OESOPHAGEAL SQUAMOUS CELL CARCINOGENESIS:

A STUDY OF CELL CYCLE REGULATORY PROTEINS BY IMMUNOHISTOCHEMISTRY

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Dissertation submitted in fulfilment of Part III of the requirements for the degree of Master of Medicine (Anatomical Pathology), University of Cape Town

July 2004.

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RYAN SOLDIN

JULY 2004

ACKNOWLEDGEMENTS

My sincere thanks and appreciation to the following people:

Professor Pauline Hall, my supervisor, for her ideas, guidance, support and help with editing.

Heather McLeod and Nafiesa Allie for the long hours preparing all the slides and doing the immunohistochemical stains.

CANSA for partially funding this study.

Dr Jo-Ann McLoughlin and Dr Sedick Isaacs for statistical analysis.

My family for their love and support.

TABLE OF CONTENTS

LIST OF GRAPHS AND TABLES:

CHAPTER ONE:

CHAPTER TWO:

CHAPTER THREE:

LIST OF FIGURES: Page

CHAPTER TWO:

CHAPTER THREE:

LIST OF ABBREVIATIONS:

OSCC	-	oesophageal squamous cell carcinoma
WHO	-	World Health Organisation.
CANSA	-	Cancer Association of South Africa.
HPV	-	human papillomavirus
TOC	-	tylosis oesophageal carcinoma
HCC	-	hepatocellular carcinoma
IHC	-	immunohistochemistry
DNA	-	deoxyribonucleic acid
RNA	-	ribonucleic acid
Rb	-	retinoblastoma
DLC	-	deleted in lung and oesophageal cancer
CDK	-	cyclin dependant kinase
PCNA	-	proliferating cell nuclear antigen
SSCP	-	single strand conformation polymorphism
VEGF	-	vascular endothelial growth factor

ABSTRACT

Oesophageal squamous cell carcinoma (OSCC) is a highly malignant tumour that has a poor prognosis and shows marked regional variation in its incidence, implicating environmental factors. South Africa is one of several countries that has areas of high incidence. The exact aetiopathogenesis of OSCC is not well understood. Current environmental risk factors include alcohol, tobacco, human papillomavirus (HPV) infection and nutritional factors including; low intake of Vitamins A, C and riboflavin, lack of fruit and vegetables, ingestion of fungal contaminated foods and consumption of extremely hot beverages. This study was a retrospective immunohistochemical study done on paraffin embedded tissues. The histopathology, grading and staging of all resected squamous cell carcinomas over a twenty one year period from 1982 to 2002, were reviewed. Sixty eight patients were identified; all had an oesophagectomy for OSCC at Groote Schuur Hospital, a tertiary referral centre. Clinical details regarding gender, race, age, smoking or alcohol usage and survival data were collected. Survival data was updated to 23 June 2003. Two paraffin blocks representing OSCC and normal mucosa for each patient were retrieved from the archives in the Division of Anatomical Pathology. In addition 16 cases of reflux oesophagitis were included for comparison. Initial immunohistochemical staining for HPV (Dako- clone K1H8) was undertaken but the negative results necessitated a shift in the focus of this study to that of cell cycle regulatory proteins. The tissues were evaluated for p53 (Dako – clone DO-7), p21 (Novocastro - clone 4D10), cyclin D1 (Dako – clone DCS-6) and cyclin E (Novocastro – clone 13A3). Expression was interpreted as positive if 10% or more of the tumour cell population stained. Expression was also stratified into three levels (1, 2 and 3) depending on the percentage positive staining. Normal mucosa did not stain for any of the cell cycle regulators. OSCC stained as follows: 61.8% for p53, 27.9% for p21, 22.1% for cyclin E and 44.1% for cyclin D1. Reflux oesophagitis stained as follows: 31.2% for cyclin D1, 12.5% for p21 and 0% for both p53 and cyclin E. Subsequent statistical analysis failed to reveal any prognostic significance to the expression of cell cycle regulators, nor could expression or level of expression be associated with stage, grade, age, gender or alcohol use. There was however a significant relationship between cyclin D1 and smoking. In addition expression of p53 discriminated between malignant and reactive oesophageal lesions. Advancing age proved to be associated with an increased risk of mortality. Lastly, histopathological staging proved to be the most significant prognostic factor in this study.

CHAPTER ONE:

1.1 INTRODUCTION

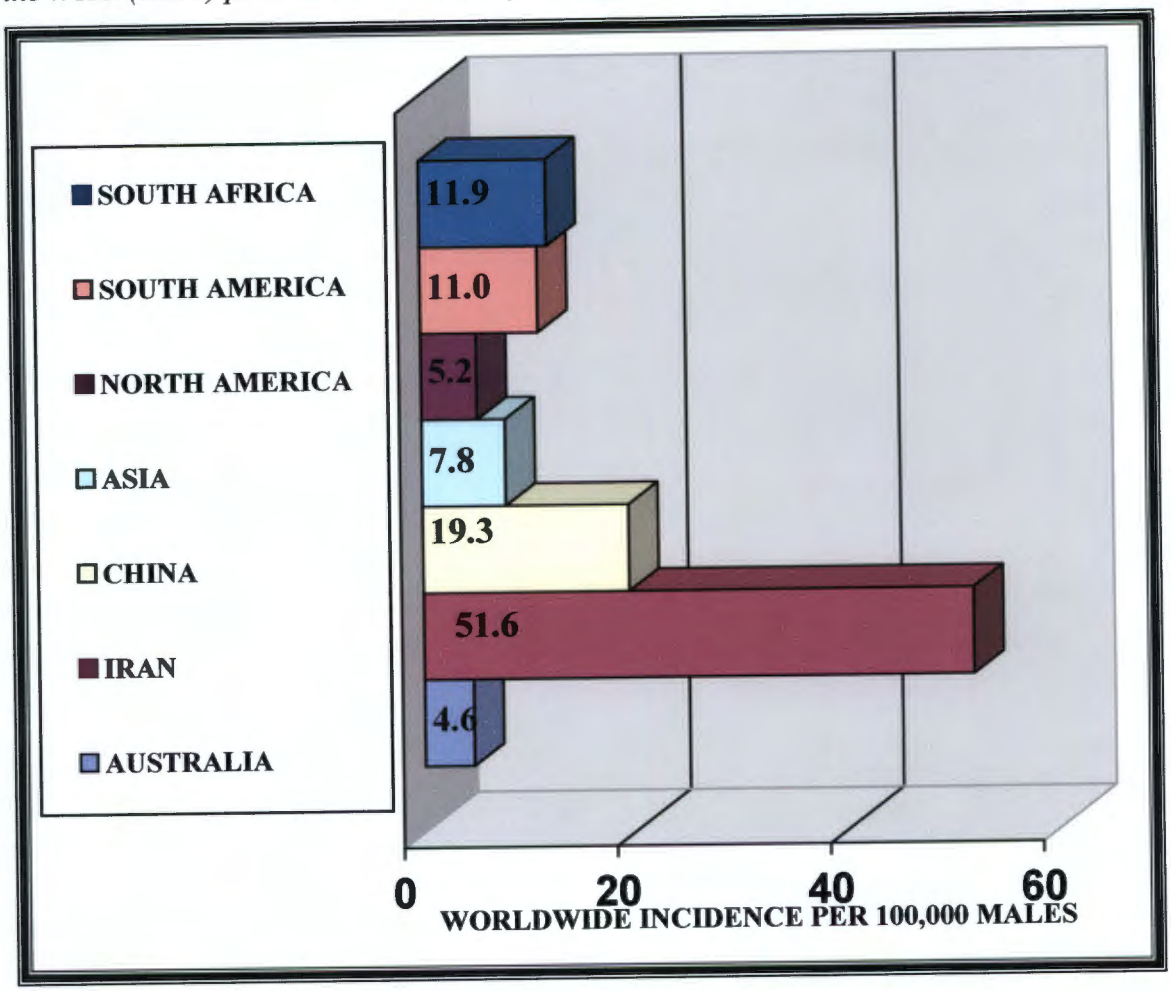
Squamous cell carcinoma of the oesophagus (OSCC) is a major health problem in the world and particularly in certain geographic areas of South Africa where the incidence is amongst the highest in the world. In the majority of South African cases the patients present late with progressive dysphagia, advanced carcinomas and are not amenable to surgical or oncological therapy thus the outcome is generally poor. As a result the incidence and mortality rates of oesophageal cancers in South Africa are almost equal. My experience as a Medical Officer in the Eastern Cape province of South Africa revealed it to be a tragic and formidable disease.

1.2 EPIDEMIOLOGY OF OESOPHAGEAL SQUAMOUS CELL CARCINOMA

In industrialised countries, the age standardized incidence does not exceed 5/100000 in males and 1/100000 in females. However there are occasional striking geographical exceptions, such as Normandy in France, where the incidence may be as high as 30/100000 in males (1). Oesophageal cancer is the eighth most frequent cancer in the world (2).

Regions of very high incidence have been identified in Iran, Central China, South Africa and Southern Brazil (Graph 1.1). Studies in the United States of America have shown that the incidence differs amongst race groups where it has been found to be 2 to 3 times more frequent in Blacks as compared to Asians, Whites or Native Americans (3). According to the WHO 2000 report, the incidence of oesophageal cancer in South Africa males is 11, 9/100000 (4).

Graph 1.1: Bar graph of worldwide annual incidence of OSCC in males/100000. *Adapted from the WHO (IARC) publication Tumours of the digestive system (4)*



However, several studies done in the former 'Transkei' region of South Africa, in an area that is now known as the Eastern Cape, show an incidence of OSCC to be in excess of 100/100000 males and more than 25/100000 in females (5-8). Interestingly, this rate of malignancy does not appear confined to the rural areas with studies from urban indigenous African populations showing similarly high age standardised incidences (9-11).

1.3 AETIOPATHOGENESIS

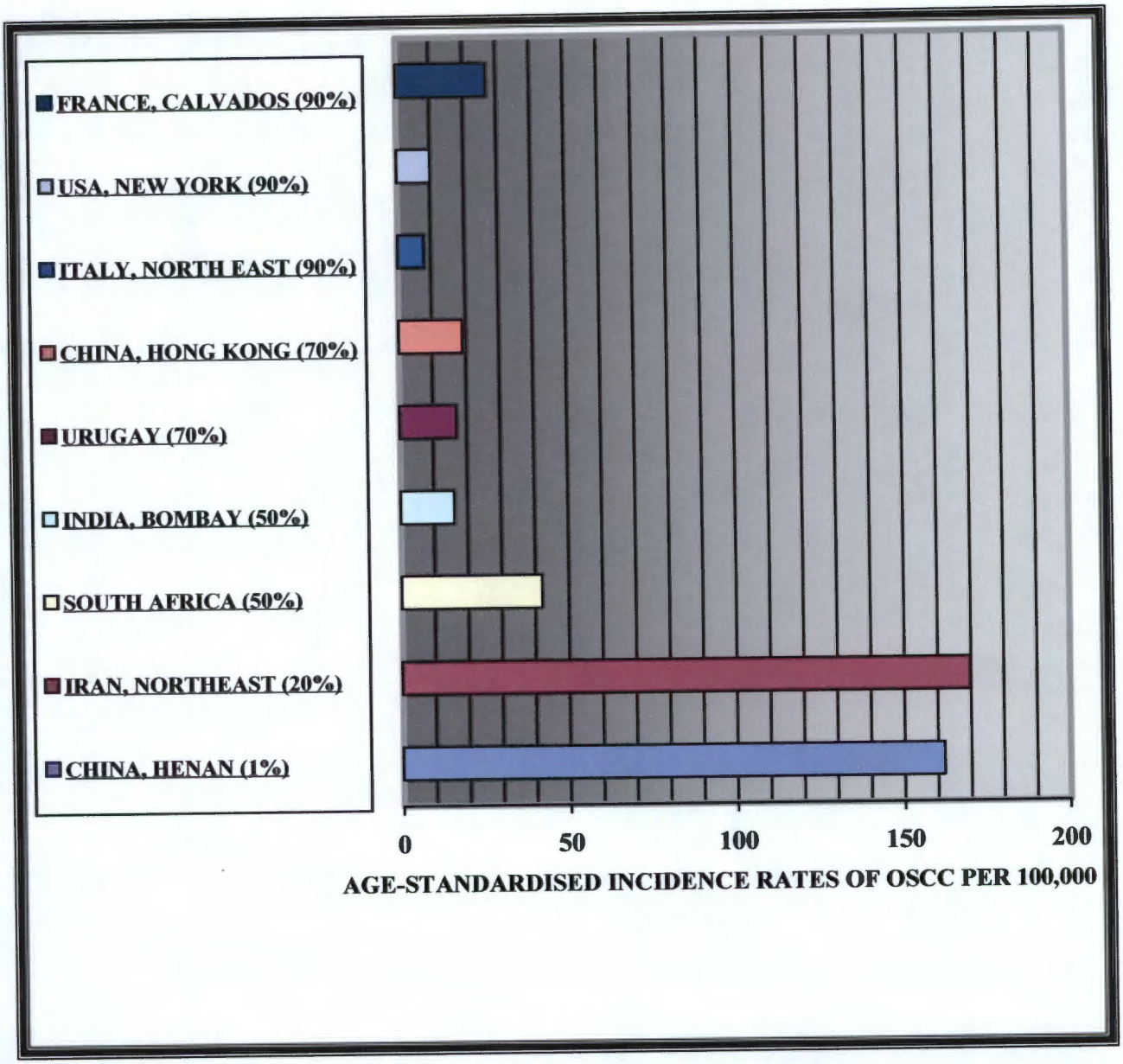
A better understanding of the aetiological agents and molecular mechanisms involved in the development of OSCC may offer opportunities to reduce exposure to environmental risk factors,

allow early diagnosis and predict response to therapy. Epidemiologic studies have identified multiple risk factors. Smoking, alcohol consumption, diets poor in fresh fruit and vegetables, consumption of foods contaminated with Fusarium verticillioides, aflatoxin and HPV infection are risk factors associated with the development of this disease in Africa (2).

1.3.1 Alcohol and tobacco:

It has been estimated that these two factors together account for approximately 80% of oesophageal cancer in males from France, USA, Japan and Latin American countries (Graph 1.2) (12-14). The risk has been shown to increase relative to the amount and type of alcohol consumed; spirits confer greater risk than wine or beer (15). A study from the UK demonstrated variations in the rates of oesophageal carcinoma which parallel total alcohol consumption (16). Chronic alcohol use interferes with the absorption and metabolism of folate, B-carotene and vitamin A thus possibly compounding carcinogenic potential (17-20). In addition home brewed alcoholic beverages in South Africa have been shown to contain carcinogenic contaminants such as mycotoxins, methans and tannins (21, 22). Studies from Japan done in people with high alcohol consumption show a polymorphism in the *aldehyde dehydrogenase 2* gene. This is significantly associated with several upper digestive tract cancers including OSCC and suggests that acetaldehyde, a carcinogenic metabolite of alcohol that has recently been shown to cause DNA damage, may play a significant role (23).

Graph 1.2: Age-standardised incidence rates of OSCC. Adapted from the *WHO (IARC)-2000, Tumours of the digestive tract*. The proportion of OSCC attributed to alcohol and tobacco is also supplied in percentages next to the relevant geographic area (4).



There is a significantly higher incidence of OSCC among smokers or those who chew tobacco when compared to those who abstain from tobacco use (24). Autopsy material from American men comparing oesophageal mucosa in smokers and non-smokers found atypical nuclei present

in approximately 6% of non-smokers while almost 80% of smokers had nuclear atypia (25). It has been suggested that in the South African high risk population, tobacco use plays a more important aetiological role than alcohol (26). Practices such as swallowing the pipe dottle from the pipe stem and scraping and chewing the residue in these pipes are cultural practices that prevail in many of the rural communities of South Africa. Similar situations exist in high incidence areas of North-eastern Iran where eating residues from opium pipes is widespread; these tobacco and opium pyrolysis products are mutagenic in the Ames test (27). Epidemiological studies in France determined chewing tobacco and hand rolled cigarettes to be major risk factors for oesophageal carcinoma. The former increasing the risk in the upper oesophagus and the latter the lower part (28).

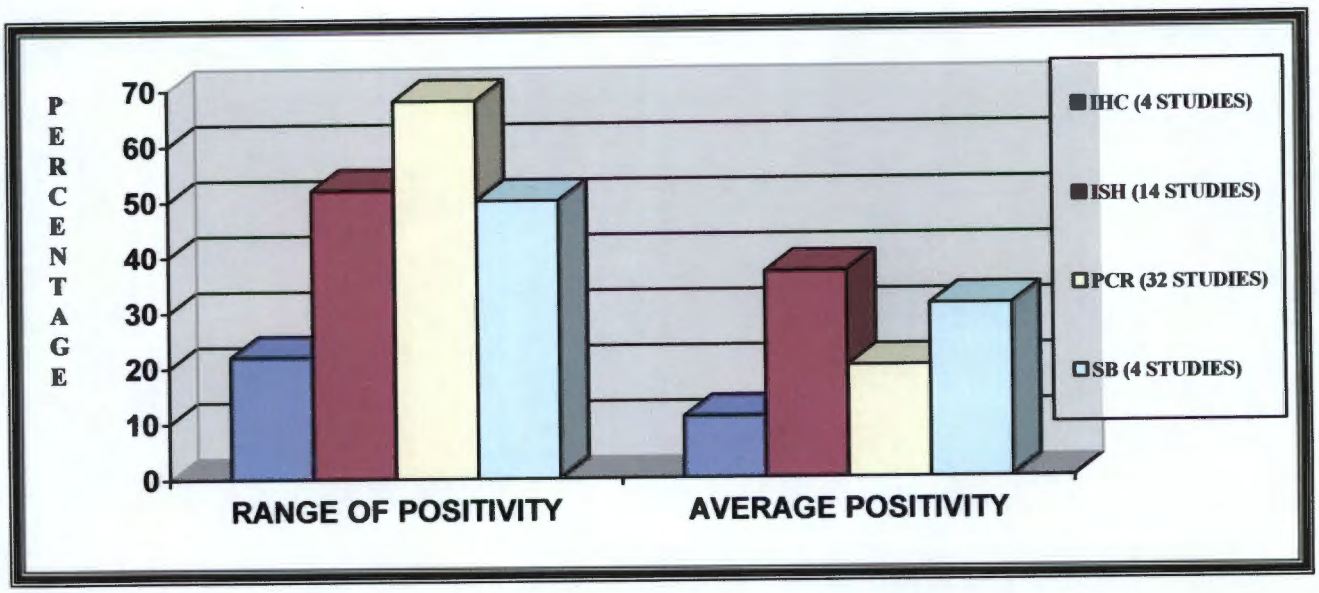
1.3.2 Diet:

Dietary surveys in Northern Iran found clear regional variations with diet, areas where diets consisted mainly of homemade bread and tea had a high incidence. These populations had low vitamin A, riboflavin and vitamin C intake (29). Similar deficiencies exist in high incidence rural areas in China and South Africa. In addition contamination of stored food by fungal species such as Fusarium, Geotrichum and Aspergillus occurs in rural areas. These fungi induce the formation of nitrosamines (30), which are potent oesophageal carcinogens in the laboratory rat (31). Fusarium species have been shown to produce the mycotoxins fumonisin B1 and B2 that are responsible for hepatic and renal cancer in rats. These mycotoxins are currently regarded as possible carcinogens in OSCC and hepatocellular carcinomas in humans. Deficiencies of elements such as zinc have also been associated with OSCC (32). Evidence that fruit and vegetables protect against OSCC is gradually accumulating (33). Another risk factor related to diet is the drinking of burning hot beverages such as Mate tea in South America which result in thermal injury and associated chronic oesophagitis and dysplasia (34-36).

1.3.3 Infectious agents:

Numerous investigators, prompted by the relevance of human papillomavirus (HPV) to squamous cell carcinoma at sites such as the cervix and anus, have searched for HPV in the oesophagus. Prevalence rates vary greatly from 0-65% in different studies (37-39). These variable results can be attributed to two factors: the different techniques used (immunohistochemistry, in situ hybridisation, polymerase chain reaction and southern blot technique - Table 1.3) and the source population. Notably within the different populations studied there is a tendency for a higher incidence of oesophageal carcinoma to be associated with a higher prevalence of oesophageal HPV infection (40, 41). However the certainty with which HPV is implicated as a carcinogenic factor in OSCC is questioned in the literature. Whether HPV is an incidental finding or relevant to OSCC remains controversial. The relatively consistent observation is that the HPV detection rate is absent (42-45) or significantly lower (40, 46) in areas of a moderate or low incidence of OSCC. While in the extremely high incidence areas HPV's detection rate is in the region of 50% or higher using techniques sensitive to detecting integrated HPV (47-49). It has been proposed that HPV replication stops before or during malignant transformation (50) and that HPV acts synergistically with other initiating events, such as environmental factors, for carcinogenesis (51). A study using oesophageal cell lines demonstrated that carcinogenesis of oesophageal epithelial cells, induced by HPV, is a multistage process, which goes through the initial, immortal, premalignant and malignant transformation stages (52). The current evidence would support a role for HPV, particularly in high incidence areas of OSCC. Graph 1.3 (below) demonstrates the commonly used methods for detecting HPV in OSCC, the range of results and the average positive result. There are important differences between the individual studies, such as the population studied, antibody clone or specific primers used that the table does not account for. What it attempts to highlight is the difference in sensitivity and average positivity between differing detection methods.

Graph 1.3: The prevalence of HPV in OSCC by: (1) IHC- immunohistochemical (37, 38, 53), (2) ISH- in-situ hybridisation (48, 54-66), (3)PCR- polymerase chain reaction (40-47, 49, 54, 67-88), (4)SB- southern blot analysis (39, 43, 77, 78).



The possibility that other viruses (herpes simplex virus, cytomegalovirus, and Epstein-Barr virus) are potential aetiological factors has also been raised (77).

1.3.4 Genetic factors:

Individuals with autosomal dominantly inherited keratosis plantaris and palmaris (Tylosis) are at high risk for OSCC; the authors suggest affected people have a 90 to 95% chance of developing OSCC by 65 years of age (89). Ashworth and colleagues studied two Tylotic families and compared them to inflammatory controls and found a significant increase in single cell keratinisation in the Tylotic group (90). Abnormalities in the tylosis oesophageal carcinoma (TOC) gene have been found in both Tylosis associated and sporadic OSCCs (91). Studies from high incidence areas of Northern Iran, showed that 47% of OSCC patients gave a positive family history of OSCC, compared to only 2% from low incidence areas of Iran (92). The tylosis gene has not been studied in South African patients with OSCC.

1.3.5 Other factors:

OSCC occurs with increased frequency in patients with achalasia (93), oesophageal diverticulae (94, 95), Plummer-Vinson syndrome (96), coeliac disease (97), duplication cysts of the oesophagus (98, 99), therapeutic radiation involving the oesophagus (100)) and stricture formation following lye (crude sodium hydroxide with sodium carbonate) ingestion (101-103). All of these diseases have the common link of being associated with chronic oesophagitis.

1.4 CLINICAL ASPECTS:

The clinical symptoms and signs are mainly due to narrowing of the oesophageal lumen by tumour growth and include dysphagia, weight loss, epigastric or retrosternal discomfort and regurgitation. Certain populations of central, eastern, and southern Africa display very high incidence rates of OSCC, presenting a serious health burden to the continent. Most patients are diagnosed at a late stage because of the asymptomatic development of the disease, with associated poor prognosis. Squamous cell carcinoma is the most prevalent oesophageal malignancy worldwide and tends to occur with a higher incidence in developing countries. OSCC is found predominately in the lower and middle third of the oesophagus. Approximately 10% are located in the upper third of the oesophagus.

1.4.1 Special investigations:

Oesophagography: Using radiological contrast media (usually barium) lesions may be detected within the lumen of the oesophagus. The sensitivity of this method is questionable and given that patients require a biopsy for a histological diagnosis of malignancy, it is becoming less popular.

Endoscopy: This procedure allows the investigator to identify and sample the lesion simultaneously. Malignant lesions may be divided into three types endoscopically: flat, ulcerated or polypoid. In addition chromoendoscopy, using either toluidine blue or Lugol's iodine spray, may be of value particularly in identifying early, small lesions. Toluidine blue, a metachromatic stain that binds to DNA and RNA, helps identify areas richer in nuclei than the

surrounding normal oesophageal mucosa (104). Lugol's iodine reacts with glycogen in the normal oesophageal mucosa giving a purplish colour while dysplastic, malignant, reactive lesions and heterotopic tissue do not stain (105).

Endoscopic ultrasonography: Oesophageal carcinoma is generally viewed as a localised or diffuse wall thickening with a predominately echo poor or inhomogeneous pattern. This technique is mainly used to evaluate depth of invasion and paraoesophageal lymph node involvement (106)

Computed tomography and magnetic resonance imaging: These investigations are useful when advanced lesions are suspected giving information on local and systemic spread. In particular, lymph node enlargement may be accurately assessed (107)

Cytology: Endoscopic brush cytology is slowly gaining acceptance as a useful screening procedure for premalignant and malignant lesions particularly in areas of high incidence. This technique is dependant on the technology employed to obtain and prepare the sample, and the skill and experience of the examining cytopathologist or technologist.

Histology: Biopsy and histopathological examination is a relatively simple procedure and remains the gold standard for the diagnosis of oesophageal malignancy.

1.5 PATHOLOGY:

Oesophageal carcinoma occurs as two main subtypes, squamous cell carcinoma (more prevalent in developing countries) and adenocarcinoma (more common in developed countries and usually associated with reflux oesophagitis complicated by Barrett's disease). These account for more than 90% of oesophageal malignancies. Other histological types of carcinoma seen in the oesophagus include adenosquamous, mucoepidermoid, adenoid cystic, small cell neuroendocrine and undifferentiated. On rare occasions other malignant lesions such as melanomas, leiomyosarcomas, carcinoid tumours, Kaposi sarcoma, gastrointestinal stromal tumours, rhabdomyosarcomas and lymphomas may present primarily in the oesophagus. OSCC develops

as the result of a sequence of histopathological changes that typically involves oesophagitis, atrophy, mild to severe dysplasia, carcinoma in situ and finally, invasive cancer.

1.5.1 Macroscopic:

The typical appearance of OSCC is that of an exophytic, ulcerated and/or infiltrating lesion that often results in a stricture. However, when the malignancy undermines the surrounding normal mucosa it may appear as a smooth annular stenosis. Submucosal extension is often present and may result in satellite nodules. Multifocal primary carcinomas are not uncommon but it is important to exclude submucosal or intramural spread before making this diagnosis.

1.5.2 Microscopic:

OSCC is defined as the penetration of neoplastic squamous cells through the basement membrane and extension into the surrounding lamina propria or deeper tissues of the oesophagus. Along with vertical tumour growth a horizontal proliferation is usually present that undermines the adjacent normal mucosa at the tumour periphery. The carcinoma often shows early invasion into intramural lymphatic channels and veins. In advanced lesions the carcinoma invades the muscular layers, the adventitia and may extend beyond by invading adjacent organs or tissues. The formation of oesophago-tracheal or oesophago-bronchial fistulae is not uncommon. The degree of stromal desmoplasia and inflammatory response are variable. Thus OSCC is variable in its appearance and attempts to categorise these appearances histologically led to the development of a grading system.

1.5.3 Grading:

OSCC is currently graded according to the traditional parameters of degree of differentiation, anisonucleosis and mitotic activity. These parameters are subjective and as no objective acceptable criteria exist the grading of OSCC results in considerable inter-observer variation (108-112).

Well differentiated: These tumours show both cytological and histological features that are similar to the normal oesophageal squamous epithelium. Well differentiated tumours are composed predominantly of large differentiated, keratinocyte-like squamous cells with a low

proportion of basal-type cells. The malignant cells are often arranged in nests with central keratinisation and intercellular bridges between the cells. These intercellular bridges are an integral part of normal squamous epithelium and are referred to as 'prickle cells'. Interestingly, keratinisation has been interpreted as a sign of differentiation even though the normal oesophageal mucosa is non-keratinising.

Moderately differentiated: This is the most common type of OSCC and lies between well and poorly differentiated tumours in its appearance.

Poorly differentiated: These tumours consist predominantly of basal-type cells that have a high mitotic rate, they usually lack squamous differentiation/maturation but when present, it is minimal and focal (4). The malignant cells often are show basaloid, sarcomatoid/spindled or discohesive morphology. Immunohistochemical and ultrastructural studies are often required to help confirm the diagnosis.

1.5.4 Staging:

The TNM (tumour, node and metastasis) system established by the International Union Against Cancer is the most widely used system. The usefulness of this system in treatment planning and prognostication has been validated (113-116).

TNM staging of oesophageal carcinoma:

T-primary tumour

TX- primary tumour cannot be assessed.

T0- No evidence of a primary tumour.

Tis- Carcinoma in-situ.

pT1- tumour invades lamina propria or submucosa.

pT2- tumour invades muscularis propria.

pT3- tumour invades the adventitia.

pT4- tumour invades adjacent structures.

N-Regional lymph nodes

NX- Regional lymph nodes cannot be assessed.

pN0- no regional lymph node metastases.

pN1- regional lymph node metastasis present.

M-Distant metastasis

MX- distant metastasis cannot be assessed.

M0- no distant metastasis.

M1- distant metastasis are present, where M1a = metastasis in celiac lymph nodes. M1b = other distant metastasis.

Staging is therefore as follows:

Stage 0 = Tis N0 M0

Stage I = T1 N0 M0

Stage IIA = T2 N0 M0

or T3 N0 M0

Stage IIB = T1 N1 M0

or T2 N1 M0

Stage III = T3 N1 M0

or T4 any N M0

Stage IVA = any T any N M1a

Stage IVB =any T any N M1b.

1.6 MOLECULAR PATHOLOGY:

Genetic changes associated with the development of OSCC include mutation of the *p53* gene, disruption of cell-cycle control in G1 by several mechanisms (inactivation of *p16MTS1*, amplification of *cyclin D1*, alterations of *retinoblastoma*), activation of oncogenes (*HST-1, HST-2, EGFR, c-MYC*), and inactivation of several tumor suppressor genes. Amplification of *cyclin D1* is frequently detected in cancers that retain expression of retinoblastoma (Rb) protein consistent with the notion that these two factors cooperate within the same signalling cascade

(117). Inactivation of cyclin dependant kinase 2A occurs in advanced OSCC and is thought to be due either to homozygous deletion or de novo methylation. Loss of heterozygosity on chromosome 17q25 has been linked with tylosis (nonepidermolytic palmoplantar keratoderma), a rare autosomal dominant syndrome associated with high predisposition to OSCC. The TOC gene has been mapped to chromosome 17q25 a tumour suppressor gene which shows abnormalities in both tylosis associated/hereditary and sporadic OSCC (91). Chronic oesophagitis is a frequent histological finding in OSCC specimens and in some instances, patches of positive cells in areas of oesophagitis at the margins of tumours were found to contain a mutation in the *p53* gene. This observation is consistent with field cancerization in the oesophagus and suggests that oesophagitis may represent an interesting target for early detection of OSCC as well as for intervention strategies (118).

Other possible important genetic events include inactivation of the *fragile histidine triad (FHIT)* gene, an assumed tumour suppressor gene at 3p14 (117). 'Frequent loss of heterozygosity occurs at 3p21.3 in OSCC' and analysis of clones 'led to the discovery of a novel gene called *DLC1* (deleted in lung and oesophageal cancer-1)' (119). Reverse transcriptase-PCR experiments indicate that approximately 33% of primary OSCC lack *DLC1* transcripts entirely or contain increased levels of non-functional DLC1 mRNA. There is also evidence that loss of heterozygosity at 5p15 occurs in a large portion of OSCC suggesting a possible tumour suppressor function (120).

How these genetic events interrelate and result in carcinoma, with phenotypic variations, remains speculative.

1.7 PROGNOSTIC FACTORS:

The overall prognosis of OSCC is dismal with an average 10% 5-year survival rate. One met-analytical review revealed that 'of 100 patients with OSCC, 39 will be surgically resectable, 26 will leave hospital and only 4 will be alive after 5 years' (121). Local recurrence is common following surgical resection (122). Currently the best chance at cure is achieved by early

focused on surgical outcome while studies on other forms of therapy have shown a survival advantage to those receiving combined chemoradiotherapy when compared to those treated with radiotherapy alone (123). However chemotherapy alone has been shown to result in tumour regression (124, 125).

1) *Staging*: This is the single most important prognostic factor currently known. Several studies have shown that the depth of invasion and presence of nodal metastases are both independent predictors of survival (113, 115, 116). The majority of studies have used the TNM classification to determine the prognostic significance but the current TNM system does not differentiate between tumours confined to the mucosa and those involving the submucosa. This has been shown to be of considerable prognostic significance (126, 127).

2) *Differentiation*: Poor standardisation of grading criteria has led to equivocal results, while some studies have shown grade to convey prognostic significance (128, 129), the majority did not find any significance to grading (108-112).

3) *Vascular and lymphatic invasion*: Several studies have shown these are poor prognostic factors (113, 130).

4) *Lymphocytic response*: The presence of an intense lymphocytic response has been associated with a better prognosis (108, 131).

5) *Ploidy*: Flow cytometry or image analysis studies have found that aneuploidy is present in 55-95% of OSCCs and that patients with diploid tumours usually have a longer survival. However prognostic significance independent of tumour stage has only been shown in some studies (132-134).

6) *Molecular factors*: In addition to cell cycle regulatory proteins (discussed below), a number of molecular factors have shown prognostic significance.

Epidermal growth factor receptor has been shown as a potential prognostic factor with over expression conferring a worse prognosis. The few studies done in this regard have shown conflicting results (135, 136).

Redox defence system components (137, 138) and *matrix proteinases* (139-141) have been reported as conveying prognostic value.

Caspase-3 expression correlated with a significantly favorable prognosis in primary resected OSCC in multivariate analysis (129).

None of the above molecular factors are used in routine clinical practice.

1.8 CELL CYCLE REGULATORY PROTEINS:

1.8.1 P53:

P53 is a critical controller of normal growth and homeostasis of cells and tissues (142). The *p53* gene is located on the short arm of chromosome 17 and is responsible for the production of the p53 protein. This is a phosphoprotein with a molecular weight of 53 kilodaltons. It is a tumour suppressor gene whose inappropriate function may lead to disordered growth and malignancy. Normal or 'wild type' p53 protein acts as the 'guardian of the genome' by inhibiting mitosis in cells with damaged DNA. The p53 protein acts on other genes to arrest the cell cycle until the damaged DNA is repaired or to cause apoptosis. Normal *p53* is upregulated in cells with damaged DNA, such as that caused by UV light, ionising radiation, chemotherapeutic agents or following hypoxia. P53 inhibits progression into the S phase of the cell cycle by inhibition of cyclin dependant kinases (CDK's) due to upregulation of $p21^{CIP1}$ protein, and proliferating cell nuclear antigen (PCNA). The GADD45 (growth arrest DNA damaged) protein is also up-regulated which in turn also binds to PCNA. The cell is thereby arrested in the G1 phase. If DNA repair fails then wild type p53 can trigger apoptosis, although apoptosis may also be triggered by other means. The loss of the normal function of *p53* usually occurs by a two-step process with a mutation of one allele and a deletion or inactivation of the second allele. Chen

and colleagues (143) used generated cell lines and showed that the cellular level of p53 can dictate the response of the cell such that lower levels of p53 result in arrest, whereas higher levels result in apoptosis; nevertheless they found DNA damage can heighten the apoptotic response to p53 without altering the protein level of p53 in cells. They also demonstrated that arrest and apoptosis are two genetically separable functions of *p53* because a transcriptionally incompetent p53 can induce apoptosis but not arrest, whereas induction of $p21^{WAF1}$, which is a major transcriptional target of p53, can induce arrest but not apoptosis. A full apoptotic response to p53 requires both its amino and carboxyl terminus, suggesting there is synergism between transcription-dependent and -independent functions of p53 in apoptosis. *P53* is mutated in more than 50% of human cancers (142).

Normal cells are in a balance between growth promotion, cellular differentiation and growth restraint. In cancers, mutations of *p53* result in progression of cells through the cell cycle. Patients with the rare Li-Fraumeni syndrome inherit germline mutations of *p53* on one allele. The subsequent loss of the only wild type *p53* allele leads to multiple tumours (adrenal cortex, brain, breast, sarcomas and various types of leukaemia (144-146). However, several common malignancies that frequently have *p53* mutations, such as colorectal carcinoma do not occur with increased frequency in Li-Fraumeni syndrome. This is probably due to the fact that *p53* loss is a relatively late event in colorectal carcinogenesis, after Ki-ras activation and loss of *'deleted in colorectal carcinoma' (DCC)* gene (147).

P53 activity is influenced by both the structure of the molecule and its concentration. The normal molecule contains three domains which each have a specific function. The N-terminal region mainly controls transactivation and the C-terminal region controls oligomerisation, the assembly of active tetramers (148). Mutations in the C-terminal region may lead to it relocating from the nucleus to the cytoplasm. An additional regulatory region controlling the allosteric switch from a latent form to an active form, for sequence specific binding is also located in the C-terminal (149). The intervening portion of *p53* is highly conserved between species and the majority of mutations occur in this region. This is the region with DNA binding activity that interacts with target sequences of transcriptionally activated genes. Mutations in this region

interfere with protein folding and therefore its interaction with DNA. P53 binds in a sequence specific manner via its central domain as a tetrameric protein (150).

Interestingly, the study of *p53* mutations has shown that specific mutations are more common in certain tumours. In high-risk areas of hepatocellular carcinoma (HCC) a well known risk factor is aflatoxin. These HCC's are associated with a specific *p53* mutation while HCC's in low risk areas have more variable mutations (151). In addition, oesophageal adenocarcinomas and squamous cell carcinomas have different patterns of DNA change in the *p53* gene. Squamous cell carcinoma of the oesophagus, in high risk areas, tends to show a transversion - a change from one nucleotide to another (e.g. from a G:C base pair to a T:A base pair) in the *p53* core domain. While transitions of dinucleotides predominate in oesophageal adenocarcinomas (152). 'Coincidentally' squamous cell carcinomas of the lung tend to show transversions in their mutated *p53* as well, implicating tobacco as a common carcinogen (153).

Immunohistochemical detection of p53 has been studied intensively and several different anti-p53 monoclonal antibodies are available. Wild-type p53 is virtually undetectable in normal cells because it has a half-life of approximately 20 minutes. Positive immunoreactivity has been considered to equate with altered protein structure or function, as a result of mutation or viral oncogene activation (154). However, if antigen retrieval is used (microwave or pressure cooker) either wild type and mutant protein can be detected, or at least the threshold for detection is lowered (155).

P53 in OSCC:

A number of immunohistochemical studies have consistently demonstrated over expression of p53 in OSCC ranging from approximately 40 - 70% in the tumours analysed. There was no correlation between p53 over expression and stage, tumour differentiation, lymph node metastases, and patient's survival (156-160). Coggi et al (159) also found that p53 aberrations do not independently predict prognosis in oesophageal tumours. They also analysed *p53* mutations in exons 5-8, which were detected in 53% of the carcinomas whereas p53 accumulation was observed in 57% of cases. Comparison of single strand conformational polymorphism (SSCP) and immunohistochemical (IHC) analysis revealed 27 discordant cases

(38%). Overall, only 27% did not display *p53* mutation and/or p53 accumulation. P53 protein accumulation and *p53* gene mutation were not related to patient survival by univariate or multivariate analysis in oesophageal carcinomas.

Coexpression of p53 and vascular endothelial growth factor (VEGF) has been found. This suggests that mutant p53 expression is associated with angiogenesis and distant metastasis in OSCC, and that the coexpression of p53 and VEGF may play an important role in angiogenesis, and have important clinical significance (161). A second study looking at these two proteins in OSCC differed in its conclusion finding no significant association between p53 or VEGF expressions and prognosis (162).

An interesting study on 153 Chinese oesophagectomy subjects looking at p21 and p53 expression showed there was no significant correlation between the expression of p21 and the abnormal accumulation of p53. The prognosis of patients with absent p21 expression was better, while the survival rates of patients was worse if they had expression of both p21 and p53. Consequently, in this study, p21 and p53 had prognostic value for OSCC (163). There are conflicting reports about the value of p53 expression as a prognostic indicator for patients with OSCC. A low level of p53 expression has been correlated with a long term survival (greater than 3 years) (164).

One study revealed that p53 expressing cells in OSCC correlated with the distribution of Ki-67 expression and was confined to the basal layer in dysplastic lesion and in the deeper portions of invasive tumours (165).

A step wise over expression of p53 related to normal mucosa, chronic oesophagitis, low grade dysplasia, high grade dysplasia and carcinoma has also been documented (166, 167).

1.8.2 P21:

The *p21* gene is thought to play a central role in tumour suppression. $P21^{WAF1/CIP1}$ *(p21)* inhibits the activity of the cyclin/cyclin dependant kinase complex and controls the G1 to S cell phase transition. The gene product binds and inhibits kinase activity of members of the cyclin dependent kinase family thereby preventing progression into the S phase of the cell cycle. It is

kinase inhibitor blocks cell cycle transition and replication in response to DNA damage. Although required for p53-mediated cell cycle arrest, p21 expression can also be initiated via p53-independent pathways. The *p21* gene encodes a cyclin-dependent kinase inhibitor which is induced by wild-type, but not mutated *p53* gene product. P21 is also associated with cellular senescence (168) and terminal differentiation (169).

The expression of p21 in OSCC is reported to range between 14% and 70% via immunohistochemical technique, the variability may be related to the clone used (163, 170-173).

An immunohistochemical study of distribution patterns of p21 and p53 in OSCC and the neighbouring non-cancerous squamous epithelia found p21 protein in the majority of well-differentiated OSCC, where the p21-positive cells were located mainly in the interior/central ("maturing") layers of the cancer nests. Conversely, p53-positive cells were found mostly in the peripheral layers. Cells containing both p21- and p53-positive immunostaining were not observed in a double-immunostaining experiment (174). P21 protein accumulation has been reported to show an inverse distribution to that of p53 protein. In areas where both p53 and p21 proteins were accumulated, few apoptotic cells were observed. Particularly in cases of mucosal tumours, p53 protein was prominently accumulated in the lower layer of the tumour, whereas p21 protein accumulation was confined to the upper layer (175). Researchers have found increased p21 with irradiation exposure. P21 production was independent of p53 status (176). Shirakawa and colleagues in 2000 found that p21-expressing cells shifted to the upper layers of the epithelium with the progression of dysplasia. They also concluded that p21 plays a critical role in the differentiation process (165). Ohashi et al. examined p21 expression immunohistochemically using surgically resected tissues from 25 patients, and analyzed the relationship with alteration of *p53* gene(171). All *p53*-mutation positive cases were negative for p21 expression, whereas 61% of mutation negative cases showed positive p21 expression. This result implies that p21 expression is dependant on an intact *p53* gene. P21 expression also correlated with tumour grade, well differentiated tumours over expressed p21 and poorly differentiated tumours were negative. The frequency of apoptotic cells was significantly higher in p21 positive cases than negative cases. P21 expressing cells were distributed mainly in the middle layers of the invading nests, especially around areas of keratinization, which was similar

to the distribution of apoptotic cells. The results of this study suggest that expression of p21 in OSCCs is induced by a *p53*-dependent pathway and affects apoptosis and differentiation of carcinoma cells.

The prognostic significance of p21 was investigated immunohistochemically in samples of normal oesophageal squamous epithelium, dysplasia, carcinoma in situ, permanent OSCC cell lines, and invasive squamous cell carcinomas treated either by potentially curative resection or by combined modality therapy (radiochemotherapy +/- surgery). P21 expression was present in the normal epithelium but restricted to a few cells adjacent to the basal cell layer, p21 over expression was frequently found in preneoplasias and invasive carcinomas. Expression of p21 in invasive carcinomas did not correlate with tumour differentiation or stage. Among carcinomas treated by potential curative resection p21 expression in greater than 50% of cells correlated strongly with poor overall survival by univariate ($P = 0.0025$) and multivariate ($P = 0.0081$) analysis. This group (>50%) also did not respond to combined modality radio/chemotherapy. Univariate survival analysis ($P = 0.0006$) revealed the same prognostic influence in the group of patients treated by combined modality therapy. This study therefore concluded that over expression of p21 protein is a frequent event in preneoplasia and neoplasia of the oesophagus and immunohistochemical examination of p21 expression may provide important prognostic information for decision-making in the treatment of patients with oesophageal cancer (177). Similar conclusions have been reached in other immunohistochemical retrospective studies of OSCC who underwent potentially curative resection therapy (111, 163). P21 expression in OSCC has also however been shown to be a favourable prognostic indicator (173).

Loss of p21 expression in OSCC without *p53* alternations has been demonstrated indicating that other mechanisms are also involved in turning off the gene (163). Several studies analysing the *p21* gene and its association with oesophageal cancer found underlying genetic polymorphism at codons 149 and 31 resulted in an increased susceptibility for development of cancer. This polymorphism was germline in origin and occurred at a higher frequency in patients with premalignant and malignant lesions than in the control population (178, 179). The majority of the patients with this polymorphism and OSCC expressed wild type p53 (180). A study performed using normal mucosa, reactive change, low-grade dysplasia, high-grade dysplasia and invasive carcinoma specimens and immunohistochemical detection of p21 found a stepwise reduction of p21 ($P=0.0189$) with progression to carcinoma (181).

invasive carcinoma specimens and immunohistochemical detection of p21 found a stepwise reduction of p21 (P=0.0189) with progression to carcinoma (181).

1.8.3 Cyclin D 1:

The *cyclin D1*$^{bcl1/PRAD-1}$ gene, located on chromosome 11q13, is frequently rearranged in parathyroid neoplasms and follicular lymphoma. This is currently known to be one of the earliest proteins expressed at the start/initiation of the cell cycle. It is a proto-oncogene that has a critical role in G1 progression of the cell cycle; it promotes progression of the cell through the G1 phase of the cell cycle by cyclin-dependent kinase (CDK) 4 and 6 mediated phosphorylation of the retinoblastoma protein. Its protein product is therefore expressed at high levels during the G1 phase of the cell cycle. The *p16* gene and the *cyclin D1* gene cooperatively regulate CDK 4-mediated phosphorylation of retinoblastoma protein in the cell cycle of normal cells. Cyclin D1 functionally competes with the tumour suppressor genes *retinoblastoma (Rb)* and *p16*^{INK4} (182). Inhibition of cyclin D1 therefore arrests the cell before the S phase, while over expression contributes to uncontrolled proliferation. P21 is involved in cyclin D1 inhibition. Cyclin D1 over expression ranges approximately between 20 and 52% in OSCC (183, 184).

Polymerase chain reaction to evaluate *cyclin D1* gene amplification showed amplification in 22% of primary OSCCs and 25% within the lymph node metastases. Lymph node metastases tended to be more common in patients with *cyclin D1* amplification (70%) than in those without amplification (37%). *Cyclin D1* amplification and over expression by immunohistochemistry was associated with decreased survival in several studies of OSCC (184-197).

Cyclin D1 over expression has also been shown to be a significant indicator of lymph node metastases (184-187, 196-198); although there are sporadic studies that have refuted this finding (199).

Zheng and colleagues examined the *cyclin D1* gene in 21 pairs of primary OSCC and the mucosa adjacent to the cancers and also in four oesophageal cancer cell lines by means of molecular biological and immunohistochemical techniques. Amplification of *cyclin D1* gene was found in

12 out of 21 primary cancers and in oesophageal mucosa adjacent to the tumours in five of the cases, suggesting this is an early event in the carcinogenesis (183).

Shama et al. looked at 36 squamous dysplasias and 34 early OSCC. The frequency of cyclin D1 over expression was similar in dysplasias and early cancers (30% vs. 35%). This study found that cyclin D1 over expression starts early in dysplasia and could be a useful marker for its malignant potential (200).

An unusual study linked the Epstein-Barr virus ED-L2 promoter to the human cyclin D1 cDNA and utilized this transgene to generate founder lines. This transgene was transcribed specifically in the tongue and oesophagus both sharing a stratified squamous epithelium. The transgene protein product localized to the basal and suprabasal compartments of these squamous epithelial tissues, and mice from different lines developed dysplasia, a prominent precursor to carcinoma, by 16 months of age in contrast to age-matched wild-type mice. This transgenic model is useful in demonstrating that normal cyclin D1 may be a tumour initiating event in aero-upper digestive squamous epithelial tissues (201).

A subgroup of patients with OSCC get multiple primary lesions, about 50% present with synchronous lesion, whereas the remainder usually present with a second tumour within the first 3 years following the initial presentation. Therefore, a study was conducted, examining the expression of 2 proteins, cyclin D1 and p53, in an attempt to predict the occurrence of multiple primary malignant neoplasms (MP). Monoclonal antibodies to cyclin D1 (DCS-6) and p53 (DO-7) proteins were used. Resection specimens from 47 patients, 12 patients with MP, and 35 patients with a single primary tumour, were analyzed. Those in single primary malignant neoplasm group had longer than 3 years follow-up to ascertain the absence of MP. Tumour over expression of cyclin D1 was significantly associated with the development of MP ($P<.01$). Tumour over expression of p53 was also frequent in patients with MP although statistical significance was not achieved. The combination of these two parameters was an even greater predictor of MP ($P<.001$). Over expression of cyclin D1 and p53 proteins was highly correlated with the development of MP (202).

Studies on eight OSCC cell lines suggest that the over expression of cyclin D1 can confer oesophageal cancer cells with enhanced malignancy through increases in anchorage-independent growth and VEGF production, and down-regulation of Fas expression, thus suggesting novel functions of the cyclin D1 protein in tumour progression (203).

1.8.4 Cyclin E:

There is a limited amount of information regarding cyclin E and OSCC in the literature. It is involved in the cell cycle down stream to cyclin D1 and its function is activation of cyclin dependant kinase 2 (CDK2). Cyclin E's expression is due to phosphorylation of Rb and E2F which first requires cyclin D1 activity. Cyclin E/CDK2 together with cyclin D/CDK4,6 propels the cell through the initial phase of the cycle into the DNA synthesis phase. Cyclin E has a half-life of approximately 20 minutes (204).

Studies examining the relationship between immunohistochemically detected expression of cyclin E protein in patients with OSCC found a 30% positive rate and could not prove any significant relationship with clinicopathological features or a prognostic significance (190, 205, 206). However the study by Ohbu et al using 22 normal mucosa, 17 reactive change, 22 low-grade dysplasia, 15 high-grade dysplasia and 22 invasive carcinoma specimens revealed a stepwise over-expression of cyclin E with progression of oesophageal carcinogenesis, correlating with the increased cell proliferation observed with Ki67 labelling (181).

In conclusion, p53 and p21 proteins inhibit the cell cycle, while cyclin E and cyclin D1 proteins are necessary for progression to the DNA synthesis phase of the cycle. Cell cycle regulatory proteins p53, p21, cyclin D1 and cyclin E have been shown to be over expressed in OSCC in other parts of the world and other population groups and to a limited extent the South African population. There are conflicting reports on whether they convey a prognostic significance to OSCC, although the weight of the literature would support cyclin D1 as an independent prognostic factor. Whether the expression of these cell cycle regulatory proteins is related to aetiological risk factors alcohol and tobacco in OSCC merits further investigation. The reports on HPV and its possible involvement in the carcinogenesis of OSCC are inconsistent.

CHAPTER TWO:

2.1 AIMS:

1. To determine the expression of cell cycle regulatory proteins, cyclin E, cyclin D1, p21 and p53, in normal, reactive and malignant oesophageal tissue.

2. To determine whether the expression, or increasing level of expression, of any, or all, of the cell cycle regulatory proteins is related to age at presentation, smoking and/or alcohol use and whether expression/over expression of any of these proteins predicts survival.

3. To ascertain whether there is a relationship between the expression of p53 and p21; and the expression of cyclin D1 and cyclin E

4. To explore prognostic indicators for survival in a sample of patients with surgically resected oesophageal squamous cell carcinoma (OSCC).

5. To determine, by immunohistochemical analysis, whether any human papillomavirus (HPV) subtypes, are present in OSCC, normal oesophageal mucosa from patients with OSCC or reactive oesophageal lesions.

2.2 HYPOTHESES

1. Over expression of cyclin D1 will be associated with a worse survival.

2. HPV subtypes will be detected in OSCCs in South African patients.

3. The stage of the tumour will probably remain the best predictor for survival in this sample of 68 patients from Groote Schuur Hospital, South Africa.

2.3 PATIENTS AND METHODS:

Ethics approval for this study was obtained from the Research Ethics Committee, University of Cape Town.

2.3.1 Clinical data

A central clinical data base was consulted for: age, sex, race, survival or death and a history of smoking or alcohol use. Some cases were lost to follow up; survival was then recorded as the time between the date of surgery and the date of the patient's last clinical visit (Table 2.1). No doubt the survival of some patients was underestimated.

Table 2.1: The clinical data retrieved from the database for this study.

Case	Age	Sex	Race	Stage	Grade	Smoking	Alcohol	Survival	Status (27.06.03)
	Years	Female =0 Male=1	Black=0 Coloured=1 White=2	Stagel=1 Stagella=2 Stage IIb=3 Stage III=4	Well =0 Moderate =1 Poor =2	Smoker=1 non-smoker=2	Nil=0 Alcohol=1 Unknown =2	Days	Deceased=0 Alive=1 Lost to follow up=2
1	54	0	0	2	1	1	1	6001	0
2	41	1	0	4	1	1	0	2785	0
3	52	1	2	3	1	1	1	4998	1
4	57	1	1	2	1	1	1	2575	0
5	60	1	0	4	1	1	1	8	0
6	50	1	1	2	2	1	1	4712	1
7	47	1	0	4	1	1	2	4488	0
8	31	0	0	2	1	1	0	3892	2
9	57	1	0	4	0	1	1	477	0
10	67	1	1	2	0	1	1	737	2
11	57	1	1	2	0	1	1	262	0
12	66	1	0	2	1	1	1	182	0
13	58	1	2	4	2	1	1	620	0
14	63	1	0	4	0	1	1	116	2
15	53	1	1	2	0	1	1	39	0
16	64	1	1	2	1	1	1	74	0
17	59	1	2	4	1	1	1	47	0

Case	Age	Sex	Race	Stage	Grade	Smoking	Alcohol	Survival	Status
	Years	Female =0 Male=1	Black=0 Coloured=1 White=2	StageI=1 StageIIa=2 Stage IIb=3 Stage III=4	Well =0 Moderate =1 Poor =2	Smoker=1 non-smoker=2	Nil=0 Alcohol=1 Unknown =2	Days	Deceased=0 Alive=1 Lost to follow up=2
18	46	0	1	4	0	1	0	401	0
19	56	1	0	2	0	1	0	1667	2
20	63	1	1	2	1	1	1	231	2
21	41	0	0	2	2	1	1	1993	0
22	39	1	1	4	1	1	2	26	0
23	51	1	1	4	1	1	1	42	2
24	61	1	1	2	1	1	0	3212	0
25	42	0	0	4	1	0	2	1200	2
26	48	1	0	4	1	1	2	858	0
27	57	1	1	3	0	1	1	636	0
28	37	1	1	2	1	1	1	1180	0
29	55	1	1	2	1	1	1	45	0
30	44	1	0	4	2	1	0	652	0
31	66	1	1	2	1	1	0	19	0
32	49	1	0	2	1	1	1	16	0
33	59	1	1	4	0	1	0	131	0
34	45	1	0	2	1	1	1	201	2
35	57	1	0	2	1	1	1	1376	0
36	59	1	1	4	1	1	1	16	0
37	80	1	1	4	1	1	1	112	0
38	58	0	1	4	1	0	0	85	0
39	53	1	1	2	1	1	0	3244	1
40	51	1	0	4	2	1	1	195	0
41	45	0	1	4	1	1	1	36	0
42	50	1	1	4	1	1	1	148	0
43	74	0	2	4	1	0	1	165	0
44	52	1	1	1	0	0	0	3119	1
45	59	1	1	4	1	1	1	372	0
46	58	1	0	1	1	1	1	39	0
47	74	0	2	2	2	1	0	2779	1

Case	Age	Sex	Race	Stage	Grade	Smoking	Alcohol	Survival	Status
	Years	Female =0 Male=1	Black=0 Coloured=1 White=2	StageI=1 StageIIa=2 Stage IIb=3 Stage III=4	Well =0 Moderate =1 Poor =2	Smoker=1 non-smoker=2	Nil=0 Alcohol=1 Unknown =2	Days	Deceased=0 Alive=1 Lost to follow up=2
48	34	0	1	4	1	1	0	598	0
49	55	1	2	2	1	1	1	55	0
50	53	0	0	4	1	0	1	302	0
51	58	1	0	4	2	1	1	318	0
52	42	0	0	2	0	0	0	2387	1
53	44	1	0	2	1	1	1	348	0
54	61	1	1	2	0	0	0	2247	1
55	50	1	1	2	1	1	1	520	0
56	52	1	0	2	1	0	1	433	0
57	58	0	2	1	1	1	1	1715	1
58	41	0	0	3	1	0	1	175	0
59	52	1	0	3	0	0	0	429	0
60	45	1	2	4	2	1	1	126	0
61	61	0	1	4	1	1	0	92	0
62	48	0	1	2	1	1	0	1443	0
63	50	0	1	3	2	1	1	102	0
64	60	1	0	2	1	1	1	109	0
65	48	0	1	2	1	0	0	1086	0
66	23	1	1	3	1	0	0	952	2
67	53	1	1	2	2	1	1	232	0
68	30	0	1	2	1	1	0	1012	1

2.3.2 Pathology

The histopathology and staging, of all resected squamous cell carcinomas over a twenty one year period from 1982 to 2002, were reviewed. Sixty eight patients were identified in a data base; they had all had an oesophagectomy for OSCC, at Groote Schuur Hospital, a tertiary referral centre. Slides and paraffin blocks were retrieved form the archives in the Division of Anatomical Pathology. Haematoxylin and eosin (H&E) sections of the tumours were graded according to the WHO (2000) criteria. Figure 2.1 illustrates typical examples of well differentiated (Fig.2.1A), moderately

differentiated (Fig.2.1B) and poorly differentiated (Fig. 2.1C) OSCCs. A second pathologist also performed a randomised review of the slides as a confirmatory investigation.

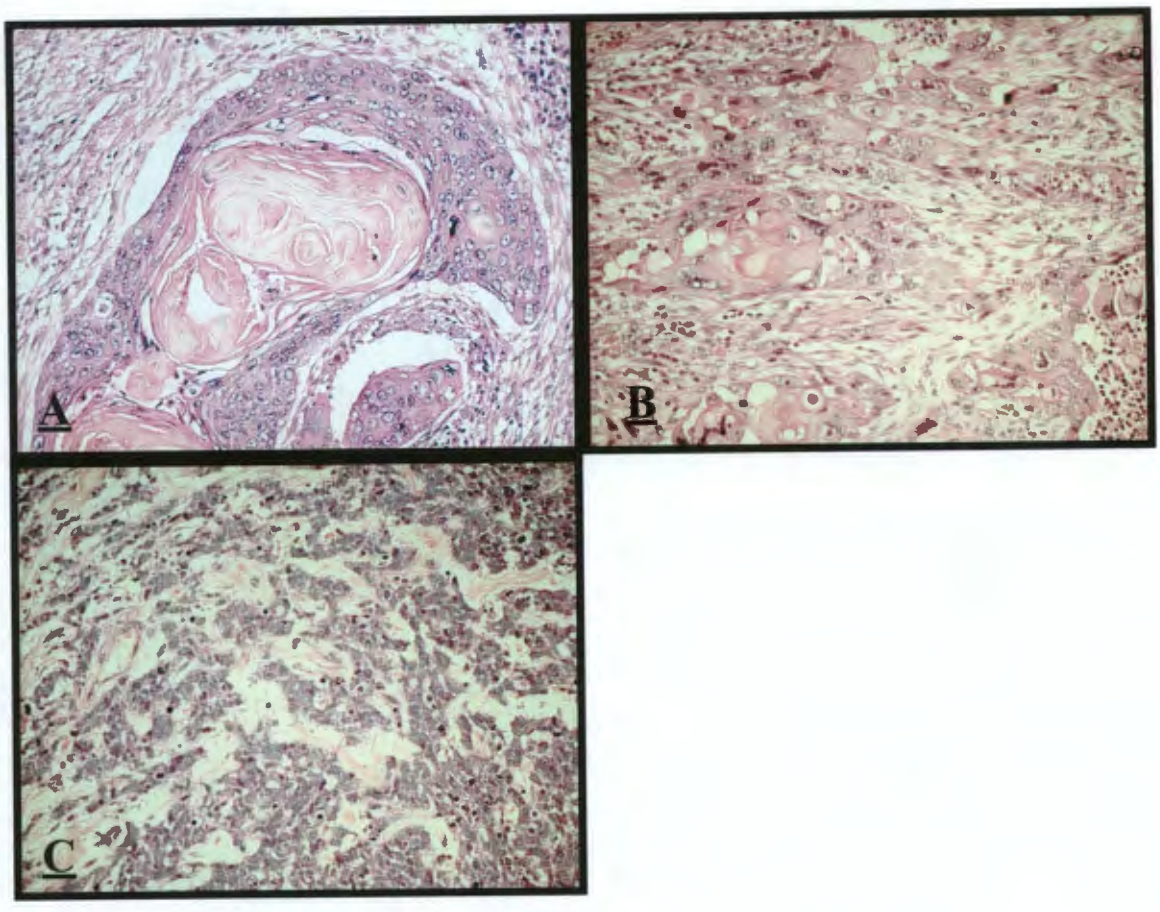


Figure 2.1: A - a well differentiated tumour, **B** - a moderately differentiated tumour and **C** - a poorly differentiated OSCC. H&E, x 100.

In addition, 16 cases of reflux oesophagitis, that showed reactive changes in the squamous epithelium, were chosen in order to compare and contrast any changes in the cell cycle regulatory proteins.

2.4 IMMUNOHISTOCHEMISTRY

Two paraffin embedded tissue blocks were then selected from each case, one of tumour and one of normal epithelium. Representative blocks of paraffin-embedded tissues were re-cut to provide two 4um thick sections of tumour and normal mucosa, and where possible, the sections were placed on the same slide. APES-coated slides were used for the immunohistochemical studies. For APES-coating slides were washed in Teepol solution, thoroughly rinsed in running tap water, rinsed in distilled water and air dried overnight. Slides were then 'coated' by immersing them in a 3% solution of APES in acetone for 2 minutes, rinsing in two changes of acetone with a final rinse in distilled water. The silanised slides were dried at 37° overnight.

The slides were heat fixed on a hot plate at 75° for 30 minutes and allowed to cool to room temperature. For de-waxing slides were placed in xylene for three minutes each, with three changes. Followed by two changes in 100% alcohol of two minutes each, then followed by two changes in 96% alcohol of two minutes each, and followed by one change of two minutes in 70% alcohol then rinsed in running water, and rinsed in distilled water.

Antigen retrieval was done with a pressure cooker. The appropriate buffer (Table 5) was brought to boiling point in a pressure cooker without a seal. The slides were generously spaced in a metal rack and lowered into the boiling buffer. The pressure cooker was then sealed and full pressure maintained for three minutes. The cooker was then removed from the heat source and cooled under running water. The lid was then removed and the slides were placed in running tap water to avoid drying. Heat-mediated antigen retrieval was performed for all five antibodies.

The five monoclonal antibodies used in this study were anti-p53 (DAKO-clone D0-7), anti-p21 (Novocastra-clone 4D10), anti-cyclin D1 (DAKO-clone DCS-6), anti-cyclin E (Novocastra-clone 13A3) and anti-HPV (DAKO-clone K1118). The HPV antibody is known to bind subtypes 6, 11, 16, 18, 31, 33, 42, 51, 56 and 58, which include the 'high grade'

subtypes (16, 18, 31 and 33) implicated in cervical carcinoma. The cell cycle regulatory protein antibodies are currently thought to be highly specific.

Endogenous peroxidase was blocked with 1% H_2O_2 in methanol for 20 minutes to prevent non-specific staining and slides were rinsed in water. They were then treated with normal goat serum (DAKO) for 15 minutes to prevent non-specific binding. Without rinsing they were then incubated with individual antibodies at calculated dilutions and for appropriate times (Table 2.2). Slides were washed with 0.1M PBS and 0.05% Tween-20(MERCK).

Cyclin E, p21, p53 and HPV were stained using the Dako Envision system (goat anti-mouse/peroxidase), which was applied for 30 minutes. Slides were then rinsed in PBS/Tween solution.

Cyclin D1 was stained using LSAB detection system. Biotinylated goat anti-mouse was applied as a secondary antibody for 30 minutes, followed by rinsing in PBS/Tween solution. This was followed by avidin peroxidase application for 30 minutes and a rinse in PBS/Tween.

Following peroxidase application, 'Liquid DAB (DAKO)' was then applied to all the slides for 5 minutes after which they were again rinsed in PBS/Tween. Sections were then washed in running water, counterstained with haematoxylin, dehydrated in alcohol, cleared in xylene and mounted.

Table 2.2: The antibodies used, antigen retrieval, dilutions, incubation times and controls.

Antibody	Clone	Buffer for Antigen Retrieval	Dilution	Incubation Time with Antibody	Controls (Figures 2.2.1 – 2.2.5)
p21	4D10	0.01M Citrate pH6.0	1:25	120 min	Squamous cell carcinoma
p53	DO-7	0.01M Citrate pH6.0	1:50	60 min	Squamous cell carcinoma
CyclinD1	DCS-6	0.01M Citrate pH6.0	1:150	60 min	Tonsil
CyclinE	13A3	0.001M EDTA pH8.0	1:40	120 min	Placenta
HPV	K1H8	0.01M Citrate pH6.0	1:50	60 min	Cervix(CIN1)

Staining was confirmed with known specified positive and negative controls in each batch of slides. P21 staining required increasing the incubation time from 60 to 120 minutes for positive control to show adequate antibody binding. The initial incubation time of 60 minutes resulted in weak antibody binding but after increasing the incubation time the antibody was clearly detectable with crisp nuclear staining.

2.4.1 Positive controls for immunohistochemistry:

Recommended positive controls were used in this study; see Figures 2.2.1 - 2.2.5.

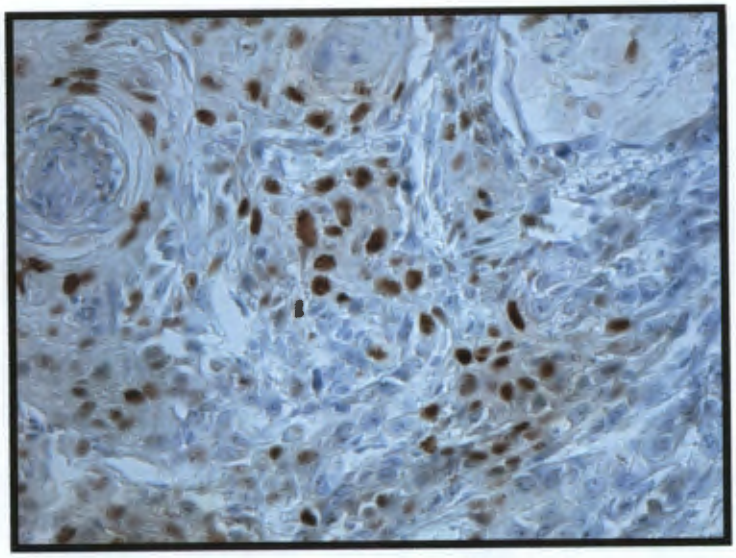


Figure 2.2.1: P21 control. Metastatic squamous cell carcinoma to a lymph node revealing distinct nuclear staining in malignant squamous cells. X400.

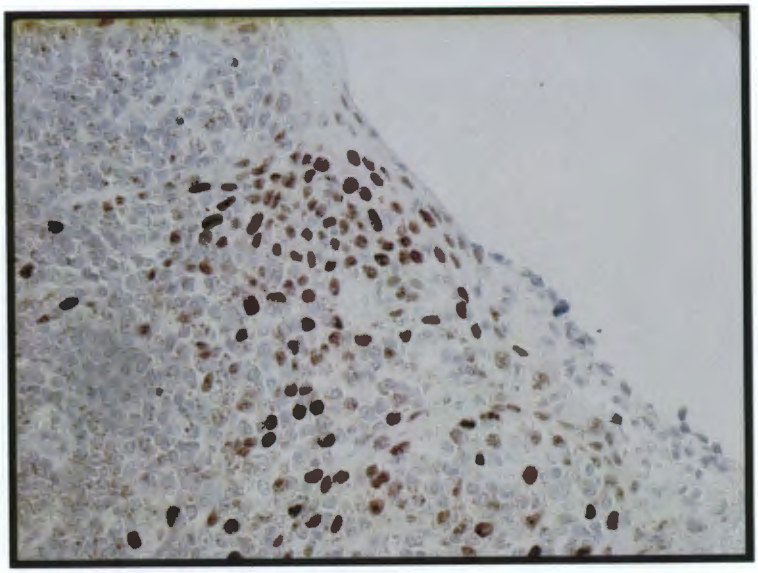


Figure 2.2.2: Cyclin D1. Normal tonsillar cells with distinct nuclear staining. X100.

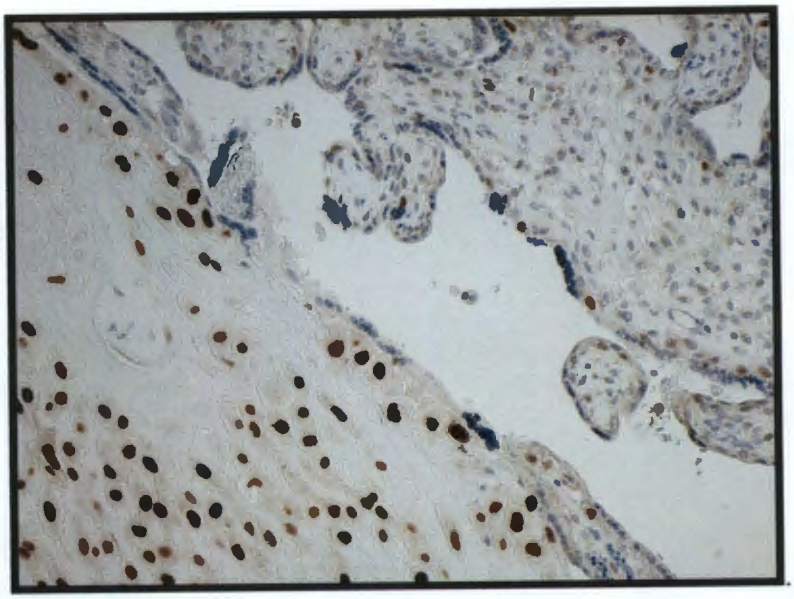


Figure 2.2.3: Cyclin E positive control, normal placenta showing crisp nuclear staining of trophoblastic cells. X100.

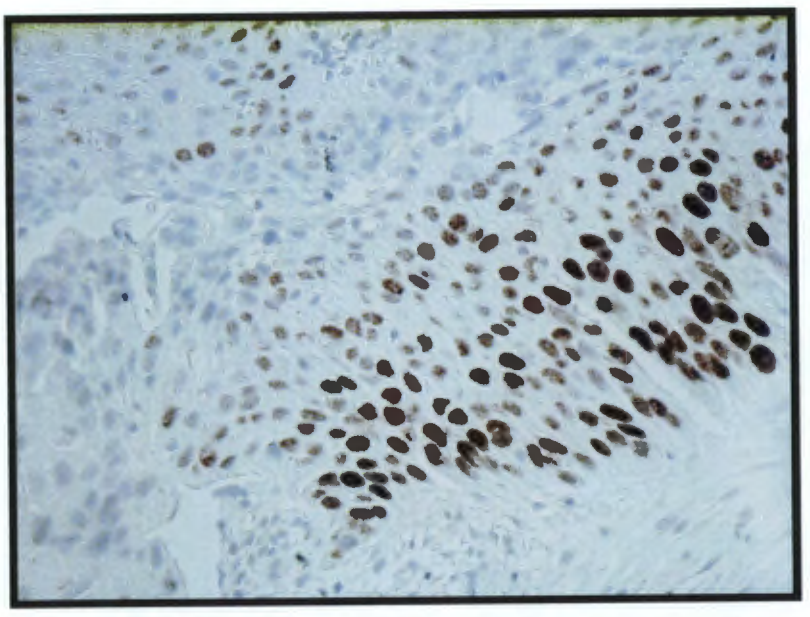


Figure 2.2.4: P53 positive control. A squamous cell carcinoma of the skin revealing clear nuclear staining in the malignant squamous cells particularly the basal cells. X100.

2.5 STATISTICAL ANALYSIS

The initial associations were analysed using the Spearman rank correlation test. Univariate associations between binary categorical variables were analysed using the Chi squared test or the Fishers exact test whilst multiple logistic regression was used for multivariate analysis. The post-operative period was measured from the date of oesophagectomy to the date of death. Patients who were lost to follow-up or confirmed alive at the end of the study (23rd June 2003) were considered censored for the purposes of survival analysis. Survival ranged from 8 days to 6001 days with a median survival of 387 days. The Kaplan-Meier method was used to estimate death from oesophageal cancer, and the log-rank test was used to estimate statistical significance. Univariate and multivariate survival analysis was analysed using the Cox proportional hazard model. Statistical significance was determined using the 95% confidence interval; P values of less than 0.05 were considered statistically significant. All of the statistical analyses were conducted using the STATA version 7.0 software package.

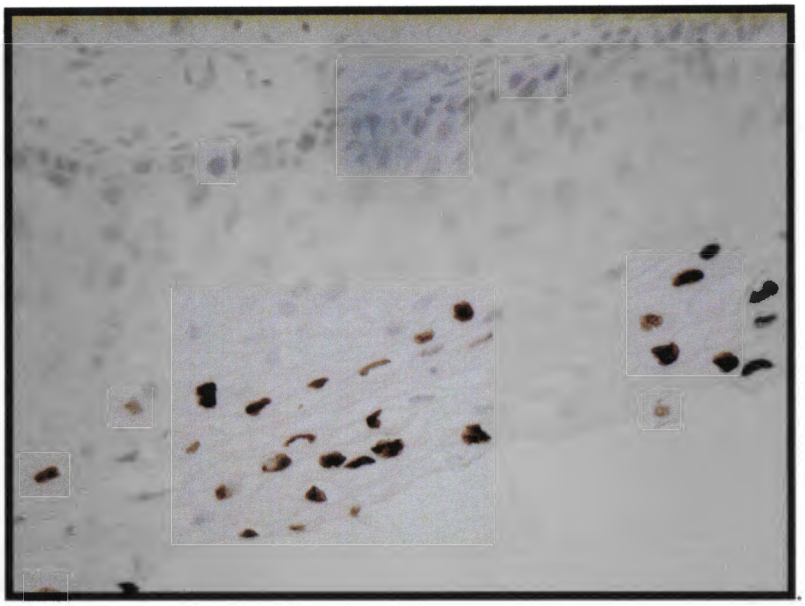


Figure 2.2.5: HPV. Positive control, low grade cervical intra-epithelial neoplasia showing strong binding to the nuclei of infected cells. X100.

Sections of the tissues used as positive controls were also used as negative immunohistochemical controls by processing the sections without addition of the primary antibody.

2.4.2 Interpretation of staining:

Immunostaining was evaluated in coded slides, without knowledge of the tumour stage, grade or clinical parameters. In addition, a random audit of the immunohistochemical results was carried out by a second pathologist to assess the quality of the stains and for confirmation of the results.

p53, p21, cyclin E and cyclin D1: As in other studies, nuclear staining in over 10% of tumour cells was considered a positive result (165, 181, 207-209). One hundred consecutive tumour cells where counted in two different representative fields (using 400 times magnification) and the number of positive cells was then divided by two to get a fairly objective percentage. In addition, positive cases were stratified into three groups according to the level of expression 10-25%, 26-50% and lastly those with more than

CHAPTER THREE:

3.1 RESULTS: CLINICAL DATA

3.1.1 Gender:

19 (27.9%) of the patients were female and 49 (72.1%) of the patients were male.

3.1.2 Race:

26 (38.2%) of the patients were black, 34 (50.0%) were mixed race and 8 (11.8%) were white. This distribution possibly reflects surgical selection criteria and the drainage area of Groote Schuur Hospital rather than the epidemiology of OSCC.

3.1.3 Age:

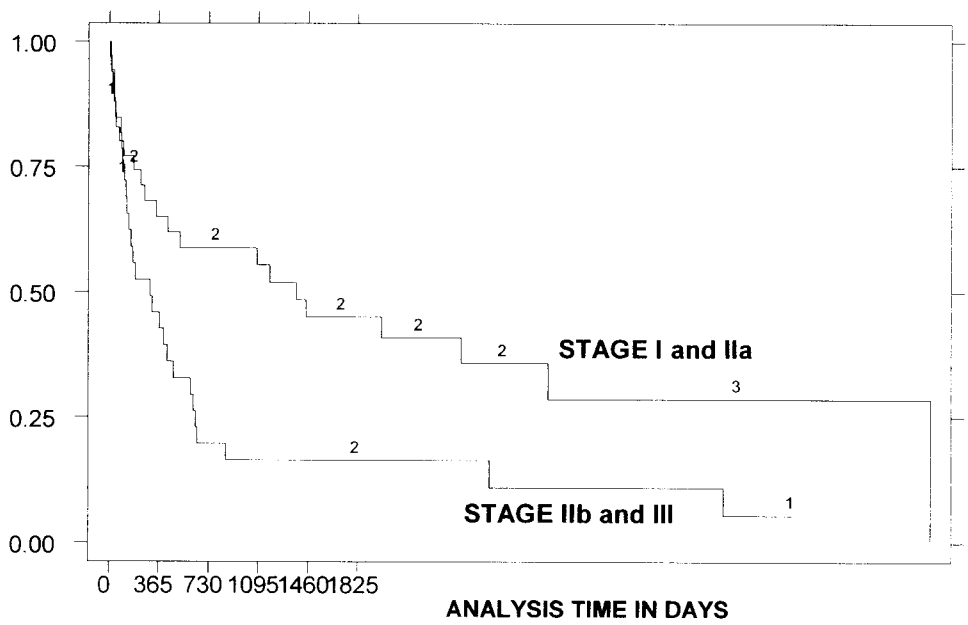
Age ranged from 23 to 80 years with a mean age of 52.7 years and a standard deviation of 10.3 years. Thus the mean age at the time of oesophagectomy for this group of patients was 52.7 years (Table 3.1).

Table 3.1: Shows the age distribution for this cohort of patients.

3.1.4 Stage:

Three patients had (4.4%) stage 1 disease, 32 (47.1%) had stage 2a disease, six (8.8%) had stage 2b disease, and 27 (39.7%) had stage 3 disease. For statistical analysis stage was stratified into those with no metastatic disease (stage I and IIa) and those with metastatic disease (stage IIb and III) (Table 3.2).

Table 3.2: Demonstrating survival for those with and without metastatic disease.



Kaplan-Meier survival estimates, by STAGE

3.1.5 Grade:

13 (19.1%) had well differentiated tumours, 45 (66.2%) had moderately differentiated tumours and 10 (14.7%) had poorly differentiated tumours.

3.1.6 Survival status:

59 (73.5%) of the patients had died by the end of the study period (January 1982 – 27 June 2003), 9 (13.2%) were alive at the end of the study period and 9 (13.2%) were lost to follow up.

3.2 RESULTS: CELL CYCLE REGULATORY PROTEINS

All four markers, p53, p21, cyclin E and cyclin D1, were tested on normal oesophageal tissue from the 68 patients. The normal tissue was consistently negative for expression of the marker i.e. < 10% of cell population analysed. Almost all the samples of normal mucosa were totally negative (0% of cells positive).

Sections of tissue in which the diagnosis of OSCC was histologically confirmed showed that 42 (61.8%) of the tumours stained positively for p53, 19 (27.9%) stained positively for p21, 15 (22%) stained positively for cyclin E and 30 (44.1%) stained positively for cyclin D1 (Table 3.3). Figures 3.1 - 3.8 are representative examples of staining, while appendix 1 lists the complete results).

Table 3.3: Levels 1, 2 and 3 of expression of cell cycle regulatory proteins in OSCC tissue

Level of expression	**P53 n=68**	**P21 n=68**	**Cyclin E n=68**	**Cyclin D1 n=68**
0 (0-9%)	26 (38.2%)	49 (72.1%)	53 (77.9%)	38 (55.9%)
1 (10-25%)	15 (22.0%)	16 (23.5%)	12 (17.7%)	15 (22.0%)
2 (26-50%)	5 (7.4%)	3 (4.4%)	1 (1.5%)	11 (16.2%)
3 (> 50%)	22 (32.4%)	0 (0%)	2 (2.9%)	4 (5.9%)

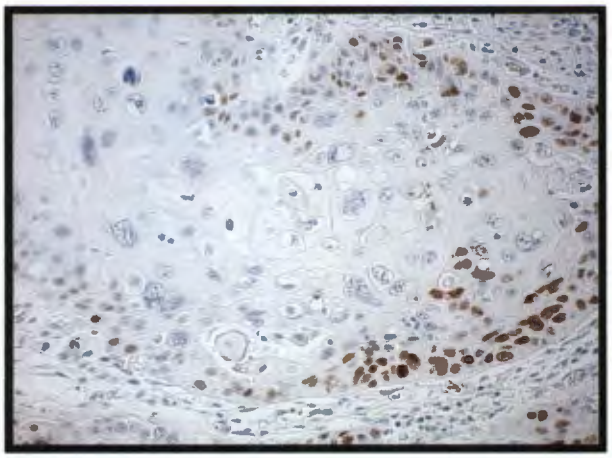


Figure 3.1: P53, level 1 staining (10 – 25% of cells positive) staining of OSCC with p53. Note the basal pattern of staining, while the more differentiated tumour cells are negative. X400.

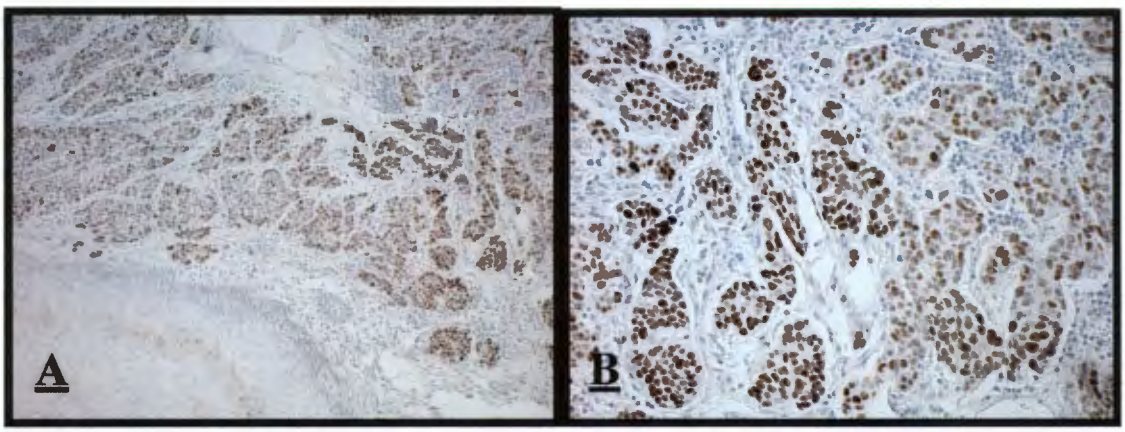


Figure 3.2: P53 showing level 3 staining (greater than 50% positive) of OSCC.

A: Shows some normal epithelium present in the lower left corner with an infiltrating OSCC showing strong p53 expression seen in the upper half of the picture. X40. **B:** Shows strong nuclear p53 expression. X100.

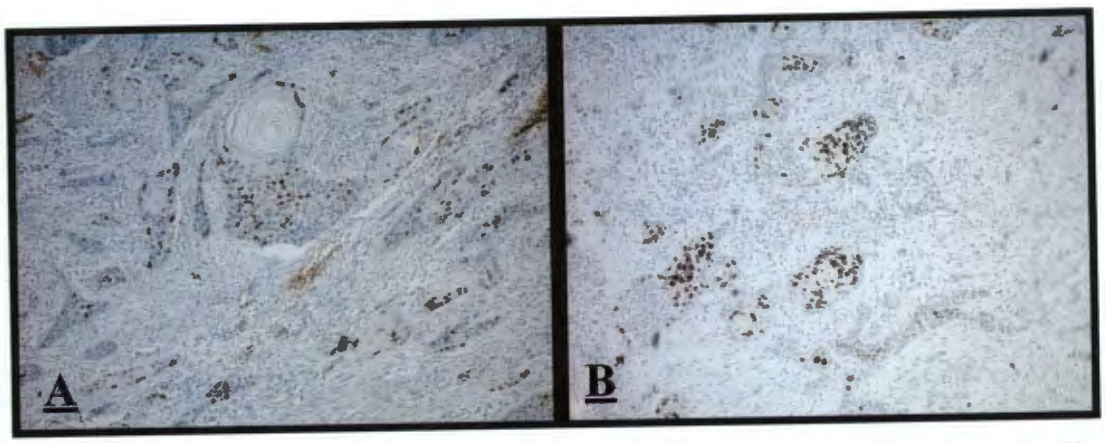


Figure 3.3: P21. **A:** Shows level 1 staining (10-25% positive) staining X40. **B:** Shows level 2 staining (26 – 50%), with p21 in OSCC. X40.

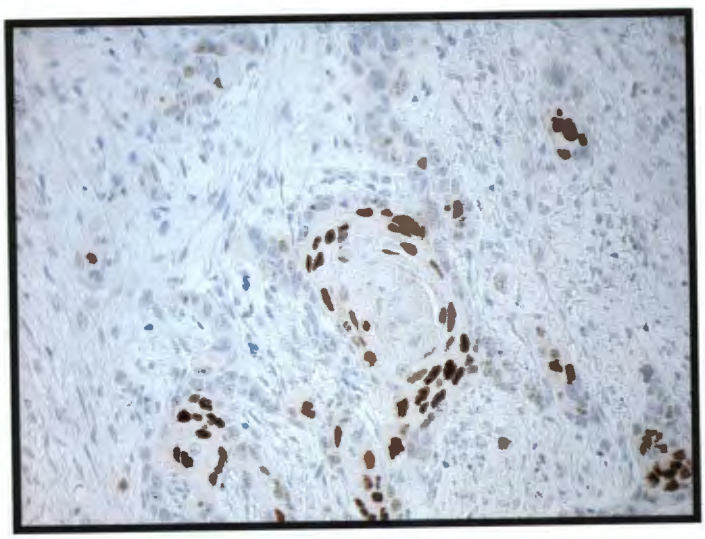


Figure 3.4: P21, level 2 staining in OSCC. The positive staining is confined to the more differentiated cells of the squamous nests, while the basal cells tend to be negative. X100.

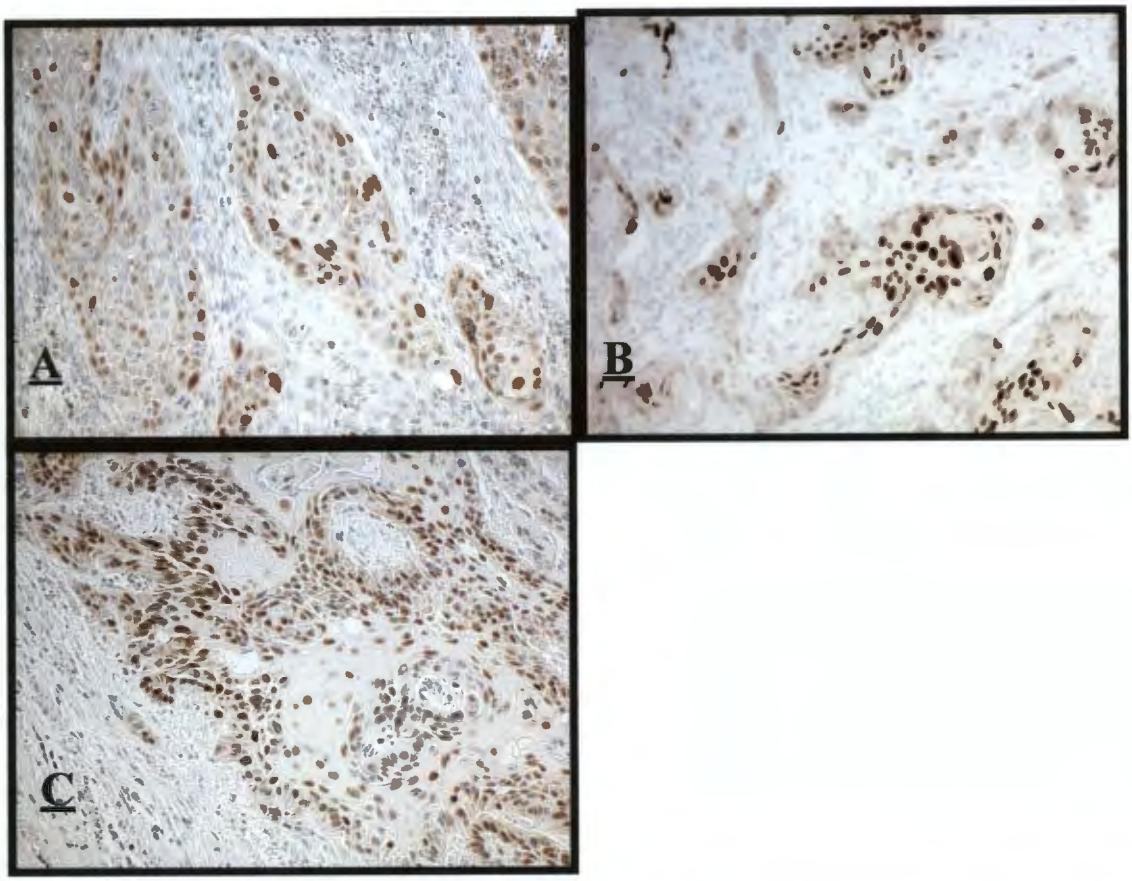


Figure 3.5: Cyclin E, staining level 1, 2 and 3 (**A, B** and **C** respectively) in OSCC. X100

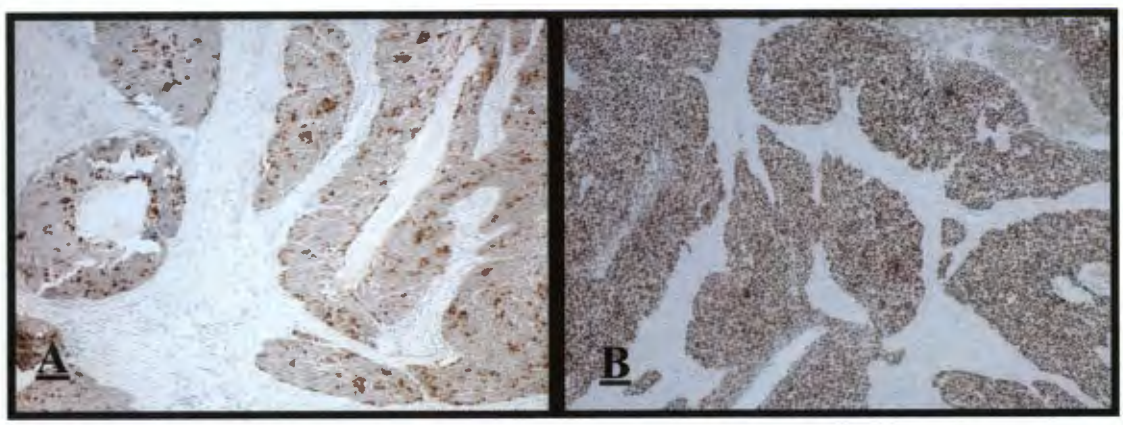


Figure 3.6: Cyclin D1, staining level 1 and 3 staining, in **A** and **B** respectively, in OSCC. X40.

Figure 3.6: Cyclin D1, staining level 1 and 3 staining, in **A** and **B** respectively, in OSCC. X40.

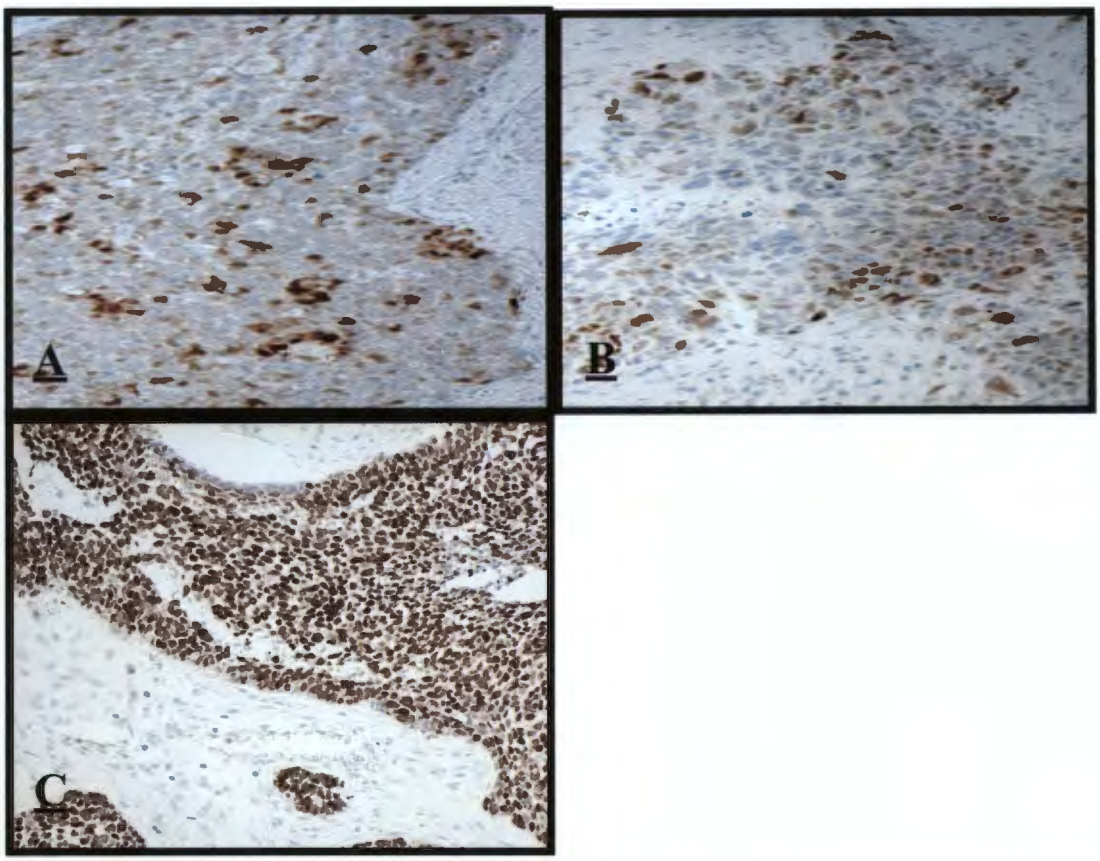


Figure 3.7: Cyclin D1, level 1, 2 and 3 staining (**A, B** and **C** respectively) in OSCC, showing the number of positive cells per 100 tumours cells. X100.

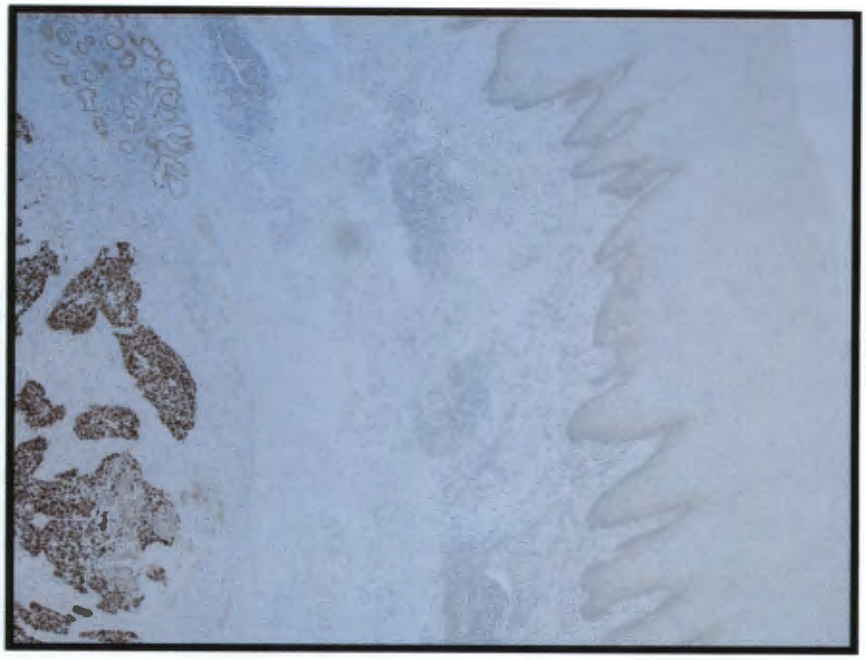


Figure 3.8: Cyclin D1, level 3 staining in the OSCC on the left side, seen undermining the overlying benign epithelium on the right which is negative. X25.

The same antibodies were tested on tissue from 16 inflammatory lesions of the oesophagus. In this instance both p53 and cyclin E were 100% negative whilst five were positive for p21 and two were positive for cyclin D1 (Table 3.4 and appendix 2).

Table 3.4: Levels of expression in reactive oesophageal squamous epithelium

Level of expression	P53 n=16	P21 n=16	Cyclin E n=16	Cyclin D1 n=16
0 (0-9%)	16 (100%)	14(87.5%)	16 (100%)	11 (68.8%)
1 (10-25%)	0	2(12.5%)	0	5 (31.2%)
2 (26-50%)	0	0	0	0
3 (> 50%)	0	0	0	0

Analysis of the relationship between expression of the proteins in cancerous versus inflamed oesophageal mucosa showed a significant difference in the expression of p53. P53 was not

expressed in any of the reactive squamous epithelia but was expressed in 61.7% of the tumours ($p<0.000$)(Table 3.5).

Table 3.5: Pearson's Chi squared analysis of p53 expression in OSCC and reactive tissue.

TISSUE TYPE	P53 STATUS +	-	Total
OSCC	42	26	68
REACTIVE	0	16	16
Total	42	42	84

Pearson chi2(1) = 19.7647 Pr = 0.000
Fisher's exact = 0.000
1-sided Fisher's exact = 0.000

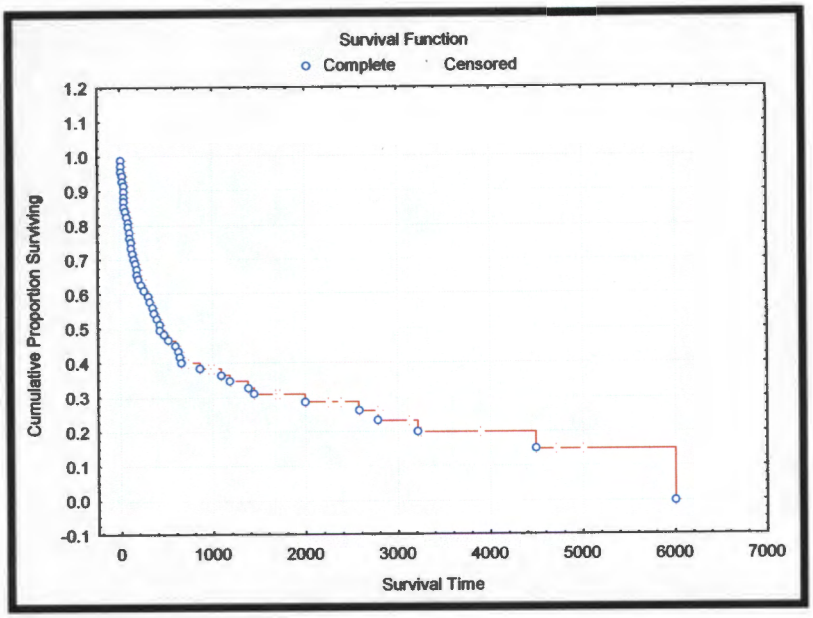
Cyclin E expression in neoplastic compared to reactive tissue approaches significance at the 5% level ($p=0.063$, Fishers exact test). Cyclin D1 and p21's expression was not significantly different between malignant and reactive tissue ($p=0.409$ and $p=0.336$ respectively).

3.3 PROGNOSTIC INDICATORS

The overall 5 year survival rate of this sample of patients was 30%. The 5 year survival in patients without lymph node metastases was 44.9% whilst those with nodal metastases had a 16.4% 5 year survival. This difference is highly significant ($p=0.0074$).

Table 3.6 shows the survival data of the patients included in this study.

Table 3.6: Survival graph of the entire group of patients with OSCC plotted in days from day 0 - 6000.



Univariate analysis using the Cox proportional hazards model confirmed that those with nodal metastases were 2.2 times (95% confidence interval 1.2 to 3.9 times) more likely to die from oesophageal cancer post surgery than those without metastases (p=0.009).

However, univariate analysis of each of the proteins showed that none of them independently predicted survival (Table 3.7). When adjusted for age, gender, stage, grade, smoking, alcohol and each other, the proteins remain as non-predictive for prognosis in multivariate analysis.

Table 3.7: Univariate and multivariate analysis of cell cycle regulatory proteins.

Variables	Univariate analysis of survival.		Multivariate analysis (full model)	
	Hazard Ratio	P value	Hazard Ratio	P value
P53	1.199	0.533	1.147	0.672
P21	1.545	0.175	1.862	0.132
CyclinD1	1.200	0.527	0.899	0.767
CyclinE	1.391	0.341	1.188	0.653

Multivariate analysis model building using a forward stepwise approach showed that when all the variables were considered (age, sex, stage, grade, smoking, alcohol, p53, p21, cyclin E and cyclin D1) only stage and age had prognostic significance (Table 3.8, below). The effect of alcohol on survival is not quite significant (p=0.08).

Table 3.8: Stage and age where the only two variables of significance in the multivariate survival analysis.

Variables	Multivariate analysis		
	Hazard Ratio	P value	Confidence Interval
Stage	2.439	0.003	1.345-4.421
Age	1.032	0.030	1.003-1.062

Thus, when adjusted for age, those with nodal metastases are 2.4 times more likely than those without metastases to die from oesophageal carcinoma post surgical intervention. Univariate and the Spearman's correlation coefficient test and multiple logistic regression showed that there was no significant association between mutant expression and stage or when adjusted for each other and stage. Additionally, there was no correlation between mutant expression and grade.

A noteworthy finding was an association between smoking and cyclin D1. In this study smokers were 8.6 times more likely than non-smokers to express cyclin D1. Though not

wide, the 95% confidence interval shows that this relationship is significant (CI: 1.0 – 73.7, p=0.049).

3.4 RESULTS: HUMAN PAPILLOMAVIRUS DETECTION

None of the 68 cases of OSCC, their normal mucosal counterparts, or any of the 16 examples of reactive oesophageal mucosa showed any immunohistochemical positive staining for HPV.

CHAPTER FOUR:

4.1 DISCUSSION

This study investigated 68 patients who underwent oesophagectomy for OSCC. The tumours in these patients were assessed for stage, grade, levels of p53, p21, cyclin D1, cyclin E, as determined by percentage expression, detected immunohistochemically in their cancerous and normal oesophageal tissue. The HPV status of each patient's oesophagus was also elucidated. For comparison, the same immunohistochemical stains were also done on 16 reactive oesophageal lesions from different patients.

In this study OSCC was more common in males than females (ratio 2.6:1); the racial breakdown showed that people of mixed race most commonly underwent oesophagectomy at Groote Schuur Hospital, followed by black and then white patients. However, the apparent predominance of people of mixed race is due to the geographic area drained by the hospital, and the fact that the majority of black patients with OSCC come from rural areas and present at advanced stages with tumours that are not amenable to surgical intervention. Consequently this study was done on a group of patients selected for surgery because the disease was thought to be 'early'; those with clinically advanced malignancy are not considered as surgical candidates. Thus, this study is on a highly selected group of patients and the findings cannot invariably be extrapolated to all cases of OSCC.

This study group had a 'Guassian' age distribution (mean age = 52.7 years) and age proved to be statistically significant with regards to predicting survival (p=0.03, 95% confidence interval =1.003-1.06). This meant for every advancing year there was statistically a 3% increase in the risk of death.

In this study, the stage, in particular the presence of nodal or metastatic deposits was shown to be the single most significant prognostic factor in survival analysis (p=0.003, 95% confidence interval =1.3 – 4.4, hazard ratio 2.439).

The 5-year survival rate for the entire group was 30%. Patients without lymph node metastases had a 5-year survival of 44.9%, but only 16.4% with nodal metastases survived for 5 years. This is in keeping with most large retrospective reports of surgically resected OSCC in which the 5 year survival rate is between 29 and 38% (127, 210-212). Factors such as increased age and advanced stage are associated with a worse 5 year survival (210). Early stage is consistently associated with a better 5 year survival rate as noted in a recently published report from China, where active oesophageal screening cytology is taking place. Wang and colleagues (213) report an 86% 5 year survival for 420 patients who underwent oesophagectomy for superficial OSCC (involving mucosa and submucosa).

In this study, as in many others (108-112) the grade of the tumour failed to show any prognostic significance. A distinctive pattern of staining could however be seen with p53 and p21 in the well differentiated, and to some degree, in the moderately differentiated OSCCs. P53 was largely confined to the basal layers (Fig. 3.1) while p21 was evident just prior to keratinocytic apoptosis, and was particularly evident in the squamous whorls (Fig. 3.4). These patterns of expression have been previously reported (174, 175).

There was no significant relationship between survival and over expression of the various cell cycle regulatory proteins; nor between the expression of these proteins and the grade and tumour stage. In this study increased p53 expression on its own did not convey any prognostic significance as previously reprted (156-160). The subgroup of p21 positive p53 positive OSCC did not have a worse prognosis compared to the rest of the group as previously noted (163). None of the 68 malignancies analysed showed level 3 staining for p21 (>50% of tumour cells showing positive staining) and therefore the previous report that this conferred a worse prognosis could not be confirmed (177). The finding that cyclin D1 expression did not convey any significance in predicting survival in this study was unexpected. However, other immunohistochemical studies of cyclin D1's significance as an independent prognostic indicator were conducted on different population groups, using differing antibody clones and antigen retrieval methods (186,

188, 190, 192). In this context the findings of this study fail to confirm published reports of cell cycle regulatory proteins conveying prognostic relevance in OSCC.

Positive expression of p53 was seen in the OSCCs (61.8%) but not in the normal or reactive oesophageal mucosa. Of note was that 22 of the 68 OSCC (32%) expressed p53 in over 50% of their tumour cells. In addition, p53 was shown to be a significant factor in discriminating between reactive lesions and neoplastic lesions ($p<0.000$). Similar over expression of p53 was found in this study in areas of full and partial thickness dysplasia (n=3) adjacent to invasive OSCCs. Expression of p53 in dysplastic, preinvasive lesions of the oesophagus has been described (214). In addition, stepwise over expression of p53 in oesophagitis, dysplasia and neoplasia has also been described in oesophageal squamous cell lesions (165). These findings suggest that p53 over expression (in > 10% of tumour cells) in formalin fixed, paraffin embedded tissue is specific for neoplasia, but unfortunately the sensitivity was only 62%.

Cyclin E was also over expressed in OSCC (22.1%), but negative in normal and reactive mucosa. The expression of cyclin E was less useful, than p53, in the discrimination between reactive and neoplastic lesions but approached significance ($p= 0.063$). In view of the small number of cases showing cyclin E positive staining the statistical significance may not be reliable. A larger number of cases may provide more reliable results. If the study group was larger cyclin E expression may well have proved significant as none of the reactive lesions expressed cyclin E. The positive expression of p21 (27.9%) and cyclin D1 (44.1%) in OSCC was increased when compared to the normal mucosa (100% negative). The expression of p21 and cyclin D1 proteins was not able to significantly discriminate between reactive and neoplastic lesions. Although the reactive group was small none of the reactive lesions showed greater than 25% expression (level 1) in the mucosal cells of p21 and cyclin D1. Strong cyclin D1 expression (>26% of cells/level 2) was seen in 22.1% of OSCC. Thus, level 2 or greater expression of cyclin D1 could be useful in distinguishing between reactive and neoplastic lesions.

More importantly, cyclin D1 was noted to be significantly over expressed in those patients who smoked compared to non-smokers. This finding may well warrant further investigation in OSCC and other smoking related lesions. Over expression of cyclin D1 has been documented in oral cavity dysplasia/malignancy and in squamous lesions in the bronchus (metaplasia, dysplasia and neoplasia) (209, 215). Cyclin D1 expression is clearly related to smoking. Importantly, future studies of cyclin D1 and survival should consider whether smoking is a confounding factor when assessing the independent prognostic significance of cyclin D1. Cyclin D1 over expression is currently considered an early event in the carcinogenesis of OSCC (199) and its expression may well be related to chronic irritation which results in increased cell turnover.

No relationship could be demonstrated between the presence or absence of expression of the various cell cycle proteins in the neoplastic tissue. In particular, p53 and p21 did not demonstrate inverse or related expression. Neither could a relationship between cyclin D1 and cyclin E expression be found.

The initial aim of this study was to investigate the role of HPV, using immunohistochemical staining, in OSCC but the negative results necessitated a shift in the focus of this study to that of cell cycle regulatory proteins. The absence of immunohistochemically detectable HPV in this study was an unexpected result. Particularly, since this finding was discordant with that of fellow investigators who examined tumour tissue from many of the same patients, using PCR techniques, and found an approximate 20% prevalence of HPV in their sample (unpublished data, personal communication with Professor I Parker, Department of Medical Biochemistry, University of Cape Town).

This adds to the observation that the different method used to detect HPV result in differing prevalence rates. The clone used in this study (K1H8-Dako) was a monoclonal mouse anti-HPV antibody delivered in a fetal calf serum tissue culture supernatant. The antibody reacts with the non-conformational internal linear epitope of a major capsid protein (VP1) that is broadly expressed amongst the different HPV subtypes. It has been

shown to identify HPV 1 in ELISA and to detect HPV 1 and 6 by indirect immunoflourescence in infected frozen tissues. In addition, the antibody has been shown to be immunoreactive with paraffin sections of formalin fixed tissue demonstrated by Southern blot hybridisation to be infected with HPV types 6, 11, 16, 18, 31, 33, 42, 51, 52, 56 and 58 (216). Hence, if full viral particles of these types are formed with an intact capsid they should be detectable by immunohistochemical methods. However, if the virus has been integrated into the cellular genome/deoxyribonucleic acid (DNA) and is not replicating it would be unlikely that this particular antibody would detect it. PCR would most likely identify any viral DNA sequences present. The vast majority of PCR studies from areas of high incidence have found a high prevalence of HPV DNA in OSCC. Other immunohistochemical studies, that have identified HPV in OSCC used different clones (37, 38), while another study demonstrating positive staining used a different anti-HPV capsid clone (53).

There is now sufficient 'literature' to support the hypothesis that HPV does infect the oesophageal mucosa and becomes integrated into the genome of some patients; recent publications report that high grade cervical dysplasia is associated with, and requires the integration of HPV into the genome (217, 218). Once integrated into the genome, HPV is capable of modulating the p53 and retinoblastoma gene products and contributing to carcinogenesis. Therefore, the absence of immunohistochemically detectable HPV capsid antigen in the 68 patients studied would imply that there was no active viral replication. This does not exclude the possibility that HPV is incorporated into the genome and may play an important role in aetiopathogenesis of OSCC. The patients included in this study were from an urban and peri-urban environment predominantly and not the high incidence areas of the Eastern Cape.

While the cell cycle regulatory proteins were not predictive of survival they have been shown in several studies to be useful in predicting a response to therapy. Studies of the predictive value of p53, to determine whether the effectiveness of radiotherapy could be predicted, showed over expression of p53 was been associated with an unfavourable treatment response and negative p53 expression with a favourable response (219-221).

The efficacy of combined chemo-radiotherapy in relation to p53 expression, found a high level of p53 expression in operated specimens was associated with unfavorable tumour response to preoperative treatment. Therefore, immunohistochemical detection of p53 protein was predictive of the outcome of preoperative therapy (222).

Potential therapeutic interventions have been studied analysing adenoviral-mediated wild-type *p53* gene therapy on oesophageal cancer cell lines with promising results. A significant growth suppression following an Ad5CMV-p53 infection was observed in both cancer cell lines. A Western blot analysis confirmed the presence of both exogenous p53 protein expression and p21 protein induction. Apoptotic cell death was observed with TUNEL staining (223).

P21 and p53 have been studied in patients who received preoperative chemotherapy for OSCC and subsequently underwent oesophagectomy with lymph node dissection. The positive rate of p53 and p21 expression was 56.7% and 36.7%, respectively. Preoperative chemotherapy was ineffective in all the patients who expressed p53, but not p21. In contrast, chemotherapy was effective in patients with lymph nodes metastases that were p53 negative but p21 positive. These findings suggest that p21 positive expression, in the absence of p53, is associated with favorable effects of preoperative chemotherapy in patients with OSCC (224).

One study looked at a recombinant adenoviral approach and gene gun technology to introduce *p21* into oesophageal cancer cells in order to assess the effect of *p21* on cell growth. Infection with the p21 adenovirus (AdV) resulted in inhibition of malignant oesophageal squamous cell lines. The levels of involucrin, which is a marker of squamous epithelium differentiation, markedly increased at 48 h and 72 h after *p21* AdV infection. These results indicate that p21 plays an important role in oesophageal cancer cell proliferation. Over expression of the *p21* gene can inhibit cell growth and induce differentiation in oesophageal cancer cells. *P21* gene therapy may prove beneficial in the treatment of oesophageal cancer (225).

Cyclin D1 expression may also be important regarding patients receiving chemo-radiotherapy. Among 34 patients with OSCC, differences in the responsiveness to chemoradiotherapy were correlated with cyclin D1 immunoreactivity assessed in the biopsy specimens. This study found that the cyclin D1 negative group responded favourably compared to the positive group. Thus the cyclin D1 protein may be a useful predictor of sensitivity to concurrent chemo-radiotherapy for OSCC (226).

4.2 CONCLUSION:

In conclusion, this study confirmed that expression of the cell cycle regulatory proteins cyclin D1, cyclin E, p21 and p53 did not convey any prognostic significance in a group of patients who underwent oesophagectomy for OSCC. Nor is expression of these proteins related one to another, or to stage, age, grade and alcohol use.

Cyclin D1 was found to be over expressed in smokers versus non-smokers.

P53 is specific, but not sensitive, for discriminating between reactive and neoplastic oesophageal lesions. The expression of cyclin E may also be useful in this regard.

Differences between this study and previous reports of cell cycle regulators conveying independent prognostic significance may be related to the population studied, antigen retrieval methods, different antibody clones, methodology used for antibody binding and in some cases differing methods of statistical analysis.

HPV could not be demonstrated, using immunohistochemical techniques, in this group of patients with OSCC.

Younger patients had a better survival than older patients, with increasing age significantly increasing the risk of mortality. Lastly, histopathological staging, in particular the presence of nodal or other metastatic disease, remains the most important

prognostic indicator in these patients. Any clinical intervention enabling early detection of OSCC should be wholeheartedly endorsed.

4.3 RECOMMENDATIONS:

1. Exploration of the relationship between cyclin D1 and smoking; this might shed light on understanding the molecular pathology of smoking related neoplastic lesions.

2. Prospective studies to confirm whether expression of p53, p21 and cyclin D1 determines a response group to chemo- and/or radiotherapy.

3. The various methods used to detect HPV should be compared using the same tissue samples taken from high incidence areas of OSCC and the biological reason for varying detection explored.

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APPENDIX:

1. IMMUNOHISTOCHEMICAL RESULTS FOR OSCC:

Where, n = normal tissue and t = tumour.

Case	p53n	p53t	p21n	p21t	cyclinEn	cyclinEt	cyclinD1n	cyclinD1t
1	0	0	0	0	0	0	0	0
2	0	0	0	1	0	1	0	2
3	0	1	0	0	0	0	0	2
4	0	0	0	1	0	0	0	3
5	0	2	0	0	0	0	0	1
6	0	3	0	0	0	0	0	0
7	0	0	0	0	0	0	0	1
8	0	0	0	0	0	0	0	2
9	0	3	0	0	0	0	0	0
10	0	3	0	0	0	0	0	1
11	0	3	0	1	0	0	0	1
12	0	0	0	0	0	0	0	1
13	0	1	0	0	0	0	0	0
14	0	2	0	1	0	0	0	1
15	0	1	0	0	0	0	0	0
16	0	0	0	0	0	0	0	3
17	0	0	0	1	0	1	0	1
18	0	3	0	0	0	1	0	2
19	0	0	0	0	0	0	0	0
20	0	3	0	1	0	1	0	0
21	0	1	0	0	0	1	0	0
22	0	1	0	1	0	0	0	0
23	0	1	0	2	0	1	0	1
24	0	0	0	0	0	0	0	0
25	0	3	0	0	0	1	0	0
26	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0
28	0	1	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0

Case	p53n	p53t	p21n	p21t	cyclinEn	cyclinEt	cyclinD1n	cyclinD1t
30	0	0	0	0	0	0	0	0
31	0	3	0	0	0	1	0	0
32	0	0	0	1	0	0	0	1
33	0	0	0	0	0	0	0	0
34	0	3	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0
36	0	3	0	1	0	0	0	0
37	0	3	0	0	0	1	0	0
38	0	3	0	0	0	0	0	0
39	0	3	0	0	0	0	0	2
40	0	3	0	0	0	0	0	0
41	0	2	0	0	0	0	0	2
42	0	1	0	0	0	0	0	2
43	0	3	0	0	0	0	0	0
44	0	0	0	0	0	0	0	0
45	0	0	0	0	0	0	0	3
46	0	1	0	0	0	0	0	2
47	0	2	0	1	0	0	0	1
48	0	0	0	1	0	0	0	1
49	0	3	0	1	0	0	0	3
50	0	1	0	0	0	0	0	0
51	0	1	0	0	0	2	0	0
52	0	3	0	0	0	0	0	0
53	0	3	0	0	0	0	0	1
54	0	3	0	0	0	0	0	0
55	0	0	0	0	0	0	0	2
56	0	1	0	0	0	3	0	0
57	0	1	0	0	0	0	0	0
58	0	0	0	1	0	1	0	1
59	0	3	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0
61	0	1	0	1	0	0	0	2
62	0	0	0	1	0	0	0	2
63	0	3	0	0	0	1	0	0

Case	p53n	p53t	p21n	p21t	cyclinEn	cyclinEt	cyclinD1n	cyclinD1t
64	0	1	0	2	0	3	0	0
65	0	0	0	0	0	0	0	1
66	0	0	0	2	0	0	0	0
67	0	3	0	1	0	0	0	1
68	0	2	0	0	0	1	0	0

2. IMMUNOHISTOCHEMICAL RESULTS FOR REACTIVE OESOPHAGEAL TISSUE.

case	Cyclin D1	Cyclin E	P21	P53
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	1	0	0	0
5	0	0	0	0
6	0	0	1	0
7	1	0	0	0
8	0	0	0	0
9	1	0	0	0
10	1	0	1	0
11	0	0	0	0
12	1	0	0	0
13	0	0	0	0
14	0	0	0	0
15	0	0	0	0
16	0	0	0	0
total	5	0	2	0