7.2, 15% glycerol. 200µl aliqouts of competent cells were placed into microcentrifuge tubes and stored at -70^{0}C.

6.4.2 Transformation of Competent Cells

XL1-Blue competent cells were thawed on ice and transformed with 1µl of plasmid construct (pCDNA3 HPV11 E6, or pCDNA3 HPV16 E6, or pCDNA3 empty vector, or pCMV-ß-galactosidase). The cells/DNA mixture was incubated on ice for 30 minutes and heat shocked for 2 minutes at 42^{0}C, then incubated on ice for 2 minutes. To each transformation reaction 1ml of Luria broth (pre-warmed to 37^{0}C) was added and incubated at 37^{0}C for 1 hour. Aliquots of the transformation mix were plated onto Luria Broth ampicillin (LB-Amp) agar plates and incubated at 37^{0}C for 16 hours.

6.4.3 Small-scale Plasmid DNA Preparation

Colonies from the LB-amp agar plates were selected and used for the small-scale preparation of plasmid DNA. The bacterial colonies selected were removed with a sterile pipette tip, inoculated into 3 ml of Luria Broth containing ampicillin and incubated overnight with shaking at 37^{0}C.

The cells were collected by centrifugation at 3000 rpm in a Beckman TJ-6 benchtop centrifuge for 10 minutes at 4^0C. The cell pellets were resuspended in 100µl of Solution 1 (section 6.11). 200µl of freshly prepared Solution 2 (section 6.11) was then added to the lysate and the solution mixed by vigorous vortexing. Solution 2 hydrolyses the bacteria and disrupts the cell membrane. Neutralisation of the mixture was achieved by adding 150µl of Solution 3 (section 6.11) followed by centrifugation at 3000rpm for 5 minutes at 4^0C and the supernatant was transferred to a clean 1.5ml-microfuge tube. The plasmid DNA was precipitated by adding 600µl of isopropanol and placed at -20^0C for 1hour. The DNA was pelleted by centrifugation at room temperature for 10 minutes, washed twice with 70% ethanol, dried under vacuum and resuspended in 100ul of 1X TE buffer.

6.4.4 Restriction Enzyme Analysis

To confirm the presence and size of the HPV11 E6 and HPV16 E6 fragments, a double restriction enzyme digest was performed. 10µl of the plasmid DNA was digested with the restriction endonucleases EcoR1 (10U/µl) and Bam H1 (10U/µl) in a total volume of 20µl, incubated at 37^0C for 1 hour. The digested plasmid was electrophoresed on agarose gel stained with ethidium bromide.

6.4.5 Large-scale plasmid DNA preparation by CsCl/Ethidium Bromide density gradient Ultracentrifugation.

This method of plasmid purification yields high-quality DNA free of RNA and protein contaminants. 1ml of the overnight bacterial culture used for small scale plasmid preparation was inoculated into 400 ml of LB and incubated overnight at 37^0C with shaking. Cells were harvested by centrifugation at 7000rpm for 10 min at 4^0C. The cell pellet was resuspended in 40 ml of Solution 1(section 6.11). 80ml of freshly prepared solution 2 (section 6.11) was added and the suspension mixed gently by inverting the tube 6 to 8 times. 45 ml of ice-cold Solution 3 (section 6.11) was added to the above, gently mixed and centrifuged at 7000rpm for 10 min at 4^0C. The supernatant was filtered through a gauze swab (size 10

cm X 10 cm 8 ply, ALLMED, South Africa). The plasmid DNA was precipitated by addition of 80 ml of isopropanol mixed by inversion and centrifuged at 7000rpm for 10 min at 4^0C. The DNA pellet was air dried and resuspended in 2ml of sterile distilled water.

After the pellet was completely resuspended, the solution was transferred to a 10ml Falcon tube containing 4.8g CsCl. 200µl of ethidium bromide was added to the above, mixed by vortexing and centrifuged at 3000rpm for 10 min at room temperature to clarify the solution. The supernatant was transferred to an ultracentrifuge tube (Quick-Seal ultracentrifuge tubes, Beckman,USA) and sealed per manufacturer's instructions. The samples were then centrifuged overnight at 25^0C at 50 000 rpm (Vti 65.2 Vertical tube rotar, Beckman, USA).

The plasmid band was collected by inserting an 18-gauge needle into the top of the tube, and a 21-gauge needle connected to a 2ml syringe below the plasmid band, allowing gente aspiration of the band. The plasmid band was transferred into a 14ml tube.

To extract the ethidium bromide, an equal volume of isobutanol was added, mixed vigorously and the phases allowed to settle. The isobutanol layer (top) was removed and discarded. The extraction step was repeated several times until the ethidium bromide (pink colour) was completely removed. The colourless aqueous layer was transferred into a clean 14ml tube and 1µl of RNase A (10mg/ml) was added to digest any RNA present in the plasmid DNA preparation.

Removal of CsCl was by transferring the DNA into a dialysis cassette with a 5ml syringe (Pierce, USA) and dialysed in 1L TE buffer. The beaker was then covered with foil and left at 4^oC with stirring overnight.

Following dialysis, the plasmid DNA was transferred into a clean 14ml tube and 2µl of Proteinase K (10mg/ml) and 50µl of 10% SDS (0.2% final concentration)

were added. The solution was mixed by vortexing and incubated at 37^{0}C for 1 hour. An equal volume of phenol: chloroform (1:1) was added to the sample after incubation to separated the plasmid DNA from protein. The samples were mixed and centrifuged at 14 000rpm for 2 min at room temperature. The aqueous layer was transferred into a fresh 14ml centrifuge tube and the DNA was precipitated by adding 1/10 volume of 3M NaAc (pH 5.2) and 2.5X 95% ice-cold ethanol. The solution was mixed and centrifuged at 5000rpm for 10 min at room temperature. The DNA pellet was washed with 70% ethanol, air-dried and resuspended in 500µl of distilled water.

6.4.6 Plasmid DNA Sequencing

The HPV11 E6 and HPV16 E6 constructs were subjected to automated plasmid DNA sequencing. The nucleotide sequence of the constucts was determined using the T7 sequencing primer. Automated sequencing was according to the manufacture's instructions by automated BigDye Terminator using the ABI 373A sequencer (Applied Biosystems, Foster City, CA).

6.5 TRANSFECTION OF PLASMID DNA

6.5.1 Propagation of Cells

The cell lines used were the WI-38: a human embryonic lung fibroblast cell line (ATCC) and SV-WI-38: an SV40 immortatilsed human embryonic lung fibroblast cell line.

In T-75 (Greiner Labotechnik) culture flasks 10ml of Dulbecco's Modified Eagles Medium (DMEM) containing antibiotics (penicillin plus streptomycin) and 10% heat inactivated foetal calf serum (FCS) was inoculated with 1ml of thawed cells. All the cell lines were incubated at 37^{0}C with 5% CO_2. Upon reaching confluency, the cells were washed with 3ml of PBS (section 6.11) for 15 to 30 seconds and the solution aspirated. 3ml of fresh trypsinisation solution was added to the cells and allowed to lift of from the flask surface. Trypsinisation was stopped by

addition of fresh DMEM containing 10% FCS and cells aspirated several times to disrupt clumps. 1ml of the cell suspension was used to inoculate 10ml fresh DMEM, and the rest of the cells were centrifuged at 800rpm for 5min at 4^0C. The cell pellet was resuspended in freezing down solution and stored for 24 hours at -70^0C before being archived in liquid nitrogen.

6.5.2 Preparation of Cells for Transfection

WI-38 cells were grown to confluence in 10ml DMEM supplemented with 10% foetal calf serum (FCS) and antibiotics (penicillin-streptomycin) at 37^0C in a 5% CO_2 incubator. The day prior to the transfection, the cells were plated at a density of $1X10^6$ in 35mm dishes and allowed to grow overnight to 60 -80% confluence.

6.5.3 Transient Transfection

Cells that had reached 60 - 80% confluence were co-transfected with 200ng of one of the expression constructs (HPV11 E6, or HPV16 E6, or empty vector) and 100ng of pCMV-ß-gal as internal control. Transfections were carried out using the FuGENE 6 transfection reagent (Roche) as per manufacture's instructions. Cells were incubated at 37^0C for 24 hours, thereafter treated with 0.34µM doxorubicin (Sigma) and incubated for the indicated times. Cells were harvested at two different time points following doxorubicin treatment (12 or 24 hours).

6.5.4 Preparation of Cells for Immunostaining

Cells were grown overnight on coverslips at 37°C in DMEM containing 10% FCS. The cells growing on coverslips were transfected as described in section 6.5.3. Following transfection, cells were fixed in acetone for 5 min, air-dried and washed in PBS. They were subsequently immunostained with either p53 or p73 antibodies as described in sections 6.9.5 and 6.9.6, respectively.

6.5.5 Preparation of Cell Extract

The cells were washed three times with cold PBS and incubated for 5 min at room temperature in 1ml of TEN (section 6.11). The rubber bottom plunger of a 1ml syringe was used to scrape the cells from the dishes and the cells transferred to a clean 1.5ml microfuge tube. The cells were pelleted by centrifugation at 13 000rpm for 10 min at 4^0C. Cell pellets were resuspended in 200µl of 0.25M Tris/HCl pH 8.0. The cells were disrupted by three rapid freeze-thaw cycles: 1 min in liquid nitrogen and 2 min at 37^0C water bath followed by vortexing. Cell extracts were obtained by collecting the supernatants after centrifugation at 13 000rpm for 10 min at 4^0C

6.5.6 ß -galactosidase (ß-gal) Reporter Assay

The ß-gal activity was measured using O-nitrophenyl-ß-D- galactopyranoside (ONPG) as substrate. The reaction mixture contained 10µl cell extract, 500µl Z buffer (section 6.11) and 100µl ONPG. The reactions were incubated at 37^0C until a yellow colour was observed. The reaction was stopped by the addition of 250µl of 1M Na_2CO_3 stopped the reaction. The absorbance at 420nm was measured on a Beckman DU 650 Spectrophotometer (Beckman Instrument, USA).

6.6 CELL CYCLE ANALYSIS

Transfected cells were treated with 0.34µM doxorubicin (Sigma). Prior to treatment, the medium was removed and fresh DMEM containing 0.34µM doxorubicin was added to the cells. The cells were incubated at 37^0C until ready for harvesting. At 12 hours or 24 hours after treatment with doxorubicin, the cells were harvested by removing the medium and rinsing the cell layer with PBS (section 6.11). The cells were trypsinised and pelleted by centrifugation at 800rpm for 5 min. Cells were resuspended in 200µl ice-cold 1X PBS and 4ml of 70% ethanol was added before storage at -20^0C until required.

When ready for analysis, the cells were pelleted at 2000 rpm for 10 min and the supernatant removed. 100µl RNase (1mg/ml), 20µl propidium iodide (1mg/ml) in a final volume of 1ml PBS was added, vortexed and incubated in the dark for 30 min at 37^{0}C. Samples were transferred to 4^{0}C and immediately sorted on a Becton Dickinson Facscan to determine the distribution of cells in the various stages of the cell cycle.

The distribution of cells was captured using Cell Quest and analysed by Modfit as per manufacturer's instructions

6.7 WESTERN BLOT ANALYSIS

6.7.1 Total protein lysates

The cells were washed three times with cold PBS. 50µl of 2X SDS buffer (section 6.11) was added to the cells. Cells were scraped from the dishes and transferred to a clean 1.5ml eppendorf tube, sonicated to break up the DNA and stored at 4^{0}C until required.

6.7.2 Protein Determination in Cell Lysates

Protein determination was performed using the BCA Protein assay kit (Pierce) as per manufacturer's instructions. Briefly, the micro well plate protocol was followed: 25µl of protein standard and samples were pipetted onto the wells; 200µl of working reaction (WR) was added, mixed using the pipette tip and incubated at 37^{0}C for 30 min. The plates were allowed to cool to room temperature and absorbency of each well was measured at 595 nm. Absorbency readings were plotted against BCA concentration to construct a standard curve, to calculate the protein concentration PRISM version 3.0 software was used.

6.7.3 Polyacrylamide Gel Electrophoresis (PAGE)

The gel apparatus were assembled according to the manufacturer's instructions (30cm X 40cm plates, Biorad). Gel solution (section 6.11) was prepared in a final volume of 50ml with distilled H_2O. Before pouring the gel, 250µl of 10%

ammonium persulphate (APS) (section 6.11) and 25µl of TEMED were added to the gel solution to allow polymerisation. Using a 50ml syringe, the gel solution was poured between the glass plates leaving about 10 cm for the stacking gel. The gel was allowed to set at room temperature before the stacking gel (section 6.11) was poured, and the well comb inserted.

After the stacking gel had set, the comb was removed and the wells thoroughly rinsed. The gel was assembled using the Biorad system, and the tank filled with protein electrophoresis buffer (section 6.11). Prior to loading the samples, cell lysates were boiled and bromophenol blue added. The amount in microlitres of protein to be loaded was determined by the concentration 50µg (section 6.7.2)

6.7.4 Transfer of Protein onto Nitro-cellulose Membrane

The nitro-cellulose membrane (0.45µm Hybond - C protein membrane was used. After electrophoresis, the gel was placed on 3 pieces of Whatman 3MM paper, cut to the same size as the gel and pre-soaked in 10 X transfer buffer (section 6.11). A piece of 0.45um Hybond -C nitro-cellulose membrane (Amersham), also pre-soaked in 10 X transfer buffer and cut to the same size as the gel, was placed on top of the gel, and finally another 3 pieces of Whatman 3MM paper were placed on top of the nitro-cellulose membrane. The sandwich was assembled was placed in a transfer cassette and tank filled with transfer buffer (section 6.11). Electrophoretic transfer of protein from gel to membrane was performed at 100 V for 1 hour in a cold room.

6.7.5 Probing of Nitro-cellulose Membrane

Immobilised proteins were probed with anti-p53 (DAKO) antibody, stripped and reprobed with anti-tubulin (Promega). The membrane was washed twice in PBS containing 0.1% Tween 20. Non-specific binding was blocked by incubating the membrane while shaking in blocking solution (section 6.11) at room temperature for 1 hour. Next, it was placed in a blocking solution containing the primary antibody (1:500 for anti- p53) and incubated with shaking at 4^0C overnight. The

blot was washed three times in PBS containing 0.1% Tween 20 and incubated in secondary antibody (1:3000 anti-goat IgG, Pierce) Antigens were identified by luminescent visualisation kit (Pierce) as per manufacturer's instructions.

6.8 CYTOPATHOLOGY

6.8.1 Cytostaining of Brush Biopsies

Cytotologic material from the surface of the entire oesophagus was obtained using the Nabeya capsule, smeared on glass slides, sprayed with a cytological fixative containing ether, alcohol, and polyethylene glycol (Fencott, South Africa). The smears were air-dried, transported to the laboratory and stained using the Papanicolaou method as follows:

Hydration: 80%, 70%, 50% alcohol for 15 sec each, followed by tap water rehydration for 15 sec. Nucleus staining: Gill haematoxylin for 2 min followed by tap water rinse to remove excess stain. 1% Litthium (blue nucleus stain) for 15 seconds. Preparation of smears for alcohol based stain (cytoplasm): 70%, 96% alcohol each for 15 seconds. Cytoplasmic stain one: OG-6 (Polychem, United Scientific) for 1 min and rinsed twice in 96% alcohol for 15 seconds. Cytoplasmic stain two: EA-65 (Polychem, United Scientific) for 1.5 min and excess stain removed in two washes of 96% alcohol for 15 sec each. Dehydration: three washes of 15 seconds in 100% ethanol. Clearing of cells: three wahes of xylol for 15 seconds. Finally, mount slides using Entellen (Merck) and examine slide under microscope (Zeiss) for morphologic changes.

6.9 IMMUNOHISTOCHEMISTRY

6.9.1 Cutting of Tissue Sections

Paraffin embedded tissue sections were cut to 3μM thick sections using a microtome (Jung). The sections were mounted onto 3-aminopropyltriethoxysilene (APES) coated slides and heat fixed in an oven overnight at 60^0C.

6.9.2 Coating of slides for Immunohistochemistry

Slides were first washed in 1% liquid detergent. The slides were washed for 1 hour by rocking and rinsed in running tap water for 1 hour. Thereafter, rinsed in three changes of distilled H_2O, dried at 37^0C overnight and coated by dipping into the following: 3-aminopropyltriethoxysilene (APES) ten times, Acetone ten times, distilled H_2O ten times. Excess H_2O was drained by gently blotting the slides on blotting paper and dried overnight at 37^0C.

6.9.3 Immunohistochemistry using anti-HPV

Tissue sections were dewaxed in 4 changes of xylol followed by 4 changes (5min) of 96% ethanol. Retrieval of antigen was achieved by pressure cooking the sections in 10mM citrate buffer (pH 6.0) for 2 minutes. Immunostainig was done using automated DAKO universal stainer and IHC software. Briefly, endogenous peroxidase within the tissues was blocked by incubating the sections in 1% hydrogen peroxide. The sections were blocked for non-specific binding using normal goat serum (DAKO) for 20 min. Incubation with primary antibody (anti-HPV, DAKO) 1:50 dilution using 1XPBS for 1 hour. For detection, a secondary antibody (anti-rabbit) Envision™ rabbit peroxidase (DAKO) was used. Visualisation was by means of diaminobenzene staining (DAB), counter stained in haematoxylin, hydrated in four changes of alcohol, cleared in xylol and mounted in Entellen. Examination of HPV antigens was carried out using a microscope (Zeisis).

6.9.4 Immunohistochemistry using Cytokeratin antibody

IHC staining was done using an automated universal stainer (DAKO)as above. Monoclonal Mouse Anti-Human Cytokeratin, Clone MNF 116, (DAKO) was used as the primary antibody.

6.9.5 Immunohistochemistry using p53 antibody

Monoclonal Mouse anti-Human p53, Clone DO-7 (DAKO) was used as primary antibody. The tissue sections were stained using the automated DAKO universal

stainer and IHC software (DAKO). Paraffin sections were dewaxed and rehydrated using standard procedures. Retrieval of antigen was achieved by pressure cooking the sections in 10mM citrate buffer (pH 6.0) for 2 minutes. After blocking endogenous peroxidase activity, non-specific binding was reduced by incubation with normal goat serum for 30 min. Slides were then incubated for 1hour with primary antibody, diluted at 1:50.

Unbound antibody was removed and washed in 1XPBS. The antibody signal was then amplified by incubation in goat antirabbit-HRP (DAKO). Visualisation was achieved using Diaminobenzene (DAB Substrate-Chromagen, DAKO). After immunostaining, sections were washed in water, lightly counterstained in haematoxylin (15 seconds), dehydrated in three washes of alcohol, cleared in three xylene washes and mounted in Entellan (Merck),

6.9.6 Immunohistochemistry using p73 antibody

The tissue sections were dewaxed in 4 changes (5min each) of xylol followed by 5min four changes of 96% ethanol. The p73 antigen was retrieved by pressure cooking the sections in EDTA pH8.0. Briefly, the EDTA buffer was brought to boil in an open pressure cooker pot. When boiling the sections were immersed and the pot sealed to reach full pressure. The pressure was maintained for 2min and then released. Slides were then rinsed in tap water for 5min.

Endogenous peroxide was blocked by incubating the sections in 1% hydrogen peroxide for 20min and then rinsed in tap water followed by PBS rinse. Using normal goat serum (DAKO) for 20 min blocked non-specific binding. The serum was drained off and anti-p73 primary antibody clone H-79 (Santa Cruz), was diluted 1:200 in antibody diluent (DAKO) and added to the sections, incubated at $4^{O}C$ overnight.

After overnight incubation with primary antibody, the sections were washed thoroughly with PBS at room temperature. The Envision detection system

(DAKO) was used to detect primary antibody as per manufacture's instructions. The development of colour was achieved using Diaminobenzidine (DAB substrate chromagen, DAKO). After the colour had developed, the slides were washed and the signal enhanced by incubating the slides in a 1%copper sulphate solution (section 6.11) and then lightly counterstained in Haematoxylin (section 6.11). Scotts tap water was used to "blue" the sections, after which the sections were dehydrated through graded alcohols, cleared in xylol and mounted in Entellen (Merck).

6.10 STATISTICAL ANALYSIS

The data were computerised, captured on an Excell spreadsheet. Statistical tests were performed using the statistical package STATISTICA. The tests used were chi-square test for comparison of investigated factors and Spearmans Rank correlation test was used for correlation analysis. These tests were considered significant when *P* value was less than 0.05.

6.11 SOLUTIONS

DIGESTION BUFFER FOR PARAFFIN WAX EMBEDDED TISSUE

50mM Tris/HCl, pH 8.0
1mM EDTA
0.5% Tween 20

DIGESTION BUFFER FOR FRESH TISSUE AND CELLS

100mM NaCl
10mM Tris/HCl, pH 8.0
25mM EDTA
0.1mg/ml Proteinase K (added fresh with each use)

CHLOROPHORM: ISOAMYL ALCOHOL (49:1)

49ml chloroform

1ml isoamyl alcohol

1 X PCR BUFFER

10mM Tris-Cl, pH 8.3

50mM KICl

1.5mM $MgCl_2$

FORMAMIDE LOADING BUFFER

95% Formamide

10mM EDTA

0.05% Bromophenol blue

0.05% Xylene cyanol

LURIA BROTH

10g/l Tryptone

5g/l Yeast extract

5g/l Nacl

LURIA BROTH AMPICILLIN AGAR PLATES (LB-Amp)

10g/l Tryptone

5g/l Yeast extract

5g/l NaCl

15g/l Agar

1ml Ampicillin (10mg/ml)

AMPICILLIN SOLUTION (10mg/ml)

10mg/ml Ampicillin trihydrate salt in sterile water

Filter sterilise using 0.22µM syringe filter

Store in the dark at 4^0C

PLASMID PREPARATION (SOLUTION 1)

25mM Tris/HCl, pH 8.0

10mM EDTA, pH 8.0

50mM Glucose

PLASMID PREPARATION (SOLUTION 2)

0.2N NaOH

1% SDS (w/v)

PLASMID PREPARATION (SOLUTION 3)

3M Potassium acetate

11.5% Glacial acetic acid

TRYPISINISATION SOLUTION

0.5g Trypsin

in Ca^{++} and Mg^{++} in free PBS

PENICILLIN/STREPTOMYCIN SOLUTION

Add 5 million units sodium benzylpenicillin to 5ml PBS.

Separately add 5g streptomycin sulphate to 15ml PBS. Pool the two antibiotics and make up volume to 500ml with PBS. Aliquot in 5ml stocks and store at -20^0C, ready for use in 500ml of medium.

TEN BUFFER

1M Tris/HCl, pH8.0

0.5M EDTA

5M NaCl

O-NITRPHENYL-ß-D-GALACTOPYRANOSIDE (ONPG)

(4mg/ml): measure 0.04g ONPG and add 10ml of 0.1M $NaPO_4$

Aliquots in 1ml, cover with foil and store at -20^0C

Z-BUFFER

60mM Na_2HPO_4

40mM NaH_2PO_4

10mM KCl

1mM $MgSO_4$

50mM ß-mercaptoethanol (add when ready to use)

TE BUFFER

10mM Tris-Cl, pH 8.0

1mM EDTA, pH 8.0

10 X TBE BUFFER

890mM Tris

890mM Boric Acid

25mM EDTA

pH to 8.3

PHOSPHATE BUFFERED SALINE

137mM NaCl

2.7mM KCl

4.3mM $Na_2HPO_4.7H_2O$ (pH 7.4)

1.4mM KH_2PO_4

TRIS/HCl (pH 8.0)

50mM Tris salt

pH using 1M HCl

2 X SDS BUFFER

1M Tris (pH 6.8)

10% SDS

ß-mercapto-ethanol

Glycerol

30% ACRYLAMIDE

28.5% Acrylamide

2% Bisacrylamide

2% Ion exchanger V

Add H_20 to a final volume of 100ml. Stir for 16 hours, filter and store at 4^0C

SDS POLYACRYLAMIDE GEL (12%)

30% acrylamide - 4.8ml

3ml resolving gel buffer (36.2g Tris + 0.8g SDS to final volume of 200ml, pH 8.9)

4.2ml distilled h2O

120µl 10% APS

12µl TEMED

STACKING GEL

30% acrylamide - 1ml

1.5ml stacking gel (5.9g Tris + 0.4g SDS to final volume of 100ml)

3.5ml distilled H2O

60µl of 10% APS

6µl TEMED

TRANSFER BUFFER (Protein to membrane) 10 x SOLUTION

72g glycine

19g Tris

Make up to 1L with distilled H_2O

1 X TRANSFER BUFFER

100ml of above

700ml dH_2O

200ml Isopropanol

PBS + 0.1% TWEEEN 20

100ml (10 X PBS)

900ml dH_2O

1ml Tween 20

Stir on heating block and store at 4^0C.

BLOCKING SOLUTION (1 X PBS + 0.1% TWEEN 20 + 5% MILK)

Measure 5g of milk powder (Elite)

Make up to 100ml using 1 X PBS + 0.1% Tween 20

Stir and store at 4^0C.

HAEMATOXYLIN SOLUTION

0.3Mm Haematoxylin monohydrate

105mM Aluminium potassium sulphate dodecahydrate

0.09mM Sodium Iodate

300mM Chloral hydrate

0.4mM Citric acid

Dissolve Haematoxylin monohydrate in water and heat at 60^OC until sunset colour appears. Add individually the above in order, mix well, cool and filter before use using Whatman paper.

SCOTTS WATER

40mM $NaHCO_3$

80mM $MgSO_4.7H_2O$

mix using tap water

CITRATE BUFFER (pH 6.0)

0.01M citric acid

pH to 6 using 1M NaOH

COPPER SULPHATE SOLUTION

1% copper sulphate in distilled water

HYDROGEN PEROXIDE

1% hydrogen peroxide in methanol

APPENDIX 1

PATIENT CONSENT FORM

Project Title: Population screening and early detection of oesophageal cancer in four regions of the Transkei (Baziya, Ngangelizwe, Mhlakulo, Mbekweni).

Project leaders: Prof A Stepien

Prof R T Erasmus

Prof M I Parker

Ms T Matsha

(The subject should complete the whole of this sheet himself/herself or have answered all of these questions, if unable to fill in this form).

Have you read the patient information sheet?

Have you had an opportunity to ask questions and discuss this study?

Have you receive satisfactory answers to all your questions?

Have you received enough information about the study?

Do you understand that you are free to withdraw from the study: at any time or without having to give reason for withdrawing and without affecting your future medical care?

DO YOU AGREE TO TAKE PART IN THIS STUDY?

Counsellor----------	----------	--------
(if appropriate) Name in block letters	signature	date
Witnessed consent ----------	----------	--------
(if appropriate) Name in block letters	signature	date
Subject ----------	----------	--------

QUESTIONNAIRE: EARLY SCREENING FOR OESOPHAGEAL CANCER

PATHOLOGY DEPARTMENT

UNIVERSITY OF TRANSKEI

PRIVATE BAG X 1

UMTATA

1. Patient general information

Name and address:

Telephone no: (h)

(w)

2. Hospital/Institution information

2.1 Name Hospital/Institution

2.2 File Number

2.3 Family History of OC YES/NO

Relation

2.4 Swallowing Complaint

Since

2.5 Questionnaire completed by:

Telephone No.

Date completed

3. Patient Demographic Information

3.1 Sex

3.2 Date of Birth

3.3 Occupation Previous year

Location

Previous 5 years

Location

3.4 Anthropometry

Weight in kg

Length in m

3.5 Place of birth

Homeland

District

Location

3.6 Where did you spend most of your time for the period:

1. Age up to 18 years:

   Homeland:

   District

   Location

2. Age 19 to 35 years:

   Homeland

   District

   Location

3. Age 36 to 64 years:

   Homeland:

   District

   Location

3. Age > 64 years:

   Homeland

   District

   Location

Habits

4.1 Smoking cigarettes

Never =1; Yes = 2; Yes, but stopped =3

Average number per day

Shop-bought = 1; Home-grown = 2; Both = 3

4.2 Smoking pipe (tobacco)

Never = 1; Daily = 2; Weekly = 3; Seldom = 4

Shop bought = 1; Home-grown = 2; Both = 3

4.3 Chewing tobacco

Never = 1; Daily = 2; Weekly = 3; Seldom = 4

Shop-bought = 1; Home-grown = 2; Both = 3

4.4 Drinking beer

Never =1; Daily = 2; Weekly = 3; Seldom = 4

Shop-bought = 1; Home-made = 2; Both = 3

4.5 Drinking spitits

Never = 1; Daily = 2; Weekly = 3; Seldom = 4

Shop-bought = 1; Home-made = 2; Both = 3

4.6 Tribal medicines

Never = 1; Daily = 2; Weekly = 3; Seldom = 4

Shop-bought = 1, Home-made = 2, Both = 3

4.7 Inducing vomiting

Never = 1; Daily = 2; Weekly =3; Monthly = 4; Seldom = 5

Specify method

5. Dietary Information

5.1 Main cereal in diet

Maize = 1; Sorghum = 2; Wheat = 3; Other = 4

5.2 Maize meal intake

Seldom = 1; Daily = 2; Weekly = 3; Monthly = 4

Home-grown = 1; Bought = 2; Both = 3

Amount in tea-cups per day: one = 1; two = 2 etc.

5.3 Maize meal used for home-made beer

Shop-bought = 1; Home-grown = 2; Both = 3

5.4 Home-made beer intake

Seldom = 1; Daily = 2; Weekly = 3; Monthly = 4

Amount in tea cups per day: one = 1; two = 2; etc.

5.6 Other foom components added to daily diet

Mark clearly the type of food only:

Never/Seldom = 1; Almost Daily (≥ 5X/week) = 2; 1 – 2X/week = 3

Green, leafy vegetables

Green pod vegetable

Imifino (wild/veld greens)

Beans in Samp (Umngqusho)

Fruit

Milk

Margarine or butter

Meat (beef, mutton, pork, chicken, mince, sausage)

Fish

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