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MOLECULAR ANALYSIS OF CELL PROLIFERATION AND APOPTOSIS IN OESOPHAGEAL ADENOCARCINOMA

A thesis submitted for the degree of

Doctor of Philosophy (Ph.D)

By

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At

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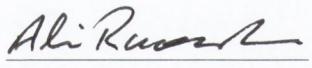
September 2002

DECLARATION

I declare that, except where otherwise acknowledged, this thesis is entirely my own work. It has not been submitted previously for a higher degree at this or any other university. The library may lend or make copies of this thesis upon request.



SUMMARY

Cancer of the oesophagus is a particularly virulent gastrointestinal malignancy with poor prognosis. Replacement of the normal squamous epithelium of the oesophagus with columnar epithelium (Barrett's oesophagus) as a consequence of chronic gastric acid reflux is considered as a premalignant condition. The carcinomas generally originate within the Barrett's area, and histological changes ranging from metaplasia to dysplasia and invasive carcinoma have been detected. The molecular events underlying progression of Barrett's oesophagus to adenocarcinoma is not well known, they therefore, represent an area of active investigation. Exploration of these molecular events may contribute not only to our understanding of their roles in the pathogenesis of Barrett's epithelium but also as regards the development of new strategies for enhancing treatment. Ongoing research at the Department of Surgery at St. James's Hospital and Trinity College Dublin has devoted considerable interest and resources to this field and this thesis is, therefore, designed to (i) investigate the stage at which aberrant expression of the p53 protein arises in neoplastic progression of Barrett's oesophagus and its relationship to the clonal evolution of cancer, (ii) explore the role of bcl-2 and bcl-2 associated bax and bcl-x gene products in the molecular regulation of Barrett's tumourigenesis and tumours' response to neoadjuvant chemoradiotherapy, (iii) assess the influence of preoperative neoadjuvant chemotherapy and radiation therapy on tumour cell proliferation and apoptosis, and (iv) elucidate (in-vitro) the mechanisms of cell death induced in oesophageal carcinoma cells in response to 5-fluorouracil, cisplatin and taxol treatment.

A total of 48 patients (36 male, 12 female) with primary oesophageal adenocarcinoma were included in this study. All patients had chemotherapy with 5-fluorouracil and cisplatin and external beam radiotherapy (40Gy) prior to oesophagectomy. Of these patients, normal oesophageal tissues, Barrett's mucosa when present, and representative blocks of tumours were subject to immunophenotype analysis for markers such as p53, Ki-67, bcl-2, bax and bcl-x proteins. In-situ end labelling technique was used to detect and quantify the apoptotic cells within the tumour samples. All pre-treatment and resection samples were analysed and all were informative for the purpose of statistical analysis. For in-vitro study, human oesophageal adenocarcinoma (JROECL-33) and squamous cell carcinoma (JROECL-21) were used. Mutation in the p53 gene was

determined by means of PCR and automated DNA sequencing. Western blotting techniques were employed for proteins detection. Light microscopy and ligation-mediated PCR was used to determine the morphological and biochemical features of the apoptotic cells following 5-flurouracil, cisplatin and taxol treatment.

This thesis demonstrates that p53 over-expression occurs along with the development of dysplasia in Barrett's oesophagus and that its accumulation is associated with increasing proliferative activity. The observed association between p53 and cell proliferation in severe dysplastic Barrett's and adenocarcinomas are similar. An increasing degree of dysplasia in Barrett's mucosa to adenocarcinoma was significantly associated with the reduction of bcl-2 expression, while bax and bcl-x gene products demonstrated a consistently high level of expression. We documented (Raouf *et al*, 2001) that preoperative chemotherapy and radiation therapy significantly increases apoptotic cell death and significantly decreases cell proliferation. Analysis of our data indicated that the frequency of spontaneous apoptosis and tumour growth activity at resection is a prognostic biomarker associated with tumours' response to neoadjuvant therapy and tumours' metastatic potential, respectively. The expression level of p53, bcl-2 and its associated protein bax and bcl-x did not influence or predict the response or resistance of oesophageal adenocarcinoma to neoadjuvant chemoradiotherapy.

This thesis also demonstrates (in-vitro) that the mutational status of p53 is associated with the chemosensitivity of oesophageal carcinoma cells to 5-fluorouracil and that the presence of the mutant p53 protein may provide cells with pathways against apoptosis. In addition, cisplatin and taxol were demonstrated to be inducers of apoptosis in oesophageal carcinomas but through different molecular mechanisms. This study also shows that apoptosis and its modulation by the 5-fluorouracil, cisplatin or taxol occurs independently of bax and bcl-x_L, the dominant splice variant of bcl-x, protein.

In conclusion, we demonstrated that defective regulation of epithelial cell proliferation and death are major contributing factors associated with oesophageal adenocarcino-genesis and response of tumours' to preoperative chemoradiotherapy. It must be hoped that future investigation will be conducted to further uncover molecular events that potentiate tumour development and predict response to neoadjuvant regimens so that therapy can be tailored toward maximising treatment benefit and minimising complications.

DEDICATION

This thesis is dedicated to my parents and my sisters, and most especially to my wife Bayan and my daughters Zainab and Tara

"Proclaim (or read) in the name of thy Lord and Cherisher who created –
Created man, out of a (mere) clot of congealed blood:
Proclaim! And thy Lord is most Bountiful, –
He who taught (the use of) the pen,
Taught man that which he knew not."
(The Qur'an, 96: 1-5)

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ACKNOWLEDGMENT

In submitting this thesis in fulfilment of the requirements for the degree of Doctor of Philosophy, I am conscious that it represents the fruit of some five years of research which though undertaken alone for the most part, was - at critical times - shared with colleagues and friends to whom I would now like to express my profound gratitude and appreciation.

A very special word of thanks is owing to Professor John Reynolds of the Department of Surgery, St. James's Hospital who made me most welcome in his laboratory and who provided invaluable encouragement, advice and assistance throughout these past three years. Without his support I could not have contemplated, much less completed, this work.

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ASSOCIATED PUBLICATIONS

MANUSCRIPTS

Raouf A, Evoy D, Carton E, Mulligan E, Griffin M, Sweeney E, Reynolds JV. Spontaneous and inducible apoptosis in oesophageal adenocarcinoma. *British Journal of Cancer 2001; **85**: 1781-1786.*

Raouf AA, Evoy DA, Carton E, Mulligan E, Griffin MM, Reynolds JV. Loss of Bcl-2 expression in Barrett's dysplasia and adenocarcinoma is associated with tumor progression and worse survival but not with response to neoadjuvant chemoradiation. *Diseases of the Esophagus 2003; **16**: 17-23.*

Raouf AA, Evoy DA, Reynolds JV. Mechanisms of cell death induced in oesophageal carcinoma cells in response to 5-fluorouracil, cisplatin and taxol treatment. *(manuscript in preparation).*

PRESENTATIONS

A. Raouf, D. Evoy, E. Carton, E. Mulligan, M. Griffin, E. Sweeney, JV. Reynolds. Bcl-2, bax and bcl-x expression in normal epithelium, Barrett's epithelium and adenocarcinoma of the oesophagus. *Irish Society of Gastroenterology (2001 meeting), Cork, Ireland. Irish Association of Cancer Research (2001 meeting), Cork, Ireland. Digestive Disease Week (2001 meeting), Atlanta, USA.*

A. Raouf, D. Evoy, E. Carton, E. Mulligan, M. Griffin, E. Sweeney, JV. Reynolds. Prognostic significance of apoptosis and proliferation associated Ki-67 antigen expression in oesophageal adenocarcinoma before and after chemoradiotherapy. *Irish Society of Gastroenterology (2001 meeting), Cork, Ireland. Irish Association of Cancer Research (2001 meeting), Cork, Ireland.*

A. Raouf, D. Evoy, M. Griffin, JV. Reynolds. Taxol induced apoptosis, p53 and bcl-2 associated bax and bcl-x protein expression in oesophageal adenocarcinoma - in vitro analysis. *Irish Society of Gastroenterology (2001 meeting), Cork, Ireland. Irish Association of Cancer Research (2001 meeting), Cork, Ireland. American Association of Pharmaceutical Scientists, 2001 Annual Meeting, Denver, USA (AAPSPharmSci 2001; Vol. 3, No. 3).*

E. Carton, **A. Raouf**, E. Mulligan, A Bane M. Griffin, JV. Reynolds. Expression of bcl-2 in oesophageal adenocarcinoma. *Sylvester O'Halloran Meeting (1999) Limerick, Ireland. Irish Journal of Medical Sciences 1999: **168**.*

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ABBREVIATIONS

A	Adenine
ABC	Avidin-biotin complex technique
AO	Acridine orange
AP	Alkaline phosphatase
APAAP	Alkaline phosphatase anti alkaline phosphatase
APES	3-aminopropyltreithoxysaline
APS	Ammonium persulfate
ATP	Adenosine triphosphat
bp	Base pair
BrdU	5-bromo-2 deoxyuridine
BSA	Bovine serum albumin
C	Cytosine
Ca^{2+}	Calcium
CD	Cluster designation
DAB	3'-3'diaminobenzidine tetrahydrochloride
DPAS	Diastase periodic acid schiff stain
DMSO	Dimethlysulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide transferase
EB	Ethedium bromide
ELISA	Enzyme linked immunosorbent assay
FCS	Foetal calf serum
G	Guanine
GTP	Guanosine triphosphate
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
H&E	Haematoxylin and eosin
IgG	Immunoglobulin G
IL	Interleukin
Kb	Kilobase
kDa	Kilodaltons
LI	Labelling index

LM-PCR	Ligation mediated PCR
mAb	Monoclonal antibody
MAP	Micrpotubule associated protein
Map	Mitogen-activated protein
MgCl	Magnesium chloride
pAb	polyclonal antibody
PAP	peroxidase-antiperoxidase
PAS	Periodic acid schiff stain
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PKC	Protein kinase c
PMSF	Pheyl methyl sulphonyl fluoride
PVDF	Polyvinylidene difluoride
R_f	Relative mobility
RNA	Ribonucleic acid
SDS-page	Sodiumdodecyle sulphate polyacrylamie gel electrophoresis
ssDNA	Single stranded deoxyribonucleic acid
T	Thymine
TdT	Terminal deoxynucleotidyl transferase
TEMED	N,N,N',N'-tetramethylethylene-diamine
TSB	Tris buffer saline
TSBT	Tris buffer saline tween
U	Uracil

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

OESOPHAGUS – BARRETT'S OESOPHAGUS AND BARRETT'S ASSOCIATED NEOPLASIA: PATHOLOGY AND MOLECULAR BIOLOGY

1. INTRODUCTION

1.1 OESOPHAGUS: Normal Histology and Function

The oesophagus is a muscular tube which is in a collapsed state most of the time although it distends to allow the passage of food, saliva, or gastric content during eating, swallowing, or regurgitation. The swallowing mechanism consists of relaxation of the sphincters accompanied by the passage of peristaltic waves of contraction through both striated and smooth muscle segments. The oesophagus extends from the pharynx at the level of the 6th cervical vertebra to the level of the diaphragm at the 11th or 12th thoracic vertebra. It measures 24-30 cm in the adult, from 1.3-3 cm in its lateral diameter and approximately 1.9 cm in its anterior-posterior diameter. The entrance is encircled by the crico-pharyngeus muscle which acts as the upper oesophageal sphincter. The lower oesophageal sphincter at the gastro-oesophageal junction is a physiological entity rather than an anatomic structure and it can be regarded as a closing segment. The purpose of the sphincter is to prevent regurgitation of gastric contents into the oesophagus and to facilitate relaxation during swallowing in order to allow the passage of food. The oesophageal wall is composed of four layers-mucosa, submucosa, mascularis propria and adventitia (Figure 1.1) (McGee *et al*, 1992; Sternberg, 1992).

1.1.1 Mucosa

The mucosa consists of a nonkeratinised stratified squamous epithelium, lamina propria and muscularis mucosa. The epithelium is divided into the basal and suprabasal cell layers. The basal layer occupies approximately 10-15% of the epithelium; being 1-3 cells thick; however, in the distal 3cm, approximately 60% of normal individuals (without evidence of gastro-oesophageal reflux) may show basal cell hyperplasia of greater than 15% (Weinstein *et al*, 1975). The upper extent of the basal zone has been arbitrarily defined as the level where the nuclei is separated by a distance equal to their diameter (Groben *et al*, 1987; Cooper *et al*, 1989). The basal cells are responsible for epithelial regeneration and compensate the suprabasal cell layers which consist of glycogen-rich cells that become progressively flatter toward the surface. Occasional

lymphocytes, plasma cells, and basophilic granulocytes were shown to be constituents of the normal oesophageal mucosa (Seefeld *et al*, 1977). As in the rest of the gastrointestinal tract, intra-epithelial lymphocytes are OKT3 (antihuman T cell antibody)/OKT8 (antihuman cytotoxic T cell) positive, indicating suppressor/cytotoxic function. Langerhans cells, which are OKT6 (antihuman thymocyte) and OKIa (Ia like antigen) positive, are also located in the suprabasal location with similar function to that of skin cells (Seefeld *et al*, 1977; Geboes *et al*, 1983).

The lamina propria is the nonepithelial portion of the mucosa above the muscularis mucosa consisting of areolar connective tissue and containing vascular structure, scattered inflammatory cells and mucus-secreting glands. In adults, the presence of scattered inflammatory cells including lymphocytes and plasma cells is considered a normal finding and does not correlate with acid reflux (Goldman *et al*, 1982; Groben *et al*, 1987). Lymphocytes identified in the lamina propria are both OKT 4 and OKT 8 positive with the T4 population predominantly (Seefeld *et al*, 1977). IgA-producing B-cells (plasma cells) predominate, with a smaller population of IgG- and IgM- producing B cells (Seefeld *et al*, 1977). Fingerlike extensions of lamina propria, termed papillae, extend into the epithelium, with the maximum depth of extension allowable in the normal oesophagus varying from 50-75% (Goldman *et al*, 1982; Brown *et al*, 1984). In the distal 3 cm of the oesophagus, however, up to 60% of individuals without reflux demonstrated papillary lengths that may exceed these values (Weinstein *et al*, 1975).

The muscularis mucosa is composed of smooth muscle bundles oriented longitudinally (Goyal, 1985), rather than having both a circular and longitudinal arrangement as in the stomach. The muscularis mucosa begins at the cricoid cartilage of the pharynx and becomes thicker distally. At the gastro-oesophageal junction, the oesophagus muscularis mucosa is thicker than that of the stomach. This thicker appearance along with the longitudinal arrangement can sometimes be used to identify an oesophageal origin for biopsy. The differences between the muscularis mucosa of the stomach and oesophagus can be used to identify the muscular gastro-oesophageal junction (Sternberg, 1992).

1.1.2 Submucosa

The submucosa consists of loose connective tissue containing vessels, nerve fibers, lymphatic and submucosal glands. The submucosal glands are considered to be a

continuation of the minor salivary glands of the oropharynx and are scattered throughout the entire oesophagus, but are more concentrated in the upper and lower regions (Enterline *et al*, 1984). These glands consist of mucinous cells with or without a minor serous component and they produce acid mucins. The glands are drained by ducts, initially lined by a single layer of cuboidal epithelium, becoming stratified squamous in type, which penetrate the muscularis mucosa and epithelium to open into the oesophageal lumen. The presence of submucosal glands is indicative of an oesophageal origin because these glands are not present in the stomach (Sternberg, 1992).

1.1.3 Muscularis propria

The muscularis propria is composed of two well-defined muscle layers. The inner layer is circular and the outer layer longitudinal. A thin layer of connective tissue lies between the two muscle layers. In the upper third of the oesophagus the muscularis propria consists primarily of striated skeletal muscle. In the middle third, both layers exhibit a mixture of smooth muscle, while, in the lower third of the oesophagus, only smooth muscle is found (Meyer *et al*, 1986; Sternberg, 1992).

1.1.4 Adventitia

The adventitia of the oesophagus consists of a loose connective tissue layer that blends with the adventitia of the trachea and the surrounding structure. Adipose tissue, large blood vessels and nerves forming the neurovascular bundle are present in the adventitia (Eroschenko, 1996).

1.2 BARRETT'S OESOPHAGUS

1.2.1 Definition and Histological Features

Barrett's oesophagus is a condition in which the normal squamous epithelium is replaced by a metaplastic columnar epithelium (Figure 1.2) (Spechler *et al*, 1987; Phillips *et al*, 1991). It develops as a complication in approximately 10-12% of patients with chronic gastro-oesophageal reflux and predisposes to the development of adenocarcinoma of the oesophagus (Spechler *et al*, 1987; Hamilton *et al*, 1988; Phillips *et al*, 1991). Three types of columnar mucosa may be identified in Barrett's oesophagus; (1) intestinal - type mucosa, (2) junctional - type mucosa, and (3) fundic - type mucosa.

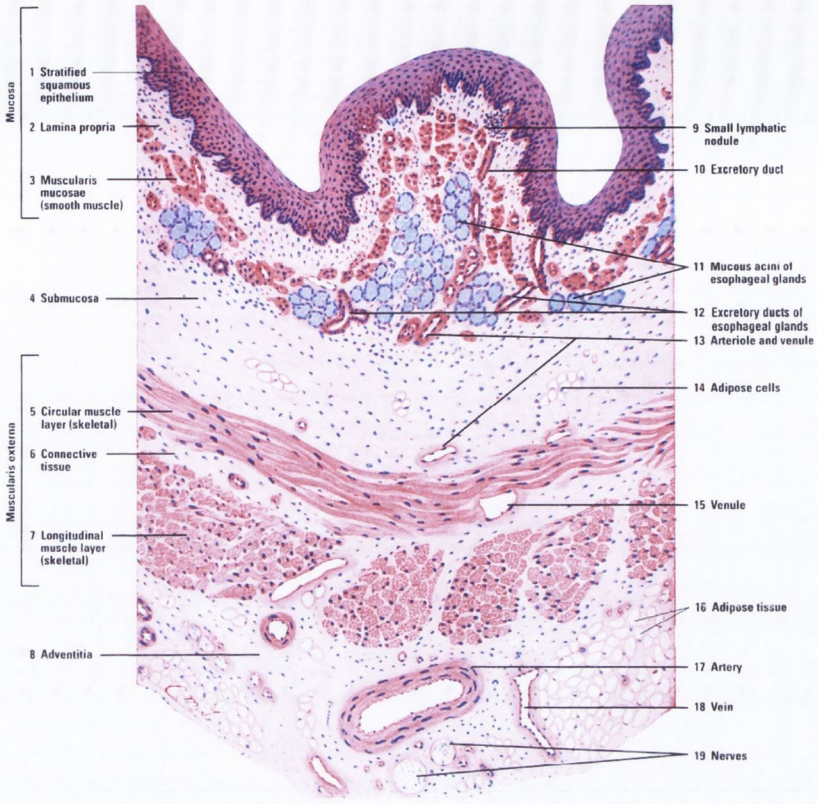

Figure 1.1. Microscopic view of a transfer section of the normal oesophageal wall. Section stained with haematoxylin and eosin (Eroschenko, 1996).

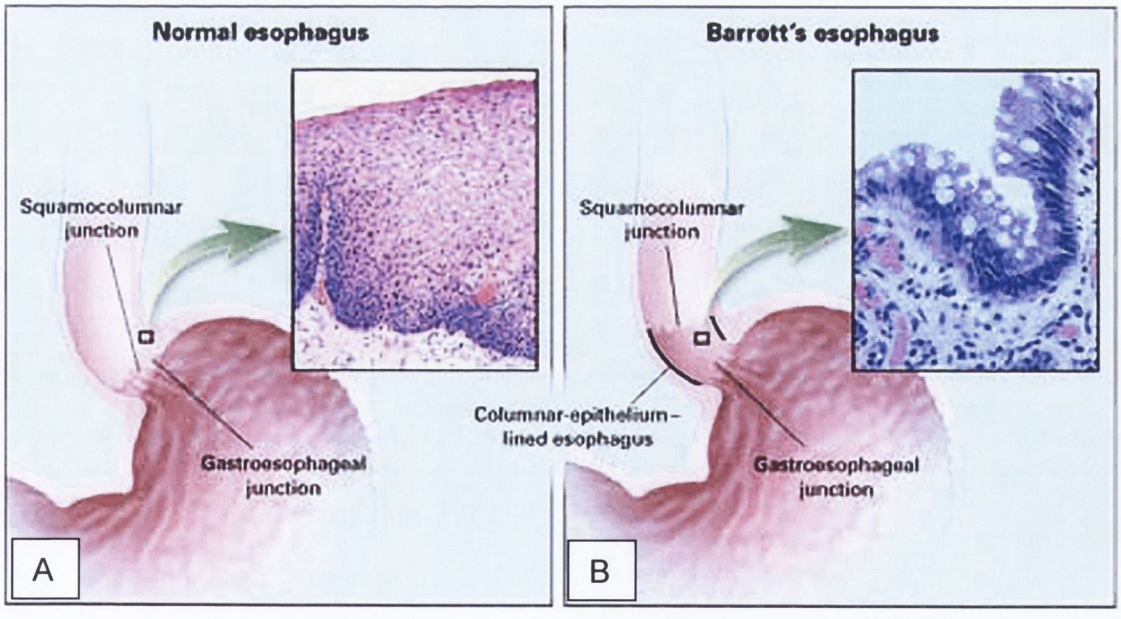

Figure 1.2. Illustration of the oesophago-gastric junction and microscopic view of transfer section of (A) normal oesophageal squamous mucosa and (B) Barrett's mucosa. (Spechler, 2002).

The intestinalised (specialised) columnar epithelium is by far the most frequent epithelium type identified and is clearly associated with the malignant transformation (Paull *et al*, 1976; Thompson *et al*, 1983). Specialised - type epithelium is characterised by the presence of goblet and columnar cells. The goblet cells contain acid mucins, thus staining positive with alcain blue at pH 2.5. The columnar epithelial cells may resemble either small intestinal absorptive cells (complete intestinal metaplasia) or gastric foveolar cells (incomplete intestinal metaplasia). These metaplastic cells resemble abnormal features, which distinguish them from their normal counterparts. In the case of complete intestinal metaplasia, the small intestinal-like columnar cells may demonstrate a brush border, but it is not well developed and lacks the uniform enzymatic activity normally found in the brush border of the small intestine (Trier, 1985). Ultrastructurally, these metaplastic cells have been shown to contain mucin granules, which are not present in the absorptive cells of the small intestine (Trier, 1985; Reid, 1987; Reid *et al*, 1988). The gastric foveolar-like columnar cells frequently contain alcian blue positive acid mucin contrary to those that normally populate the stomach, which contains alcian blue negative, neutral mucines (Trier, 1985).

The junctional-type mucosa is indistinguishable from normal gastric cardia mucosa. In cases where Barrett's mucosa extended proximally for more than 3cm, junctional-type mucosa is usually present with specialised-type epithelium (Reid *et al*, 1988).

The fundic type mucosa is uncommon and if present, resembles the transitional epithelium seen at the junction of the gastric body with the antrum or cardia with a scattering of parietal and chief cells. Fundic type mucosa without intestinalisation and resembling normal gastric fundus mucosa can be seen in childhood Barrett's oesophagus. The lack of intestinalised mucosa is a feature usually confined to children; it is likely that specialisation occurs later in early adult life (Sternberg, 1992).

Grossly, a well-defined area of red velvety mucosa similar to the adjacent gastric mucosa usually represents Barrett's mucosa. It has irregular margins and may contain islands of residual squamous, pearly white oesophageal mucosa, or it may be ulcerated. It is usually limited to the lower third of the oesophagus, but in severe cases, it may extend to the middle and upper oesophagus. The endoscopic diagnosis of Barrett's oesophagus may be challenging, especially if the gastroesophageal junction is difficult to identify (Woolf *et al*, 1989).

Metaplastic Barrett's mucosa has been further characterised for dysplasia (development of neoplastic epithelium) as being non-dysplastic or showing low-grade or high-grade dysplasia. (Riddell *et al*, 1983; Ming *et al*; 1998). Low-grade dysplasia is distinguished (1) by enlarged nuclei that remain stratified and occupy less than 50% of the overall cell size, and (2) by general preservation of overall crypt architecture. High-grade dysplasia, in contrast, is characterized by nuclei that are more than 50% of cell size, with (1) loss of basilar stratification, (2) distorted and branched glands and crypts, (3) frequent mitotic figures, and (4) loss of mucin. If features of dysplasia are present but do not extend to the surface epithelium or if these changes are associated with severe inflammation, this will be classified as indeterminate (Hamilton, 1989; Antonioli, 1993).

The exact mechanism by which the squamous epithelium is replaced by the metaplastic mucosa is not certain. However, it seems that initially, following erosion of the squamous mucosa by the acid-peptic action of the gastric content, undifferentiated progenitor cells migrate into the denuded areas. These cells are multipotential stem cells that, in the presence of persistent gastro-oesophageal reflux, selectively differentiate into columnar mucin-secreting epithelium. Alternatively, metaplasia can occur simply by upward migration of the columnar epithelium from the stomach to reepithelialise the ulcerated mucosa (Hamilton, 1985; Sternberg, 1992).

1.2.2 Etiology and Pathogenesis

Patients with Barrett's oesophagus acquire the columnar metaplasia in the lower oesophagus as a consequence of gastroesophageal reflux. Therefore, any condition increasing the reflux of acid from the stomach to the oesophagus represents a risk factor. These conditions include a hiatal hernia, the presence of duodenogastric reflux, delayed oesophageal acid clearance time, and decreased resting pressure of the lower oesophageal sphincter (Mulholland *et al*, 1989; Collen *et al*, 1990; Johnston *et al*, 1995). Other etiologic factors such as bile reflux following gastrectomy (Meyer *et al*, 1979) oesophageal injury (Othersen *et al*, 1993) and possibly, congenital rest of gastric epithelium (infantile Barrett's) (Sternberg, 1992) have also been associated with the development of Barrett's oesophagus. In 1990 and in effort to determine the incidence of Barrett's oesophagus in the general population, a study from the Mayo Clinic (Cameron *et al*, 1990) compared cases diagnosed by endoscopy (clinically evident

cases) with those diagnosed at autopsy (clinically silent cases). Investigators reported a rate of clinically diagnosed Barrett's oesophagus at 22 per 100,000 people and an autopsy rate of 376 per 100,000 a 17-fold difference. It has also been shown that 18% of patients undergoing upper gastrointestinal endoscopey for any reason are found to have Barrett's oesophagus (Speckle *et al*, 1996). The implications of these observations are significant in that if more than 90% of cases of Barrett's oesophagus remain undetected, treatment and surveillance of known cases will have little impact on the overall death rate from this disorder.

The prevalence of Barrett's oesophagus increases with age (although childhood cases have been described, most notably in children with such comorbidities as neurologic impairment, chronic lung disease, and oesophageal atresia). The median age at diagnosis is the mid 40s, and the curve plateaus at ages in the 60s. The male-female ratio is roughly 2:1. There is also a correlation with increased duration of reflux symptoms (Cameron, 1997).

1.2.3 Prevention and Regression of Barrett's Oesophagus

In the treatment of Barrett's oesophagus the aim of intervention strategies are to diminish complications, most notably progression to cancer. To date, no treatments have been shown to reverse the progression of Barrett's oesophagus completely or to alter its natural history once it has developed (Barr *et al*, 1996). Pharmacological approaches including the use of prolonged high-dose proton pump inhibition or successful antireflux surgery indicated that fewer than 10% of Barrett's cases regress and progression to cancer may occur over a short span of 3 years (Sagar *et al*, 1995; Prach *et al*, 1997). Moreover, persistent genetic abnormalities in Barrett's mucosa were reported following photodynamic therapy (Krishnadath *et al*, 2000). Experimental approaches to arresting Barrett's progression are in the preliminary stages and include the use of difluoromethylornithine (eflornithine hydrochloride [Ornidyl]), heat-shock protein-27 and cyclooxygenase-2 (COX-2) inhibitors (Heath *et al*, 2000; Buttar *et al*, 2002). Ablation therapy utilising genetic approaches that target genetically defected pre- or neoplastic- cells are in progress and a phase 1 study has been reported (Cohen *et al*, 2001). Molecular approaches to the treatment of Barrett's and oesophageal adenocarcinoma are further discussed in (section 1.6).

1.2.4 Cancer Risk in Barrett's Oesophagus

Barrett's oesophagus predisposes to the development of adenocarcinoma. However, cancer is not common in absolute terms, and other factor such as alcohol, smoking, diet and *Helicobacter pylori* infection may play a role in the neoplastic development (Levi *et al*, 1990; Abbas *et al*, 1995; Wolfgarten *et al*, 2001). The risk of malignancy is about 30-40 times higher among patients with Barrett's oesophagus than in the normal population (Spechler *et al*, 1984; Van der Veen *et al*, 1989). Thus, once the diagnosis is made, periodic endoscopy with biopsy and histological examination is recommended for detection of dysplasia and early carcinoma.

1.3 MALIGNANT PROGRESSION IN BARRETT'S OESOPHAGUS

It has been hypothesized that neoplastic progression develops as a consequence of an acquired genetic instability and the subsequent evolution of clonal populations with an accumulation of genetic errors (Nowell, 1976). Accordingly, human cancer and some premalignant lesions contain multiple genetic abnormalities not present in the normal tissue from which the neoplasms arose (Fearon *et al*, 1990; Boland *et al*, 1995). Adenocarcinoma develops in Barrett's oesophagus by a multistep process in which the metaplastic epithelium progresses to low- high -grade dysplasia and eventually to invasive adenocarcinoma (Figure 1.3) (Hameeteman *et al*, 1989; Reid, 1991; Reid *et al*, 1992). The exact sequence of genetic and epigenetic events is not known and neither it is uniform. Nevertheless, there is substantial evidence that the progression to adenocarcinoma in Barrett's oesophagus is associated with a process of clonal evolution and progressive accumulation of genetic abnormalities. The most important molecular changes include impaired regulation of the cell cycle, altered function of known oncogenes and tumour-suppressor genes, as well as mismatched repair and cell-adhesion molecules (Figure 1.4).

1.3.1 Cell Proliferation and Cell-Cycle Regulatory Genes

In a multistep progression from Barrett's oesophagus to fully developed carcinoma, accelerated proliferation may indicate genomic instability and, therefore, may be an important factor in the pathogenesis and prediction of malignant transformation. Several studies have used Ki-67 (MIB-1) and proliferating cell nuclear antigen (PCNA) antibody to study the proliferative properties in normal and neoplastic cell populations.

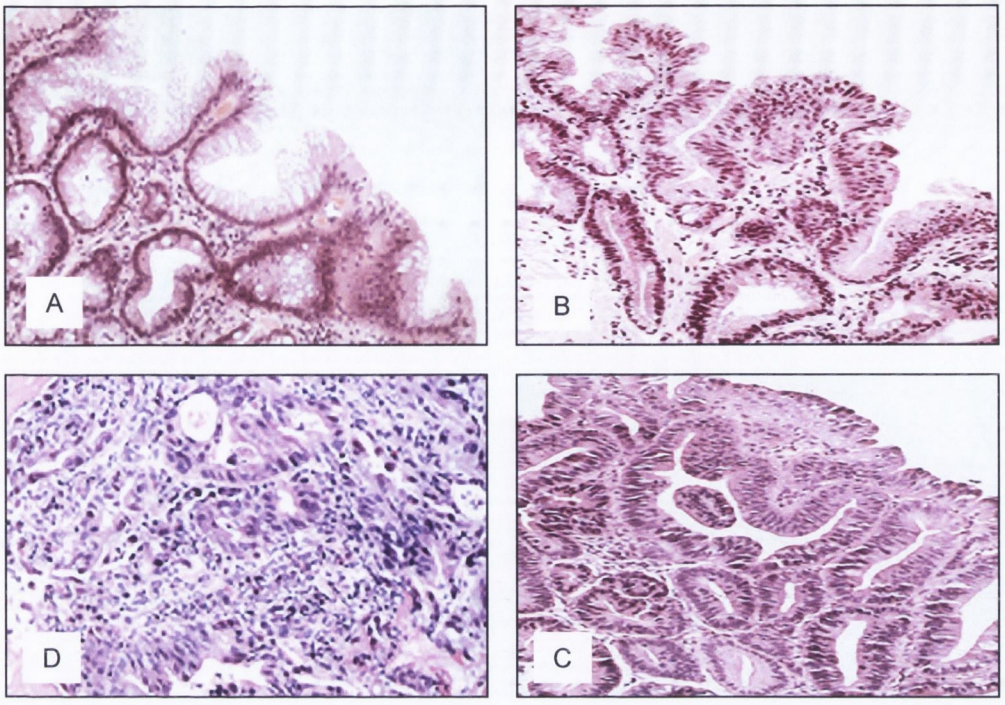

Figure 1.3. Representation of the proposed metaplasia (A) - dysplasia (B- low grade & C- high grade) – carcinoma (D) sequence of the evolution of Barrett's oesophagus to oesophageal adenocarcinoma.

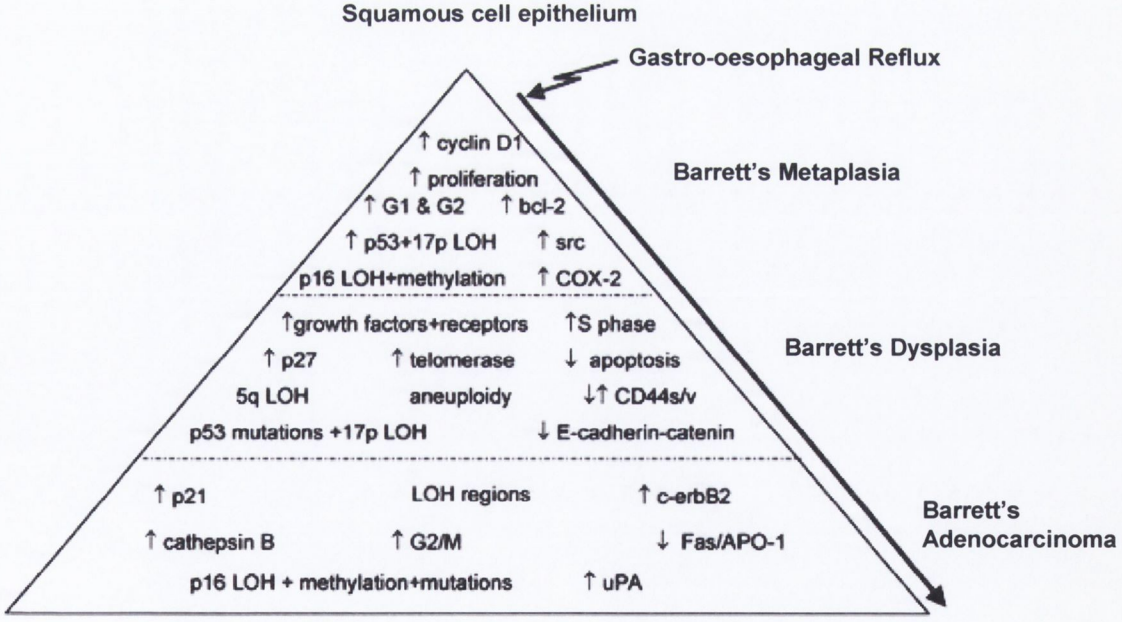

Figure 1.4. Schematic diagram of genetic alterations involved in the progression of Barrett's metaplasia toward Barrett's adenocarcinoma. Alterations that occur in the early stages are usually also present in the advanced histologic stages. Increased expression (↑) , decreased expression (↓).

An increased number of proliferating cells and an expansion of the proliferative compartment were shown in Barrett's oesophagus and adenocarcinoma (Gillen *et al*, 1994; Soslow *et al*, 1999; Whittles *et al*, 1999). Expression of proliferating cell nuclear antigen (PCNA) was mainly seen in the basal cells of the neck/foveolar epithelial compartment of the glands in Barrett's oesophagus. However, in mucosa with high-grade dysplasia, the proliferative compartment extended upward into the superficial layers of the glands (Hong *et al*, 1995; Lauwers *et al*, 1997). The expression of Ki-67 also correlated with the histological findings in Barrett's epithelium. The number and the localisation of Ki-67-positive nuclei were significantly different between nondysplastic and low-grade and high-grade dysplastic Barrett's lesions (Polkowski *et al*, 1995; Yacoub *et al*, 1997; Rioux *et al*, 1999).

The mammalian cell cycle is divided into several distinct phases M, G1, S and G2. A normal cell has a chromosome number of 2N, for which the term diploid is applied. Cells reproduce by duplicating their content (4N) and then dividing in two (Figure 1.5). The cell cycle checkpoints are controlled by cyclin dependent kinases (CDK) which are composed of two proteins - a cyclin (structural protein) and a kinase (enzyme). A succession of kinases (CDK4, CDK2, and CDC2) is expressed along with a succession of cyclins (D, E, A, and B) as cells go from G1 to S to G2 to M (Figure 1.6).

It has been reported that the evolution from normal squamous epithelium to metaplastic Barrett's epithelium is frequently associated with abnormal DNA content (aneuploidy) and an increased G2/M fraction of the metaplastic cells (Reid *et al*, 1987; Levine *et al*, 1989; Reid *et al*, 1992). Moreover, abnormal DNA content shows a correlation with the histological diagnosis of dysplasia and carcinoma (Montgomery *et al*, 1996; Gimenez *et al*, 1998). A flow cytometry analysis (Reid *et al*, 1992) showed that 70% of patients with aneuploidy or increased G2/tetraploid fraction in biopsy specimens obtained during initial endoscopic evaluation developed high-grade dysplasia or cancer, whereas no patients without abnormalities showed progression to high-grade dysplasia or invasive carcinoma. In addition, several studies have reported that Barrett's adenocarcinoma with abnormal DNA content is associated with increased lymph node metastasis, advanced disease and poorer survival (Schneeberger *et al*, 1990; Nakamura *et al*, 1994; Bottger *et al* 1999). Cyclin D1 abnormalities, either gene amplification or overexpression, lead to constitutive activation of the cyclin D1-CDK4/6 pathway.

Expression of cyclin D1 is observed in Barrett's metaplasia especially in intestinal-type lesions and in 22% to 64% of oesophageal adenocarcinomas (Roncalli *et al*, 1998; Arber *et al*, 1999; Arber *et al*, 1996, Kuwahara *et al*, 1999). Amplification of the cyclin D1 gene was observed in 16-26% of oesophageal adenocarcinomas (Shimada *et al*, 1997; Morgan *et al*, 1999).

Another cell-cycle regulatory gene is p27. Overexpression of p27 induces a block during G1 in the cell cycle. P27 protein expression and p27 mRNA was found to be increased in intensity and distributed throughout the glands of high-grade dysplastic Barrett's epithelium, indicating transcriptional upregulation of p27. In contrast, low p27 protein expression but elevated levels of p27 mRNA were found in 83% of oesophageal adenocarcinomas, possibly because of post-transcriptional regulation of the gene (Singh *et al*, 1998). In addition to nuclear immunoreactivity, cytoplasmic staining of p27 was noted in 48% and 26% of cases of dysplasia and carcinoma, respectively (Singh *et al*, 1998). Loss of nuclear or cytoplasmic staining for p27 correlated with higher histological grade, depth of invasion, presence of lymph node metastasis, and shorter survival (Singh *et al*, 1998). These findings suggest that the cell cycle inhibitor p27 may be overexpressed to counteract proliferative stimuli in Barrett's-associated dysplasia. Loss of p27 or altered subcellular localization as the process becomes invasive suggests an important role for this CDK inhibitor in preventing the progression of Barrett's oesophagus to adenocarcinoma. (Ellis *et al*, 1997).

The G1-S phase of the cell cycle can also be downregulated by inhibition of CDKs by p21WAF1/CIP1 (p21). Nuclear expression of p21 is upregulated by the wild-type p53 tumour suppressor but not by mutated p53. Expression of p21 protein was found to be elevated in Barrett's epithelium classified as indefinite for dysplasia, low-grade dysplasia, high-grade dysplasia, and Barrett's adenocarcinoma, but not in Barrett's oesophagus negative for dysplasia (El-Deiry *et al*, 1993). No relationship between p21 and p53 staining in oesophageal adenocarcinomas was found, indicating that there are also p53-independent pathways for the upregulation of p21 (El-Deiry *et al*, 1993; Moskaluk *et al*, 1996). It has also been reported that elevated nuclear p21 expression in Barrett's and oesophageal adenocarcinoma probably does not represent mutated p21 protein and could be regarded as a prognostic factor (Shiohara *et al*, 1994; Kuwahara *et al*, 1999).

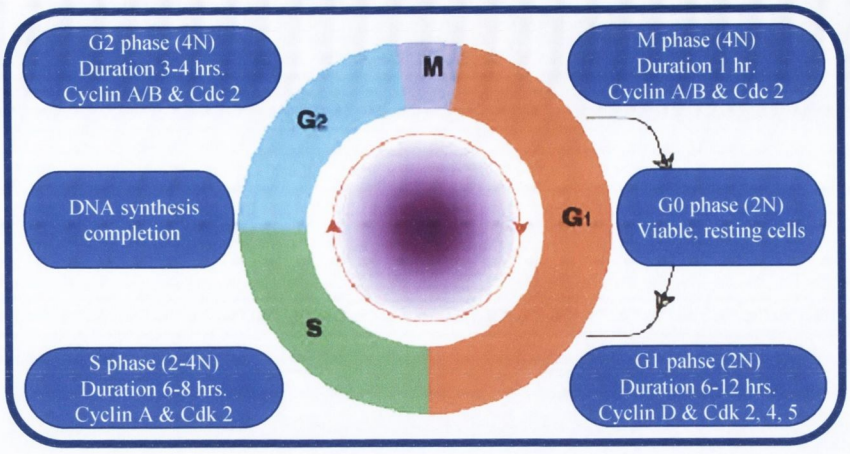

Figure 1.5. Schematic diagram of the cell cycle. Mitosis (M phase) is the process of nuclear division. Replication of DNA occurs in the S (synthesis) phase. The interval between M phase and S phase is called the G1 (gap) phase, and the interval between the end of the S phase and the beginning of the M phase is the G2 phase. Cells can exit the cell cycle and enter the G0 phase, which is the quiescent state.

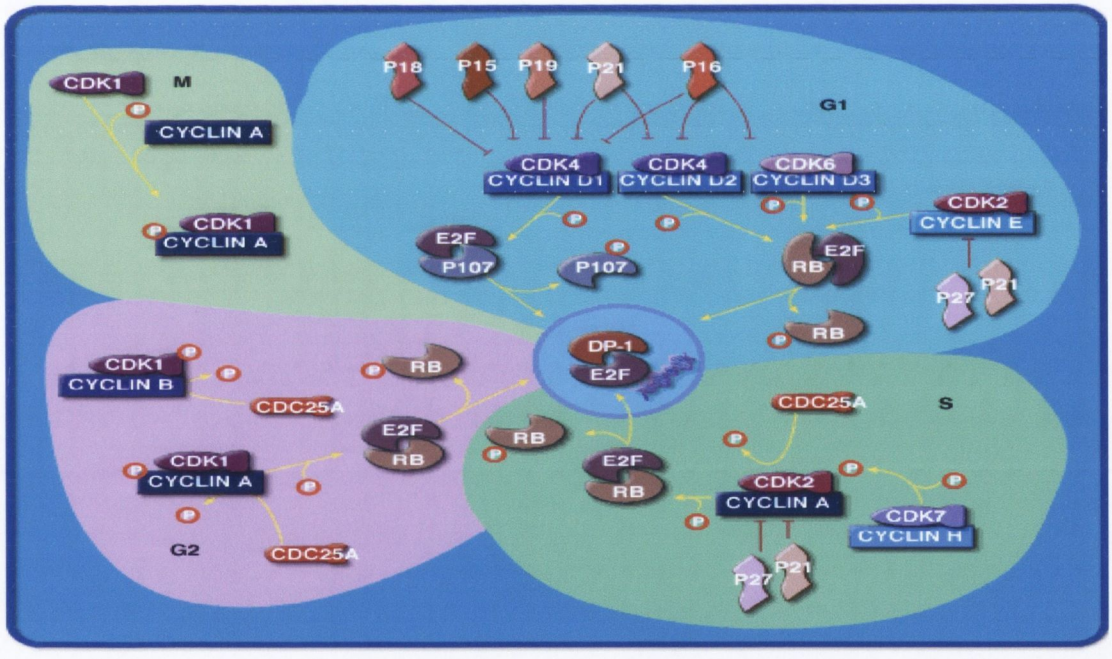

Figure 1.6. Schematic diagram of the cell cycle in G1-S-G2 and M phases and the interplay of many associated proteins. Key among these are the cyclins which are expressed and then degraded in a concerted fashion to drive the stages of the cell cycle. Cyclins combine with cyclin dependent kinases (cdks) to form activated kinases that phosphorylate targets leading to cell cycle regulation. A breakdown in the regulation of this cycle can lead to out of control growth and contribute to tumour formation. Defects in many of the molecules that regulate the cell cycle have been implicated in cancer. Key among these are p53, the cdk inhibitors (p15, p16, p18, p19, p21, p27), and Rb, all of which act to keep the cell cycle from progressing until all repairs to damaged DNA have been completed.

1.3.2 Chromosomal Abnormalities and Microsatellite Instability

Karyotyping and in situ hybridization studies of Barrett's and oesophageal adenocarcinoma have identified several chromosomal alterations including loss, aneusomy, translocation, trisomy and structural rearrangements. The most consistent numeric chromosomal abnormalities found in dysplastic Barrett's oesophagus and adenocarcinoma is the loss of the Y chromosome (Garewal *et al*, 1989; Raskind *et al*, 1992;). The frequency of Y chromosome loss in Barrett's oesophagus increased in line with the grade of dysplasia and a frequency loss of 31%-93% were reported in the tumours (Krishnadath *et al*, 1995). Aneusomy of chromosome 6, 7, 11 and 12 was found to be an early event, frequently present in both Barrett's oesophagus and dysplastic regions. Trisomies for chromosome 5 and 7 and translocation involving chromosome 3 and 6 in Barrett's oesophagus have been described (Garewal *et al*, 1989). Structural rearrangments in the 1p, 3q, 11p-13 and 22p regions have also been reported in Barrett's and oesophageal adenocarcinoma (Menke *et al*, 1996). Loss of chromosomes 4, 17, 18 and 21 and overrepresentation of chromosome 6, 1, 8, 11 and 12 are also frequent chromosomal aberrations observed in oesophageal adenocarcinoma (Krishnadath *et al*, 1994; Menke *et al*, 1996; Persons *et al*; 1998).

Microsatellite instability, althoght it is not frequent, has been reported in Barrett's and adenocarcinoma of the oesophagus. Microsatellite instability at one or more chromosomal loci of the five dinucleotide microsatellite repeats has been found in 7% of patients with Barrett's metaplasia and in 22% with oesophageal adenocarcinoma (Meltzer *et al*, 1994). The microsatellite instabilities were shown to be associated with the diploid component of the tumour, suggesting that these may develop as an early event in the progression of Barrett's to adenocarcinoma. Several studies (Keller *et al*, 1995; Gleeson *et al*, 1996; Barrett *et al*, 1996; Dolan *et al*, 1998) have also confirmed the low prevalence of microsatellite instabilities in Barrett's adenocarcinoma but as yet, it is not well elucidated which mismatch repair genes are responsible for this deficiency which subsequently leads to a genome-wide accumulation of mutations and genetic errors.

1.3.3 Tumour Suppressor Genes

As their name suggests, these genes are involved in preventing the development of malignancy. These are cellular genes that primarily are involved in cell proliferation, apoptosis, cell adhesion and gene expression regulation (Figure 1.7). They are recessive

genes, which implies that both gene copies need to be inactivated to contribute to tumourigenesis. Functional inactivation of tumour suppressor genes can be caused by genetic as well as by epigenetic phenomena; mutation, deletion of part of the gene, and epigenetic silencing through promoter methylation. Studies of genetic abnormalities in oesophageal adenocarcinoma and its precursor Barrett's epithelium have shown allelic losses in multiple chromosomal loci of known tumour suppressor genes such as 17p (p53), 5q (APC), 13q (Rb) and 9p (p16) (Huang *et al*, 1992; Barrett *et al*, 1996; Hammound *et al*, 1996; Montesano *et al*, 1996; Gonzalez *et al*, 1997; Wu *et al*, 1998).

The p53 tumour suppressor gene plays an important regulatory role in cell cycle control and apoptosis (Figure 1.8) (Levine, 1997; Lundberg *et al*, 2000; Vogelstein *et al*, 2000). Investigators have detected increased frequency of p53 mutation and p53 protein accumulation that parallel an increasing degree of dysplasia in Barrett's epithelium (Blount *et al*, 1992; Kim *et al*, 1997, Wu *et al*, 1998). Identical mutations between adjacent areas of dysplasia and adenocarcinoma have been reported which supports clonal expansion as the mechanism of carcinogenesis (Gleeson et al, 1998; Reigman *et al*, 2001). Accumulation of chromosomal losses in 17p-location of p53 gene-has been reported during the malignant transformation of Barrett's adenocarcinoma (Wu *et al*, 1998). Wu and colleagues also reported that adenocarcinomas with allelic loss of 17p had worse survival than cancers with no allelic loss. Together, these observations indicate that analysis of p53 status may serve as an objective biomarker during the neoplastic progression of Barrett's epithelium. The role of the p53 tumour suppressor gene in the neoplastic progression of Barrett's mucosa and tumour response to chemoradiotherapy are further discussed in (chapter 3).

Loss of heterozygosity of the adenomatous polyposis coli (APC) locus on 5q has been found in oesophageal adenocarcinoma and in the surrounding high-grade dysplastic Barrett's epithelium. Moreover, the patterns of allelic loss of the gene were identical in all stages of neoplastic progression, suggesting the emergence of a clonal population of cells (Zhuang *et al*, 1996; Wu *et al*, 1998). However, loss of heterozygosity has not been found in Barrett's metaplasia and low-grade dysplatia (Gonzalez *et al*, 1997). Promoter hypermethylation appears to be another means of inactivating the APC gene. APC hypermethylation has been repoted to occur in 92% of patients with oesophageal adenocacinoma and in 39% of patients with Barrett's metaplasia, whereas, no

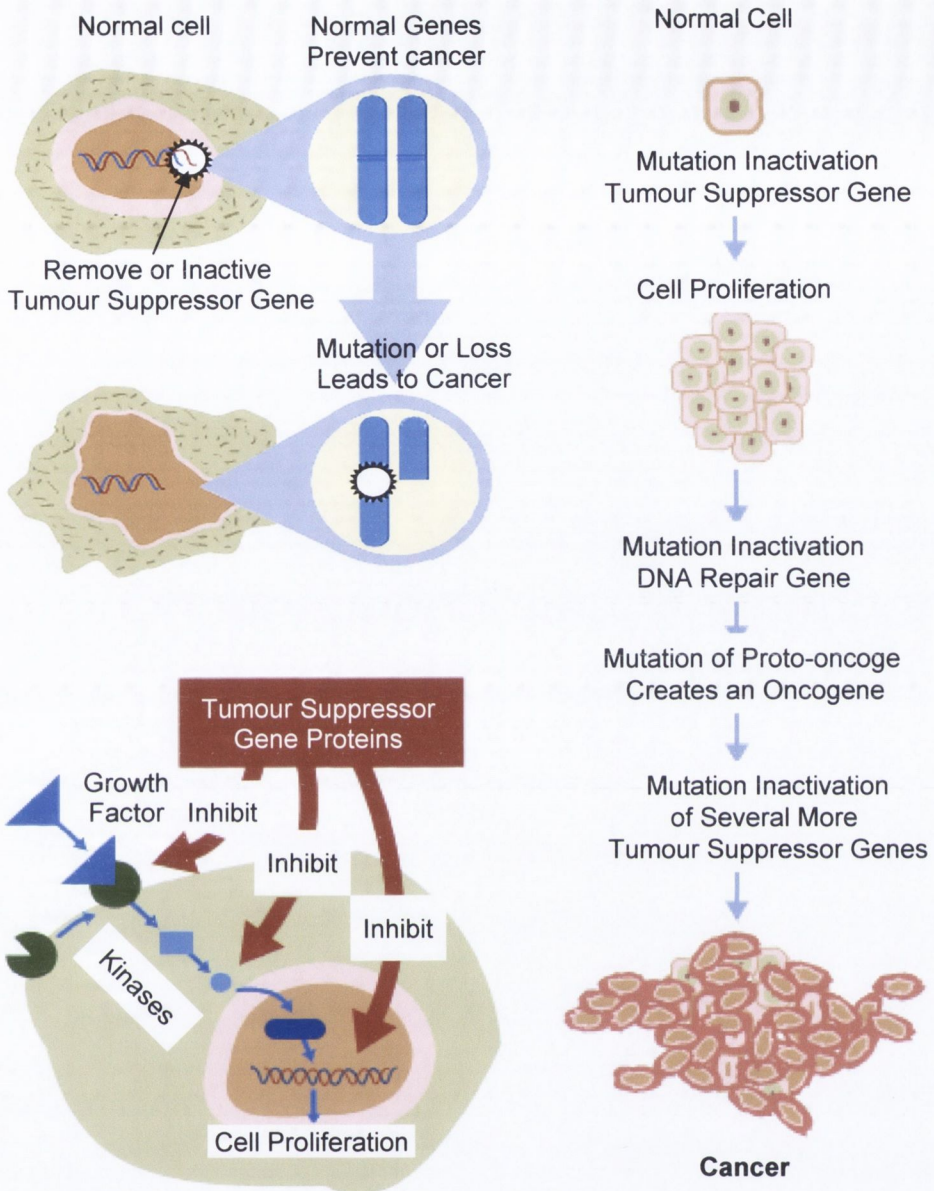

Figure 1.7. Schematic representation of the function of tumour suppressor genes. Tumour suppressor genes are a family of normal genes that instruct cells to produce proteins that restrain cell growth and division. The functional loss of such proteins allows a cell to grow and divide in an uncontrolled fashion. The proliferating cells then tend to acquire subsequent mutations involving a DNA repair gene and several other tumour suppressor genes. The accumulated damage yields a highly malignant and metastatic tumour.

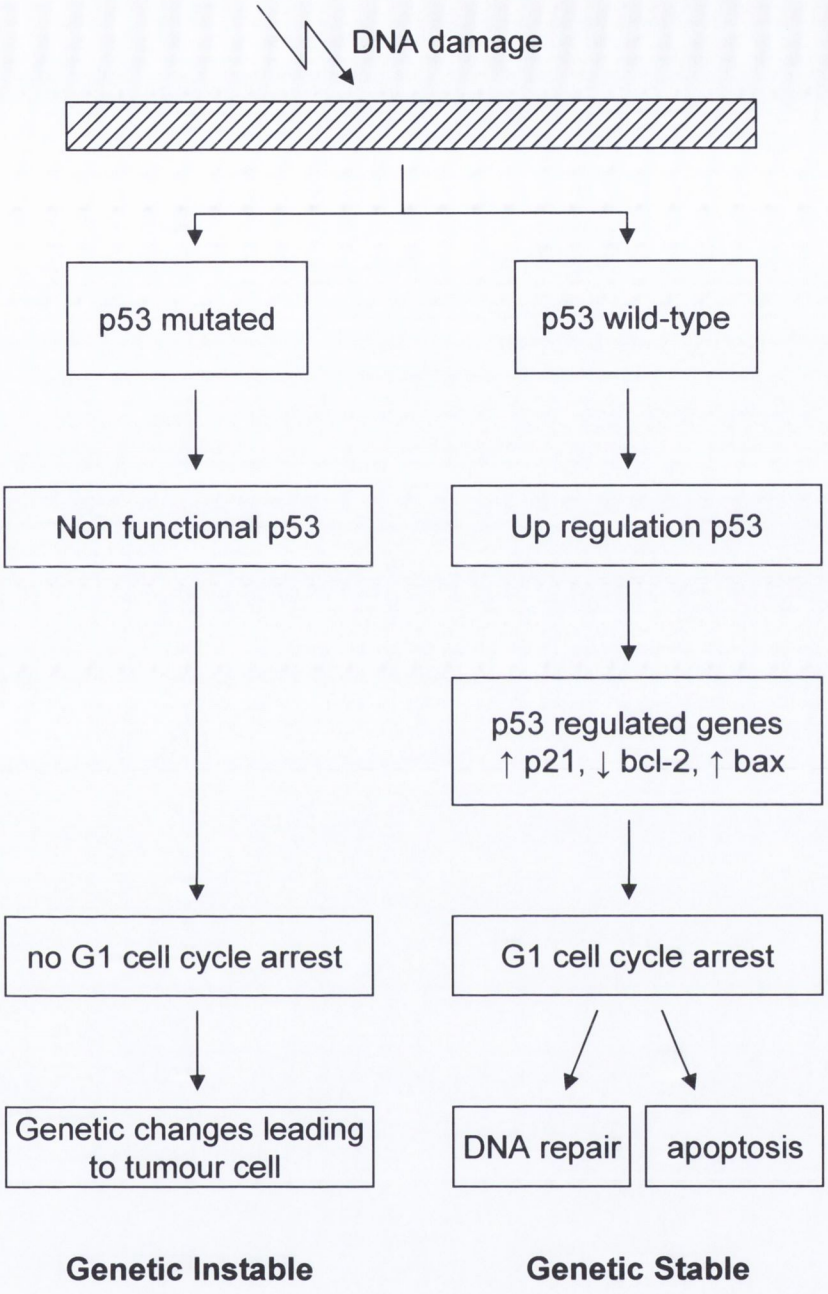

Figure 1.8. Schematic diagram of the normal function of wild-type p53. In response to DNA damage, wild-type p53 activates other genes (e.g. p21, bax, bcl-2), that control the cell cycle. In the absence of or with a mutant p53, there is no G1 arrest and therefore a possibility of increased mutation or genomic instability arises.

hypermethylation was observed in normal oesophageal tissues (Kawakami *et al*, 2000). These observations indicate that inactivation of the APC gene due to promoter hypermethylation may occur in the early development of oesophageal adenocarcinoma while loss of heterozygosity and mutations may develop later. Allelic loss of 17p (p53) appeared to precede allelic loss of 5q (APC) during neoplastic progression of oesophageal adenocarcinoma and may be occurring before the development of recognisable morphological change (Blount *et al*, 1993).

The protein encoded by the normal retinoblastoma (Rb) gene is a critical regulatory molecule in the G1 phase of the cell cycle. Mutation in Rb gene results in uncontrolled cell proliferation and predispostion to cancer. Loss of heterozygosity of 13q (locus of Rb-gene) has been shown in oesophageal adenocarcinoma (Boynton *et al*, 1991) and was associated with an unfavorable survival rate (Roncalli *et al*, 1998). Loss of normal Rb protein expression was observed as the Barrett's metaplasia progressed to dysplasia and carcinoma, indicating early accumulation of unstable aberrant protein (Coppola *et al*, 1999; Soslow *et al*, 1999).

Abnormalities involving the p16 gene (also known as cyclin-dependent kinase N2 [CDKN2], p16 [INK4a], or MTS1) are highly prevalent in oesophageal adenocarcinomas. Loss of heterozygosity at 9p21 chromosome (locations for p16) is frequently observed in the Barrett's and oesophageal adenocarcinoma (Barett *et al* 1996, Gonzalez *et al*, 1997; Galipeau *et al*, 1999). During neoplastic progression of Barrett's oesophagus 9p21 allelic losses and p16/CDKN2 mutations develop as early lesions in diploid cells before aneuploidy and cancer (Barett *et al* 1996). Like the APC gene, p16 promoter methylation (with or without loss of heterozygosity) is a common mechanism of its inactivation during neoplastic progression in Barrett's oesophagus and is already present in nondysplastic premalignant Barrett's (Wong *et al*, 1997; Klump *et al*, 1998). The association between p16 and the Rb gene is that p16 gene product can form complexes with the cyclin-dependant kinases CDK4 and CDK6 thereby inhibiting their ability to phosphorelate the Rb protein. Unphosphorelated Rb protein prevents the cells from entering the S phase of the cell cycle resulting in the accumulation of uncontrolled cell proliferation and subsequent clonal expansion. Therefore, p16 inactivation may be a useful biomarker to stratify the risk of pregression of Barrett's metaplasia to adenocarcinoma (Galipeau *et al*, 1999).

1.3.4 Proto-oncogenes

Proto-oncogenes are dominant genes that all act in signal transduction from extracellular stimuli to the nucleus or in regulation of gene expression. On activation of proto-oncogenes by mutation, amplification, translocation and so forth, these genes turn into oncogenes with unregulated, constitutive activity (Figure 1.9). The protein products of proto-oncogenes and their transforming variants are involved in a variety of cellular processes linked to intracellular signalling and cell-cycle control and they can be grouped into distinct classes based on their subcellular localization and biochemical activity. These include (1) growth factors and their receptors with tyrosine kinase activity; (2) cytoplasmic protein with tyrosine kinases; (3) membrane-associated guanine nucleotide-binding proteins; (4) soluble cytoplasmic serine-threonine-specific protein kinases; (5) nuclear proteins and (6) cytoplasmic proteins that affect cell survival (Figure 1.10).

Elevated levels of the epidermal growth factor receptor, a growth-factor-receptor tyrosine kinase, and/or its cognate ligands have been identified as a common component of multiple cancer types and appear to promote solid tumour growth. Epidermal growth factor (EGF) has a stimulatory effect on epithelial cell proliferation in the gastrointestinal tract and has been shown to be overexpressed in Barrett's and oesophageal adenocarcinomas (Jankowski *et al*, 1993; Yacoub *et al*, 1997). Although EGF is expressed in Barrett's epithelium, the expression of EGF does not discriminate between dysplastic and neoplastic epithelium. In contrast, transforming growth factor-alpha (TGF-α), which is structurally and functionally related to EGF expression is increased in metaplastic, dysplastic and neoplastic tissue of the oesophagus compared with normal mcosa (Jankowski *et al*, 1993). Overexpression of epidermal growth factor receptor (EGFR) in the oesophagus correlates with the degree of mucosal dysplasia and the occurrence of adenocarcinoma, suggesting that high expression levels may reflect increased malignant transformation potential in Barrett's oesophagus (Jankowski, *et al*, 1993; Yacoub *et al*, 1997).

In contrast to TGF-α, transforming growth factor-beta (TGF-β) is a potent inhibitor of cell proliferation, an inducer of differentiation in epithelial cells of the intestine, and a suppressor of genomic instability (Glick *et al*, 1996). There is evidence that the TGF-β signalling pathway is involved in the initiation and progression of oesophageal

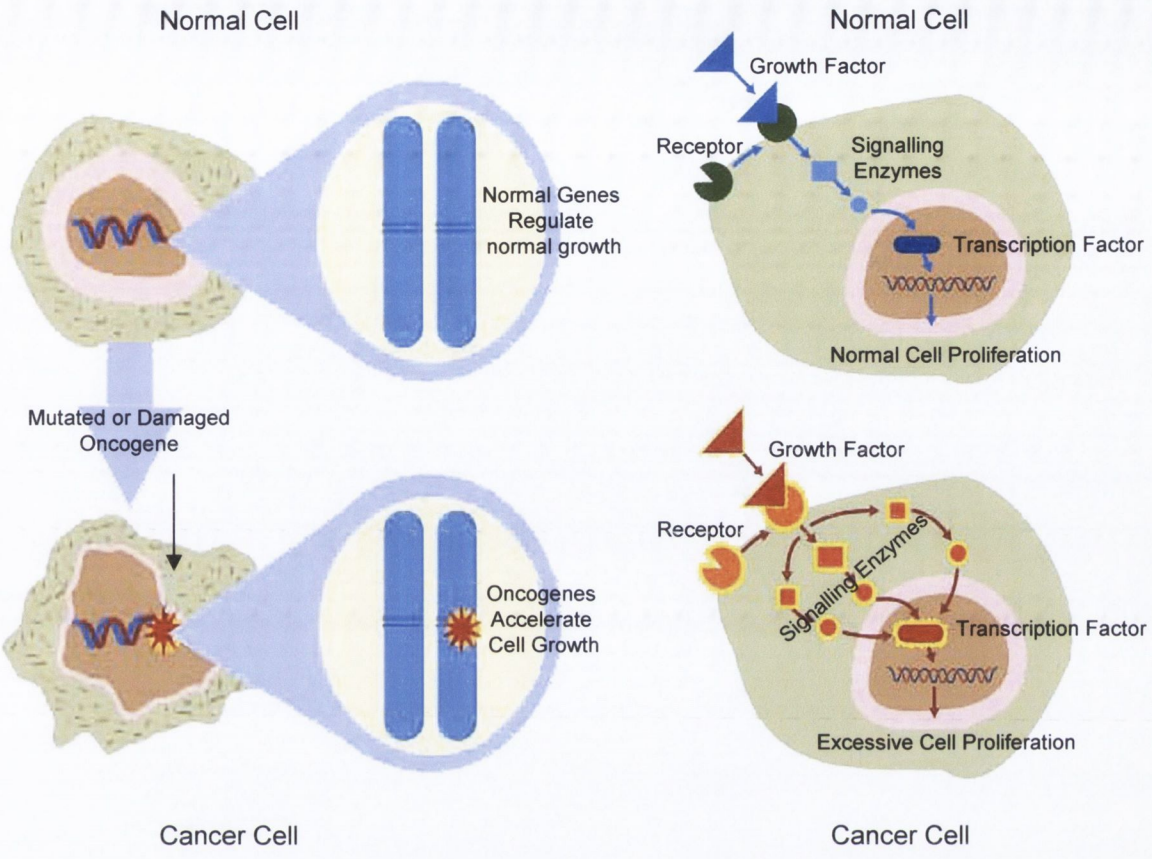

Figure 1.9. Schematic representation of the function of the proto-oncogenes. Proto-oncogenes are a family of normal genes that code mainly for proteins involved in a cell's normal growth-control pathway. Cell growth and division is normally controlled by proteins called growth factors, which bind to receptors on the cell surface. This binding activates a series of enzymes inside the cell, which in turn activates special proteins called transcription factors inside the cell's nucleus. The activated transcription factors turn on genes required for cell growth and proliferation. Many components of this pathway, for example, growth factors signalling enzymes, receptors, and transcription factors, are encoded by proto-oncogenes. Oncogenes arise from the mutation of proto-oncogenes. Oncogenes code for an altered version (excessive quantities) of the growth-control proteins, thereby disturbing a cell's growth-signalling pathway.

adenocarcinomas. Expression of TGF-β has been reported in non-dysplastic Barrett's as well as oesophageal adenocarcinomas (Triadafilopoulo *et al*, 1996; Ellis *et al*, 1997). Inactivating mutations of MADR2 gene, an important component of the signalling pathway for TGF-β, which is frequently associated with loss of heterozygosity in chromosome 18q21 (location of MADR2 gene) was found in oesophageal adenocarcinomas. Loss of expression of the functional receptor for TGF-β (TGF-β receptor type II) is shown to be associated with Barrett's oesophagus and oesophageal adenocarcinomas (Triadafilopoulo *et al*, 1996; Souza *et al*, 1996; Garrigue *et al*, 1996).

The c-erbB2 proto-oncogene (HER2/neu; chromosome 17q21) encodes a transmembrane glycoprotein with intrinsic tyrosine kinase activity that is homologous to, but distinct from, EGFR. C-erbB2 protein overexpression or amplification of the c-erbB2 receptor gene occurs in approximately 10-70% of oesophageal adenocarcinomas (Nakamura *et al*, 1994; Duhaylongsod *et al*; 1995; Hardwick *et al*, 1997, Polkowski *et al*, 1999) Overexpression of c-erbB2 was not demonstrated in dysplastic Barrett's epithelium, suggesting it is a late event in the dysplasia-to-carcinoma sequence (Hardwick *et al*, 1995).

The fibroblast growth factors (FGFs) are potent mitogens that possess angiogenic properties and the ability to regulate the growth and differentiation of various cell types. The expression of acidic and basic FGF (aFGF, bFGF) has been studied in oesophageal adenocarcinoma and Barrett's metaplasia. Fibroblast growth factors are generally sequentially accumulated in the progression from metaplasia to neoplasia. Enhanced expression of aFGF mRNA and protein but not of bFGF, has been detected in high-grade dysplastic Barrett's and oesophageal adenocarcinomas compared with low-grade dysplasia and normal control epithelium (Soslow *et al*, 1997; Soslow *et al*; 1999).

The ras families of proto-oncogene (H, K and N) encode specific proteins that appear to be essential components in normal cell division and differentiation. Ras proteins act as signal-transducing molecules in the cytoplasm. Increased H-ras expression in Barrett's carcinoma and amplification of the K-ras gene in oesophageal adenocarcinomas has been reported (Abdelatif *et al*, 1991; Jankowski *et al*, 1992; Sorsdahl *et al*, 1994). Point mutations in K-ras in Barrett's oesophagus and in oesophageal adenocarcinomas have

also been reported, although these were rare (Trautmann *et al*, 1996; Casson *et al*, 1997). Activation of the ras proto-oncogene seems to be of little importance in Barrett's adenocarcinomas, contrary to what has been observed in other carcinomas of the gastrointestinal tract.

The c-myc gene is located on chromosome 8q24 and encodes a nuclear protein thought to regulate the transcription of other genes that are important for cell growth (Dang *et al*, 1999). Activation of the c-myc gene may contribute to tumour progression by preventing cells from entering the resting phase (G0). Studies suggest that c-myc is the target gene of the chromosome 8q high-level amplifications found in oesophageal adenocarcinomas (Lu *et al*, 1988; Persons *et al*, 1998; Moskaluk *et al*, 1998; Van Dekken *et al*, 1999). Using in situ hybridization, enhanced c-myc expression was reported in dysplastic Barrett's oesophagus and adenocarcinomas but not in nondysplastic Barrett's mucosa (Abdelatif *et al*, 1991). It is unclear whether amplification or mutation of c-myc plays a significant role in the malignant progression of Barrett's oesophagus, but it appears to be a late event in the tumourigenesis (Miyazaki *et al*, 1992; Walch *et al*, 2001).

The cellular oncogene c-src and its viral homolog v-src encode 60-kD, cytoplasmic, membrane-associated, protein-tyrosine kinases. A close correlation exists between elevated specific kinase activity and cell transformation. Src may deregulate cell adhesion by anchorage-dependent growth control, thereby maintaining cells in the proliferative state (Takekura *et al*, 1992). Src activity was found to be three to four fold greater in Barrett's oesophagus and six fold greater in oesophageal adenocarcinomas than in control tissues (Kumble *et al*, 1997) suggesting a role for src in the malignant transformation of Barrett's oesophagus.

Proteins encoded by bcl-2 family genes are important regulators of programmed cell death (apoptosis). For example; bcl-2, mcl-2 and bcl-x_L prevent cells from entering apoptosis, whereas bax, bak, bad and bcl-x_S can induce cell death (Hockenbery *et al*, 1990; Korsmeyer, 1992; Boise *et al*, 1993). The susceptibility of cells to apoptotic stimuli is thought to be controlled by the relative ratios of the different bcl-2 family proteins (Reed, 1994; Sato *et al*, 1994). Bcl-2 has been shown to be involved in the

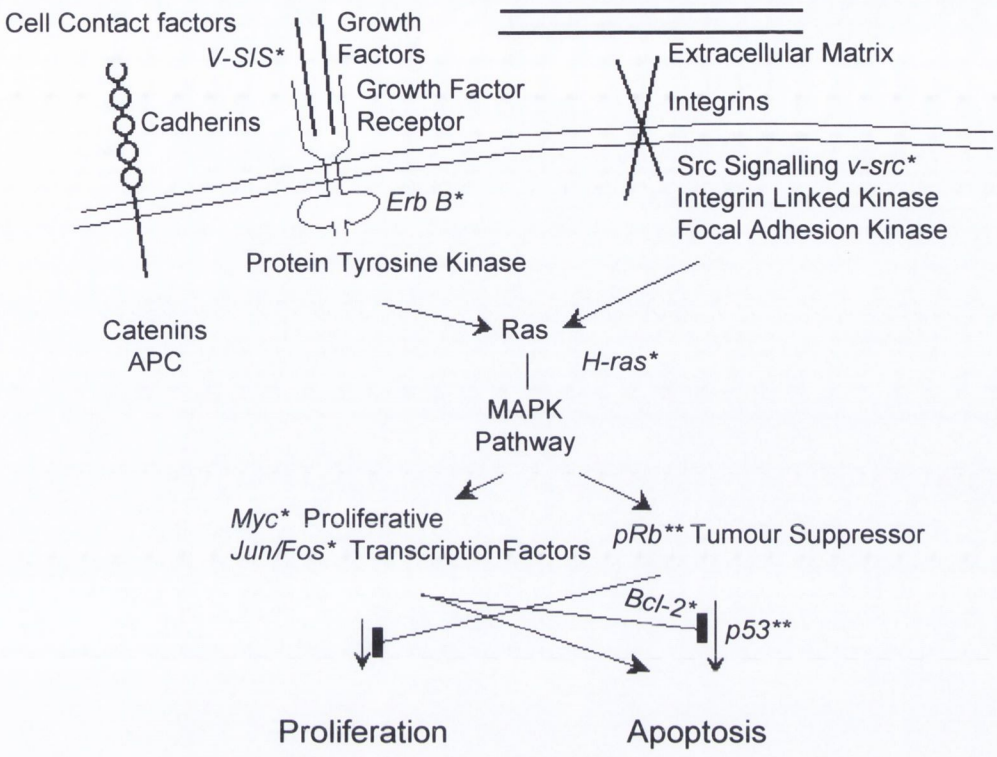

Figure 1.10. Schematic diagram of signal transduction from the cell membrane to the nucleus and the proteins (protooncogene products) involved. Activation (e.g., mutations) of the genes encoding growth factors, their receptors, or the signal transduction pathway genes (ras, src, myc, bcl-2) can lead to constitutive activation of the cell cycle. EGF, epidermal growth factor; TGF, transforming growth factor. Oncogenes showing transformation by gain in function or overexpression are marked with an asterisk symbol. Tumours suppressors marked with a double asterisk symbol are transforming by virtue of repression or loss of function.

development of follicular lymphoma via a chromosomal translocation t(14;18) (Tsujimoto *et al*, 1986), however, little is known about its function in non-hematolymphoid neoplasms. Overexpression of bcl-2 can contribute to neoplastic growth by prolonging cellular life span. The bcl-2 gene family has been studied in various human cancers such as gastric (Krajewska *et al*, 1996); colorectal (Ogura *et al*, 1999), lung (Chen *et al*, 1999) prostate (Krajewska *et al*, 1996), ovarian (Marone *et al*, 1998) and cervical (Ferrandian *et al*, 2000), but its regulation in oesophageal adenocarcinoma has not, as yet, well elucidated. The role of bcl-2 and bcl-2 associated bax and bcl-x proteins in the molecular regulation of Barrett's tumourigenesis and tumours' response to neoadjuvant chemoradiotherapy are further discussed in (chapter 4).

1.3.5 Cell-cell Adhesion

It has long been known that cell-cell adhesion is generally reduced in human cancers. Reduced cell-cell adhesiveness removes the contact inhibition of proliferation, thus allowing escape from growth control signals. Moreover, invasion and metastases, which are life-threatening properties of malignant tumours, are considered to be later but critical carcinogenic steps.

The e-cadherin-catenin complex is the prime mediator of calcium-dependent cell-cell adhesion in normal epithelial cells. In non-malignant epithelia, e-cadherin and the catenins show a membranous localisation at intercellular borders. In Barrett's adenocarcinomas, reduced membranous expression of e-cadherin as well as the catenins is observed in 60-80% of tumours (Jian *et al*, 1997; Krishandath *et al*, 1997; Bailey *et al*, 1998). Moreover, reduced expression of e-cadherin and alpha-and beta-catenin correlated significantly with unfavourable tumour stage, grade, lymph node metastases, and patients' survival (Krishandath *et al*, 1997). Reduced expression of e-cadherin has also been shown to be associated with greater degrees of dysplasia in Barrett's epithelium (Bailey *et al*, 1998, Washington *et al*, 1998). This suggests that the e-cadherin-catenin complex may be useful as a biomarker for neoplastic progression from Barrett's metaplasia to adenocarcinoma and metastases. Frequent loss of heterozygosity of the e-cadherin locus at 16q22 in oesophageal adenocarcinomas has also been reported (Wijnhoven *et al*, 1999).

The serine protease system has been shown to play an important role in the invasive potential of a variety of tumours by breaking down the extracellular matrix. Urokinase plasminogen activator is a serine protease. High levels of urokinase plasminogen activator were found in oesophageal adenocarcinomas (Sier *et al*, 1993; Hewin *et al*, 1996) and correlated with, tumour stage, lymphatic invasion, and survival (Nekarda *et al*, 1998). Therefore, urokinase plasminogen activator antigen content could identify oesophageal adenocarcinoma patients who will develop early tumour recurrences, thus providing a more accurate estimation of prognosis.

CD44 is a family of glycoproteins involved in cell-cell adhesion and cell-matrix interactions. As a result of alternative splicing of 10 exons (v1-10), more than 20 isoforms have been described. CD44 standard (CD44s) and its abnormal transcripts (CD44v) have been detected in oesophageal adenocarcinoma. In Barrett's oesophagus, CD44s expression increases along with dysplasia and the proliferation rate, and increased CD44v6 was seen in an early stage of malignant transformation (Lagorce-Pages *et al*, 1998). Increased CD44s expression was seen in 50% to 66% of oesophageal adenocarcinomas and a significant correlation between CD44s ,v6 and v10 expression and clinicopathologic characteristics has also been reported (Castella *et al*, 1996; Bottger *et al*, 1998; Lagorce-Pages *et al*, 1998).

The cysteine protease cathepsin B (CTSB) gene, which maps to 8p22, codes for a lysosomal enzyme that has been shown to be overexpressed or to exhibit altered localisation in cancers (Keppler *et al*, 1996). Overexpression or altered localisation of CTSB is thought to result in degradation of the basement membrane, facilitating tumour invasion and metastasis. Abundant extracelluar expression of CTSB protein was found in 73% oesophageal adenocarcinoma specimens (Hughes *et al*, 1998). These data support an important role for CTSB gene amplification and CTSB protein overexpression in oesophageal adenocarcinomas.

1.4 CURATIVE MANAGEMENT OF OESOPHAGEAL ADENOCARCINOMA

1.4.1 Single-Modal Therapy versus Multi-Modal Therapy

Adenocarcinoma of the oesophagus is a highly malignant disease with dismal prognosis. With a marked increase in its incidence, the majority of cases, even in localized disease, present with lymph node metastases at the time of diagnosis (Lund *et*

al, 1989; Powell *et al*, 1992; Walsh *et al*, 1996, Daly *et al*, 1996; Devesa *et al*, 1998; Cohen *et al*, 1999). Although, the role of preoperative concurrent chemoradiotherapy remains to be defined, this strategy seems to result in a higher rate of pathologically complete responses and longer survival rates compared to single-modality therapy i.e. chemotherapy (Jonker *et al*, 1999), radiation therapy (Cooper *et al*, 1999) or surgery alone (Walsh *et al* 1996, Forastiere *et al*, 1997). In addition, neoadjuvant chemoradiotherapy given before surgery may reduce the incidence of micrometastasis, increase resectability and control systemic disease, all of which might influence decisions on and management of postoperative treatment. Explanation of why chemotherapy or radiation therapy alone fails to achieve local control has been attributed to intrinsic cell resistance to the chemotherapy or radiotherapy, hypoxic cell resistance and repopulation as a result of increasing cell proliferation. To some extent, these mechanisms can be, and have been, overcome by the application of combined chemoradiotherapy. The rationale for combination therapy relies on spatial cooperation or interaction between modalities. Interactions may take place (I) at the molecular level, with altered DNA repair or modification of the lesions induced by drugs or radiation, (II) at the cellular level, notably through cytokinetic cooperation arising from differential sensitivity of the various compartments of the cell cycle to the drug or radiation, and (III) at the tissue level, including reoxygenation, increased drug uptake or inhibition of repopulation or angiogenesis.

I- Interaction at the Molecular Level

Antitumour drugs may provide various mechanisms of interaction with radiation including DNA repair inhibition, cell-cycle redistribution, altered cytokinesis or apoptosis. The relative importance of these mechanisms has seldom been evaluated, even for in vitro studies. Ionising radiation induces a wide range of lesions in the DNA of target cells, including base damage, alkali-labile sites, single-strand breaks (SSB) and double-strand breaks (DSB). It has long been shown that these lesions are rapidly repaired, with the noticeable exception of DSB for which the $t_{1/2}$ for rejoining extends over 55 minutes or more, in repair-proficient cells. Unrepaired DSBs are consistently regarded as lethal lesions (Foray *et al*, 1997). However, the contribution to induced cell kill of the oxidative stress associated with low-low energy transfer radiation (LET) and employing poly (ADP-ribose) polymerase for its repair, should not be underestimated (Fernet *et al*, 2000). Many chemotherapeutic drugs also target DNA, creating adducts,

SSB or DSB. Cisplatin, for example, exerts its cytotoxic effect through the chelation of guanine residues, yielding monofunctional adducts and intrastrand or interstrand cross-links. The bulk adducts are repaired through the excision repair pathway (Huang *et al*, 1994), but mismatch repair is also involved in the processing of cisplatin adducts by the cell (Fink *et al*, 1998). The possibility exists that the presence in close vicinity in DNA, of both a cisplatin adduct and a radiation-induced SSB may result in a mutual impairment of proper repair. This model is supported by calculations made to estimate the probability of interaction between cisplatin adducts and radiation-induced SSB (Begg, 1990) as well as by experimental data (Yang *et al*, 1995).

DNA synthesis and DNA repair often share common pathways. This has provided a rationale for investigating the potential of DNA synthesis inhibitors in combination with radiation. DSB are repaired through two main pathways, namely, non-homologous end-joining (NHEJ) and homologous recombination. Yet, it is intrinsically error-prone, NHEJ largely prevails in somatic mammalian cells. The main enzyme complex in NHEJ is the DNA-dependent serine/threonine protein kinase (DNA-PK). DNA-PK is formed from three subunits known as Ku70, Ku86 and DNA-PKcs. Deletion or mutation in any of these genes confers extreme sensitivity to radiation (Denekamp *et al*, 1989; Lees-Miller *et al*, 1995). Like many other enzymes involved in DNA damage signalling or repair (ataxia-telangiectasia mutated (ATM), ataxia telangiectasis Rad3-related (ATR), FRAP, TRRAP), DNA-PKcs has a catalytic domain with high homology to phosphatidylinositol 3-kinase (PI3-K). DNA-PKcs may, therefore, be inhibited to near completion by PI3-K inhibitors, most notably wortmannin (Sarkaria *et al*, 1998). In vitro, wortmannin is not toxic per se, but it increases dramatically the cytotoxicity of radiation (Boulton *et al*, 1996; Okayasu *et al*, 1998). PI3-K inhibitors, however, are highly toxic in vivo.

The drugs that affect nucleoside and nucleotide metabolism are among the most effective and most widely used agents to sensitise tumour cells to radiation treatment, including fluoropyrimidines (5-fluoro-2'-deoxyuridine (5-FU), 9-β-D-arabinofuranosyl-2-fluoroadenine monophosphate (fludarabine), 2',2'-difluoro-2'-deoxycytosine (gemcitabine)), thymidine analogues (5-bromo-2'-deoxyuridine (BrdUrd), 5-iodo-2'-deoxyuridine (IrdUrd)) and hydroxyurea.

5-FU inhibits thymidylate synthase and depletes the pool of nucleotide triphosphates, leading to cell cycle redistribution, DNA fragmentation and cell death (Pinedo *et al*, 1988). The incorporation of 5-FU into DNA and RNA as fluoro-deoxyuridine, contributes to its cytotoxicity. 5-FU is a well known radiosensitiser. There is evidence suggesting that 5-FU-induced radiosensitisation relies on the 5-FU fraction, which is incorporated into DNA (Lawrence *et al*, 1994; McGinn *et al*, 1996). Radiosensitisation correlates with a decrease in the rate and extent of repair of radiation-induced DSB and the addition of thymidine to the culture medium reverses, in part, the susceptibility to radiation. Leucovorin, however, likely due to the enhanced trapping of fluoro-dUMP by thymidylate synthase, also acts as an enhancer of radiosensitisation by 5-FU, suggesting that an imbalanced deoxynucleoside triphosphate pool may be a major pathway of altered DNA repair. However, cell cycle redistribution following 5-FU exposure may also explain the enhanced radiation susceptibility (Miler *et al*, 1992).

Gemcitabine is a pyrimidine analogue with a wide range of activity against solid tumours. It acts to deplete the deoxynucleoside triphosphate pool and is incorporated into DNA, in the same way as 5-FU. A clear correlation between incorporation into DNA and cytotoxicity, with inhibition of DNA synthesis and probably DNA repair, has been demonstrated (Huang *et al*, 1991). Gemcitabine has been found to exert a major radiosensitising effect in colon, pancreatic and squamous cell carcinoma cell lines in relation to the S-phase cell content (Lawrence *et al*, 1996; Robertson *et al*, 1996; Rosier *et al*, 1999). The effect was observed for relatively low drug concentrations, reaching a maximum 24 hours after the onset of drug exposure (Shewach *et al*, 1994) and persisted for more than 48 hours after contact with the drug (Lawrence *et al*, 1997). Depletion of the deoxynucleoside triphosphate pool contributes to enhanced radiation susceptibility (Lawrence *et al*, 1996; Shewach *et al*, 1994) but no increase in the incidence or repair of DNA strand breaks by gemcitabine could be evidenced in an analysis of $\geq$ 200 kbp fragments by pulsed field gel electrophoresis (Gregoire *et al*, 1997; Lawrence *et al*, 1997). This seems to rule out any direct inhibition of DNA repair pathways. In spite of this, major toxicity has been reported during the preliminary clinical trials of the gemcitabine-radiation combination (Scalliet *et al*, 1998), such that it has been recommended that this association should not be used outside of carefully designed clinical trials.

BrdUrd and IdUrd readily substitute for deoxythymidine and incorporate into DNA, thus inducing potent radiosensitisation with enhanced DNA damage and decreased DNA repair (Dkordevic *et al*, 1960). BrdUrd and IdUrd are believed to act both through an enhanced yield of radical damage at the C-5 position of the pyrimidine ring (Danzinger 1968; Rivera *et al*, 1983). The magnitude of the effect in terms of survival consistently correlates with the amount of drug incorporated into DNA (Miller *et al*, 1992). However, halopyrimidines can hardly be used as adjuvants to radiotherapy in view of their general toxicity.

The mechanisms underlying the use of Taxanes as radiosensitisers include cell synchronisation and reoxygenation. It has been suggested (Makino *et al*, 2001) that paclitaxol may induce cell death by triggering apoptosis. In addition, it enhances the effect of ionizing radiation, possibly by blocking cells in the G2 phase, that phase of the cell cycle in which cells are most radiosensitive (Das *et al*, 2001; Ozkan *et al*, 2002).

II- Interaction at the Cellular Level

Cytokinetic cooperation: It has long been known that radiosensitivity changes with the progression of cells through the cell cycle. The S phase is most radioresistant, and the G2-M phase is usually most radiosensitive (Terasima *et al*, 1966; Sinclear *et al*, 1966). For this reason, a large increase in radiation susceptibility is observed as proliferating cells are exposed in close temporal proximity with radiation, to drugs which specifically kill cells in S phase. This is the case for the camptothecin and camptothecin analogues acting as topoisomerase I poisons (Hennequin *et al*, 1994), and possibly also for gemcitabine. It is best in these instances to consider that the effect proceeds from cytokinetic cooperation rather than radiosensitisation, since the drug is inactive against non-S phase cells and does not affect the radiation response among survivor cells (Hennequin *et al*, 1994; Mattern *et al*, 1991; Falk *et al*, 1992; Szumiel *et al*, 1995).

Synchronisation: Maximum radiosensitivity is usually observed in the G2-M phase of the cell cycle. Therefore, synchronisation of the cells in G2-M, if it occurs, is expected to elicit the maximum response to radiotherapy. For a few years, this was proposed as a rationale for the use of paclitaxel in conjunction with radiotherapy.

Paclitaxel and docetaxel bind with a high affinity to microtubules and alter their dynamics (Schiff *et al*, 1980; Parness *et al*, 1981; Arnal *et al*, 1995). At high cytotoxic doses, both drugs were shown to inhibit the formation of the mitotic spindle and consistently to block the progression of cells in mitosis, between prophase and metaphase (Robert *et al*, 1990, Gupta 1985). In fact, both drugs act through disruption of the centrosome network, thus inducing faulty mitosis and cytokinesis (Paoletti *et al*, 1997). Pioneering studies of radiation interaction with paclitaxel indicated that increased radiosensitivity occurred at the time of the G2-M block (Tishler *et al*, 1992; Steren *et al*, 1993; Hei *et al*, 1994). However, further studies showed that enhanced radiation cell kill by taxanes does not work in all cell lines, and it was recognised that prometaphase arrest upon prolonged contact with drugs, if it occurs, may not be a sufficient condition for increased radiation sensitivity (Geard *et al*, 1994; Minarik *et al*, 1994; Stomberg *et al*, 1995;). Moreover, in some cell lines paclitaxel and docetaxel at low doses may induce protection against radiation-induced cell kill, presumably through alteration of signal transduction pathways (Hennequin *et al*, 1996; Ingram *et al*, 1997). In vitro radiosensitisation of cells by fludarabine (Grégoire *et al*, 1994) and 5-fluoro-2'-deoxyuridine (FdUrd) (Miller *et al*, 1992) has also been proposed to proceed from the accumulation of cells in a radiosensitive compartment of the cell cycle.

Whether cell cycle redistribution might be used to increase tumour response to radiotherapy is open to discussion (Steel 1994). The usefulness of this approach can be questioned for many reasons. First of all, tumours are heterogeneous, with an uncontrolled amount of cells in the quiescent (G_0) state, and the possibility of induced cell synchronisation by antimetabolites or DNA polymerase inhibitors in humans is extremely limited. Secondly, as normal tissue surrounding the tumour may also be a target for cell synchronisation, such synchronisation may not necessarily result in an increased therapeutics index. Furthermore, conflicting data have been reported as to whether G2-M cells are more prone to apoptosis than cells in other phases of the cell cycle (Milross *et al*, 1996).

Promotion of apoptosis: Cells may undergo lysis or lose reproductive ability through various unscheduled (immediate, mitotic and delayed cell death) or programmed (apoptosis and senescence) mechanisms. Mitotic cell death, involving abortive mitosis and oncosis (referred to as necrosis), is one of the modes of cell death in epithelial

tumours. Recent developments have shed light on the basic mechanisms of mitotic cell death, such as defects in the control of centrosome replication (Sato *et al*, 2000; Schatten *et al*, 2000). However, for the last decade interest has turned mainly to apoptosis, simply because it is the safest mode of cell death that could be modulated to counter tumours' proliferation activity and it is also the most amenable to studies based on molecular biology and genetics.

Lymphocytes, thymocytes, prostate cells, salivary acini, endothelial cells and intestinal crypts, are the most sensitive cells to apoptosis in response to DSB induction, oxidative stress or hypoxia, ceramide, contact with some cytokines or deprivation of growth factors. The p53 protein, due for the most part to its role in increased (decreased) transcription of the pro-apoptotic (anti-apoptotic) mitochondrial proteins Bax (Bcl-2), is mandatory for radiation-induced apoptosis (Hale *et al*, 1996). Conversely, p53 mutation or deletion reportedly promotes apoptosis after paclitaxel treatment (Wahl *et al*, 1996). A kind of a cooperation depending on the p53 status and the cytotoxic agent could thus be proposed, yet this question is still very much a matter of debate (Rakovitch *et al*, 1999).

Apoptosis undoubtedly plays a major role in cell killing by radiation or DNA-nicking drugs applied alone, e.g. for topoisomerase I- or II-targeting agents. However, evidence in favour of increased apoptosis as a general mechanism to account for increased response to combined treatment, is not firmly established. For example, increased apoptosis might account for radiosensitisation by gemcitabine in cell lines that are prone to radiation-induced apoptosis (Lawrence *et al*, 2001); however, a radiosensitising effect of gemcitabine is also observed in apoptosis-resistant cells.

III- Interaction at the Tissue Level

The way to a more efficient anticancer treatment would be to target tumours with treatments eliciting minimal response in surrounding, dose-limiting normal tissue. Radiotherapy takes advantage of differential sublethal damage repair in tumours versus normal tissue. Unfortunately, there is, as yet, no convincing evidence to show that normal tissue sparing is retained when the chemoradiotherapy combination is used, and, in fact, randomised trials for the appreciation of the late toxicity of chemo-radiotherapy are still lacking. Such studies should be encouraged, taking into account some well-

known properties of solid tumours that make them resistant to treatment. In particular, (I) tumours are frequently hypoxic, in relation to defective angiogenesis, and respond poorly to radiotherapy; (II) some tumours contain a large proportion of quiescent cells, which are usually resistant to chemotherapy; (III) in contrast, target tumours for radiotherapy are often rapidly proliferating, with a high amount of radioresistant S-phase cells; (IV) epithelial tumour cells overexpress growth factor receptors, or may grow independently of growth factors; (V) tumour cells are mutated or deleted for genes involved in genome maintenance or cell cycle control.

Reoxygenation and tumour shrinkage: A reduction in tumour volume after treatment with one modality may result in an improved blood supply to the tumour, leading to reoxygenation and increased radiosensitivity and chemosensitivity. This was clearly demonstrated in a human tumour xenograft treated with paclitaxel prior to irradiation (Milas *et al*, 1995). Reoxygenation correlated with an increased radiation response, irrespective of whether cells accumulated in G2-M or not (Milas *et al*, 1995). Similar reoxygenation and radiosensitisation has been reported following gemcitabine treatment (Mason *et al*, 1999). Fractionated irradiation, through a reduction of the tumour volume, may also increase the tumour blood flow and facilitate drug access to the tumour. Indeed, it has been demonstrated that radiation increases the uptake of some drugs, like carboplatin and 5-FU (Yang *et al*, 1995; Young *et al*, 1981).

Inhibition of tumour proliferation: Tumour repopulation is often invoked to account for the failure of radiotherapy (Fowler *et al*, 1992). Although the mechanisms involved in tumour regrowth are not completely understood, the role of growth factors is likely to be of major importance (Trott *et al*, 1985). Modulation of tumour proliferation may be achieved by epidermal growth factor receptor (EGFR) inhibition, either with a monoclonal antibody directed against the receptor (C225 mAb) or through the inactivation of the tyrosine-kinase activity of EGFR (Mendelsohn *et al*, 2000, Huang *et al*, 1999). Preclinical studies have demonstrated the ability of C225 to enhance in vitro radiosensitivity. Several mechanisms have been proposed to explain this observation, including inhibition of cell proliferation, of DNA damage repair (Bandyopadhyay *et al*, 1998), of tumour angiogenesis (Perrotte *et al*, 1999) or, in contrast, promotion of radiation-induced apoptosis. The inhibition of EGFR seems to be a promising way to increase the cytotoxic effect of radiation. However, due to the fact that repopulation of

normal, rapidly responding tissues is the rule after irradiation, acute toxicity may be feared. Careful phase I studies with EGFR inhibitors in combination with radiation, should therefore be planned before any phase II or III studies.

Inhibition of angiogenesis: Angiogenesis is essential for tumour growth. Consistently, the search for compounds endowed with antineoangiogenic activity is flourishing, including angiostatin, combretastatin, flavone derivatives or kinase inhibitors. The combination of angiostatin and irradiation was found to have a major antitumoral effect in a human tumour xenograft model (Mauceri *et al*, 1998). This synergy may arise from a cytotoxic effect on endothelial cells. Others compounds, such as TNP-470, may also elicit a radiosensitising potential (Lund *et al*, 2000).

Specificity for tumour tissues: Another way to increase specificity against tumour tissue is to use drugs which are capable of targeting a particular organ. For instance, estramustine has proven highly specific for prostate tissues and may act as a radiosensitiser (Kim *et al*, 1994; Rockwell, 1995). Clinical trials have started with this molecule in prostate cancer, in the hope that a specific radiosensitisation of these tumours may be obtained (Zelefsky *et al*, 2000).

1.4.2 Response to Preoperative Chemoradiotherapy

The ability to predict a major or complete pathological response to neoadjuvant therapy has enormous importance; to both enhance treatment outcomes in responding patients and to obviate the requirement for costly and potentially toxic therapy in non-responders. Therefore, a pathological and molecular understanding of what influences tumour sensitivity or resistance to neoadjuvant therapy would be of obvious clinical benefit. Based on multivariate analysis; specific pathological variables were found to be independent prognostic factors associated with better outcome. A study (Dunne *et al*, 2001) from our Unit at St. James's Hospital reported that patients who are identified at diagnosis with negative loco-regional lymph node should benefit considerably from neoadjuvant therapy. Similarly; (Rice *et al*, 2001) reported poorer survival in patients with no evidence of down-staging (pN1) following induction of chemoradiotherapy prior to surgery. Poorer disease-free survival in patients whose tumours showed no evidence of pathological regression has also been reported (Mandard *et al*, 1994). At the molecular level; there are few data available that accurately identify those tumours that

eventually have complete or maximum response to neoadjuvant therapy. Understanding the role of apoptosis or other active mechanisms of cell death and proliferation following treatment with anti-cancer agents is of fundamental importance in determining the effectiveness of the therapy. Equally important is the identification of genes and biofactors that regulate these mechanisms. We documented (Raouf *et al*, 2001) that pre-therapeutic tumours with high propensity to apoptosis have a greater response rate to chemotherapy and radiation therapy than tumours with low apoptotic indices, and tumours with high proliferation activity are at higher risk of developing lymph node metastasis. High level of expression in two of the platinum markers (GST-pi [glutathione S-transferase-pi], P-glycoprotein [P-gp or multidrug resistance]) and one marker of possible 5-fluorouracil association TS (thymidylate synthase) were identified to be predictors of early recurrence and decreased survival following chemoradiotherapy (Harpole *et al*, 2001). A correlation between c-erbB2 negativity and residual microscopic disease at resection has also been reported (Duhaylongsod *et al*; 1995) suggesting that c-erbB2 may be a marker of tumour susceptibility to preoperative chemoradiotherapy.

1.5 MOLECULAR STRATEGIES TO IMPROVE TREATMENT OF OESOPHAGEAL CANCER

Molecular analysis of malignant transformation in Barrett's epithelium provides insight into the temporal nature and significance of individual genetic events during multistep oesophageal carcinogenesis. Potential targets for intervention in oesophageal neoplasms include mutations involving retinoblastoma (Rb) and p53 tumor-suppressor pathways as well as tyrosine kinase cascades, which are known to promote cell cycle progression. Data from recent experiments provide the preclinical rationale for novel molecular and pharmacological interventions in established oesophageal cancers, and suggest strategies for prevention in patients at risk for the development of these neoplasms. Genetic ablation of dysplastic p53 deficient Barrett's epithelium currently in phase 1 study; genetically engineered adenovirous ONYX-015 were utilised in a preclinical trial and were shown to have the ability to replicate in and kill pre and neoplastic cells with defected p53 gene. In recent experiments, Schrump *et al*, 1999 evaluated the effects of p53 gene replacement in oesophageal cancer cells mediated by retroviral or adenoviral vectors. Retroviral p53 had a modest growth-inhibitory effect in cultured epidermoid oesophageal cancer cells. In contrast, an adenoviral p53 construct (Adp53) mediated

pronounced inhibition of proliferation as a result of more efficient transfer and expression of the p53 transgene; growth inhibition mediated by Adp53 correlated with cell cycle arrest and apoptosis. Additional experiments revealed that oesophageal adenocarcinoma cells could be transduced with retroviral vectors (Schrump *et al*, 1996) but were highly refractory to transduction by adenoviral vectors, thus diminishing the utility of adenovirus for gene therapy of oesophageal adenocarcinomas and Barrett's oesophagus.

Overexpression of cyclin D1 has been observed in approximately 40% of oesophageal adenocarcinomas and in nearly 30% of biopsy specimens from precancerous Barrett's epithelia (Jiang *et al*, 1992; Adelaide *et al*; 1995; Arber *et al*, 1996). Retroviral vector expressing an antisense cyclin D1 sequence to inhibit cyclin D expression in HCE-7 epidermoid oesophageal cancer cells has been utilised (Zhou *et al*, 1995). The antisense construct mediated significant growth inhibition without obvious cell arrest or apoptosis. Collectively, these data confirm the significance of cyclin D1 overexpression during oesophageal carcinogenesis and suggest that strategies designed to abrogate this overexpression may be efficacious in the treatment of oesophageal cancers and premalignant oesophageal lesions.

Inactivation of the p16 gene resulting from point mutation, allelic deletion or promoter hypermethylation occurs in a significant percentage of pre- and neoplastic- oesophageal lesions (Tarmin *et al*, 1994; Barrett *et al*, 1996). A recombinant adenoviral vector (Adp16) to restore p16 expression has been used in oesophageal epidermoid cancer cells which markedly inhibited the proliferation and tumourigenicity of these cancer cells (Schrump *et al*, 1996). Interestingly, the degree of growth inhibition after p16 gene transfer appeared similar to that observed after p53 gene replacement in these cells despite the fact that p16 mediated G1 arrest without inducing programmed cell death. Furthermore, the extent of cell cycle arrest in HCE-7 oesophageal cancer cells after Adp16 transduction was markedly greater than that observed after retroviral transduction with an antisense cyclin D1 construct (Zhou *et al*, 1995).

The use of pharmacological agents, which can achieve desired molecular end points without complexities and limitations to a delivery system such as vectors, has also been subject to experimental studies. The effect of 17-allylaminogeldanamycin (17-AAG) as

an inhibitor of erbB2 (p185) expression in cultured oesophageal cancer cells has been reported (Schrump *et al*, 1999). Dose dependent inhibition of p185 protein expression and proliferation was observed with an IC_{50} of 160nM for oesophageal adenocarcinoma cells. In addition, 17-AAG doses of 20-40nM potentiate the effect of paclitaxol in oesophageal cancer cells irrespective of histology by pronounced G_2/M arrest and apoptosis (Nguyen *et al*, 1999).

1.6 MOLECULAR STRATEGIES TO IMPROVE TREATMENT OF SOLID TUMOURS

The use of novel molecular (biological) response modifiers in clinical studies may improve chemo- radio- therapeutic results. For example, synthetic membrane-permeable alkyl-lysophospholipids (ALPs) exert a potent and preferential cytotoxic effect on malignant cells, strongly enhance the radiation-induced cell kill and interfere with angiogenesis in vitro (Ruiter *et al*, 2001). Unlike most currently available cytostatic drugs, ALPs target the plasma membrane and interfere with normal phospholipid metabolism and mitogenic signal transduction pathways. These biological properties, combined with the toxicological and pharmacological profile of ALPs, make these compounds attractive model substrates to be combined with radiotherapy in patients. A phase I clinical study combining oral ALP and radiotherapy in patients with advanced solid tumours has recently been initiated (Bartelink *et al*, 2002).

Another strategy that has been pursued to enhance radiation response involves blockading of growth factor receptors, including the epidermal growth factor receptor (EGFR), a member of the ErbB receptor tyrosine kinase family. Overexpression of the EGFR has been correlated with increased radioresistance, more aggressive behaviour, and poor clinical outcome (Grandis *et al*, 1998). Conversely, blockading of the EGFR by the monoclonal antibody C225 (Cetuximab) increased the in vitro radiosensitivity of various squamous cell carcinoma cell lines (Huang *et al*, 1999). Additional preclinical data confirming these studies were generated in nude mice bearing A431 squamous cell carcinoma xenografts (Milas *et al*, 2000). A more than 3-fold increase in tumour response after irradiation was observed when these animals received concurrent systemic C225. Possible cellular mechanisms, by which C225 enhances the response to radiation, include (1) inhibition of proliferation, (2) induction of cell-cycle arrest, (3) enhancement of radiation-induced apoptosis, (4) inhibition of radiation-induced DNA

damage repair, and (5) inhibition of angiogenesis (Huang *et al*, 2000). Based on these preclinical studies, several clinical phase I/II trials have been successfully completed. Currently, a multicentre phase III trial is being conducted, randomising between radiotherapy with concomitant C225 and radiotherapy alone. Preliminary data indicate a high response rate in advanced head and neck squamous cell carcinoma with only mild, non-overlapping side-effects (Harari *et al*, 2001).

Mutated oncogenes offer an alternative approach for molecular targeting and radiosensitisation. An example of this is the ras gene which is mutated in 30% of human tumours. These mutations are associated with increased radioresistance. For functional activity, the ras protein requires post-translational prenylation. Inhibitors of this process, e.g. farnesyl transferase inhibitors (FTI) can reverse the transformation and cause increased radiosensitivity. Some inhibition of tumour growth also occurs which is independent in tumours that do not harbour a mutated ras gene and that do not over express the ras protein (Cohen-Jonathan *et al*, 2000). FTIs in combination with radiotherapy have recently entered clinical trials (Brown *et al*, 2001).

Concomitant chemotherapy and radiotherapy has resulted in a major step forward in the treatment of patients with advanced tumours. Identification of less toxic chemotherapeutic agents and the use of biological modifiers could have greater therapeutic efficacy. Improved understanding of molecular factors for the development of tumour resistance may allow the prediction of clinical response to neuadjuvant treatments. Furthermore, the identification of oncogenes involved in tumour resistance has already led to in vitro approaches which successfully inactivated these genes using ribozymes or antisense oligodeoxynucleotides, thus restoring drug sensitivity. It is conceivable that these strategies, once transferred to a clinical setting, may have the potential to enhance treatment for a great variety of malignancies and thus more fully exploit the antineoplastic agents and its curative potentials.

CHAPTER 2

GENERAL METHODS

CHAPTER 2
GENERAL METHODS

2.1 PATIENT SELECTION

A total of 48 patients (36 male, 12 female) presenting with primary oesophageal adenocarcinoma between 1990 and 2001 who were treated at the Department of Surgery - St. James's Hospital, Dublin were included in this study. Patients' ages ranged from 45 to 77 years (65 median). All patients had oesophagogastro-endoscopy and all were subject to histological confirmation of diagnosis prior to treatment with chemotherapy (5-fluorouracil and cisplatin) and radiotherapy (CRX) followed by oesophagectomy (Walsh *et al*, 1996). All patients were treated with curative intent and were followed up at regular intervals.

2.2 HISTOPATHOLOGICAL ASSESSMENT

For each case, pathology reports of pre-treatment biopsy and surgical resections were reviewed. Non–metaplastic, non-tumourous oesophageal resection margins were used as normal oesophageal samples. Barrett's epithelium was defined as specialised intestinal metaplasia within the biopsy or resection specimen. This was further defined as being non-dysplastic or showing low-grade dysplasia or high-grade dysplasia (Riddell *et al*, 1983; Ming *et al*; 1998). Tumours were staged according to the AJCC/TNM system (Beahrs *et al*, 1992; Sobin *et al*, 1997) and the final status was given at resection. Most informative blocks (2-4) of tumours were selected for immuno - phenotype analysis. A complete pathological response (CPR) to CRX was defined when no tumour cells were identified microscopically at resection including the regional lymph nodes. Patients were defined as non-responders (NPR) when there was no evidence of pathological response in the resection specimens e.g. the presence of macroscopic tumours, lymph node metastasis and lympho-vascular permeation. A third intermediate group was defined as having major pathological response (MPR) to CRX. These patients were all lymph node negative and had only microscopic residual tumour cells in any part of the oesophageal wall.

2.3 STATISTICAL ANALYSIS

All continuous data presented in this thesis were of non-parametric distribution. Statistical calculations were carried out using the StatView software package version 4.5 (Abacus Concepts, Berkeley, CA). The significance of association among variables i.e. apoptotic index, Ki-67 labelling index, p53, bcl-2, bax and bcl-x expression and the clinicopathological parameters including tumour differentiation, depth of invasion and lymph node status was determined (as appropriate) by the Mann-Whitney or Kruskal-Wallis test. The association between p53, bcl-2 family expression and type of response to CRX was determined either by the Chi-square or Fisher's exact test. For paired comparison of pre- to post- CRX values, the Wilcoxon test was used. Statistical significance was defined as $P < 0.05$.

2.4 IMMUNOHISTOCHEMISTRY

This section describes the most commonly used immuno-enzymatic staining methods for detection and quantification of specific antigens. There are many staining methods which can be used i.e. PAP (peroxidase-antiperoxidase) or APAAP (alkaline phosphatase anti alkaline phophatase); in this study we performed the three step avidin-biotin peroxidase method for detection of p53, cell-cycle related Ki-67, bcl-2 and bcl-2 associated bax and bcl-x protein expression (Naish *et al*, 1989). In this method; an unconjugated primary antibody binds to the specific structural binding sites (epitopes) of targeted antigen. A biotinylated secondary antibody then allowed to binds to the primary antibody. The sequence of the reaction is then completed by performing an avidin-biotin complex using horseradish peroxidase as a label enzyme. The reaction product of peroxidase will be visualized by a chromagen that results in distinct brown staining within the positive cells.

2.4.1 Tissue Preparation

Archival formalin fixed paraffin embedded tissue samples was used. Four μm sections of the normal oesophageal tissues, Barrett's mucosa and representative 2-4 blocks of tumour were sliced using the microtome. Tissue sections were mounted onto slides which had been pre-treated with 3-aminopropyltreithoxysaline (APES) [Appendix 1] and air-dried overnight at 37°C. Prior to immunostaining, sections were deparaffinized in two changes of xylene and rehydrated through graded alcohols to tap water.

2.4.2 Antigen Retrieval

Formalin fixation causes the masking of tissue antigens by forming methylene bridges (epitopes cross link) between reactive sites on different portions of the same molecule or on adjacent proteins. The extent of masking is related to fixation concentration, time in fixative and temperature (Cattoretti *et al*, 1993). The antigen unmasking can be achieved by high-temperature antigen retrieval methods such as microwave or autoclave pretreatment of tissue sections (Shi *et al*, 1995). Non-enzymatic unmasking was achieved by boiling the slides in a microwave oven in 10mM citrate buffer pH 6 [Appendix 1] at high power for 20 minutes and then cooled down at room temperature for 15 minutes before rinsing in tris buffer saline for 5 minutes.

2.4.3 Blocking Endogenous Peroxidase Activity

Endogenous peroxidase activity may be produced in cells, particularly, leucocytes and erythrocytes yielding false positive results (Bancroft *et al*, 1990). To quench Endogenous peroxidase activity, sections were treated with a hydrogen peroxide solution (0.3% H_2O_2 in methanol) for 30 minutes and then rinsed in water for 5 minutes.

2.4.4 Blocking Non-Specific Antibody Binding

Cross-reactivity of an antibody generally denotes its specific interaction with an individual epitope found on two or more different antigen molecules. Undesired reaction may occur if the secondary antibody cross-reacts with endogenous immunoglobulins in the specimens. The interaction of an antibody with dissimilar epitopes can be eliminated by using preabsorbed antiserum that is raised against the species from which the antibody is obtained (Naish *et al*, 1989). In this study bovine serum albumin was used to block non-specific antibody binding. Sections were incubated with 0.1% bovine serum albumin for 10 minutes prior to its incubation with primary antibodies.

2.4.5 Incubation with Primary and Secondary Antibody

The optimal working conditions of the primary and secondary antibodies are important to obtain a consistently good quality of staining. This will be achieved by the assessment of different antibody titrations, incubation time and temperature. An

antibody titer is defined as the highest dilution of an antibody which results in optimal specific staining with the least amount of background. A dilution range is usually recommended by manufacturers. Correct dilution will then be determined by performing of a series of experimental dilutions in fixed times. Depending on specimens' size, the application of sufficient solution per section is also important to achieve homogenous distribution of the antibody on the surface of the slide. For maximum equilibration consistent incubation time is important. Generally there is an inverse relationship between incubation time and antibody titer - the higher the antibody titer the shorter the incubation time. In addition, higher affinities between antigen and antibody allow for the shortening of the incubation time. Optimal incubation time has been reported following 1 hour, for primary antibody this may be extended to 24 – 48 hours to achieve maximum antigen saturation with the antibody. It is not known whether temperature selectively promotes the antigen-antibody reaction rather than the reaction that gives rise to the background. Increased temperature (i.e. 37°C or room temperature) may shorten the reaction time. A temperature of 4°C is also used in combination with overnight or longer incubation. The optimal working dilution, incubation time and temperature of the primary and secondary antibody used in this study are described in [Appendix 2] and the steps applied were as follows: -

1. Sections were incubated with 100-200 μl of primary antibody.
2. Sections were washed with tris buffer saline and covered in buffer for 10 minutes.
3. Decant, wipe excess buffer and incubate sections with the secondary antibody.
4. Sections were washed with tris buffer saline and covered in buffer for 10 minutes.

2.4.6 Formation of Peroxidase Complex and Chromogen Formation

Currently, two avidin-biotin methods are in frequent use – the avidin-biotin complex (ABC) and the labelled avidin-biotin (LAB) technique. These methods utilize the high affinity of avidin or strept-avidin for biotin (dissociation constant 10^{-19} M) (Elias *et al*, 1989). Avidin has four binding sites for biotin. However, due to the molecular orientation of the biotin-binding sites, fewer than four molecules of biotin will actually bind (Naish *et al*, 1989). In this study; the strepavidin-horse radish peroxidase (Vectastatin- 6100 Elit ABC kit) was used as a labelling enzyme. Strepavidin is isolated from *streptomycin avidinii* which is uncharged at a neutral pH, thus reducing the possibility of non-specific binding (Larsson 1988). In this step; the open sites of avidin

bind to the biotin which is covalently attached to the secondary antibody (the linker antibody) resulting in avidin-biotin-enzyme complex (ABC) formation. The reaction products of the peroxidase were then visualized with 3'-3' diaminobenzidine tetrahydrochloride (DAB) [Appendix 1]. The DAB chromogen produces a brown end product (stain) which is highly insoluble in alcohol and other organic solvent. Oxidation of DAB also causes polymerisation, resulting in increase in its electron density and staining intensity (Naish *et al*, 1989). The technique applied was as follows: -

1. Sections were incubated in avidin-biotin complex reagent for 30 minutes.
2. Sections were washed with tris buffer saline and left covered in buffer for 10 minutes.
3. Sections were incubated in DAB solution in a clean container for 3-10 minutes.
4. Sections were washed in running water for 5-10 minutes.

2.4.7 Counter Staining

Section were briefly counterstained in Mayer's Haematoxylin for 2 minutes and allowed to blue in tap water. Sections were then dehydrated through graded alcohols, cleared in two changes of xylene and cover-slipped with a permanent mounting medium. Sections were then evaluated by light microscopy.

2.4.8 Controls and Reproducibility Testing

Reagent and procedure controls are necessary for the validation and proper interpretation of immunohistochemical staining. Of all the components used in this staining system, the primary antibody is the most critical, although other reagents may need to be optimised. Tissue controls; either positive, negative or built in (internal) controls serve as indicators of specimen handling, processing and demonstration of the immunoreactivity of the antibody being used. Positive tissue controls must contain target protein. In some cases, it will be advantageous to have this control tissue stain marginally positive, so as to monitor not only for the presence of the antigen, but also for any possible loss of sensitivity. Negative tissue controls are specimens which on being tested were found not to be containing the target protein. Thus processing such specimens could assess non-specific binding of the antibody. The presence of internal control such as peripheral nerves or lymphocytes within the unknown section is of additional advantage and obviates the use of separate positive tissue control. To test the reproducibility of our stained slides; each batch of 10 slides contained known positive

and negative tissue controls. Sections from colon adenocarcinoma, tonsil, follicular lymphoma, prostate carcinoma and intestinal type gastric adenocarcinoma were used as tissue controls for p53, Ki-67, bcl-2, bax and bcl-x antibodies, respectively. The presence of muscle cells and lymphocytes served as internal positive tissue controls for bcl-2, bax and bcl-x. Replacement of the primary antibody and secondary antibody with tris buffer during the staining procedure provided negative control for reagents.

2.4.9 Interpretation and Labelling Index

Sections demonstrating distinct moderate to intense brown nuclear (p53 and Ki-67) or cytoplasmic (bcl-2, bax and bcl-x) were considered to be immunopositive. The labelling index (LI) was obtained for each case using a protocol described by (Sullivan *et al*, 1993). This was defined as the number of cells showing positive immunostaining as a percentage of total cells counted. In this study up to 2000 cells were counted by light microscope at 400x magnification using an eyepiece graticule to define the areas being counted. Sections demonstrating weak staining with a labelling index <5% was considered to be immunonegative.

2.5. CELL TISSUE CULTURE

2.5.1 Source of Cell Lines

Human oesophageal cancer cell lines JROECL-33 and JROECL-21 were dispatched from European Collection of Animal Cell Cultures (ECACC), Salisbury, Wiltshire, UK. JROECL-33 and JROECL-21 cell- lines were established from stage IIA (T2-3, N0, M0) poorly differentiated adenocarcinoma of the lower oesophagus (Barrett's metaplasia) of a 73 year old Caucasian female patient and from stage IIA moderately differentiated squamous cell carcinoma of the middle oesophagus of a 74 year old Caucasian male patient, respectively (Rockett JC, *et al*; 1997). Both cell lines grew as monolayer and showed pleomorphic-epithelial morphology with some multinuclear giant cells.

2.5.2 Maintaining and Growing Cell Cultures

As recommended by ECACC; Cell lines were grown in complete medium, consisting of RPMI-1640 medium supplemented with 10% filtered foetal calf serum (FCS), 100 units/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine and at 37°C [appendix 1] (all cell culture reagents were obtained from GIBCO BRL, Life Technology Ltd.). The

medium was stored at 4°C and used within 2 weeks, as glutamine can become enzymatically converted by serum enzyme (Griffith, 1992). A stock solution of penicillin/streptomycin and L-glutamine was prepared and stored in aliquots at -20°C [Appendix 1]. FCS was incubated at 56°C in a water-bath for 1 hour to heat inactivate complement, and stored in aliquots at -20°C until required. Cells were cultured in supplemented RPMI medium at a density of $1x10^4$ cells/cm^2 and maintained at 37°C in a 5% CO_2 incubator. Cells were inspected regularly by phase contrast microscopy to determine their condition prior to experimental use. Confluent adherent cells were detached from the culture flasks using 0.25% trypsin-EDTA; culture media, which contained dead cells and metabolites, were carefully removed from the flasks. To remove any remaining cell debris, cells were washed twice with Hank's balanced salt solution (HBSS) containing 1M HEPES. Cells were then incubated with 0.25% trypsin-EDTA at 37°C and were monitored until all the attached cells were lifted from the flask. Cells were collected in a sterile tube and centrifuged at 1000 RPM for 5 minutes. Cells were re suspended at complete medium and seeded at recommended concentrations.

2.5.3 Enumeration and Viability of Cells

Cell number and viability were assessed by fluorescence microscopy, using ethidium bromide and acridine orange (EB/AO) (Lee *et al*, 1975). Suspended cells were diluted 1:10 in EB/AO working solution [Appendix 1], vortexed and then pipetted under a coverslip on a haemocytometer (Hudson and Hay, 1976). Fluorescent cells were viewed using a Leits Dialux microscope with ultraviolet filter. Viable cells emit green fluorescence due to the presence of EB. Non-viable cells emit red fluorescence due to the presence of AO.

2.5.4 Preservation and Resuscitation of Frozen Cells

Stocks of viable cells were maintained by cryopreservation according to a protocol provided by ECACC (Liss, 1988). Adherent Cells growing at exponential phase were trypsinised, washed twice in HBSS and resuspended at 2 x 10^6 cells/ml in filtered FCS containing 10% dimethyl sulphoxide (DMSO) [Appendix 1]. Cells in 1 ml aliquots in sterile cryostat tubes (Sarstedt, Numbrecht, Germany) were Frozen at -80°C for 24 hours prior to transfer to a gaseous phase liquid nitrogen storage vessel (-185 °C). When required; frozen cells were easily reconstituted by rapid thawing of an aliquot and resuspention in 10 ml complete medium. It is important to thaw cells quickly to

minimise any damage to the cell membranes. Cells were then centrifuged at 1000 rpm for 5 minutes, washed twice with HBSS, resuspended in 5ml of complete medium in a $25cm^3$ tissue culture flask and incubated in a 5% CO_2 incubator at 37°C. Cells between 3rd and 15th passages were used for experiments.

2.6 EXTRACTION OF CELLULAR PROTEINS

2.6.1 Cell Preparation and Total Cell Lysis

At the end of each experiment adherent and detached cells were collected. Adherent cells were scraped from the surface of culture plate using a cell scraper and approximately 2 x 10^7 cells were collected by low speed centrifugation (1000 rpm for 5 minutes at room temperature). The supernatant was carefully removed and the pellets washed with PBS at room temperature and again collected by low speed centrifugation. Cells were then incubated with 1 ml ice-cold lysis buffer (Ripa buffer) with freshly added protease inhibitors (Research Application - Santa Cruze Biotechnology) [Appendix 1]. To shear the DNA, the mixture was passed through a 21-gauge needle and transferred to a microcentrifuge tube and placed on ice for 30-60 minutes - vortexed every 5 minutes. The microcentrifuge tube was then centrifuged at 10,000g for 10 minutes at 4°C. The supernatant fluid is the total cell lysate which was aliquoted and stored at -70°C.

2.6.2 Estimation of Protein Concentration

Protein concentration of each cell lysate was determined using the Bradford assay (Bradford, 1976). A standard curve was established by using a stock solution of bovine serum albumen (BSA) [Appendix 1]. The standard solution contained 0, 25, 50, 75 or 100μg BSA /100μl of solution (25mg BSA in 25ml buffer gives a stock solution of 1μg/1μl and the range of the standards were prepared from this). The sample concentration was determined within this range. Standard and test solutions were prepared in the PBS as indicated in (Table 2.1). The Bradford reagent [Appendix 1] was filtered immediately before pipetting 5ml into each tube. The tubes were vortexed and colour development was allowed to proceed for 30 minutes. Absorbance was read at 595nm in a 1 cm light path against a reagent blank. A standard curve was plotted from a BSA standard which allowed for estimation of the sample's protein concentration.

2.6.3 Protein Precipitation

Acetone precipitation was performed for preparing protein samples for Polyacrylamide Gel Electrophoresis (PAGE). Sufficient volume was aliquoted to yield 50μg protein per sample per lane. This volume was then diluted 1:5 with ice-cold acetone (stored at -20°C), vortexed and allowed to incubate at -20°C for a minimum of 30 minutes. The precipitated protein was micocentrifuged at 15,000g for 2 minutes and the supernatant was then removed. Excess acetone was allowed to evaporate at room temperature for 15 minutes. To the precipitated protein 20 μl of 1X sample buffer [Appendix 1] was added, briefly mixed and incubated in a boiling water bath at 95°C for 5-10 minutes. At this point, samples can be applied directly to an SDS-PAGE gel or they can be stored at -20°C for latter use.

2.7 ELECTROPHORESIS AND WESTERN BLOTTING

2.7.1 Preparation of SDS-PAGE Gels

The gel equipment was set up according to the manufacturer's specification (ATTA-Medical Supplies, Japan). Gasket and glass plates were thoroughly washed with detergents. The gel-plates were further cleaned with ethanol to remove protein residues or other debris from previous experiments which might have interfered with the running of the gel and protein samples. The resolving (running) gel [Appendix 1] was marked (depth 6.5cm, width 9cm). The composition of the running gel (Table 2.2) is dependant on the molecular weight of the proteins of interest. In this study, targeted proteins were ranged between 18 - 53 kDa and a 10% polyacrylamide gel was used. Depending on the number of gels, the appropriate volumes required to make up the gels were prepared and mixed. The polymerising agents -ammonium persulfate (APS) and the TEMED- were added last. The mixture was swirled gently to a mix, since excessive agitation incorporates oxygen into the mixture which inhibits polymerisation. The mixture was then pipetted into the casting stand, allowing about 2cm at the top for the stacking gel. A layer of water-saturated butanol [Appendix 1] was layered on the top of the gel preventing contact with oxygen and then allowed to polymerise for 1 hour. When the running gel set the water saturated butanol was completely removed by washing with water, until butanol was no longer detected. At this stage the gel can be overlaid with resolving buffer and stored at 4°C overnight. Alternatively, the stacking gel can be prepared as indicated in table 2.2 and poured to fill the casting stand. A clean comb was inserted carefully into the stacking gel and allowed to polymerise for 30 minutes. Prior

Table 2.1. Preparation of standard solution for Bradford assay.

Solution (µl)	Volume of Diluents PBS (µl)	Total Volume (µl)	Bradford solution (ml)
BSA (µg/100µl)			
0	100	100	5
25	75	100	5
50	50	100	5
75	25	100	5
100	0	100	5
Samples			
10	90	100	5

Table 2.2. Composition of 10% gel for SDS-PAGE.

Component	Resolving gel (10%)	Stacking gel (10%)
Distilled water	8.23 ml	5.55 ml
Resolving buffer	5 ml	---
Stacking buffer	----	3.05 ml
Acrylamide	6.66 ml	1.33 ml
10% SDS	200 µl	100 µl
10% APS	100 µl	50 µl
TEMED	10 µl	10 µl
APS; ammonium persulfate; TEMED: N,N,N'-N'-tetra-methylenediamine. Composition sufficient for 2 gels.		

to running the gel, the casting stand, gasket and combs were removed. Unpolymerised gels were removed by gently rinsing the wells with distilled water, and the wells were then were straightened using a loading tip.

2.7.2 Sample Preparation and Molecular Weight Marker

Ten µl of samples that resuspended in 20µl sample buffer (section 2.6.3) were loaded to each lane. The molecular weight markers (Sigma-Aldrich, USA) were also mixed with 1X sample buffer and were incubated in water bath at 95°C for 5-10 minutes. Five µl of the molecular marker was loaded in the first lane of each gel. The molecular weight marker consists of 7 precisely sized recombinant proteins. The molecular weights of these proteins were 15, 25, 35, 50, 75, 100 and 150 kDa.

2.7.3 Polyacrylamide Gel Electrophoresis

The electrophoresis unit was filled with electrode buffer [Appendix 1] to the level of the horizontal rubber gasket. The gels were lowered in the buffer ensuring that there were no air bubbles underneath the gels. The gel plates were fixed firmly in place and the inner reservoir, formed by two gels plates, was filled with electrode buffer to the point where the buffer filled the gel wells fully. The boiled protein samples (50µg) and standard molecular weight markers were loaded onto the gel. The electrodes were connected and the gels run at 35 mAmps through the stacking gel and 50 mAmps through the resolving gel. When the sample front reached the end of the gel, the power supply was disconnected and the proteins on the gels could, at this point, be stained by Coommassie Blue (section 7.4.5) or transferred to Biotrace PVDF (polyvinylidene difluoride) membrane (Gelman Sciences Inc., MI, USA).

2.7.4 Transfer of Proteins to PVDF Membrane

Proteins were transferred by semi-dry transfer as described by (Towbin, 1997). The graphite electrodes of the transfer system were saturated with distilled water for 30 minutes prior to transfer. Six sheets of (6.5 x 9 cm) Whatman filter paper and one PVDF membrane (6.5 x 9 cm) were cut for each gel. The PVDF membrane was soaked in methanol for 2 minutes and then in the transfer buffer [Appendix 1] for 20 minutes before use (Harlow and Lane, 1988). The resolving gel was removed from the plates and rinsed with transfer buffer to remove SDS which may interfere with the transfer of

proteins. The filter papers were soaked in transfer buffer immediately before assembly of the transfer sandwich. Three sheets of soaked filter paper were placed on the cathode bottom electrode, the PDVF membrane was placed on top, then the resolving gel and, the remaining three filter papers were placed over the gel. Air bubbles trapped between the layers and any residual buffer surrounding the transfer sandwich which, interferes with transfer efficiency were removed. Finally, the anode, top electrode, was placed in contact with the last filter paper. Transfer was performed at (0.8 mAmps/cm^2) for 1 hour. Once the transfer was completed the PVDF membrane and gel were soaked separately in PBS. Individual lanes in the PVDF membrane were marked and the lane (molecular weight strip) containing the standard weight markers was removed and stained with coomassie blue for 5 minutes followed by detaining with 50% methanol. The remaining membrane was processed for protein detection.

2.7.5 Coomassie Staining and Determination of Molecular Weight of Proteins

The efficiency of semi-dry transfer was assessed by Coomassie staining the gel post transfer. Gels were immersed in 0.1% Coomassie Blue [Appendix 1] and allowed to stain overnight at room temperature. After staining was completed, excess stain was removed by diffusion distaining. The distaining solution [Appendix 1] was renewed until an acceptable level of background staining was achieved. The gels were then transferred to 7% acetic acid solution for storage. Visualization of positions of the standard protein bands (molecular weight strip) following transfer was used to determine the molecular weight of the sample proteins. The relative mobility (R_f) values of specific proteins were determined by dividing the migration distance of the protein from the top of the gel to the centre of the protein band, by the distance of migration of the tracking dye front from the top of the gel. The R_f value of the standard proteins was then plotted against its known molecular weight on semi-logarithmic paper and the standard curve allowed for estimation of the molecular weight of unknown bands.

2.7.6 Blocking of PVDF Membrane

Non-specific binding sites were blocked by incubating the PVDF membrane with blotto-tween [Appendix 1] for 1 hour at room temperature. Alternatively, the membrane may be blocked at 4°C overnight in a covered container, using blotto without tween.

2.7.7 Western Blotting

Once the non-specific sites were blocked, proteins of interest were probed with specific antibodies. The primary antibodies (dilution 1:1000) were those described previously (section 2.4.5) and the secondary antibodies (dilution 1:3000) were Horse-Radish Peroxidase conjugated anti mouse/anti rabbit. The PVDF membranes were incubated with the primary antibody for 1 hour with gentle agitation at room temperature. Membranes were then washed twice with PBS each wash lasting 10 minutes and then incubated with HRP conjugated secondary antibody for 45 minutes at room temperature. The PVDF membrane was washed with PBS as described above. The membrane was then ready for chemiluminescence detection.

2.7.8 Enhanced Chemiluminescence Detection

The antigen-antibody complex was detected by incubating the membrane in a western blotting luminol reagent [Appendix 1] for 1 minute at room temperature. The membrane was then transferred face down to clingfilm, any air bubbles were excluded and the membrane carefully wrapped. Blots were exposed to X-ray film for 10-60 seconds. Exposed films were then developed, fixed and allowed to air dry.

2.8 EXTRACTION OF GENOMIC DNA

2.8.1 Extraction of DNA from Tissue Culture Cells

Total genomic DNA was extracted from treated and control oesophageal carcinoma cell lines using QIAamp DNA Blood Mini Kit (QIAGEN). Following the manufacturer's instruction; maximum $5x10^6$ cells were harvested and resuspended in 200 µl PBS and transferred to a 1.5ml sterilized microcentrifuge tube. To the suspended cells; 20µl Qiagen protease, 20µl of DNase free RNase A (stock of 20mg/ml) and 200µl lysis buffer (buffer AL) were added. Samples were mixed by pulse-vortexing and incubated at 56°C for 10 minutes. In order to ensure efficient lysis, it is essential that the sample and the lysis buffer are mixed thoroughly to yield a homogenous solution. Incubating the sample at 95°C for an additional 15 minutes after cell lysis can inactivate infectious agents; however, extended incubation at such a high temperature can lead to some DNA degradation. After complete cell lysis 200 µl ethanol (96-100%) were carefully added to the sample, mixed by pulse-votexing for 15 seconds and briefly centrifuged to remove drops from the inside of the lids of the microcentrifuge tube. The mixture was then

carefully applied to the QIAamp spin column without wetting the rim and centrifuged at 6000g (8000 rpm) for 1 minute. The QIAamp spin column was placed in a clean 2ml collection tube (provided with Kit) and 500µl washing buffer (AW1) was added to the QIAamp spin column and centrifuged at 6000g for 1 minute. The tube containing the filtrate were discarded and the QIAamp spin column was placed in a clean 2ml tube with 500µl of washing buffer (AW2) being carefully added to the spin column and centrifuged at 20,000g (14000rpm) for 3 minutes. To eliminate any chance of possible buffer AW2 carryover the QIAamp spin column was placed in a clean 2ml tube and centrifuged at 20,000g for 1 minute. The QIAamp spin column was then placed in 1.5ml sterile microcentrifuge tube and 100µl elution buffer (Buffer AE) were added and the tubes were incubated for 5 minutes at room temperature. To elute the DNA the spin column was centrifuged at 6000g for 1 minute. The eluted DNA (the filtrate) was collected and stored at -20°C for later use.

2.8.2 Determination of DNA Concentration

DNA yield is determined from the concentration of DNA in the eluate, measured by absorbance at 260 nm. To be accurate, absorbance reading at 260 nm should fall between 0.1 and 1.0 and the sample dilution should be adjusted accordingly. Sterilized-filtered water was used to dilute samples and to calibrate the spectrophotometer. DNA samples were diluted (1:100) and the absorbance measured at 260 nm ($1A_{260}$= 50 ng DNA/µl sample). For example, a sample containing 25ng DNA/ µl will read A_{260} of 0.5. The purity of the DNA sample was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an A260/A280 ratio of 1.7-1.9.

2.9 POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) is an in-vitro technique that enables the amplification of specific sequences of nucleic acids (DNA or RNA). Although invented by Kary Mullis (Mullis *et al*, 1986) and described originally by (Saiki *et al*, 1985) the principle had been described over a decade earlier by Kleppe and Panet (Kleppe *et al*, 1971; Panet *et al*, 1974). PCR technology is unique in its ability to locate and exponentially amplify a small quantity of a specific nucleotide sequence which is ''lost'' against a large background of total nucleic acid. This feature of PCR has made possible the development of a vast number of experimental and diagnostic molecular

biology techniques, which were previously extremely time consuming or, in many cases, impossible to perform. In the PCR (Figure 2.1), two primers (short single stranded DNAs) are used that are complementary to opposite strands of the DNA sequence to be amplified. After heat mediated denaturation of the template DNA, the primers bind to their respective sequences (annealing) on the template DNA and a DNA polymerase synthesizes a complementary strand in the 5' to 3' direction (extension). Each round of denaturation, annealing, and extension is known as a cycle. Theoretically, with each cycle the amount of the template DNA sequence amplified doubles. Therefore, after 10 cycles the target sequence within the template DNA is multiplied by a factor of one thousand and after 20 cycles by a factor of more than 1 million. However, an "amplification plateau" will eventually be reached when additional cycles will not lead to any further increase in amplified product. This amplification plateau results from the exhaustion of reagents such as the dNTPs and primers. The optimum temperature at which each of these steps (denaturation, annealing, and extension) proceeds is different and therefore the reaction is best performed in a thermal cycler, which makes the temperature changes automatically.

2.9.1 Amplification of Exons 5 - 8 of P53 Gene

Polymerase chain reaction (PCR) technique was utilized to amplify exons 5-8 of p53 gene using HotStarTaq Master Mix Kit (QIAGEN). The PCR products were then used for mutation analysis and automated DNA sequencing. PCR was carried out in 50µl reaction volume containing <1 µg of genomic DNA, 200µM of each deoxynucleotide (dATP, dTTP, dCTP and dGTP), 1.5 mM $MgCl_2$, 2.5 U HotStarTaq DNA polymerase and 15pmole of each primer. Specific oligonucleotide primers homologous to sequences in the adjacent introns were used (Hsu *et al*, 1991). The sequences of p53 primer pairs were listed in (Table 2.3). The PCR was performed in a heated lid DNA thermal cycler (MJ Research PTC-100) with the following temperature profile. The reaction was started with an initial heat activation of Taq DNA polymerase at 95°C for 15 minutes followed by 3 step/cycle for total of 35 cycles: denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute for exon 5 and 8 and 64°C for exon 6 and 7, extension at 72°C for 1 minute. The reaction was completed with a post-cycling extension at 72 °C for 10 minutes. The final PCR products were stored in sterile 0.5ml tubes at –20°C for subsequent analysis.

2.9.2 Verification of PCR Products

To verify proper PCR setup and to detect possible contamination a negative control that lacks the template nucleic acid was run together with the samples. To verify that the expected sized products were amplified, 5 µl of each PCR product (including a negative control - set of PCR reaction without DNA template) were mixed with 1µl gel loading buffer [Appendix 1] and run on a 2% agrose gel containing 0.5µg/ml ethidium bromide. A lane containing a 100bp DNA ladder (Boehringer Mannheim, Germany) was run alongside the samples. The product sizes expected for amplified regions were as follow: exon 5 = 310bp, exon 6 = 223bp, exon 7 = 237bp and exon 8 = 231 bp.

2.9.3 Purification of PCR Products

After amplification, the PCR sample may contain a complex mixture of specific PCR product and residual reaction components such as primers, unincorporated nucleotides, enzymes, salts and probably non-specific amplification products. Before the specific PCR product can be used in subsequent experiments, it is often necessary to remove these contaminants. In this study the PCR products were purified using a QIAquick PCR Purification kit (QIAGEN). With this kit, PCR fragments ranging from 100bp to 10kb can be purified from primers, polymerasese and other residual components. According to the manufacturer's protocol the clean up procedure was as follow: -

1. Add 5 volumes of buffer PB (provided) to 1 volume of the PCR reaction and mix.
2. Place a QIAquick spin column in a provided 2ml collection tube.
3. Apply the samples to the QIAquick column and centrifuge at 13000 rpm for 1 min.
4. Discard flow-through and place the QIAquick column back into the same tube.
5. Add 750µl buffer PE to the QIAquick column and centrifuge at 13000 rpm for 1 min.
6. Discard flow-through and place the QIAquick column back into the same tube.
7. Centrifuge the column for 1 minute at maximum speed (14000 rpm).
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µl buffer EB to the centre of the QIAquick tube.
10. Incubate for 1 minute and centrifuge the column for 1 minute at 13000 rpm.
11. Eluted DNA (PCR product) was stored in sterile tubes at –20°C for later use.

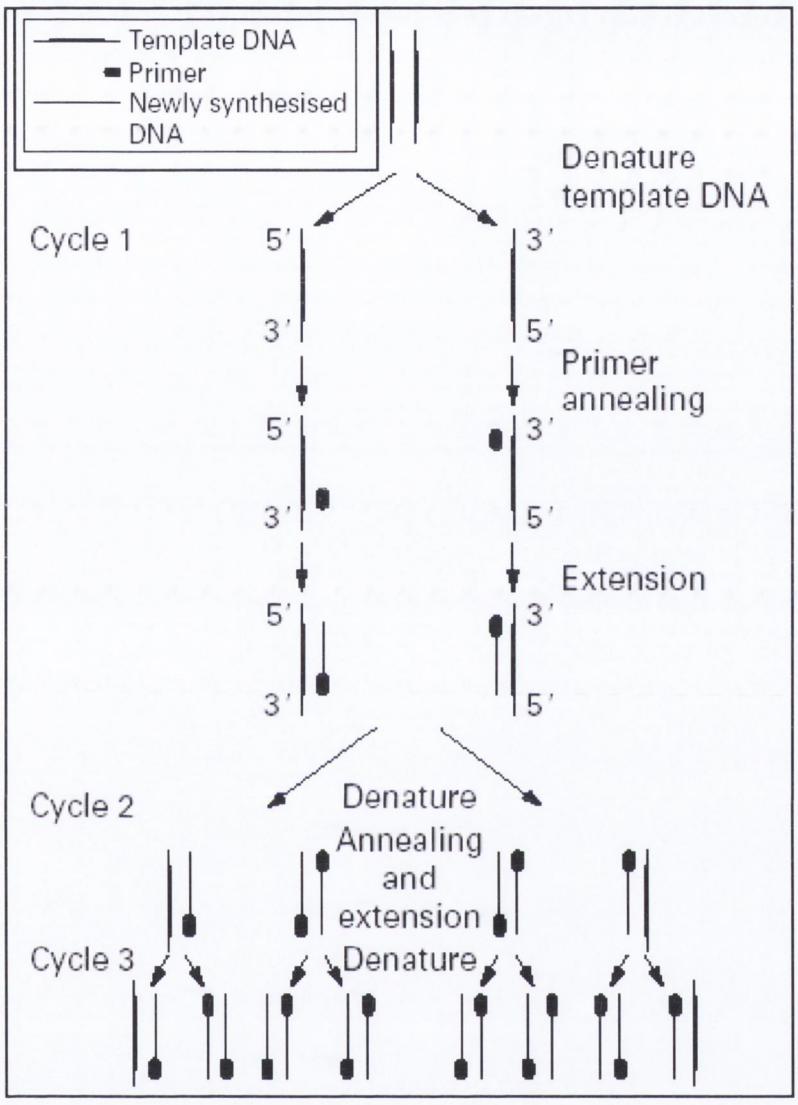

Figure 2.1. Schematic diagram of Polymerase Chain Reaction. Three cycles are shown here, most PCR reactions occur over 25-30 cycles.

Table 2.3. PCR amplification and sequencing primers for p53, exons 5-8.

p53	Primer sequences	Application: PCR and Sequencing
Exon 5	5′-TGTTCACTTGTGCCCTGACT-3′	Forward PCR and sequencing primer
	5′-AGCAATCAGTGAGGAATCAG-3′	Reverse PCR and sequencing primer
Exon 6	5′-TGGTTGCCCAGGGTCCCCAG-3′	Forward PCR and sequencing primer
	5′-GGAGGGCCACTGACAACCA-3′	Reverse PCR and sequencing primer
Exon 7	5′-CTTGCCACAGGTCTCCCCAA-3′	Forward PCR and sequencing primer
	5′-AGGGGTCAGCGGCAAGCAGA-3′	Reverse PCR primer
	5′-TGTGCAGGGTGGCAAGTGGC-3′	Reverse sequencing primer
Exon 8	5′-TTCCTTACTGCCTCTTGCTT-3′	Forward PCR and sequencing primer
	5′-AGGCATAACTGCACCCTTGG-3′	Reverse PCR and sequencing primer

Sequencing analysis of exons 5, 6 and 8 used the same primers as those used for PCR amplification. For exon 7, an internal primer was used in the reverse direction to give optimal sequencing results.

2.10 WAVE NUCLEIC ACID FRAGMENT ANALYSIS SYSTEM

Detection of p53 mutations and polymorphisms in the oesophageal carcinoma cell lines were determined by means of PCR and Temperature Modulated Heteroduplex Analysis (TMHA). TMHA employs the formation of heteroduplexes between wild-type (reference) and mutated (variant) DNA, which are efficiently separated on the Wave DNA Analysis System (Transgenomic, Inc., San Jose, CA, USA). TMHA relies upon the physical changes in DNA molecules induced by mismatched heteroduplex formation during re-annealing of wild type and mutant DNA. Individuals heterozygous for a polymorphism have a 1:1 ratio of wild-type to mutant DNA. When the PCR product from such an individual is heated to 95°C and slowly cooled, the DNA strands separate and randomly re-anneal to form a mixture of three species: a mutant homoduplex, a heteroduplex, and a wild-type homoduplex. The same results can be achieved with DNA from a homozygous mutant by first mixing it with wild type DNA and then allowing the denaturing and hybridisation to occur (Underhill *et al*, 1996). Material from a homozygous wild-type individual, however, will only ever form one species: the wild-type homoduplex. When mutant and wild-type DNA strands re-anneal to form a heteroduplex, the molecule will be physically altered. At the region of base pair mismatches, a "bubble" will form yielding a short linear region of single-stranded DNA (Figure 2.2). This structural alteration can be used to distinguish and separate the heteroduplex from the homoduplex species and ultimately identify the polymorphism. The Wave System platform is based upon ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) and can be operated in three modes as determined by the temperature at which separation is performed (Figure 2.3). Under-denaturing temperatures, sequence independent fragment separation is possible and it allows high-resolution separation of fragments for length polymorphism assays, cloning, RT-PCR. At fully denaturing temperatures, single stranded molecules (RNA or DNA) can be separated. This mode is particularly suitable for oligonucleotide quality control and purification, where oligos can be effectively separated from failure sequences or for primer extension genotyping assays. The third mode of operation involves the separation of fragments under partially denaturing conditions. Sequence variations in PCR product creates mismatched heteroduplexes during re-annealing of wild type and mutant DNA. The resulting differences in melting temperature between heteroduplexes and homoduplexes allows for separation by IP-RP-HPLC, thereby identifying variations (mutations). To achieve the best possible separation the region

that contains the mutation should have a helical fraction between 30-99%. The melting profile of the oligo-duplex fragment(s) is determined by Wave system utility software (Wave Maker software version 4.0, Transgenomic). Using the normal sequence of the DNA fragment (PCR product in this study), the automatically selected temperatures are precisely maintained by the wave system oven during the analysis. The Wave Maker usually chooses temperatures that result in 75% helicity of DNA fragment when performing mutation screening. If hetroduplexes are present, additional peaks or shoulders will be observed in the chromatogram. The central algorithm to describe this process by which the melting point is determined is based on the Fixman and Friere implementation of Poland's algorithm, which calculates the probability that a base is in the helical duplex form or the non-helical, single-stranded form. In order to optimise the analytical condition in our experiments the following procedures were performed.

2.10.1 Sample Preparation and PCR Amplification

Total genomic DNA was extracted from JROECL-33, JROECL- 21 cell lines as described previously (section 2.8) and was subjected to PCR amplification for exon 5-8 of p53 gene (section 2.9.1). Colon adenocarcinoma cell line HCT-116 known to express wild-type p53 gene (known homozygous) was used as reference DNA. A ratio of 1:1 of unknown (JROECL -33 or -21) and control DNA sample (HCT-116) was used as templates for PCR and TMHA. Unknown and control samples were also prepared and analysed separately for individual comparison.

2.10.2 Verification of PCR Product Yield and Purity

Initial non-denaturing analysis of PCR products at 50°C is performed to determine the size, yield and quality of PCR products prior to mutation detection analysis on the Wave System. In this step the PCR products should appear as single, clean peaks of the expected size at 50°C. If the chromatogram shows more than a peak, or a peak with a shoulder, this indicates that there is more than one fragment of DNA in the sample and the PCR protocol, therefore, needs to be optimised. Seven µl of the final product (s) were analysed at 50°C along with the X174/HaeIII DNA standard marker. The DNA size marker contained 11 fragments of sizes 72, 118, 194, 234, 271, 281, 310, 603, 872, 1087, and 1353 bp.

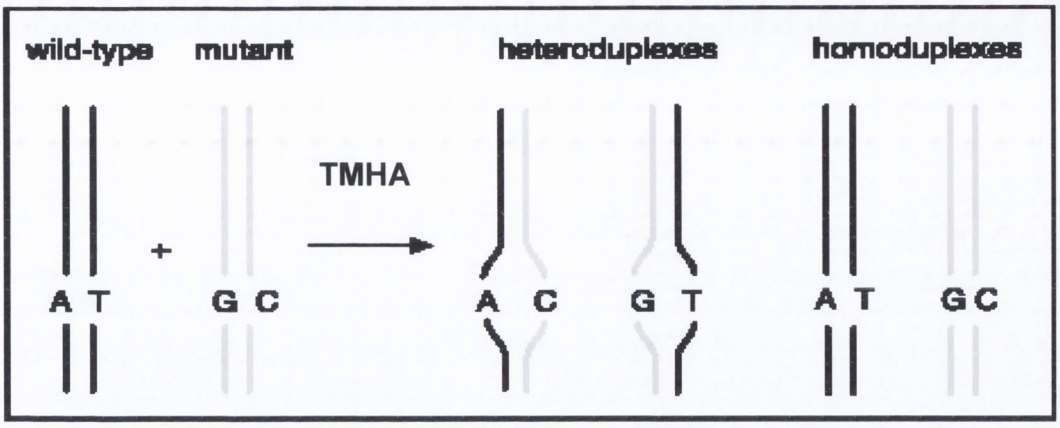

Figure 2.2. Heteroduplex formation through hybridisation of a wild-type with a mutant DNA.

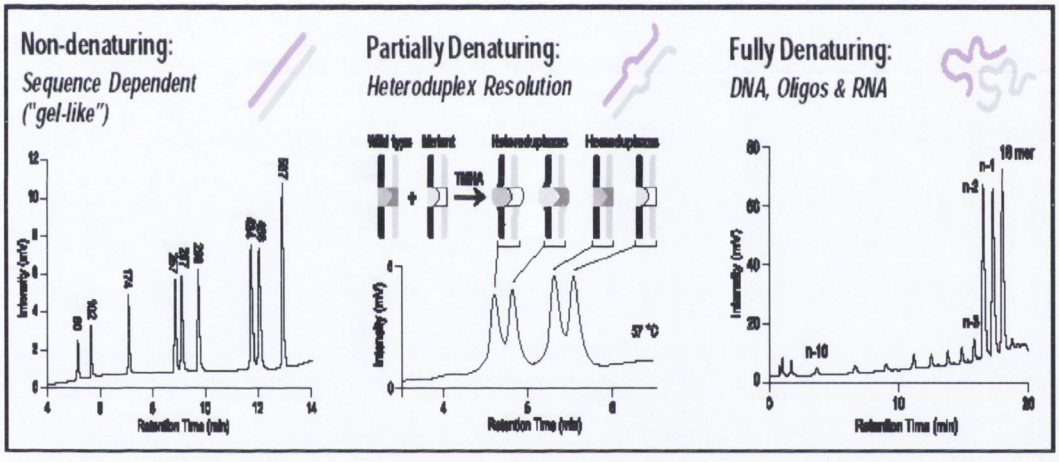

Figure 2.3. Three modes of operation of the Wave Nucleic Acid Fragment Analysis System. The positively charged double stranded molecules (homoduplex) have stronger interaction with the column than heteroduplex species and are, therefore, retained for longer in the matrix, leading to the separation of the two species.

2.10.3 Hybridisation to Form Heteroduplexes

Heteroduplex formation was performed with PCR products under conditions where the reference (wild type) and variant (mutant) DNA were mixed in a ratio of 1:1. The PCR product was then subject to denaturation at 95°C for 5 minutes and then slowly re-annealed by ramping the temperature down to 25°C at rate of 0.1°C/4 second. This thermocycle step can be performed after the initial PCR implications. Samples can be stored at 25°C prior to Wave Analysis. Hetroduplexes/mutations could be detected using the above approach even at 500-fold excess of wild type allele.

2.10.4 Heteroduplex Melting Profile

For a given sequence (exons 5-8) of p53 gene; the melting characteristics of each fragment was determined using Wave Maker software version 4. The software predicts the average melting temperature (Tm) for the entire fragment and is used as a midpoint for a temperature titration. Wave System recommends an initial 5°C temperature titration in 1°C increments to screen 2°C above and below the temperature predicted by Wave Maker software. The temperature titration of exon 5-8 of p53 gene used to detect mutations in our samples is displayed in (Figure 2.4). The separation of heteroduplex and homoduplex were performed at partially denaturing temperature as recommended by Wave System.

2.10.5 Chromatogram Interpretation

The observation of heteroduplex peaks in a chromatogram indicates the presence of mutations, while samples without mismatches resolve as homoduplexes. Heteroduplex peaks elute earlier than homoduplexes, and can be observed as separate peaks or as shoulders on the leading edge of homoduplex peaks. The manner in which a heteroduplex peak resolves is influenced by the specific nucleotide mismatch present and the melting characteristics of the surrounding bases. Elution profiles that differ from wild type or reference DNA indicate the presence of mutations or polymorphisms.

2.11 SEQUENCING ANALYSIS AND POLYMORPHISM IDENTIFICATION

Automated DNA sequencing with ABI PRIZM 310 Genetic Analyser (Perkin-Elmer-Applied Biosystems, USA) was performed to identify the type of p53 mutations detected by Wave Nucleic Acid Fragment Analysis System. The ABI sequence software (DNA sequencing analysis, version 3.0) was used for sequence analysis. Sequencing

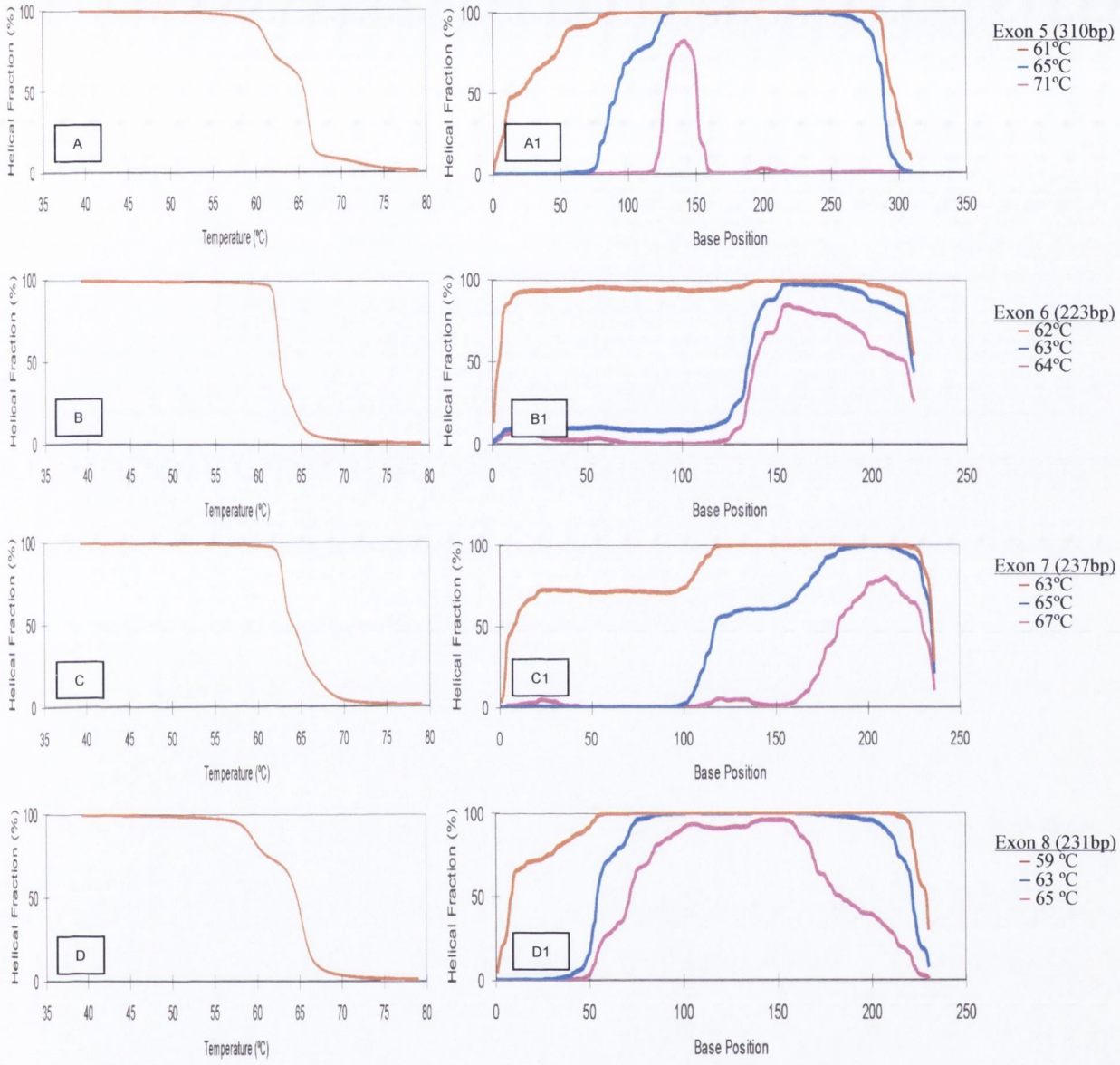

Figure 2.4. Illustration of three-temperature melting profile of exons 5-8 of p53 gene. A-D shows the predicted temperature range for the transition (melting) of the fragments from double strand DNA (100% helical) to single stranded DNA (0% helical). A1-D1 shows the pattern of denaturation along the fragment at the temperatures entered in the partial-denatured fields. For each fragment, three melting temperatures were determined by Wave Maker Software to screen for mutation in a helical fraction between 30-99%.

traces in electorpherogrames were visually inspected, and variants were identified by comparison with traces of the wild type human p53 sequence relative to the reference GenBank sequence (Accession number U94788 - National Centre for Biotechnology Information) [Appendix 3]. Each variant position was confirmed by reamplification and resequencing of the variant site from the two DNA strands (Forward and reverse sequence).

2.11.1 Templates Preparation and Sequencing Primers

Purified PCR products of exons 5-8 of p53 gene (section 2.9.3) were used as DNA templates for sequencing. Sequencing primers were the same as those used for initial PCR amplification except that exon 7 an internal primer with following sequence (5′-TGTGCAGGGTGGCAAGTGGC -3′) was used in the reverse direction. The amounts of purified template and sequencing primer used in a sequencing reaction were 3-10ng and 3.2pmol, respectively.

2.11.2 Sequencing Reagents

Cycle sequencing PCR was performed using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer-Applied Biosystems). This kit provides the dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA polymerase-FS, r*T*th pyrophosphatase (a component in AmpliTaq DNA Polymerase-FS), magnesium chloride and buffer (Tris-HCl pH 9.0) which are premixed and ready for use. The cycle sequencing kit also provides a control double-strand DNA template (pGEM-3Zf(+) control DNA) and a -21 M13 control forward sequencing primer. The results from the control can help determine the quality of the sample template and sequencing reaction. The partial sequence of pGEM from the -21 M13 forward primer, followed by the ensuing 1000 bases is shown in [Appendix 4] "control DNA Sequence."

Description of Reagents

The dye structure contained a fluorescent donor dye, e.g., 6-carboxyflourescein (6-FAM), linked to a dichlororhodamine (dRhodamine) acceptor dye. The excitation maximum of each dye label is that of the fluorescent donor, and the emission spectrum is that of the dRhodamine acceptor. The donor dye is optimised to absorb the excitation energy of the argon ion laser in the DNA sequencing instruments. The BigDye terminators are labelled with following dRhodamine acceptor dyes:

A- Dye Terminator labelled with dichloro[R6G]→ green colour on electropherogam.

C- Dye Terminator labelled with dichloro[ROX]→ red colour on electropherogam.

G- Dye Terminator labelled with dichloro[R110]→ blue colour on electropherogam.

T- Dye Terminator labelled with dichloro[TAMAR]→ black colour on electropherogam.

The deoxynucleoside triphosphates (dNTP) mix includes dATP, dCTP, dITP in place of dGTP to minimise band compression. The dNTP mix also uses dUTP in place of dTTP. dUTP improves the incorporation of the T terminator and results in a better T pattern.

The sequencing enzyme AmpiTaq DNA polymerase, FS is a variant of *Thermus aquaticus* DNA polymerase that contains a point mutation in the active site. This results in less discrimination against dideoxynucleotides. This enzyme also has a second mutation in the amino terminal that virtually eliminates the 5′ → 3′ nuclease activity of the AmpliTaq DNA polymerase. The enzyme has been formulated with a thermally stable inorganic pyrophosphatase to eliminate problems associated with pyrophosphorolysis.

2.11.3 Cycle Sequencing PCR

Cycle-sequencing reactions are made up in a final volume of 20μl. The volume includes 8μl Terminator Ready Reaction Mix, 8μl DNA template (3-10ng of initial PCR product), 1μl primer (3.2 pmol), 3μl Deionised water. Cycle sequencing PCR was performed on the GeneAmp PCR system 2400 thermal cycler (Perkin Elmer-Applied Biosystems) with the following temperature protocol:

1. Place the tubes (samples) in a thermal cycler, and set the volume to 20μl.
2. Repeat the following steps (a-f) for 25 cycles (rapid thermal ramp is 1°C/Second).
   a. Rapid thermal ramp to 96 °C.
   b. 96 °C for 10 seconds.
   c. Rapid thermal ramp to 50 °C.
   d. 50 °C for 5 seconds.
   e. Rapid thermal ramp to 60 °C.
   f. 60 °C for 4 minutes.
3. Rapid thermal ramp to 4°C.
4. Samples can be held at this temperature until ready to purify.

2.11.4 Isopropanol Precipitation of Extension Products

Excess dye terminators in sequencing reactions obscure data in the early part of the sequence and can interfere with base calling. Unincorporated dye terminators were removed before electrophoresis and the extension products were purified using 75% isopropanol. The following steps were followed:

1. Place the entire contents of the extension reaction into a 1.5ml microcentrifuge tube.
2. Add 80 µl of 75% isopropanol, close the tube and vortex briefly.
3. Leave the tube at room temperature for 15 minutes (precipitate extension products).
4. Mark the orientation of the tube and spin for 20 minutes at 14000rpm.
5. Carefully aspirate the supernatants and discard. Pellets may or may not be visible.
6. Add 250 µl of 75% isopropanol to the tubes and vortex briefly.
7. Place the tubes in the microcentrifuge in the same orientation as in step 5.
8. Spin for 5 minutes at maximum speed (14000rpm/minutes).
9. Carefully aspirate the supernatants and dry the sample (pellets) in a vacuum centrifuge for 10-15 minutes or to dryness. Alternatively, place the tubes with the lids open in a heat block or thermal cycler at 90 °C for 1 minute.

Notes:

Step 2, precipitation times shorter than 15 minutes will result in the loss of very short extension products. Precipitation times longer than 24 hours will increase the precipitation of unincorporated dye terminators.

Step 3, if not possible to proceed to step 4 immediately, spin the tubes for 2 minutes more immediately before performing the next step.

Step 5, the supernatant must be removed completely, as unincorporated dye terminators are dissolved in the supernatants.

2.11.5 Resuspension of the Extension Products

Purified extension products were resuspended in a Template Suppression Reagent (TSR, Applied Biosystems). TSR will keep the samples at denaturing condition (single stranded DNA) following denaturation step as described below:

1. Re-suspend each sample pellet (step 9 above) in 12-25µl TSR, vortex and spin.
2. Heat the samples at 95°C for 2 minutes then chill on ice for 10 minutes.
3. Vortex and spin the samples again.
4. Place the samples on ice until ready to run with ABI PRIZM 310 genetic analyser.

2.11.6 Electrophoresis on the ABI PRIZM 310 Genetic Analyser

Prior to running the samples with the DNA sequencer the samples should be correctly prepared and re-suspended. The computer and the instrument have been correctly configured (Refer to Instrument Setting and Configuration Manual Book, ABI PRIZM 310 Genetic Analyser). The instrument should be calibrated: spatial and spectral calibrations have been successfully run and there is sufficient space on the computer hard drive for running and collecting data for analysis. Once the DNA samples were placed in appropriate tubes or in 96-well plates on the auto-sampler, the DNA sequencer will automatically perform the following steps: The autosampler positions the capillary into the sample; the capillary (short-green capillary) is filled with POP-6 polymer, a medium that separates the DNA fragments. This polymer is pumped into and out of the capillary for each new run. The fluorescently labelled DNA is loaded into the capillary by a short period of electrophoresis called electrokinetic injection. The capillary is rinsed with water to remove sample adhering to the capillary sides. The autosampler moves and positions the capillary into the buffer chamber for electrophoresis. When the DNA fragments reach the detection window, the laser beam excites the dye molecules and causes them to fluoresce. The fluorescence emissions from the sample are collected simultaneously and spectrally separated by a reflective spectrograph. The fluorescence emissions are focused as columns of light onto the charge-coupled device (CCD) that is part of the CCD camera. The 310 Sequencing Data Collection Software reads and interprets the fluorescence data, then displays the data as an electropherogram.

2.11.7 Factors Affecting ABI PRIZM 310 Genetic Analyser Performance

There are many factors that affect instrument performance such as the quality and quantity of DNA in a reaction. The presence of residual salts, RNA, proteins, and detergents can interfere with capillary electrophoresis and electrokinetic injection. To avoid such contamination, proper template preparation-purification methods must be used. (Refer to the Automated DNA Sequencing Chemistry-Guide For Protocols).

Effect of Template Quantity: The amount of DNA template used in a sequencing reaction can affect the quality of data. Excess template makes data appear "top heavy" with strong peaks at the beginning of the run that fade rapidly. Too little template or primer reduces the signal strength and peak height. In the worst case, the noise level increases so that bases cannot be called.

Effect of Residual Salts: The 310 Genetic Analyser is especially susceptible to salt in samples, either from template preparation or from cycle sequencing reactions. The negative ions in salts are preferentially injected into the capillary and compete with the injection of larger DNA extension fragments during electrokinetic injection, leading to lower signal and shorter read length.

Effect of Residual RNA: Residual RNA that is present in DNA template preps competes with the DNA for injection into the capillary array and has the same effect as excess salt, that is, decreased signal and shortened read lengths.

Effect of Residual Proteins: DNA samples should be carefully purified using a proper method that removes and avoids contamination with cellular proteins. Protein, if present, in the DNA sample can be injected and adhere to the walls of the capillary, thus affecting data resolution and capillary lifetime.

Effect of Residual Detergents: Some methods of template preparation require the use of detergent such as Triton X-100 or sodium dodecyl sulphate (SDS). Negatively charged detergents may be preferentially injected over DNA during electrokinetic injection. If present at high levels, detergents such as Triton X-100 and SDS will impact the life of the capillary and the quality of the sequencing data.

2.12 IN VITRO CELL PROLIFERATION AND CHEMOSENSITIVITY ASSAY

The growth inhibition of the chemotherapeutic agents 5-fluorouracil, cisplatin and taxol on oesophaeal carcinoma cell line JROECL-33 and JROECL-21 were determined by MTS Assay (Cell Titer 96® AQueous Non-Radioactive Cell Proliferation Assay, Promega, USA). MTS is a colorimetric method for determining the number of viable cells in proliferation or chemosensitivity assays. The Cell Proliferation Assay is composed of solutions of a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H- tetrazolium, inner salt; MTS) and an electron-coupling reagent (phenazine methosulfate; PMS). PMS has enhancement chemical stability, which allows it to be combined with MTS to form a stable solution. The MTS tetrazolium compound is bioreduced by cells into a formazan that is soluble in tissue culture medium (Barltrop *et al*, 1991). The conversion of MTS into the aqueous soluble formazan is accomplished by dehydrogenase enzymes in metabolically active cells (Berridge *et al*, 1993). The absorbance of the formazan at 490nm can be

measured directly from 96 well assay plates and the quantity of formazan product, as measured by the amount of 490nm absorbance, is directly proportional to the number of living cells in the culture (Cory *et al*, 1991; Riss *et al*, 1992). In this study each experiment was repeated twice and the following protocol was used: - Cells growing in exponential phase were prepared and counted as described (section 2.5.2-3). Cells were seeded in triplicate at a density of $5x10^3$ cell/well on 96-well flat-bottomed plates. The final volume in each well after any addition was 100 µl. Cells were then incubated at 37°C for 24 hour in a humidified 5% CO_2 atmosphere. Following the 24 hour incubation, the culture medium was replaced by freshly prepared concentrations of each drug. Untreated control cells (no additive) and controls (culture medium only) to measure the MTS background absorbance were also prepared in the same manner. The plates were incubated for 24- 48 hours. At the end-time of each experiment, 20 µl/well of freshly prepared combined MTS/PMS solution was added and plates were incubated for 2 hours at 37°C in a humidified 5% CO_2 atmosphere. The absorbance of soluble formazan produced by cellular reduction of the MTS at 490nm was measured using an ELISA plate reader.

2.13 MORPHOLOGICAL ASSESSMENT OF APOPTOSIS

The morphological characteristics of apoptotic cells; cell shrinkage, condensed chromatin, reduction in nuclear size and formation of membrane-bound apoptotic bodies (Kerr *et al*, 1994) were identified by light microscopy. All the experiments were performed using the floating and attached cells. Initially cells were grown at the density of $5x10^5$cells/ml/well for 24 hours. The cells were then grow in fresh medium in either the absence (control) or the presence of chemotherapeutic agents (treated) for 24 and 48 hours. At the end of each experiment, cells were harvested and transferred to sterile tubes. A cytospin was prepared from 200 µl aliquot of $1x10^4$ cells and centrifuged at 1000 rpm for 4 minutes. Cells were then fixed in 95% ethanol for 15 minutes and haematoxylin and eosin stained. The slides were mounted in a permanent mounting medium and examined by light microscopy under 1000x magnifications.

2.14 IN SITU-END LABELLING (ISEL)

Ultrastructural changes in cellular morphology in apoptosis are usually associated with DNA fragmentation (Thiry 1992; Migheli *et al*, 1995). In situ-end labelling is a TUNEL based assay for examining apoptosis via DNA fragmentation (Gavrieli *et al*, 1992). In

this technique, DNA strand breaks induced during apoptosis are visualised by the detection of enzymatically-incorporated digoxygen labelled nucleotide to the free 3'-OH termini (Schmitz *et al*, 1991; Gavrieli *et al*, 1992). These new DNA ends that are generated upon DNA fragmentation are typically localized in morphologically identifiable nuclei and apoptotic bodies. In contrast, normal or proliferating nuclei do not have significant numbers of DNA 3'-OH ends and therefore can not be incorporated with the labelled nucleotide used in this assay (ApopTaq manual book, Intergen). ISEL technique can detect early-stage apoptosis in systems where chromatin condensation has begun and strand breaks are fewer, even before the nucleus undergoes major morphological changes (Attanasio *et al*, 1995; Chapman *et al*, 1995). The in situ labelling of apoptotically fragmented DNA and subsequent visualization by light microscopy gives biologically significant data about apoptotic cells which may be only a small percentage of the total population. Quantitative results can also be obtained. In addition, the occurrence of DNA fragmentation with regard to the cell cycle phase of apoptotic cells can be examined using this technique and flow cytometry (Gorczyca *et al*, 1992; Halicka *et al*, 1997). In this study, Apoptag®peroxidase in situ apoptosis detection kit -S7100 (Intergen Company, USA) was used for identification of apoptotic cells and bodies in paraffin-embedded tumour sections. The ApopTaq Kit is designed to label in situ the free 3'-OH DNA termini without altering the cells morphology. The digoxigenin labelled nucleotides contained in this kit are enzymatically added to the DNA by terminal deoxynucleotidyl transferase (TdT). TdT catalyzes a template-independent addition of nucleotide triphosphates to the 3'-OH ends of double-stranded or single-stranded DNA. DNA fragments which have been labelled with the digoxigenin-nucleotide are then allowed to bind an anti-digoxigenin antibody that is conjugated to a peroxidase reporter molecule. The reaction products of peroxidase were then visualized with 3'-3' diaminobenzidine (DAB) (Figure 2.5).

2.14.1 Tissue Preparation

Formalin fixed paraffin-embedded tumour sections was prepared as described in (section 2.4.1). Sections were deparaffinised as follows: -

a. Wash the specimen in 3 changes of xylene for 5 minutes each wash.

b. Wash the specimen in 2 changes of absolute ethanol for 5 minutes each wash.

c. Wash the specimen once in 95% ethanol and once in 70% ethanol for 3 minutes.

d. Wash the specimen in one change of PBS for 5 minutes.

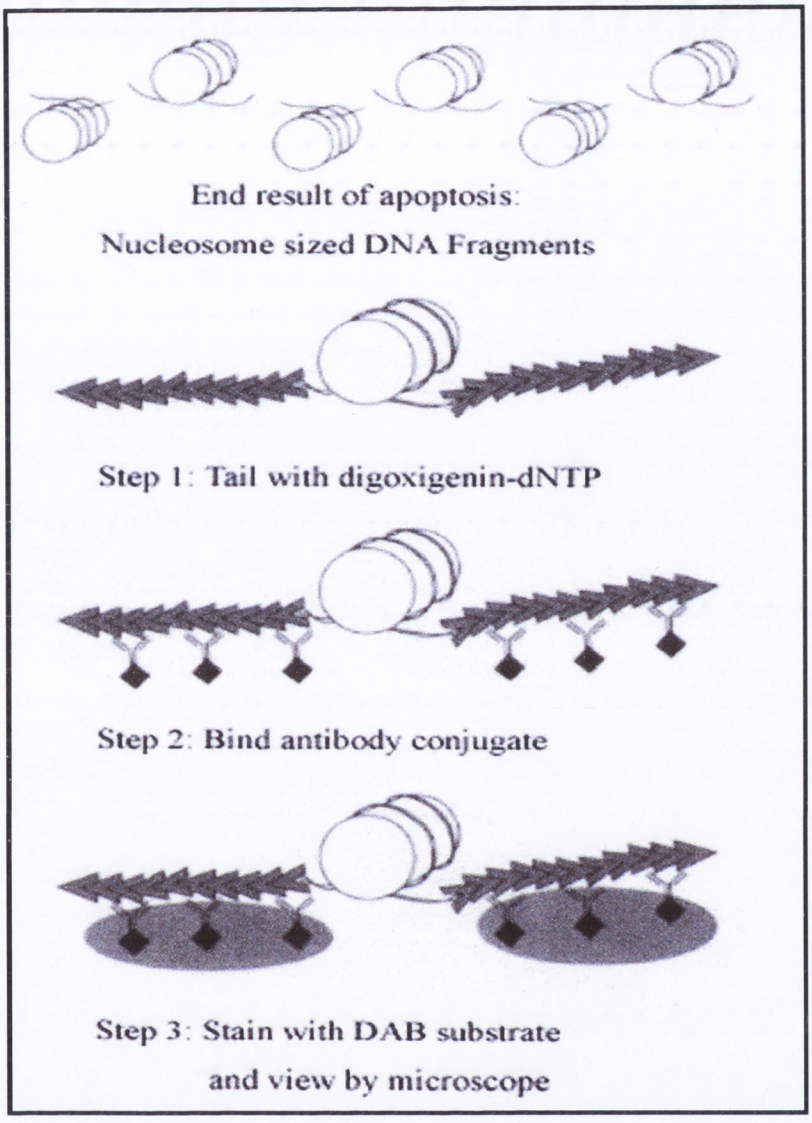

Figure 2.5. Schematic illustration of ApopTag in-situ end labelling of apoptotically fragmented DNA (Intergen Inc.).

2.14.2 Digestion of DNA-Binding Proteins

a. Apply freshly diluted protein digesting enzyme or proteinase K (20 μg/ml) to the specimen for 15 minutes at room temperature in a coplin jar or directly on the slide (~60 μl/5 cm2).

b. Wash the specimen in 2 changes of dH_2O in a coplin jar for 2 minutes each wash.

2.14.3 Blocking Endogenous Peroxidase Activity

a. Incubate the specimen in 3.0% hydrogen peroxide in PBS for 5 minutes at room temperature (either on a slide or in a coplin jar).

b. Rinse the specimen twice with PBS for 5 minutes each time in a coplin jar.

2.14.4. Apply Equilibration Buffer and Working Strength Enzyme

a. Gently tap off excess liquid and carefully blot or aspirate around the section.

b. Immediately apply 75 μl of equilibration buffer directly on the specimen and incubate for at least 10 seconds at room temperature.

c. Gently tap off excess liquid and carefully blot or aspirate around the section.

a. Immediately pipette onto the section 55 μl/5 cm2 of working strength TdT enzyme

b. Incubate in a humidified chamber at 37°C for 1 hour.

2.14.5 Apply Stop-Wash Buffer

a. Transfer the specimen in a coplin jar containing working strength stop-washing buffer, agitate for 15 seconds, and incubate for 10 minutes at room temperature.

b. Wash the specimen in 3 changes of PBS for 1 minute each wash.

2.14.6 Apply Anti-Digoxigenin Conjugate

a. Gently tap off excess liquid and carefully blot or aspirate around the section.

b. Apply 65μl/5cm2 room temperature anti-digoxigenin peroxidase to the slide.

c. Incubate in a humidified chamber for 30 minutes at room temperature.

d. Wash the specimen in 4 changes of PBS for 2 minutes per wash.

2.14.7 Apply Peroxidase Substrate and Colour Development

a. Gently tap off excess liquid and carefully blot or aspirate around the section.

b. Apply enough peroxidase substrate to completely cover the specimen (75 μl/5 cm2) and allow staining for 3-6 minutes at room temperature.

c. Monitor colour development by looking at the slide under the microscope.

d. Wash the specimen in 3 changes of dH2O in a coplin jar for 1 minute each wash.

2.14.8 Specimen Counterstaining

a. Counterstain in 0.5% (w:v) methyl green for 10 minutes at room temperature.

b. Wash the specimen in 3 changes of dH2O in a coplin jar, dipping the slide 10 times each in the first and second washes, followed by 30 seconds without agitation in the third wash.

c. Wash the specimen in 3 changes of 100% butanol in a coplin jar, dipping the slide 10 times each in the first and second washes, followed by 30 seconds without agitation in the third wash.

2.14.9 Mounting Specimens

a. Dehydrate the specimen by moving the slide through three jars of xylene, incubating for 2 minutes in each jar.

b. Remove the slides one at a time from the coplin jar. Gently tap the edge of the slide to drain, but do not allow the specimen to dry.

c. Mount under a glass coverslip in a mounting medium such as paramount.

2.14.10 Controls and Reproducibility Testing

For validation and proper interpretation of Apop Taq staining; rat mammary glands (Apoptag® positive control slides -S7115, Intergen Company) were used as positive tissue controls. Omission of the TdT during the staining procedure provided negative controls for reagents.

2.14.11 Interpretation and Labelling Index

Under the light microscopy, positive Apop Taq cells should reveal focal in situ brown staining inside the apoptotic nuclei and apoptotic bodies. This positive staining directly correlates with the more typical biological and morphological aspects of apoptosis. Therefore, it is important to evaluate Apop Taq staining results in conjugation with the morphological criteria of the cells; this was achieved with sections which were counterstained with methyl green and mounted properly. To assess the frequency of apoptosis within the tumour section the number of apoptotic cells and bodies in a total of 2000 tumour cells were counted and the percentage recorded as the apoptotic index.

2.15 LIGATION MEDIATED POLYMERASE CHAIN REACTION (LM-PCR)

During apoptosis, cellular endonucleases cleave genomic DNA between nucleosomes, producing fragments whose lengths vary by multiples of 180–200 bp (Wyllie 1980; Arends *et al*, 1990). When resolved by agarose/ethidium bromide gel electrophoresis, these DNA fragments appear as a "nucleosomal ladder" - a widely recognized hallmark of apoptotic cell death (Hale *et al.*, 1996). Traditionally, such ladders have been assayed by electrophoresing genomic DNA samples directly on agarose gels. However, in biological systems in which only a small percentage of cells are apoptotic, or in which apoptosis is occurring asynchronously, genomic DNA ladders may not be visible or may appear as a smear (Iwata 1996).

The LM-PCR assay uses PCR to specifically amplify the nucleosomal ladder (Staley *et al*, 1997), making it easier to visualize on an agarose gel. Moreover, the LM-PCR assay is semiquantitative, allowing comparison of the relative extent of apoptosis in different samples. The LM-PCR assay as shown in (Figure 2.10) includes two steps: The first step is the ligation of dephosphorylated adaptors (composed of a 12-mer and a 24-mer oligonucleotides) to the ends of the DNA fragments generated during apoptosis. In mammalian cells, such fragments generally have 5'-phosphorylated blunt ends (Staley *et al.*, 1997); thus, only the 24-mer is ligated to the DNA fragments. When the mixture of ligated DNA fragments is heated, the 12-mer is released. Next (second step), the 5' protruding ends of the molecules are filled in by a thermostable DNA polymerase. The 24-mer then serves as a primer in a PCR in which the fragments with adaptors on both ends are exponentially amplified. The resulting nucleosomal ladder can be easily visualized on an agarose gel.

In our in-vitro study, The ApoAlert LM-PCR Ladder Assay Kit (Clontech, UK) was used. The ApoAlert LM-PCR Ladder Assay Kit includes; a premade Ligation Mix contains the linker and a ligation buffer, the LM-PCR Mix which combines the primer and PCR buffer, T4 DNA Ligase and Calf Thymus DNA as positive DNA controls. Thermostable DNA polymerase (cDNA polymerase Mix, Clontech) which combines KlenTaq-1 DNA polymerase (a proofreading polymerase) and TaqStart Antibody for automatic hot start were used. Sequences of the LM-PCR adaptor oligonucleotides and the control primers are shown with (Figure 2.6). The ligation reaction and the PCR was performed according to the manufacture's instruction and as follows:-

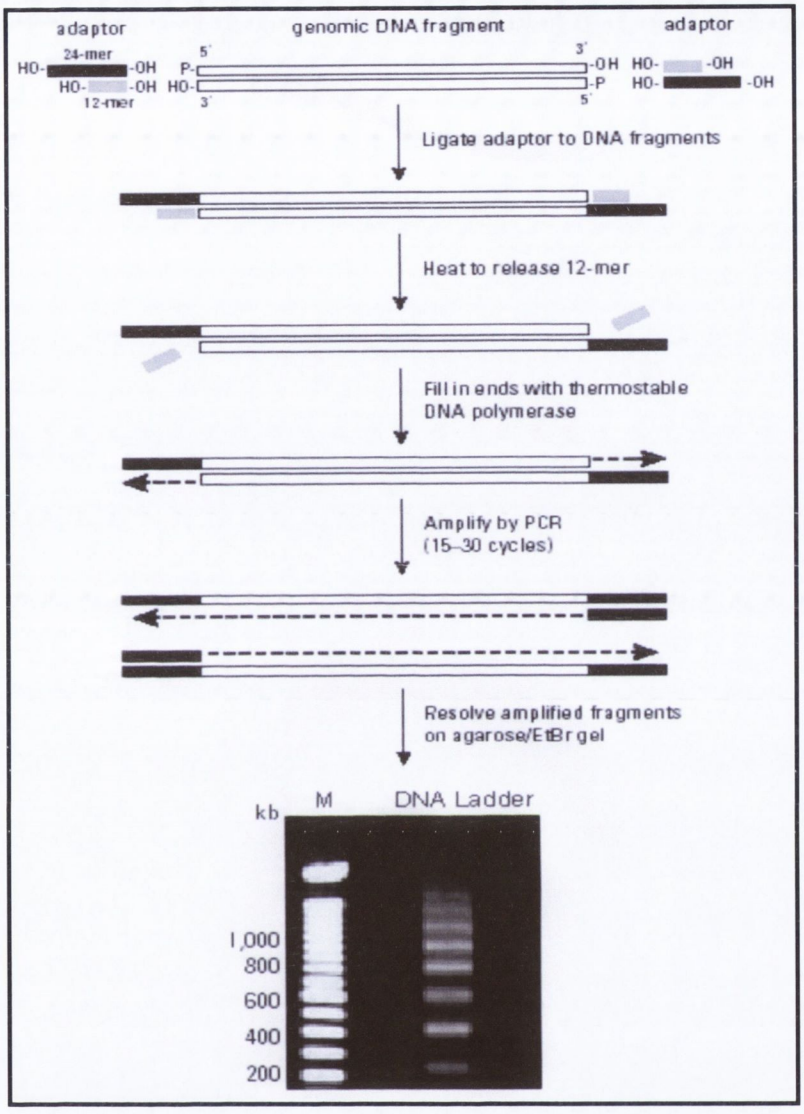

Figure 2.6. Schematic diagram of the Ligation Mediated-PCR amplification endonuclease induced DNA fragments Assay (Clontech Inc.).

Adaptor:

12-nt oligonucleotide	5'–TGCGGTGAGAGG-3'
24-nt oligonucleotide	5'–AGCACTCTCGAGCCTCTCACCGCA-3'

Control primers:

Human En-2 Control Forward Primer	5'–TCTACCCAGGTCCCAGGTCTCG-3'
Human En-2 Control Reverse Primer	5'–ACTTGCCCTCCTTGGCTGTGGT-3'

2.15.1 Extraction of Genomic DNA

At the end of the experiment, total genomic DNA was extracted as described previously (section 2.8.1-2) and 0.5 μg of DNA was used for each ligation reaction.

2.15.2 DNA Ligation Reaction

a. Mix 0.5 μg of genomic DNA with 35 μl of Ligation Mix. If more or less than 0.5 μg of genomic DNA was used, adjust the volume of Ligation Mix (approximately 70μl of Ligation Mix per1μg of genomic DNA).

b. Heat reaction mixture to 55°C for 10 minutes, flick tube to mix.

c. Allow the adaptor oligonucleotides to anneal by cooling to 10°C over approximately 1 hour. For convenience this gradual temperature ramp-down (1°C/1 minute) may be programmed into a thermal cycler.

d. Incubate reactions at 10°C for at least 10 minutes, and then add 0.5 ml of T4 DNA Ligase. Incubate reactions at 16°C for 12–16 hours (overnight).

e. Adaptor ligated DNA may be stored at –20°C until needed for PCR.

f. Calculate the concentration of DNA in your ligation reactions using the following formula: Quantity of DNA (ng) / Volume of Ligation Mix (μl) + Volume of DNA added (μl) in step 2. From this value (in ng/μl), 100ng (50-150ng) of adaptor-ligated DNA was used for PCR.

2.15.3 LM-PCR Reaction

For each LM-PCR reaction a total volume of 100μl PCR mixture was prepared by mixing :- 10X LM-PCR mix (10 μl) ; Adaptor-ligated DNA from step2f (100ng) ; 50X Advantage cDNA Polymerase mix (2 μl) ; and PCR-grade H2O to complete (100 μl) reaction volume. The PCR conditions in this protocol have been optimised using MJ Research PTC-100 DNA Thermal Cycler. The PCR was performed as follows: initial (hot start) heat activation of Taq DNA polymerase at 72°C for 8 minute; 2 steps/cycle (denaturation at 94°C for 1 minute, annealing and extension at 72°C for 3 minutes) of 25 cycles; final extensions at 72°C 15 minutes.

2.15.4 Preparation of Control Reactions

Internal control: this control confirms that equal amounts of DNA are used for PCR. A Total volume of 100 μl per reaction of control PCR mixtures was prepared as follows: -

10X control mix (10 μl) ; human En-2 forward primer (10 μl) ; human En-2 reverse primer (10 μl) ; adaptor-ligated DNA from step2f (100ng) or use the same amount of DNA used in the experimental reaction ; advantage cDNA polymerase mix (2 μl) ; and PCR-grade H_2O to complete (100 μl) reaction volume. The 3 steps / cycle of 26 cycles of the PCR was as follows: - denaturation at 94°C 1 minute; annealing at 70°C 1 minute; extension at 72°C 1 minute.

The electrophoresis of amplified fragments were performed as follows: - 10–15 μl of the PCR products was load on a 1.2% agarose / ethedium bromide gel. For the internal DNA control, each reaction should generate bands of equal intensity in addition to a single band at 290 bp that corresponds to a fragment of En-2 a single-copy gene that is conserved in most species.

Positive DNA control: ApoAlert LM-PCR Kit uses Calf Thymus as a positive DNA control. Two and a half μl of Control Calf Thymus DNA in 70 μl of Ligation Mix was prepared as positive DNA control. This mixture was processed separately with the DNA samples as described above. Electrophoresed PCR products of control DNA should result in a clear DNA laddering pattern with multiples of 180-200 bp fragments.

2.15.5 Electrophoresis of Amplified Fragments

Load 10–15 μl of each reaction (PCR product) on a 1.2% agarose / ethidium bromide at 6V/cm for approximately 2.5 hours. Resulting ladders should be comprised of bands at intervals of approximately 200bp. Because of the addition of the adaptors (48 bp total), the bands will be slightly larger than those seen in a conventional ladder. Electrophoresed PCR products of control DNA samples should result in a clear DNA laddering pattern with multiples of 180-200 bp fragments.

CHAPTER 3

THE SINGNIFICANCE OF P53 AND CELL CYCLE RELATED KI-67 EXPRESSION IN BARRETT'S AND OESOPHAGEAL ADENOCARCINOMA

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3.1 INTRODUCTION TO P53 GENE

3.1.1 P53: Normal Structure and Function

The human p53 tumour suppressor gene is located on the short arm of the chromosome 17 (17p13), it contains 11 exons spanning over 20 kilobases and encodes a 393 amino acid phosphorprotein (Lane *et al*, 1979; Isobe *et al*, 1986). This gene belongs to a family of highly conserved genes that contains, as yet identified, two other membranes, p63 and p73 (Levrero *et al*, 2000; Marin *et al*, 2000). The p53 gene appears to differ from its cousins by its unique role in tumour suppression, p53-deficient mice are developmentally normal but show a very high incidence of multiple, early tumours (Donehower *et al*, 1992), while, p73- or p63-deficient mice, show complex patterns of developmental defects and physiological dysfunctions, but do not have an elevated frequency of cancers (Mills *et al*, 1999; Yang *et al*, 2000). The unique role of p53 in protecting against cancer is also illustrated by the fact that the Li-Fraumeni Syndrome, a familial syndrome of predisposition to multiple forms of cancer, is often associated with the germ line transmission of a mutant p53 allele (Kleihues *et al*, 1997). The p53 protein has the classical architecture of a sequence-specific transcription factor, with an acidic N-terminus containing transactivation domains, a hydrophobic central core which binds to specific DNA sequences and a basic C-terminus oligomerisation and regulatory domains. Most of the mutations identified, to date, cluster in the DNA-binding domain, indicating that this particular biochemical function is crucial for the capacity of p53 to act as a tumour suppressor (Hainaut *et al*, 2000). The p53 protein is expressed in almost all tissues as a constitutively repressed protein. Several classes of signals can lead to the de-repression of p53 and to its accumulation by post-translational modifications. These signals include DNA-damaging agents (genotoxic stress), constitutive activation of growth signalling cascades (oncogenic stress), as well as other types of stress such as depletion in ribonucleotides or hypoxia (Pluquet *et al*, 2001). Thus, p53 lies at the point of convergence of several, distinct stress-response pathways. The tumour suppressor function of p53 depends principally on its ability to prevent proliferation of genetically defected cells and its response to stress stimuli that are encountered during

tumourigenic progression. Activated p53 leads to cell cycle arrest and apoptosis, and can play a role in the induction of differentiation and cellular senescence (Levine, 1997; Almog *et at*, 1997; Lundberg *et al*, 2000). P53 can also play a direct role in the repair of DNA damage through nucleotide excision repair and base excision repair (Wani *et al*, 1999; Offer *et al*, 2001; Zhou *et al*, 2001). Wild–type p53 has also been shown to inhibit angiogenesis in tumours by activating or repressing genes that regulate new blood vessel formation (Dameron *et al*, 1994; Vogelstein *et al*, 2000). The mechanisms by which p53 regulates these processes are transcriptional regulation (either activation or repression) and modulation of other proteins' activities by direct binding. The exact combination of effectors activated by p53 may differ from one cell to another depending upon the nature of the inducing signal, its intensity, the tissue considered and the degree of cell differentiation. The simultaneous activation of other pathways contributing to the control of cell growth and survival should also be considered.

3.1.2 P53 and Cell Cycle Control

The cell cycle arrest function of p53 correlates with its ability to function as a transcription factor (Crook *et al*, 1994; Pietenpol *et al*, 1994). Among p53 target genes identified to date, p21 WAF1/Cip1 stands out as playing a critical role in the induction of cell cycle arrest (E1-Deiry *et al*, 1993; Brugarolas *et al*, 1995). P21 is a cyclin-dependent kinase inhibitor that can activate both G1 and G2 cell cycle arrests similar to those seen in response to p53 induction (Agarwal *et al*, 1995; Bates *et al*, 1998). Importantly, cells deleted for p21 are deficient in both G1 and G2 arrest, and in the coupling of DNA synthesis and mitosis. These defects are also described in p53-null cells (Waldman *et al*, 1995; Waldman *et al*, 1996).

Another target of p53 that contributes to the p53-induced G2 arrest is 14-3-3 sigma (Hermeking *et al*, 1997). The 14-3-3 family proteins play a role in signal transduction and cell cycle control, in part by binding and sequestering proteins (Muslin *et al*, 2000). Although 14-3-3 sigma deficient cells could transiently arrest in G2 phase after DNA damage, they were unable to maintain the cell cycle arrest (Chan *et al*, 1999). 14-3-3 sigma can bind p53 and activate its sequence-specific DNA binding after ionising radiation (Waterman *et al*, 1998), and so may represent a positive feedback loop to p53 to prevent cell cycle progression in damaged cells. A further potential mediator of the G2 arrest includes Growth-Arrest and DNA Damage-inducible Genes (GADD) and

Reprimo gene. These genes were activated following DNA damage induced by radiation. Induction of human GADD45 by ionizing radiation depends on the normal function of p53, which binds to an intronic consensus element in the GADD45 gene. GADD45 stimulates DNA excision repair in vitro and inhibits the entry of cells into S phase (Kastan *et al*, 1992; Wang *et al*, 1999). Induction of Reprimo, (a highly glycosylated protein) in cells exposed to X-irradiation triggers G2 arrest of the cell cycle in a p53 dependent manner (Ohki *et al*, 2000). In the arrested cells, both Cdc2 activity and nuclear translocation of cyclin B1 are inhibited. These observations have established GADD45 and Reprimo as a link between the p53-dependent cell checkpoint and DNA repair.

3.1.3 P53 and Programmed Cell Death

Numerous apoptotic genes that are transcriptionally activated by p53 have been identified, suggesting that the p53 apoptotic response is multifaceted (Vousden, 2000). The first apoptotic target of p53 identified was the bax gene, a pro-apoptotic member of the bcl-2 family (Miyashita *et al*, 1995). Recently, other pro-apoptotic members of this family named Noxa (Oda *et al*, 2000) and PUMA (Nakano *et al*, 2001; Yu *et al*, 2001) have been identified as p53 targets. These proteins, as well as another p53 target gene product, p53AIPI (p53-regulated Apoptosis-Inducing Protein 1) (Oda *et al*, 2000), localize to the mitochondria and promote loss of the mitochondrial membrane potential and cytochrome c release, thus activating the Apaf-l/caspase-9 apoptotic cascade (Bossy- Wetzel *et al*, 1999). Significantly, p53-induced apoptosis was found to be inhibited by loss of Apaf-1 or caspase-9 (Soengas *et al*, 1999). Perturbation of mitochondrial integrity may also be mediated by several genes coding for redox-controlling enzymes, which were identified as p53-induced genes (PIGs) in a colon cell line undergoing p53-mediated apoptosis (Polyak *et al*, 1997). It has been suggested that reactive oxygen species (ROS) produced by these PIGs cause damage to the mitochondria which, in turn, initiates apoptosis. This model is supported by the observation that antioxidants which eliminate ROS, can inhibit p53-mediated apoptosis as well as concomitant changes in the mitochondrial membrane potential in some systems (Li *et al*, 1999). Recently a study revealed that the p53 protein itself can localize to the mitochondria presenting a potential additional transcription-independent way of mediating apoptosis (Marchenko *et al*, 2000). Through the cloning of functional p53-binding sites (p53-tagged sites) from the human genome, several novel genes

induced by over-expression of wild-type p53 have been identified (Tokino *et al*, 1994). Among them, were GML (GPI-anchored molecule-like protein), whose predicted product showed a high degree of homology to the family of the glycosyl-phosphatidylinositol (GPI)-anchored membrane proteins. The GML gene is regulated in a p53-dependent manner; its expression level is correlated with the sensitivity of oesophageal cancer cells to anti-cancer drugs (Furuhata *et al*, 1996), and the gene product enhances apoptosis induced by anti-cancer drugs and gamma-irradiation (Kagawa *et al*, 1997; Kimura *et al*, 1997). P2XM (P2X specifically expressed in skeletal muscle) is another gene isolated by this approach. It showed significant homology to members of the P2X family of ATP-gated ion channels. Expression of P2XM was reduced or lost in several sarcoma cell lines, and an aberrant splicing variant that would encode a deficient protein because it lacked part of exon 1, was predominant in some of the sarcoma cell lines (Urano *et al*, 1997).

An additional p53-target gene in the apoptosis category, PAG608, encoding a nuclear zinc finger protein, has also been identified. The product of PAG608 appears to localize preferentially to nucleoli. Transient over-expression of PAG608 in human tumour-derived cells leads to distinctive changes in nuclear morphology, and can promote apoptosis (Israeli *et al*, 1997).

P53 has also been implicated in the membrane death receptor-induced pathway of apoptosis. Expression of at least two of the death receptors, FAS/APO1 and DR5/KILLER, and one of the death receptor ligands, FASL, has been observed to be up-regulated by p53 (Owen *et al*, 1995; Wu *et al*, 1997). The DR5 promoter was shown to be a direct target of p53 (Takimoto *et al*, 2000), while cell surface expression of FAS was enhanced by p53 through promotion of its trafficking from the Golgi to the plasma membrane (Bennett *et al*, 1998). Activation of death receptors by their ligands (FAS-FASL and DR5-TRAIL) results in trimerization and recruitment of intracellular adapter molecules which initiate the caspase cleavage cascade and apoptosis (Ashkenazi *et al*, 1998). Activation of PIDD, a death domain containing protein, by p53 also induces apoptosis and is likely to function through the death receptor pathway (Lin *et al*, 2000). Loss of survival signalling can augment p53-mediated apoptosis (Canman *et al*, 1995; Lin *et al*, 1995; Prisco *et al*, 1997), while the ability of p53 to negatively regulate the

IGF pathway (Buckbinder *et al*, 1995) and inhibit intergrin-associated survival signalling may further sensitize cells to p53-induced death (Bachelder *et al*, 1999). The NF-$_K$B transcription factor has lately been shown to play an important role in p53-mediated apoptosis (Ryan *et al*, 2000), in contrast to the anti-apoptotic effect of NF-$_K$B induced in response to TNF (Van Antwerp *et al*, 1996; Phillips *et al*, 1999). However, in other systems p53 expression has been shown to be dependent on NF-$_K$B (Wu *et al*, 1994; Kirch *et al*, 1999), and the contribution of NF-$_K$B to the p53 apoptotic pathway remains unclear.

3.1.4 P53 and DNA Repair

Besides preventing cells with damaged genomes from replicating, via its apoptotic and cell cycle arrest function, p53 also participates in DNA damage repair. The current model of p53 function postulates that p53 senses DNA damage and arrests the cell cycle in either the G1 or G2 phases to allow DNA repair to take place. If repair is not successful, p53 initiates programmed cell death, thereby preventing the propagation of genetic defects to successive generations of cells. Cells lacking p53 function are deficient in nucleotide excision repair (NER), which repairs UV-induced DNA damage (Ford *et al*, 1995; Wani *et al*, 1999) and base excision repair (BER), which removes bases damaged by alkylating agents, oxygen-free radicals or hydrolysis (Offer *et al*, 2001; Zhou *et al*, 2001). The c-terminus of p53 directly binds to different forms of damaged DNA: single-stranded DNA, ends of double- strand breaks and DNA 'bulges' resulting from insertion/deletion mismatches. Also, p53 can associate with several components of the repair machinery in vitro, including XPB/ERCC3, XPD/ERCC2, p62 subunit of TFIIH, CSB, replication protein A and Ref-1. Other biochemical activities of p53, such as DNA re-annealing, DNA strand transfer and 3'-5' exonuclease activity might also play a role in its repair function (Albrechtsen *et al*, 1999; McKay *et al*, 1999). Some of the p53 target genes also participate in DNA damage repair. GADD-45 binds proliferating cell nuclear antigen (PCNA), and could inhibit replicative DNA synthesis, thus allowing DNA repair to proceed (Smith *et al*, 1994). GADD-45 null fibroblasts have defects in nucleotide excision repair similar to those seen in p53-null fibroblasts (Smith *et al*, 2000) and GADD45-dificient mice show increased radiation carcinogenesis and genomic instability comparable to that seen in p53-dificient mice (Hollander *et al*, 1999). Another transcriptional target of p53 that plays a role in DNA repair is p53R2, a ribonucleotide reductase gene (Nakano *et al*, 2000; Tanaka *et al*, 2000)

3.1.5 P53 and Angiogenesis

As normal cells progress toward malignancy, they must switch to an angiogenic phenotype to attract the nourishing vasculature that they depend on for their growth. This switch was found to coincide with loss of the wild-type allele of p53 and to be the result of reduced expression of thrombospondin-1 (TSP-1), a potent inhibitor of angiogenesis, in cultured fibroblasts from patients with Li-Fraumeni syndrome (Dameron *et al*, 1994). P53 stimulates the endogenous TSP-1 gene and positively regulates TSP-1 promoter sequences; therefore, in fibroblasts wild-type p53 inhibits angiogenesis through regulation of TSP-1. Genetic alterations of p53 are associated with neovascularization during the progression of a glioma to its more malignant form, glioblastoma. Hence, several genes transactivated by p53 are likely to function as angiogenesis inhibitors. Nishimori *et al*, 1997 isolated another novel p53-inducible gene that encodes a 1584 amino acid product that contains five thrombospondin type 1 (TSP-type 1) repeats and is specifically expressed in the brain. A recombinant protein corresponding to the TSP-type 1 repeats present in this gene product inhibited in vivo neovascularization induced by bFGF in the rat cornea. (Nishimori *et al*, 1997) also found that transcripts of this gene, named BAI1 (brain-specific angiogenesis inhibitor 1) were absent or significantly reduced in glioblastoma cell lines, suggesting that BAI1 plays a significant role as a mediator of p53 in inhibiting angiogenesis. (Duda *et al*, 2002) reported that brain-specific angiogenesis inhibitor 1 expression that was lost in a selection of human cancer cell lines could be restored by wild-type p53 adenoviral transfection and may, therefore, be considered for gene therapy and the development of efficient drugs based on endogenous antiangiogenic molecules.

3.1.6 Negative Regulation of P53

The normal function of p53 gene can be lost in a variety of ways, most commonly loss of the chromosomal region containing one allele of the gene (or of the entire chromosome 17p) and a subtle mutation involving the other allele (Greenblatt *et al*, 194; Fan *et al*, 1994). Inactivation of both alleles of p53 has been documented in a large number of human cancers, including tumors arising in the colon, brain, lung, liver and bladder. However, in some cases a missense mutation occurring in one allele of p53 is sufficient to inactivate p53 function, when such an event increases the proportion of hetero-tetramers that contain both mutant and wild-type p53 molecules; the chimeric complexes cannot activate transcription of the target gene. This 'dominant-negative'

effect may be exacerbated by the increased stability and therefore, higher intracellular concentration of the mutant protein compared with that of the wild-type p53 protein (Blagosklonny *et al*, 2000). Moreover, mutant p53 can form stable complexes with p63 and p73 members of the p53 gene family thereby blocking their transactivation capacity (Strano *et al*, 2000).

In addition to mutation in the p53 protein itself, p53 function can be affected by non-mutational events within the cell. For example, the normal MDM2 gene product can bind to and down-regulate p53 function by inhibition of transcriptional activation and subsequent degradation of the p53 protein (Momand *et al*, 1992; Chen *et al*, 1994; Haupt *et al*, 1997). Another cellular protein, JNK, was shown to bind p53 in normal, non-stressed cells. Interference with the p53-JNK association reduced p53 ubiquitination and increased p53 half-life suggesting that JNK might target p53 to ubiquitination and degradation (Fuchs *et al*, 1998). In addition to degradation by the proteasome, p53 has been shown to be cleaved by another protease, calpain, which may also serve to regulate p53 (Kubbutat *et al*, 1997; Atencio *et al*, 2000).

The p53 protein is also the target for the action of the oncoproteins of several tumour viruses. Integration of highly oncogenic human papilloma virus (HPV) into host-cell chromosomes in cervical cancer is followed by binding of HPV E6 oncoprotein to the p53gene. This process results in impaired tumour suppressor function involving DNA repair, decreased apoptosis, rapid degradation of p53 protein and eventually cell immortalisation. (Scheffner *et al*, 1990; Ferenczy *et al*, 2002).

3.1.7 Timing of P53 Mutations and Cancer Progression

Although p53 is often mutated in many types of cancer, the timing of the occurrence of the mutation during cancer progression is extremely variable from one cancer to another. P53 mutations seem to occur at an early stage in many types of cancer which are directly caused by exogenous carcinogens. This is the case for lung cancers of smokers, non-melanoma skin cancers after exposure to UV irradiation, and in most forms of oesophageal cancer. In these cancers, p53 mutations are often detectable in hyperplastic and dysplastic lesions, as well as in non-involved, apparently normal tissues surrounding the tumour (Montesano *et al*, 1997). In the now classical model of stepwise progression of cancers such as colorectal; p53 mutations and loss of alleles

preferentially occurred at the transition between late adenoma and carcinoma in situ, that is, at a relatively late stage in the histopathological development of these lesions (Fearon *et al*, 1990). There is also evidence that p53 mutation can occur at an early stage in serrated carcinomas (Hiyama *et al*, 1998). Analysis of the metaplasia-dysplasia-carcinoma sequence in the progression of adenocarcinoma of the oesophagus has shown that chromosomal imbalance and p53 mutation could occur at the metaplastic stage within Barrett's mucosa and that it primes cells for the formation of dysplastic lesion (Barrett *et al*, 1999; Walch *et al*, 2000). In contrast, absence of genomic aberrations (p53 allelic loss or mutations) in Barrett's metaplasia without dysplasia has also been reported (Gonzalez *et al*, 1997; Reigman *et al*, 2001). Another example is hepatocellular carcinoma, p53 mutations are late events in most of the cancers occurring in the western population, but are very early events in most of the cases in West Africa and Southeast Asia (Montesano *et al*, 1997). In these regions, hepatocellular carcinoma occurs as a consequence of exposure to aflatoxins, a class of potent hepatocarcinogens which contaminate traditional diet, acting synergistically with chronic carriage of hepatitis virus B. In individuals exposed to aflatoxins, p53 mutations are even detectable in cirrhotic liver before the onset of cancer. These mutations are almost exclusively transitions occurring at codon 249 in p53, a type of mutation thought to be a direct consequence of mutagenesis by aflatoxins (Aguilar *et al*, 1993). The later observations indicate that the place of p53 mutation in cancer progression is determined not only by tissue specificity, but that the nature of the mutagenic mechanism involved also plays an important role. Other factors, such as hypoxia, telomere erosion, or loss of adhesion properties, can in addition, contribute to the selection pressures acting on the p53 checkpoint (Giaccia *et al*, 1998).

3.2 INTRODUCTION TO CELL-CYCLE ASSOCIATED KI-67 GENE

3.2.1 Ki-67: Normal Structure and Function

The Ki-67 is a large nuclear protein exclusively expressed in the nuclei of proliferating cells (Gerdes *et al.* 1983). Ki-67 gene is located on chromosome 10q25 and is organized in 15 exons sized from 67 to 6845 bp and in 14 introns sized from 87 to 3569 bp (Schonk *et al.* 1989; Fonatsch *et al*, 1991; Duchrow *et al*, 1996). Immunoblots of proteins from proliferating cells, Ki-67 detects two polypeptides with the apparent molecular weight of 345 and 395 kDa (Duchrow *et al,* 1994). Two isoforms of the

cDNA with full lengths of 11.4 and 12.5 kb probably code for these polypeptide chains, solely differing in the presence or absence of a 1080 bp exon (exon 7) (Duchrow *et al*, 1996). Northern Blot analyses using mRNA from proliferating IM9 cells show distinct Ki-67 protein mRNA-specific bands corresponding to six fragments (12.5 kb, 11.4 kb, 11.2 kb, 10.2 kb, 10 kb and 8.9 kb) (Schluter *et al*, 1993). These Ki-67 protein-specific mRNAs are likely to be formed by alternative splicing in combination with the usage of three different polyadenylation sites.

Detailed cell cycle analyses have revealed that proliferation-associated Ki-67 protein is expressed in all active parts of the cell cycle (G1, S, G2, and M), but is absent in resting cells (G0) (Gerdes *et al*, 1984, Schrape *et al*, 1987, Braun *et al*, 1988; Bruno *et al*, 1992). The peak expression of Ki-67 has been observed in mitosis phase with different intranuclear distribution (Gerdes *et al*, 1984; Ross *et al*, 1995). The localization pattern of Ki-67 during mitosis revealed its exclusive location in the nucleoli of the interphase cells. During prophase the distinct nucleolar Ki-67 changed to an irregular meshwork throughout the nucleoplasm. At metaphase it appears to be distributed in a reticulate structure surrounding the condensed chromosomes, while at late telophase, a punctated staining of the entire nucleoplasm which preceded the typical nucleolar localization pattern in each of the two daughter cells was observed (Verheijen *et al*, 1989; Van Dierendonck *et al*, 1989). Furthermore, Ki-67 supports the condensation of chromosomes during mitosis and maintains the higher order of DNA during mitosis (Bruno *et al*, 1992). In addition, distinct localization of Ki-67 in the nucleoli during the G1 and S phases suggested that Ki-67 may have a structural role in the nucleoli (Ross *et al*, 1995).

The distribution of Ki-67 protein in normal tissues reflects their known cell kinetics. Thus, germinal centre cells in tonsil, basal cells of epithelium and undifferentiated spermatogonia of the testes, but not liver, kidney and brain cells, express Ki-67 antigen. Ki-67 antigen also appears in peripheral blood lymphocytes which have been stimulated to proliferate by phytohemagglutinin. It is lost from HL-60 cells (a promyelocytic cell line) after they have been induced to differentiate into resting macrophages by phorbol esters (Gerdes *et al*, 1983).

3.2.2 Ki-67 and Other Proliferation Markers

Among various more or less sophisticated ways to determine proliferation indices such as counting mitotic figures, measuring the labelling index after incorporation of ^{3}H-thymidine or bromodeoxyuridine (Brdu), PCNA expression, cycle analysis by flow cytometry; immunohistochemical detection methods have recently come of age and are increasingly applied in research and routine pathology. To date, antibodies to formalin-resistant epitopes of the Ki-67 protein are regarded as the most reliable indicator of cellular proliferation in both normal and neoplastic tissues.

The traditional approach of counting the number of mitoses in tissue section remains the easiest way to estimate the proliferation fraction as being performable on routine haematoxylin and eosin stained sections. This method has, nevertheless been repeatedly criticized for its lack of standardisation and poor reproducibility as it is constitutes only a small part of the cell proliferation cycle. In addition, fixation of sections can affect the number of mitosis discernible (Ellis *et al*, 1981; Sadler *et al*, 1989; Donhuijsen *et al*, 1990). Studies of various tumours demonstrated that Ki-67 staining and the mitotic count correlate in some, but not all, types of tumour and the relationship appears to be dependent on the tumour type, degree of differentiation and cell cycle kinetics (Sahin *et al*, 1991; Rudolph *et al*, 1998; Gontero *et al*, 2000).

The cells that are actively synthesizing DNA can be assessed by labelling of the DNA precursors (e.g. Tritiated thymidine or bromodeoxyuridine). Proliferation fraction determined by this method has been shown to correlate with the expression of Ki-67 and clinical behaviour in certain malignancies (Goodson *et al*, 1998; Borre *et al*, 1998). However, the method is laborious and time consuming and may require radiolabel substances that might lead to non specific background labelling that measures those cells in the S phase only.

Proliferating cell nuclear antigen (PCNA, 36 kD), also known as cyclin, is an auxiliary protein of DNA polymerase δ that is essential for DNA replication during the S-phase. Correlations between measurements of PCNA immunoreactivity and known prognostic variables in a range of malignant neoplasms have been reported (Konishi *et al*, 1998; Mattern *et al*, 2001). The protein is present in nucleoplasm of continually cycling cells throughout the cell cycle and its expression is limited to the proliferative compartment of many normal tissues. In many tumors, unlike Ki-67, the proportion of PCNA-positive

cells exceeds that expected for proliferating cells (Start *et al*, 1992). Increased expression of PCNA in tumors is due to growth factors that up-regulate the production of this protein. In addition, the half-life of the PCNA antigen exceeds the cell cycle time (Scott *et al*, 1991). This can lead to inaccurate estimates of the tumour growth compared to that of Ki-67.

DNA flow cytometry analysis offers accurate, rapid and objective information on cell cycle distribution (Quirke *et al*, 1986), and tumour proliferation fraction thus determined, is increasingly used in various neoplasms as an adjunct prognostic factor (Joensuu *et al*, 1990; Jones *et al*, 2001). The accuracy of this analysis is influenced by the amount of tissue necrosis and normal stromal components, as well as the presence or absence of DNA aneuploid cells. Furthermore, current optimization approaches often require dual-staining procedure prior to cytometry analysis. With a good correlation between Ki-67 staining and flow cytometry results, both approaches were used for quantitative analysis and in investigating proliferative abnormalities that develop during neoplastic progression, particularly at the transition from the G_0 to G1 phase of the cell cycle (Reid *et al*, 1993; Jones *et al*, 2001, Emri *et al*, 2001; Tsutsui *et al*, 2001). With each technique having its advantages and disadvantages the final decision may be based on which method is easily established and more reliably demonstrates the growth activity in the cell or tissue of interest.

3.2.3 Prognostic Significance of Ki-67 in Human Cancer

The proliferation activity of pre- and neoplastic- tissues is considered to provide both diagnostic and prognostic information and has become an integrative element of several grading systems. Ki-67 has proved a valuable tool to measure cell growth in human tissues, especially with regard to the progression and histopathological determination of malignancy. Here we sought to indicate the relevance of Ki-67 to some human digestive system malignancies for example: oral, oesophageal, gastric and colorectal carcinomas. Expression of Ki-67 protein showed a significant increase; during the neoplastic progression of normal oral mucosa, through dysplasia to carcinoma (Schoelch *et al*, 1999; Macluskey *et al*, 2000), and with progression of the cytological grading (Junghanel *et al*, 1998) and tumours metastatic potential (Matsumoto *et al*, 1999). However, data relating the expression of Ki-67 to radiation response and prognosis in oral cancer are conflicting (Petrasch *et al*, 2000; Stoll *et al*, 2000; Koelbl *et al*, 2001).

The progression of Barrett's metaplasia to adenocarcinoma is associated with several changes both in cellular and genetic structure. One of the earliest molecular events is the selection and propagation of the metaplastic clones with specialized intestinal metaplasia. Expression of Ki-67 best revealed the increased epithelial growth with the histological progression of this disease (Whittles *et al*, 1999; Rioux-Leclercq *et al*, 1999). Furthermore, the Ki-67 staining pattern was shown to correlate with histological finding in Barrett's mucosa and may represent an additional parameter for differentiating patients with or without dysplasia (Hong *et al*, 1995). An increase in the proliferation activity in the areas of squamous dysplasia from both cancer-free and cancerous oesophagus compared to normal or non-pathological epithelia was observed during the progression of squamous cell carcinoma (Itakura *et al*, 1996). Furthermore, oesophageal carcinomas with high proliferative activity showed considerable potential for metastasis to the lymph node (Ikeguchi *et al*, 1997; Raouf *et al*, 2001). Ki-67, therefore, may be regarded as an informative biomarker of tumour growth activity and of response to neuadjuvant therapies (Kitamura *et al*, 2000; Raouf *et al*, 2001).

Certain conditions at risk for gastric cancer show an increased cell proliferation rate. In chronic atrophic gastritis, the number of proliferating cells is increased by comparison with controls (Cahill *et al*, 1996). Higher proliferation rates have also been observed in patients with intestinal metaplasia (Yoshimura *et al*, 2000; Scotiniotis *et al*, 2000), Helicobacter pylori associated gastritis (Scotiniotis *et al*, 2000), gastric dysplasia (Miracco *et al*, 1995; Zhang *et al*, 2001) and in patients with gastric remnant resections (Lynch *et al*, 1995). Ki-67expression was shown to reflect the growth potential of early and advanced gastric carcinomas and their metastatic potential (Igarashi *et al*, 1999; Goishi *et al*, 2000). The expression status of Ki-67, however, shows no influence to the prognosis of patients with gastric cancer (Liu *et al*, 2001; Sendler *et al*, 2001).

Cell markers associated with malignant transformation within colon mucosa have been investigated with the aim of identifying a marker of malignancy. Data from recent publications indicate that Ki-67 protein appears potentially to be such a marker. The net epithelium growth assessed by Ki-67 indicates significant increase in the epithelium in the course of progression of adenoma to carcinoma (Saleh *et al*, 2000; Nomura *et al*, 2000). The distribution of Ki-67 positive cells in high grade dysplasia and carcinoma appears to be diffuse throughout the full length of the crypt, whereas low grade

dysplasia and epithelium indefinite for dysplasia, as well as regenerative epithelium, show an expanded basal zone (Andersen *et al*, 1998). Furthermore, colorectal adenocarcinoma cells that are histologically subclassified as moderately differentiated have different proliferative and metastatic activities from cancer cells that are poorly differentiated. Moderately differentiated cancer cells are associated with hematogenous metastasis to liver and high proliferative activity, and loss of tubular formation of cancer cells have been shown to be related to lymph node metastasis and infiltrative growth (Taniyama *et al*, 1996). Proliferative activity assessed by Ki-67 expression was decreased by preoperative 5-fluorouracil administration or irradiation in colon and rectal carcinoma (Porschen *et al*, 1989, Yamane *et al*, 1998).

Other malignancies of the digestive system that are known for their rapid growth are hepatic and pancreatic carcinomas. Ki-67 has been utilised as a marker of malignancy in these tumours and was shown to be a prognostic factor of tumour progression (Wu *et al*, 1999; Pizem *et al*, 2001; Buck *et al*, 2002), metastasis and patients survival (King *et al*, 1998; Ferrara *et al*, 1999; Ito *et al*, 1999; Karademir *et al*, 2000).

3.3 OBJECTIVES OF STUDY

The aims of this study were to investigate the stage at which aberrant expression of the p53 protein arises in neoplastic progression of Barrett's oesophagus, to study the relationship between p53 and Ki-67 and to establish the significance of these proteins as prognostic biomarkers to help predict tumour response to neoadjuvant therapy.

3.4 MATERIALS AND METHODS

3.4.1 Patient Cohort

Archival formalin-fixed paraffin-embedded tissue samples from 42 patients (32 male, 10 female) with primary oesophageal adenocarcinoma were included in this study. Patients' ages ranged from 48 to 77 years with a median of 66 years. All patients had oesophagogastro-endoscopy and histological confirmation of diagnosis prior to treatment with chemotherapy (cisplatin and 5-fluorouracil) and radiotherapy (CRX) followed by oesophagectomy. Surgery was undertaken at a median of 4 (range 3–6) weeks following neoadjuvant therapy. All patients were treated with curative intent and were followed up at regular intervals.

3.4.2 Histopathological Assessment

In each case, pathology reports of pre-treatment biopsy and surgical resections were reviewed. Barrett's epithelium was defined as specialized intestinal metaplasia and was further defined as being non-dysplastic or showing low-grade dysplasia or high-grade dysplasia (Riddell *et al*, 1983; Ming *et al*; 1998). Tumours were staged according to the AJCC/TNM system (Beahrs *et al*, 1992; Sobin *et al*, 1997) and the final status was given at resection. Patients were defined as complete pathological response (CPR) to CRX when no microscopic tumour cells were identified at resection ($n = 9$). Patients ($n = 29$) were defined as non-responders (NPR) to CRX when there was no evidence of pathological response in the resection specimen e.g. the presence of lymph node metastasis, lympho-vascular permeation and no down-staging (i.e. pT3) compared to pre-treatment status. A third intermediate group ($n = 4$) was defined as having a major pathological response (MPR) to CRX. These patients were all lymph node free and had microscopic residual tumour cells only, in any part of the oesophageal wall. For the purpose of statistical analysis CPR and MPR data are combined to one group ($n = 13$) versus NPR ($n = 29$).

3.4.3 Immunohistochemistry

Expression of p53 and Ki-67 protein was detected by the avidin-biotin peroxidase method (chapter 2 section 4). Immunostaining was carried out on the 4μm sections of diagnostic biopsies and representative 2-4 blocks of subsequent resections. Sections were stained with monoclonal antibody to p53 (DO-7) and polyclonal Ki-67 antibody [Appendix 2]. Sections of human colon adenocarcinoma known to be positive for p53 and tonsil sections for Ki-67 were used as tissue controls. Omission of the primary antibody during staining of sequential sections provided negative reagent controls. In each section, up to 2000 cells were counted and the percentage positivity recorded as labelling index (LI). Sections with absent or weak nuclear immunostaining with LI <5% were considered to be negative for p53. A similar scoring rationale was applied to assess immunopositivity in Barrett's mucosa samples.

3.4.4 Statistical Analysis

The association between p53 expression and clinicopathological parameters was determined either by Chi-square or Fisher's exact test. The significance of association between Ki-67 LI and the clinicopathological parameters was determined by Mann-Whitney or Kruskal-Wallis test. For paired comparison of pre- to post- CRX values, the Wilcoxon test was used. The Statistical significance of values was defined as $P < 0.05$.

3.5 RESULTS

3.5.1 Expression of P53 and Ki-67 in Barrett's Mucosa

Barrett's epithelium adjacent to oesophageal tumours was detected in 19 pre-treatment biopsies and in 22 specimens at resection. Significant correlation ($P < 0.05$) was found between the grade of dysplasia and the expression of p53 and Ki-67. In pre-treatment biopsies; non-dysplastic Barrett's ($n = 9$) were uniformly negative for p53, whereas 1 of 3 cases showing low-grade dysplasia was positive for p53. Diffuse p53 immunostaining was detected in 5 of 7 high-grade dysplastic lesions. Of these lesions, Ki-67 labelling index correlated with p53 status, higher proliferation indices being observed in dysplastic Barrett's expressing p53 (Table 3.1) (Figure 3.1). At resection, similar correlations were observed between p53 and Ki-67 LI. Non-dysplastic Barrett's ($n = 8$) were all negative for p53, 1 of 3 cases showing low-grade dysplasia was positive for p53. Diffuse p53 immunostaining was detected in 8 of 11 high-grade dysplasia. Higher Ki-67 labelling index was observed in dysplastic epithelium positive for p53 (Table 3.1).

3.5.2 Expression of P53 and Ki-67 in Oesophageal Adenocarcinoma

Before-treatment, over-expression of p53 protein was detected in 27 of 42 (64.2%) adenocarcinomas. The proliferation index, assessed by Ki-67 LI, ranged from 2-64.8% (38.1 ± 13.2 mean ± SD; 37.1 median). Of p53 positive tumours, Ki-67 LI ranged from 16.2–64.8% versus 2–38.9% of p53 negative tumours. Accumulation of p53 protein in tumour samples was significantly ($P = 0.01$) associated with high Ki-67 expression (Table 2.1) (Figure 3.2). At resection, it was not possible to determine the proliferation indices in residual carcinomas of patients having major pathological response to CRX due to an insufficient number of residual tumour cells. Over-expression of p53, however, was detected in 1of 4 (25%) residual tumours. In residual carcinomas of patients having no pathological response to CRX; over-expression of p53 was detected in 23 of 29 (79.3%) tumours. The proliferation index, assessed by Ki-67 ranged from 1 to 60% (27.4 ± 14.1 mean ± SD; 26.5 median). Of p53 positive tumours, Ki-67 LI ranged from 4–60% versus 1-36.6% of p53 negative tumours. The observed association between p53 and Ki-67 was not detected in residual carcinomas due to a significant ($P = 0.0003$) decrease in the proliferation indices (Ki-67 LI) by CRX (Table 3.2) (Figure 3.3).

3.5.3 Expression of P53, Ki-67 and Tumours' Response to Chemoradiotherapy

Pre-treatment tumour samples of the subsequent complete pathological response (n = 9), major pathological response (n = 4) and no pathological response (n = 29) to CRX demonstrated 77.7% (7/9), 50% (2/4) and 62% (18/29) p53 immunopositivity, respectively. The growth indices of these groups, respectively as above, ranged from 2-58.1%, 28-55.2% and 7.9-64.8%. Expression of p53 or Ki-67 LI did not correlate (P = > 0.05) with tumours' response or resistance to CRX. Different cut-off points of p53 and Ki-67 (together) also showed no association to tumours' response to CRX (Table 3.3).

3.5.4 Expression of P53, Ki-67 and Clinicopathological Parameters

Both before - and after- treatment, expression of p53 protein or Ki-67 LI did not statistically associate ($P > 0.05$) with patient's age or sex. No significant association was observed between the expression of p53 and the parameters such as tumour differentiation, depth of invasion or lymph node metastasis. At resection, tumours with high growth activity (high Ki-67 LI) were significantly (P = 0.008) associated with the presence of lymph node metastasis (Table 4.1).

Table 3.1. Expression of p53 and Ki-67 in Barrett's mucosa.

Parameters	No. of cases	P53 immuno-positive cases	*P* value	Ki-67 LI (%) Mean ± SD/Median	*P* value
Before-CRX					
Non dysplastic	9	0		3.5 ± 3.8 / 4.0	
Low-grade dysplasia	3	1		19.0 ± 10.1 / 21.0	0.01[B]
High-grade dysplasia	7	5	0.009[A]	27.4 ± 13.9 / 30.0	0.001*[C]
After-CRX					
Non dysplastic	8	0		4.7 ± 7.4 / 8	
Low-grade dysplasia	3	1		10.5 ± 14.7 / 2	0.01[B]
High-grade dysplasia	11	7	0.01[A]	27.2 ± 14.4 / 34	0.003*[C]

*denotes the correlation between p53 and Ki-67 labelling index. Statistical calculation was performed by chi-square [A], Kruskal-Wallis [B] and Mann-Whitney [C] test.

Table 3.2. Expression of p53 and Ki-67 in oesophageal adenocarcinoma.

Parameters	No. of cases	Ki-67 LI (%) Mean ± SD/Median	*P* value
Before-CRX			
P53 negative tumours	15	31.9 ± 7.8 / 35.2	
P53 positive tumours	27	40.9 ± 14.5 / 41.9	0.01[A]
After-CRX (NPR group)			
P53 negative tumours	6	19.0 ± 14.3 / 18.7	
P53 positive tumours	23	26.2 ± 14.0 / 27.1	0.45[A]
Labelling index (NPR group)*			
Before -CRX	29	38.8 ± 12.0 / 37.1	
After-CRX	29	24.7 ± 14.1 / 26.5	0.0003[B]

* denotes pair comparison of Ki-67 LI of the same tumour prior to and after treatment. Statistical calculation was performed by Mann-Whitney [A] and Wilcoxon test [B] test.

Table 3.3. Expression of p53 and Ki-67 and tumours response to chemoradiotherapy.

Parameters	No. of cases	P53 Immuno-positive cases	*P* value	Ki-67 LI (%) Mean ± SD/Median	*P* value
Response to CRX*					
CPR & MPR group	13	9		35.1 ± 15.7 / 33.7	
NPR group	29	18	0.73[A]	38.8 ± 12.0 / 37.1	0.35[B]

* data represent pre-treatment values of p53 and Ki-67 expression. Statistical calculation was performed by Fisher's exact [A] and Mann-Whitney [B] test.

Parameters	CPR & MPR	NPR	*P* value
Response to CRX			
P53 negative & low Ki-67 LI (*n* = 14)	4	10	
P53 positive & high Ki-67 LI (*n* = 15)	4	11	> 0.99[A]
P53 negative & high Ki-67 LI (*n* = 1)	0	1	
P53 positive & low Ki-67 LI (*n* = 12)	5	7	> 0.99[A]

n denote numbers of Pre-CRX cases fallen into the category. Median value of Ki-67 LI used as a cut-off point. Statistical calculation was performed by Fisher's exact test.[A]

Table 3.4. Expression p53 and Ki-67 in oesophageal adenocarcinoma: Correlation with the clinicopathological parameters.

Parameters	No. of cases	P53 immuno-positive cases	*P* value	Ki-67 LI (%) Mean ± SD/Median	*P* value
Before-CRX					
Gender					
Male	32	21		38.5 ± 11.6 / 36.7	
Female	10	6	> 0.99[A]	35.7 ± 17.9 / 35.7	0.45[C]
Age (years)					
< 66	19	11		41.6 ± 13.6 / 37.6	
≥ 66	23	16	0.52[A]	34.4 ± 12.2 / 35.9	0.11[C]
Type of differentiation					
Well/moderate	30	18		38.3 ± 12.6 / 36.7	
Poor	12	9	0.48[A]	36.0 ± 15.4 / 34.6	0.48[C]
After-CRX*					
Gender					
Male	23	15		38.3 ± 12.3 / 37.1	
Female	6	3	0.65[A]	40.7 ± 11.9 / 36.5	0.59[C]
Age (years)					
< 66	13	7		40.3 ± 14.2 / 37.6	
≥ 66	16	11	0.46[A]	37.5 ± 10.3 / 36.7	0.97[C]
Type of differentiation					
Well/moderate	25	15		23.8 ± 15.0 / 26.3	
Poor	4	3	> 0.99[A]	30.4 ± 4.5 / 31.0	0.18[C]
Tumours extension					
pT1	5	3		27.2 ± 7.1 / 27.1	
pT2	3	3		41.8 ± 15.4 / 36.8	
pT3	21	17	0.37[B]	21.7 ± 13.9 / 24.3	0.06[D]
Lymph node metastasis					
Negative	18	14		20.5 ± 13.8 / 22.5	
Positive	11	9	> 0.99[A]	31.7 ± 12.2 / 31.6	0.008[C]

* data represents NRP group. Statistical calculation was performed by Fisher's exact [A], chi-square [B], Mann-Whitney [C] and Kruskal-Wallis [D] test.

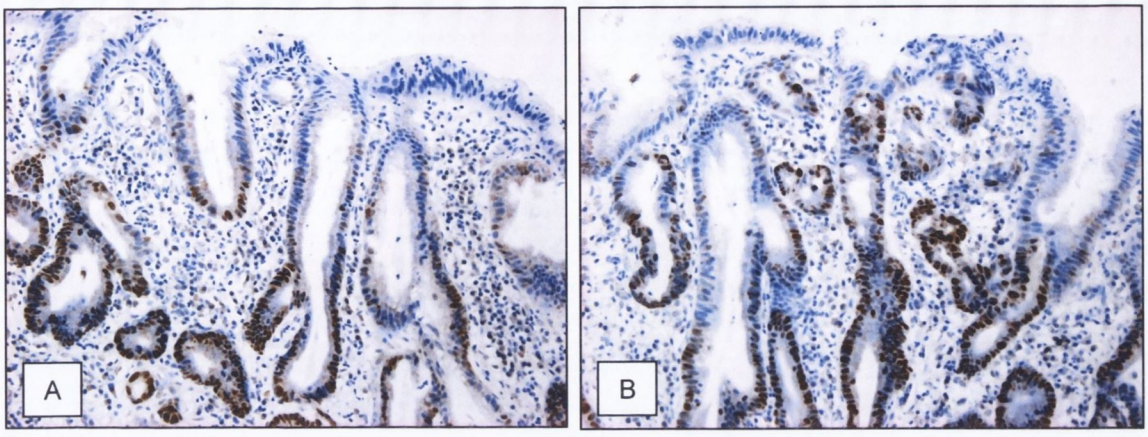

Figure 3.1. Section of Barrett's oesophagus with high-grade dysplasia demonstrating extensive nuclear immunostaining for p53 (A) and Ki-67 (B). Increased dyplasia in Barrett's mucosa is significantly associated with increasing p53 and Ki-67 expression. Sections were counterstained with haematoxylin (magnification 100x).

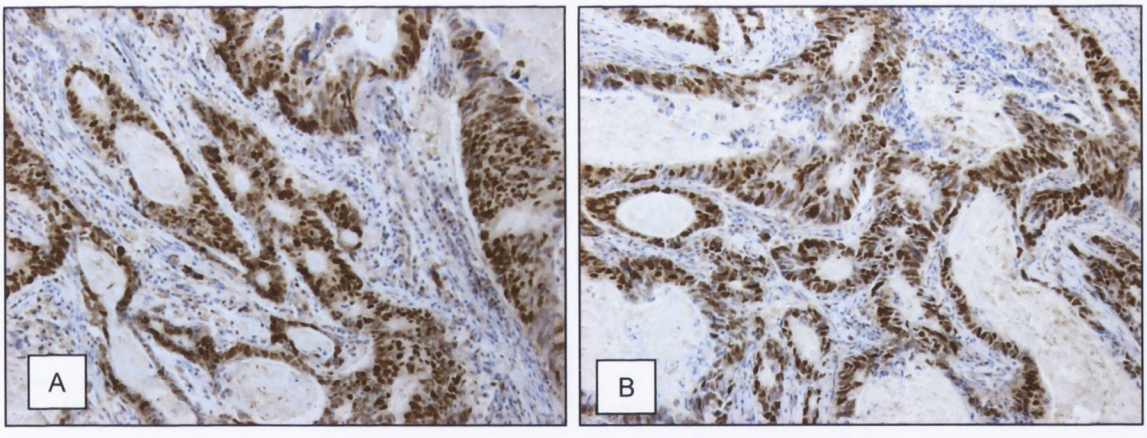

Figure 3.2. Section of moderately differentiated adenocarcinoma of the oesophagus demonstrating extensive nuclear immunostaining for p53 (A) and Ki-67 (B). Expression of p53 in tumour was associated with increasing growth activity assessed by Ki-67. Sections were counterstained with haematoxylin (magnification 100x).

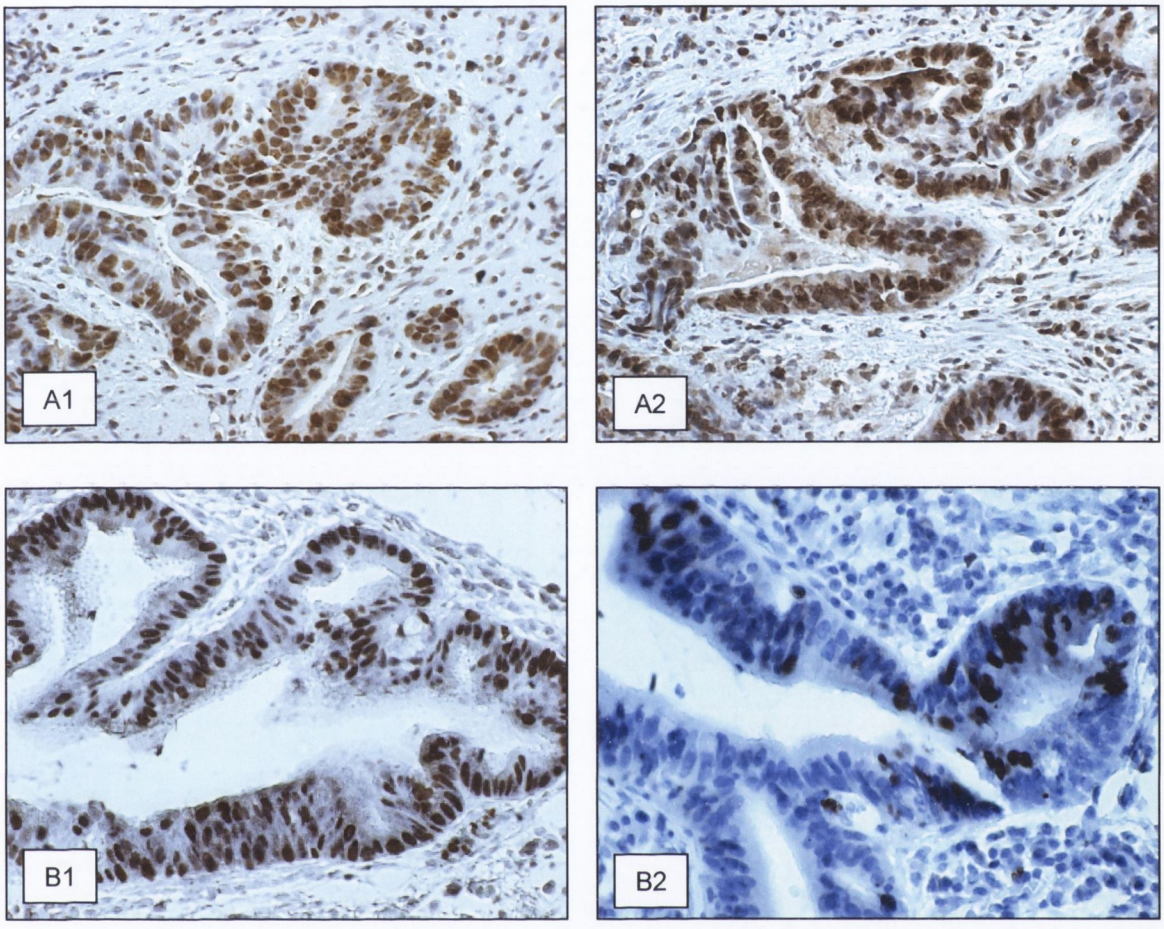

Figure 3.3. Immunohistochemistry staining of oesophageal adenocarcinoma prior to (A) and after (B) chemoradiotherapy. A1 and B1 represent p53 immunoreactivity, A2 and B2 represent Ki-67 immunoreactivity. There was a significant decrease in Ki-67 labelling index following chemoradiotherapy, whereas p53 remained unchanged.

3.6 DISCUSSION

This study identified the presence of a significant association between p53 and cell cycle related Ki-67 expression in Barrett's epithelium and oesophageal adenocarcinoma. Analysis of our data shows a pattern of increasing p53 positivity and aberrant cell proliferation, along with the various steps of the dysplasia-carcinoma sequence. The correlation between p53 accumulation and expansion of the proliferation compartment reached statistical significance. Localisation of p53 and Ki-67 positive cells was shown to correlate with the histological finding in dysplastic Barrett's epithelium. In Barrett's without dysplasia, epithelial growth was localized at the base of the oesophageal crypts and the glandular zones, which corresponds to the naturally occurring proliferative zone. Barrett's with low-grade dysplasia demonstrated a greater percentage of p53 and Ki-67 positive nuclei, particularly, in the lower crypt zone. The pattern of p53 and Ki-67 staining was significantly different in high-grade dysplasia as the percentage of positive nuclei was much higher in the upper crypt and the glandular zone. This observation supports a concept whereby proliferation stimulated by oncogene activation and loss of the tumour-suppressor function, as in other parts of the gastrointestinal tract (i.e. colon), plays a central role in the neoplastic progression. Increasing expression of p53 in Barrett's mucosa with increasing degrees of dysplasia, as shown here and in other studies (Flejou *et al*, 1995; Kim *et al*, 1997, Rioux-Leclercq *et al*, 1999) suggests that accumulation of p53 protein frequently occurs as an early event during carcinogenesis in Barrett's oesophagus. Lack of p53 expression, however, indicates that malignant progression and the loss of proliferative control, that characterizes the neoplastic progression, is possible without the involvement of p53. Further support for this suggestion is given by the lack of p53 staining in 2 cases of severely-dysplatic Barrett's and in 15 carcinomas prior to treatment. This study also confirms that over expression of p53 protein in tumours and in adjacent Barrett's epithelium does not always correspond. This was evident from p53 positive tumours which were associated with non or low - p53 negative - dysplastic Barrett's. This observation was also true in specimens where a histological continuity between Barrett's epithelium and adenocarcinoma was present. Similar to our finding a number of studies (Flejou *et al*, 1993; Hardwick *et al*, 1994) have reported a close association between p53 over-expression and adjacent highly-dysplastic Barrett's epithelium. These observations further indicate that, in some but not all cases, accumulation of p53 protein precedes the development of invasive carcinoma.

Many studies have investigated the link between p53 over-expression and tumour growth. Some indicated a positive relationship (Ikeguchi *et al*, 1997; Ozer *et al*, 1998; Liu *et al*, 2001) but others have failed to establish such an association (Vogt *et al*, 1997; Suto *et al*, 1998; Kim *et al*, 2002). The data presented here, indicate the presence of a significant association between p53 and Ki-67 expression. The cumulative expression of p53 in tumour biopsy samples was associated with increasing Ki-67 expression. In the present study, p53-spesific immunoreactivity was observed in tumours at a rate of 64.2%. Elsewhere, over expression of p53 protein has been reported in 47-90% of oesophageal adenocarcinoma (Jankowski *et al*, 1992; Duhaylongsod *et al*, 1995; Kim *et al*, 1997; Gleeson *et al*, 1998). The mean Ki-67 LI was significantly higher in p53 positive tumours compared to p53 negative tumours. The disassociation that was noted in this study was in 5 tumours with undetectable p53 and high Ki-67 LI and in 1 tumour with low Ki-67 and elevated expression of p53, these results suggest that additional factors may be at play in some neoplasms. Similar disassosiation has been noted in squamous oesophageal carcinoma (Ikeguchi *et al*, 1997) and has been attributed to possible abnormalities of c-erb B (Shiga *et al*, 1993; Kanda et al, 1994), epidermal growth factors (Chen *et al*, 1991; Kanda *et al*, 1994), cyclin (Jiang *et al*, 1993, Kanada *et al*, 1994) or other, as yet unidentified, factors.

The pattern of change of p53 and Ki-67 was unavailable in tumours that had a complete pathological response to therapy, similarly, (as regards to Ki-67) in the residual tumours having major pathological response. The observed correlation between p53 and Ki-67 in tumours which failed to respond to neoadjuvant therapy was no longer detected at resection due to a significant decrease in the Ki-67 LI by chemoradiotherapy, while the expression level of p53 was largely unchanged. Of the non-responder tumours (n = 29); 20 tumours (68.9%) (4 negative and 16 positive) sustained p53 expression. Seven tumours (24.1%) which lacked p53 before treatment showed expression of the protein afterwards, inversely 2 tumours (6.8%) lacked p53 expression after treatment. Independent of p53, the growth activity of these tumours reduced from a median of 37.1% (range 2-64.8%, 38.8 ± 12.0, mean ± SD) before treatment to 26.5% (range 1-60%, 24.7 ± 14.1, mean ± SD) at resection. The uncoupling of these parameters (p53 and Ki-67) at resection is open to a number of interpretations. It is possible, for example, that destruction of tumour cells in the vulnerable phases of the cell cycle results in therapeutic selection of that population of p53 positive cells which is in the G0

phase at the time of application of chemoradiotherapy. It is also probable that the unveiling of p53 by combination chemotherapy and radiation therapy was due to activation of the normal wild type gene in response to DNA damage, or it may be evidence of an additional iatrogenic insult to an already disordered genome. In this regard, the current study could not confirm that changes in these parameters (p53 and Ki-67) predicted either resistance or sensitivity to chemotherapy and radiation therapy.

Although, the molecular basis of p53 protein overexpression has not been determined for all human malignancies, overexpression of p53 protein in many human cancers such as colon (Rodriques *et al*, 1990), breast (Bartek *et al*, 1990; Davidoff *et al*, 1991) and lung (Iggo *et al*, 1990) has been shown to be the result of p53 gene mutation. In those cancers in which p53 protein has been measured, overexpression results from a prolonged half-life of the mutant protein which can then be detected by immunohistochemistry (Hinds *et al*, 1990; Levine 1990; Levine *et al*, 1991). It has also been established that p53 protein overexpression can occur in cells that have p53 gene mutation, even if there has not been a deletion of the remaining 17p allele (Davidoff *et al*, 1991; Varley *et al*, 1991). However, negative immunostaining may indicate loss of function of p53, especially when a stop codon mutation results in abrogation of the protein product (Gleeson *et al*, 1998). It is generally accepted that the majority of missense mutations generate a mutant protein with increased protein stability, thereby facilitating its detection (Cripps *et al*, 1994). Consistent with this, highly significant associations have been observed between missense mutation of p53 gene and diffuse p53 protein expression in oesophageal adenocarcinoma (Hamelin *et al*, 1994; Gleeson *et al*, 1998). It has also been reported that a proportion of immunonegative tumours may contain a mutation that does not result in protein stabilization (Gleeson *et al*, 1998). The occurrence of p53 mutations early in the development of oesophageal adenocarcinoma (Shiao *et al*, 1994) suggests that the gene plays a central role in the control of normal cell division. P53-dependent pathway may play a vital role in preserving molecular integrity by allowing damaged cells to undergo repair or apoptosis. Cells that have acquired a p53 mutation may have a selective advantage, being able to proliferate under conditions of DNA damage that would be inhibitory in cells with wild type p53. These actively proliferating cells may be the precursors of neoplastic clones. In addition, it has been suggested that tumours with functioning p53 may carry a better prognosis than those expressing defected protein. In support to this

theory, there are numerous studies that have demonstrated an association between p53 expression and many clinical and pathological variables (Ishida *et al*, 1997; Ogawa *et al*, 2001; Beenken *et al*, 2001; Liang *et al*, 2002). In contrast, there are also a number of studies that have not shown this association (Makinen *et al*, 1998; Nehls *et al*, 1999; Peyromaure *et al*, 2002) and it has been concluded that any adverse prognostic effect is probably small (Dowell *et al*, 1995). These findings are not surprising perhaps, since once the malignant transformation has taken place fundamental breakdown at genetic and cellular level occurs (if it has not already occurred) resulting in a heterogeneous malignant tissues that responds differently to various factors and stimuli. In our study the clinical significance of p53 as a prognostic factor appeared to be limited. Our results showed that p53 status is not a major determinant of tumours' histological grade or stage. Furthermore, we were unable to establish the prognostic significance of p53 to tumours' response to neuadjuvant chemoradiotherapy.

Recent progress in tumour cell biology has led to the description of various biologic markers of cell proliferation. Tumour proliferation rates have been considered predictive of clinical course and studies have indicated a correlation between high proliferation rates and tumour aggressiveness (Ikeguchi *et al*, 1997; Suto *et al*, 1998; Shiba *et al*, 2000; Goishi *et al*, 2000). The results of this study support the potential utility of proliferation index assessed by Ki-67 as a prognostic marker of tumour metastatic potential. We documented (Raouf *et al*, 2001) that the postoperative tumours, even if invasion of tumours was limited to the mucosa (pT1) or the mucsularis to adventitia (pT2-3), lymph node metastasis were frequently observed in patients with tumours that had high proliferation activity.

3.7 SUMMARY OF FINDINGS

This study demonstrates that p53 protein over-expression is a common feature in dysplastic Barrett's and oesophageal adenocarcinoma and it is significantly associated with growth activity. Neither p53 nor Ki67, however, predicts tumour response or resistance to chemoradiotherapy. Induction chemoradiotherapy prior to surgery significantly decreases the proliferation rate. The proliferation rate, estimated by Ki-67 expression might identify patients who are most at risk of developing metastatic disease.

CHAPTER 4

EXPRESSION OF BCL-2, BAX AND BCL-X PROTEINS IN NORMAL MUCOSA BARRETT'S MUCOSA AND ADENOCARCINOMA OF THE OESOPHAGUS

CHAPTER 4

EXPRESSION OF BCL-2, BAX AND BCL-X PROTEINS IN NORMAL MUCOSA BARRETT'S MUCOSA AND ADENOCARCINOMA OF THE OESOPHAGUS

4.1 INTRODUCTION

4.1.1 Introduction To Bcl-2 Family Genes

The bcl-2 family of proteins are important regulators of programmed cell death. Various homodimers and heterodimers formed by proteins in this family can either promote or inhibit apoptosis. Expression of some, such as bcl-2, bcl-x_L, bcl-w and bag suppresses apoptosis, while expression of others such as bax, bak, bad, noxa and diva promotes apoptosis. All members of the bcl-2 family show structural homology in two highly conserved amino acid domains in the carboxy-terminus of the molecule, BH1 and BH2. These conserved regions are thought to function in the heterodimerisation of the bcl-2 family member, resulting in inhibition or promotion of apoptosis (Craig, 1995).

Bcl-2 gene encodes a 25 kDa integral membrane protein mainly localized to the inner mitochondrial membranes, endoplasmic reticulum and the nuclear envelope (Hockenbery *et al*, 1990; Krajewski *et al*, 1993). Bcl-2 was first identified as a proto-oncogene overexpressed as a result of the t (14; 18) (q32; q21) chromosomal translocation in non-Hodgkin's lymphoma (Tsujimoto *et al*, 1986). However, subsequent studies showed that this chromosomal translocation is not a prerequisite for bcl-2 protein expression (Pezzella *et al*, 1990). Overexpression of bcl-2 plays an important role in the inhibition of apoptosis, thus contributing to neoplastic growth by prolonging cellular life span. Bcl-2 can protect against apoptosis caused by a variety of physiological and pathological stimuli (Hawkins *et al*, 1994). Antisense blocking of the bcl-2 gene has been shown to induce apoptosis and increase chemotherapeutic sensitivity in leukaemic cells even in the presence of other antiapoptotic genes (Reed *et al*, 1994; Konopleva *et al*, 2000). Bcl-2 appears to act at a critical point at which different apoptotic signals converge, but its mechanism of action remains unknown (Tsujimoto, 1996). While it is known that cytochrome c (released by mitochondria) activates caspases in apoptotic cells (Liu *et al*, 1996), recent studies have shown that bcl-2 blocks this release from the mitochondria and blocking apoptosis (Yang *et al*, 1997; Kluck *et al*, 1997).

Bcl-x is a bcl-2 related gene involved in both positive and negative regulation of apoptosis. Bcl-x protein is present in the cytosol in association with the mitochondrial periphery, a property shared with bcl-2 (Krajewski *et al*, 1994; Gonzalez-Garcia *et al*, 1994). Alternative splicing of the bcl-x mRNA generates two distinct types of protein, bcl-x_S (18 kDa, 178 amino acids) and bcl-x_L (30 kDa 241 amino acids). Bcl-x_S acts as a dominant inhibitor of bcl-2, thus inducing apoptosis, while, bcl-x_L performs a homologous function to bcl-2 by inhibiting apoptosis (Boise *et al*, 1993). Bcl-x_L has been shown to play a role in the cell's response to oxidants and resistance to chemotherapeutics and irradiation (Fang *et al*, 1995; Datta *et al*, 1995). The bcl-x gene can regulate cell death independently of bcl-2, and recent studies have shown that bcl-x_L can be more effective in inhibiting apoptosis than bcl-2 under certain conditions (Boise *et al*, 1993). On the other hand, overexpression of bcl-x_S causes sensitisation to chemotherapy - induced apoptosis in tumour cells that express high levels of bcl-2 or bcl-x_L (Sumantran *et al*, 1995).

The bcl-w gene codes for a 21 kDa protein that is widely expressed pro-survival member of the bcl-2 family. Forced expression of bcl-w, like bcl-2, renders myeloid and lymphoid cells resistant to apoptosis induced by cytokine deprivation or irradiation, but bcl-w is relatively ineffective against apoptosis induced by engagement of the CD95 (Fas) death receptor (Gibson *et al*, 1996; Takayama *et al*, 1995). Despite its widespread expression, bcl-w plays a significant role in spermatogenesis, but is not required for the normal development and function of most organs (Print *et al*, 1998; Ross *et al*, 1998).

Bag-1 (bcl-2 associated athanogene 1) gene codes for a 24.5 kDa (219 amino acids) bcl-2-binding protein which functions as a repressor of apoptotic cell death (Takayama *et al*, 1995). Bag-1 shares no significant homology with any bcl-2 family member but its coexpression with bcl-2 provides a marked increase in protection from apoptosis by comparison with either protein alone (Takayama *et al*, 1995). It has been demonstrated that inlerleukin-2 (IL-2) induced haematopoietic cell proliferation coincides with upregulations of bag-1 and bcl-2 expression (Adachi et al, 1996). There is also some evidence that bag-1 gene product may block apoptosis directly (Takayama *et al*, 1995).

Bax is another bcl-2 associated protein involved in the regulation of apoptosis. Bax encodes a 21kDa protein localized to the mitochondria and over-expression of this gene can induce cell death. Bax was the first bcl-2 related protein to be isolated, showing

homology with bcl-2 throughout two highly conserved regions (Oltvai *et al*, 1993). Bax can dimerise with itself or with bcl-2 and when overproduced promotes apoptosis (Korsemeyer *et al*, 1993). Bax was found to counter bcl-2 activity, accelerating apoptotic cell death. When bcl-2 is in excess, bcl-2 homodimers predominate and cells are protected from cell death. Alternatively, excess bax leads to bax homodimers and cell susceptibility to apoptosis (Oltvai *et al*, 1993). The ratio of bcl-2:bax represents a cell death switch which predetermines cells life or death response to an apoptotic stimulus (Korsemeyer, 1995).

Bak (bcl-2-homologous antagonist/killer) is functionally similar to bax, in that it induces apoptosis. Bak was found to be structurally similar to bcl-2 and other bcl-2 family members (Chittenden *et al*, 1995; Kiefer *et al*, 1995). The presence of a hydrophobic tail on the protein suggests that, like bcl-2, it is membrane localised (Farrow *et al*, 1995; Kiefer *et al*, 1995). The bak protein is translated from a 2.2 kb mRNA to yield a 211-216 amino acid protein with a predicted molecular weight of 23.4 kDa but it migrates by gel electrophoresis as an approximate 30 kDa protein under denaturing conditions. As well as binding to the adenovirus apoptotic repressor protein (E1B 19K) (Farrow *et al*, 1995), bak interacts with bcl-2 and bcl-x_L and certain non-members of the bcl-2 family (Kiefer *et al*, 1995). Bak induces apoptosis in many cell types, and even in the presence of survival factors, sympathetic neurons have been shown to enter the apoptotic pathway due to bak overexpression (Kiefer *et al*, 1995).

Bad a 22 kDa protein is another apoptotic member of the bcl-2 family that exhibits homology to bcl-2 limited to the BH1 and BH2 domains and can displace bax function binding to bcl-2 and bcl-x_L resulting in cell death (Yang *et al*, 1995; Zha *et al*, 1996). bad function to dimerise with bcl-x_L and with bcl-2, but not with bax, bcl-x_S or itself. In mammalian cells, bad bind with greater affinity to bcl-x_L than to bcl-2 and reverses the death repressor activity of bcl-x_L but not Bcl-2 (Yang *et al*, 1995). Dimerisation of Bad with bcl-x_L results in displacement of bax from the bcl-x_L: bax complex thereby causing restoration of bax mediated apoptosis (Yang *et al*, 1995). Survival factors such as IL-3 can inhibit the apoptotic activity of bad by activating intracellular signalling pathways that result in the phosphorylation of bad. Phosphorylated bad is then sequestrated in the cytosol by binding to 14-3-3 proteins and is unable to heterodimerize with bcl-x_L on mitochondrial membranes to induce apoptosis (Zha *et al*, 1996).

Noxa is a recently identified pro-apoptotic member of the bcl-2 family that contains the bcl-2 homology 3 (BH3) region but does not contain other BH domains (Adams *et al*, 1998; Oda *et al*, 2000). Noxa protein is shown to be selectively localised to mitochondria (Oda *et al*, 2000) and immunoprecipitation data suggest that noxa may interact with other members of the bcl-2 family, including bcl-x_L and mcl-1. Blocking the endogenous noxa results in the suppression of apoptosis. Expression of the noxa gene involves direct activation of its promoter by p53. Treating cells with noxa antisense oligonucleotide blocks radiation-induced apoptosis, and these observations suggest that noxa may be a mediator of p53-dependent apoptosis (Oda *et al*, 2000).

Diva is another pro-apoptotic member of the bcl-2 family that contains bcl-2 homology domain 1-4 (BH1-4) regions and a carboxyl-terminal hydrophobic domain (Inohara *et al*, 1998). Significantly, diva lacks critical residues in the conserved BH3 region that mediate the interaction between BH3-containing pro-apoptotic bcl-2 homologues and their pro-survival binding partners. Consistent with this, diva does not bind to cellular Bcl-2 family members such as bcl-2, bcl-x_L or bcl-w. Furthermore, mutants of diva lacking the BH3 region, fully retained their pro-apoptotic activity, confirming thereby that diva promotes apoptosis in a BH3-independent manner (Inohara *et al*, 1998). Diva interacted with Apaf-1, an adapter molecule that activates caspase-9, a central death protease of the apoptotic pathway. The expression of diva inhibited the binding of Bcl-x_L to Apaf-1, as determined by immunoprecipitation assays. Thus, diva represents a novel type of proapoptotic bcl-2 homologue that promotes apoptosis independently of the BH3 region through direct binding to Apaf-1, thus preventing bcl-x_L from binding to the caspase-9 regulator Apaf-1 (Inohara *et al*, 1998).

Other members of the bcl-2 family which function as pro-apoptotic members include, NBK (Bik), BID, Hrk, Bok, Bim. Further apoptotic inhibitors in this family are Mcl-1, Bag-1, A1 (Bfl-1), BAR and BI-1 (TEGT). Understanding of the mechanisms of differential gene regulation in different tissues and the clinical significance of the expression of bcl-2 and other members of the bcl-2 gene family is important to uncover the biological behaviour of different types of tumours and probably their responsiveness to different therapeutic or environmental insults.

4.1.2 Regulation and Interaction of Bcl-2 Family Genes

Recent studies have unearthed numerous mechanisms for regulating the function of the death agonists and antagonists of bcl-2 family proteins. In addition to the transcriptional control of gene expression, these mechanisms include posttranslational events such as phosphorylation, localisation, and the induction of conformational changes, which may either activate or inactivate these molecules. Interaction with homologous and nonhomologous proteins and specific subcellular targeting of bcl-2 proteins are other means of fine-tuning cellular response to noxious stimuli. Considerable attention has also turned to the regulation of so-called BH3-only molecules, which appear to act as stress sensors which relay signals to other pro- or anti- apoptotic family members.

At the level of transcription, numerous studies have indicated that differential expression of bcl-2 molecules can be regulated. A physiological example is provided by human neutrophils, which constitute an important first line of defence against invading microorganisms and are extremely short-lived; these cells are also known to express abundant levels of bax, yet are completely devoid of bcl-2 (Ohta *et al*, 1995). The preponderance of proapoptotic molecules may, in part, explain why these cells so readily undergo apoptosis in the absence of specific triggers (Liles *et al*, 1995; Fadeel *et al*, 1998). In addition, differentiation of myeloid leukaemic cells transpires with a decrease in bcl-2 (Delia et al, 1992). An upregulation of bcl-2 mRNA in growth factor-dependent cells by the survival factors IL-2 or IL-3 has also been observed (Otani *et al*, 1993). Treatment of some tumour cells with chemotherapeutic agents may induce a p53-dependent down-regulation of bcl-2 and a concomitant upregulation of Bax (Miyashita *et al*, 1994; Miyashita *et al*, 1995; Han *et al*, 1996). However, the level of expression of death agonists versus antagonists does not always correlate with the susceptibility of tumours to apoptosis (Jaattela, 1999), suggesting that these regulators of cell death may also be subject to posttranslational modulation.

Protein phosphorylation is the most common cellular mechanism for postsynthetic regulation of protein function, and though there is no conclusive evidence that kinases are required for the execution of the death program, it seems clear that they may amplify exogenous stimuli and/or integrate conflicting stimuli prior to commitment to apoptosis (Gjertsen *et al*, 1995; Anderson 1997). Bcl-2 family members can also be phosphorylated, although the outcome of this posttranslational modification appears to

depend on the specific stimulus. Taxol and other chemotherapeutic agents that target microtubules induce phosphorylation of bcl-2, which is associated with an abrogation of its antiapoptotic function (Haldar *et al*, 1996; Blagosklonny *et al*, 1997). It has also been proposed that bcl-2 may serve essentially as a 'guardian of microtubule integrity' (Haldar *et al*, 1997). Bcl-x_L is also phosphorylated after microtubule disruption (Poruchynsky *et al*, 1998). Exposure of cells to all-trans retinoic acid (ATRA) induces phosphorylation of bcl-2, and this has been suggested to result in a shortened half-life of the protein, perhaps by targeting of bcl-2 for degradation (Hu *et al*, 1998). In contrast, other studies have suggested that phosphorylation of bcl-2 may be required for its anti-apoptotic function in growth factor-dependent cell lines (May *et al*, 1994; Ito *et al*, 1997). Furthermore, it has been demonstrated that phosphorylation of bcl-2 may contribute to the inactivation of the antiproliferative function of bcl-2 (Poommipanit *et al*, 1999). The functional consequences of bcl-2 phosphorylation have been found to be associated with mitotic arrest rather than the induction of apoptosis (Scatena *et al*, 1998; Ling *et al*, 1998). Phosphorylation of the proapoptotic proteins may also occur, i.e. bad protein in response to IL-3 treatment; these findings have provided an illustrative example of signal transduction pathways triggered by extracellular survival signals (Zha *et al*, 1996). Dephosphorylated Bad is associated with bcl-x_L whereas phosphorylated bad translocates to the cytosol and binds to 14–3-3. This appears to sequester bad away from bcl-x_L at the mitochondrial membrane, thereby allowing bcl-x_L to exert its antiapoptotic effect. An additional report demonstrated that overexpression of bcl-2 can induce the localisation of the protein kinase Raf-1 to mitochondria where Raf-1 participates, indirectly, in the phosphorylation of bad (Wang *et al*, 1996). The serine-threonine kinase Akt may also serve to couple survival signals to the regulation of bad (Peso *et al*, 1997; Datta *et al*, 1997). Moreover, (Harada *et al*, 1999) reported that membrane-bound, cAMP-dependent protein kinase acts as a specific bad kinase. This cAMP-dependent protein kinase is tethered to the mitochondrial membrane through its association with a protein kinase A-anchoring protein, and these findings illustrate the inactivation of a proapoptotic factor at its specific target organelle in response to a survival factor.

Localisation is an important theme in signal transduction and evidence has accrued over recent years for the maintenance of specific subcellular distribution of kinases and phosphatases close to their activators and targets (Mochly-Rosen *et al*, 1995; Pawson *et al*, 1997). Similarly, evidence has been provided that the regulation of apoptotic signals

is dependent on the specific subcellular localization of bcl-2 proteins. Bcl-2 was originally reported to be an inner mitochondrial membrane protein (Hockenbery *et al.* 1990). However, other studies have demonstrated that bcl-2 resides predominantly in the outer mitochondrial membrane, the endoplasmic reticulum, and the nuclear membrane (Krajewski *et al*, 1993; Lithgow *et al*, 1994). Though the reason for these discrepancies remains unresolved, it seems clear that the antiapoptotic effect of bcl-2 is largely dependent on its membrane localization (Tanaka *et al*, 1993; Nguyen *et al*, 1994). On the other hand, the function of bcl-2 and bcl-x_L is not solely due to the prevention of cytochrome c release from mitochondria, as demonstrated in experiments with microinjected cytochrome c (Li *et al*, 1997; Zhivotovsky *et al*, 1998). In addition, it has been shown that bcl-2 protects cells from apoptosis downstream of bax-induced release of cytochrome c from the mitochondria (Rosse *et al*, 1998).

Although bax expression *per se* is not lethal to cells, the translocation of bax toward mitochondria during apoptosis was shown to be important for the ability of bax to trigger cell death (Wolter *et al*, 1997). Deletion of the carboxy-terminal hydrophobic region of bax prevents bax redistribution and abrogates its apoptosis-promoting activity, underscoring the importance of organelle binding for the activity of this molecule. In addition, enforced dimerisation of bax results in its translocation to the mitochondria and the subsequent induction of mitochondrial dysfunction and apoptosis (Gross *et al*, 1998). Furthermore, bcl-2 and bcl-x_L are present in the mitochondrial outer membrane of resting cells, whereas the proapoptotic molecules bax, bad, and bcl-x_S are targeted to these organelles during tumour necrosis factor (TNF)-induced apoptosis (Jia *et al*, 1999). Hence, it appears that the cell resets the ratio of mitochondrial proapoptotic to antiapoptotic protein in response to apoptosis triggers. Translocation of bax to the nucleus during the apoptotic process has also been reported, although the significance of these findings, at present, is not well understood (Mandal *et al*, 1998). The observation that bax is cytosolic in healthy cells suggests that the hydrophobic tail may normally be hidden within the interior of the protein or bound to other cytosolic factors, indicating that conformational changes are likely to be involved in the targeting of bax to mitochondria (Hsu *et al*, 1997).

The countervailing roles of pro- and anti- apoptotic bcl-2 family members can be regulated through protein–protein interaction. For instance, (Oltvai et al, 1993) showed

that the bcl-2 molecules can heterodimerise with the proapoptotic protein bax. Based on these and similar findings, which pointed to the delicate equipoise between bcl-2 family members as an important determinant in the decision of whether a cell should live or die, the 'rheostat' concept was formulated. This concept implies that sensitivity to death stimuli is determined by the relative ratio of agonist and antagonist homo- to hetero-dimers (Oltvai *et al*, 1994; Yang *et al*, 1996).

Mutagenesis experiments established that the bcl-2 homology (BH) domains 1 to 3 strongly influence homo- and hetero- dimerisation (Yin *et al*, 1994; Chittenden *et al*, 1995). However, it is clear that bcl-2 and bax also have intrinsic functions independent of dimerisation (Reed, 1998). Bcl-2 can bind nonhomologous proteins such as the protein kinase Raf-1, the molecular chaperone regulator bag-1, the p53-binding protein p53-BP2, and calcineurin, a calcium- and calmodulin-dependent protein phosphatase (Reed, 1998). The BH4 domain, present in all antiapoptotic family members but absent from nearly all proapoptotic counterparts, is needed for interaction with several of these seemingly unrelated proteins, including Raf-1, bag-1, and calcineurin (Wang *et al*, 1996; Shibasaki *et al*, 1997). The latter is of particular interest, considering the role of calcineurin binding for activation of the transcription factor NF-AT, and suggests that bcl-2 may play a role in cell cycle regulation (Linette *et al*, 1996).

Bcl-2 family members display homology in discrete domains designated BH domains 1 to 4. Family members that act as inhibitors of cell death harbour at least three domains (BH1, BH2, and BH3), which are important for protein–protein interaction and the suppression of apoptosis, whereas BH3 serves as the minimal 'death domain' in the proapoptotic members studied so far (Kelekar *et al*, 1998). A subfamily of proapoptotic proteins consisting of bad, bid, bik, bim, blk, and hrk, which share homology only in the latter domain, is therefore referred to as BH3-only proteins. For example, Bim, which is normally sequestered by the dynein motor complex, translocates to mitochondria on apoptosis triggering and antagonises the effect of bcl-2 (Puthalakath *et al*, 1999) and the finding that the activity of bad is controlled by phosphorylation and dephosphorylation in response to extracellular stimuli (Datta *et al*, 1997; wang *et al*, 1999). Truncated bid, as other BH3-only proteins, requires another protein such as bax or bak to exert its pro-apoptotic function (Wei *et al*, 2001; Zong *et al*, 2001). Bid was found to be associated with mitochondrial contact sites and was shown to destabilise lipid membranes in vitro

(along with bax) by increasing the permeabilization of artificial liposomes (Basanez *et al*, 1999; Kudla *et al*, 2000). Therefore, it is possible that bid can bind to the cardiolipin rich contact site membranes and destabilize them by inducing bax or bak to oligomerize. The membrane destabilization may cause a secondary effect by inhibiting the action of the proteins located there. This hypothesis would account for how Bid affects the function of many different processes without binding directly to several proteins.

4.1.3 The Role of Bcl-2 Family in Normal Physiology and Neoplasia

The precise regulation and maintenance of balance between cell proliferation and cell death in multicellular organisms is critical for tissue homeostasis. The physiological function of bcl-2 proteins turned out to be vital in this process and evidence indicates that alteration in the activity of these proteins renders neoplastic development in many human organs. In normal human tissues; expression of bcl-2 family proteins has been reported in a wide range of haematopoietic cells, non-neoplastic epithelia (both hormone-responsive and non-responsive), muscles and nerve tissues (Pezzella *et al*, 1995; Krajewska *et al*, 1996; Sarbia *et al*, 1996). Expression of apoptotic regulatory proteins in normal tissues is thought to be associated with prolonged survival, particularly in non proliferating cells thus allowing differentiation and morphogenesis to proceed. However, heterogeneous expression of bcl-2 proteins in epithelial malignancies suggests that these genes are differentially regulated. Furthermore, their expression in association with precancerous lesions suggests a role in the early stage of tumourigenesis. Expression of bcl-2 family proteins has been observed in precancerous lesion of the oesophagus as well as in gastric, colorectal, ovarian and prostate cancer, suggesting a role for bcl-2 family proteins in the genesis of epithelial tumours.

The multistep process of neoplastic transformation of Barrett's- or dysplastic squamous-epithelium to adenocarcinoma and squamous cell carcinoma of the oesophagus, respectively, was showed to be associated with sequential changes in the expression of bcl-2 protein. As neoplasia progresses from Barrett's oesophagus, bcl-2 decreases (Katada *et al*, 1997; Soslow *et al*, 1999). In contrast, bcl-2 expression increases with the progression of dysplasic epithelium to squamous carcinoma (Sarbia *et al*, 1996; Ohbu *et al*, 1997). These observations indicate that the two types of oesophageal carcinoma arise through different molecular mechanisms and that the role of bcl-2 protein in tumour progression is fundamentally different.

Aberrant expression of bcl-2 proteins has been reported in gastric carcinogenic sequence and studies have indicated that the antiapoptotic proteins bcl-2, bcl-x and mcl-1 expression is higher in carcinomas compared to normal foveolar cells, whereas the proapoptotic proteins bax and bak is reduced in tumours by comparison with normal cells. Furthermore, the expression levels of these proteins were shown to differ depending on the histological type (intestinal or diffuse) of the tumours (Lauwers *et al*, 1994; Krajewska et al, 1996).

Studies of the bcl-2 family proteins in the adenoma–carcinoma sequence of colorectal tumorigenesis indicted that bcl-x expression was increased and mcl-1 expression decreased when compared with normal mucosa or non-malignant adenomas. No differences were found for the expression of bax, while the proportion of tumours in which bcl-2 was more than or equal to that of normal colonic mucosa was significantly lower in carcinomas than adenomas (Baretton *et al*, 1996; Krajewska *et al*, 1996; Maurer *et al*, 1998). Thus, a shift from expression of the anti-apoptotic proteins bcl-2 and Mcl-1 to the bcl-x_L protein may occur during progression of colorectal tumors.

In ovarian tissues, the levels of bcl-2, bax and bcl-x expression in normal and neoplastic tissues was shown to be significantly different. Bcl-2 was higher in normal tissue whereas bax and bcl-x_L were higher in carcinoma. Of the bcl-x, bcl-x_S was present at low levels in neoplastic samples and was undetectable in normal tissue whereas, bcl-x_L was dominant in neoplastic tissues. The expression of bax and bcl-x_L was correlated with progesterone receptor level (Wehrli *et al*, 1998; Marone *et al*, 1998).

In prostatic adenocarcinoma, expression of several anti-apoptotic members of the bcl-2 gene family, including bcl-2, bcl-x, and mcl-1 increases during progression of prostate cancers, while the pro-apoptotic protein bax shows consistent expression (Krajewska *et al*, 1996; Johnson *et al*, 1998). These observations may reflect the association of bcl-2 proteins to the hormonal level and perhaps also their neoplastic phenotype. Recent studies of other malignancies such as pancreatic (Sinicrope *et al*, 1996), lung (Chen *et al*, 1999) and breast, (Eguchi *et al*, 2000) also indicates the significance of the bcl-2 proteins in the tumourogenesis of these organs.

4.2 OBJECTIVES OF STUDY

The aims of this study were to (1) explore the role of bcl-2 and bcl-2 associated bax and bcl-x protein expression in the molecular regulation of Barrett's tumourigenesis (2) examine the roles played by these genes in determining therapeutic responsiveness and (3) assess the association of bcl-2, bax and bcl-2 expression to the clinicopathological variables in patients with oesophageal adenocarcinoma.

4.3 MATERIALS AND METHODS

4.3.1 Patient Cohort

Archival formalin-fixed paraffin-embedded tissue samples from 48 patients (36 male, 12 female) with primary oesophageal adenocarcinoma were included in this study. Patients' ages ranged from 45 to 77 years with a median of 65 years. All patients had oesophagogastro-endoscopy and histological confirmation of diagnosis prior to treatment with chemotherapy (cisplatin and 5-fluorouracil) and radiotherapy (CRX) followed by oesophagectomy. Surgery was undertaken at a median of 4 (range 3-6) weeks following neoadjuvant therapy.

4.3.2 Histopathological Examination

In each case, pathology reports of pre-treatment biopsy and surgical resections were reviewed. Similar staging systems as described previously (chapter 2 section 2) were used. Non-metaplastic, non-tumourous oesophageal margins (n = 25) were used as normal oesophageal tissues. Barrett's epithelium when present, Pre-treatment biopsies and representative 2-4 blocks of the tumours or the site of a previous tumour were also analysed. At surgery, 9 cases were grouped as having complete pathological response (CPR) to CRX. No evidence of pathological response (NPR) was observed in 31 cases. A group of 8 patients was classified as having major pathological response (MPR) to CRX.

4.3.3 Immunohistochemistry

Expression of bcl-2, bax and bcl-x protein was detected by the avidin-biotin peroxidase method using microwave antigen retrieval (chapter 2 section 4). Immunostaining was carried out on the 4μm sections. Sections were incubated with mouse anti-human bcl-2, rabbit anti-human bax, and rabbit anti-human bcl-x. The latter antibody reacts with both bcl-x_S and bcl-x_L form [Appendix 2]. Biotinylated anti mouse/rabbit IgG was used as a

secondary antibody. The reaction product of peroxidase was visualised by 3`-3`diaminobenzidine-tetrachloride as a chromagen that results in brown cytoplasmic staining within the positive cells. Sections of follicular lymphoma, prostate carcinoma and intestinal type gastric adenocarcinoma were used as positive tissue controls for bcl-2, bax, and bcl-x, respectively. The presence of lymphocytes within the sections served as an internal positive tissue control. Omission of the primary antibody during the staining procedure provided negative control for reagents. In each section up to 2000 cells were counted and the percentage positivity recorded as labelling index (LI). Sections demonstrating weak immunopositivity with LI <5% were considered to be negative for bcl-2, bax or bcl-x.

4.3.4 Statistical Analysis

The significance of association between bcl-2, bax and bcl-x expression and the histopathologic parameters was determined by chi-square or Fisher's exact test. For paired comparison of pre- to post-CRX values (LI), Wilcoxon test was used. Survival curve were calculated by the Kaplan-Meier method, and the statistical significance was analysed by the log-rank test. Patients dying from other causes were excluded from the survival analysis. Statistical significance was defined as $P < 0.05$.

4.4 RESULTS

4.4.1 Expression of Bcl-2, Bax and Bcl-x in Normal Mucosa

Bcl-2, bax and bcl-x cytoplasmic immunopositivity were detected in 84% (21/25), 80% (20/25) and 76% (19/25) of the normal squamous epithelium, respectively. Bcl-2 expression was confined only to the basal-cell layer while bax and bcl-x were also detected in supra-basal cell layers of the normal oesophageal mucosa (Table 4.1) (Figure 4.1). In addition, mucous oesophageal glands, muscle cells of the muscularis mucosa and the muscularis propria all expressed bcl-2, bax and bcl-x.

4.4.2 Expression of Bcl-2, Bax and Bcl-x in Barrett's Mucosa

Barrett's mucosa was identified in 19 pre-treatment biopsies and in 22 specimens at resection. An increasing degree of dysplasia in Barrett's mucosa both before- and after-CRX was significantly associated with reduction of bcl-2 expression (P = 0.03 and 0.009, respectively). Conversely, the expression of bax and bcl-x did not vary significantly (Table 4.1) (Figure 4.2).

4.4.3 Expression of Bcl-2, Bax and Bcl-x in Oesophageal Adenocarcinoma

Before-treatment, bcl-2, bax and bcl-x expression were detected in 27% (13/48), 75% (36/48) and 87.5% (42/48) of adenocarcinomas, respectively. Pre-treatment tumour samples (n = 9) of the subsequent complete pathological responders (CPR) to CRX demonstrated 11.1% (1/9), 88.8% (8/9) and 100% (9/9) bcl-2, bax and bcl-x immunopositivity, respectively. Pre-treatment tumour samples (n = 8) of the subsequent major pathological responders (MPR) were 37.5% (3/8), 75% (6/8) and 87.5% (7/8) bcl-2, bax and bcl-x immunopositive, respectively. Tumours (n = 31) that did not respond (NPR) to CRX expressed 29% (9/31), 71% (22/31) and 83.8% (26/31) immunopositivity for bcl-2, bax and bcl-x, respectively.

4.4.4 Expression of Bcl-2, Bax, Bcl-x and Tumours' Response to Chemoradiotherapy

There was no significant association between bcl-2, bax or bcl-x expression and tumour response or resistance to CRX (Table 4.2). At resection, 22.5% (7/31) of the NPR group and 62.8% (5/8) of the MPR group tumours were bcl-2 positive (P = 0.07). There was no significant up or down regulation of bcl-2 expression by CRX. All pre-treatment bax and 5 of 6 (80%) bcl-x negative tumours were immunopositive at resection (Table 4.2) (Figure 4.3).

4.4.5 Expression of Bcl-2, Bax and Bcl-x and Clinicopathological Parameters

Both before- and after- treatment, expression of bcl-2, bax or bcl-x protein did not statistically associate with patients' age or gender, while, bcl-2 expression was significantly associated with tumour histological differentiation (P = 0.02 and 0.03, respectively) (Table 4.3). At resection, no significant association was observed between bcl-2 expression and tumour mural extension (pT) or lymph node status (pN). Expression of bax and bcl-x showed no association to any of the above-mentioned parameters (Table 4.3).

4.4.6 Expression of Bcl-2, Bax and Bcl-x and Patients Survival

At resection, bcl-2 expression was significantly associated with patients survival, whereas, bax and bcl-x showed no such association. At a median follow up of 40 months, eight out of 24 (33%) bcl-2 negative patients were alive compared with six out of 10 (60%) bcl-2 positive patients (P = 0.03). The survival curve relating bcl-2 expression at the time of resection and survival is shown in (Figure 4.4).

Table 4.1. Expression of bcl-2, bax and bcl-x protein in normal and Barrett's mucosa.

Parameters	No. of cases	Bcl-2 immuno-positive cases	Bax immuno-positive cases	Bcl-x immuno-positive cases
Normal mucosa	25	21	20	19
Barrett's Mucosa*				
Non-dysplastic	9	8	7	8
Low-grade dysplasia	3	1	3	3
High-grade dysplasia	7	2	7	7
***P* value**		0.03 [A]	0.28 [A]	0.55 [A]
Barrett's Mucosa**				
Non-dysplastic	8	7	6	6
Low-grade dysplasia	3	2	3	2
High-grade dysplasia	11	2	10	10
***P* value**		0.009 [A]	0.46 [A]	0.51 [A]

* data represent pre-treatment cases ; ** data represent post-treatment cases. Statistical calculation was performed by chi-square test. [A]

Table 4.2. Expression of bcl-2, bax and bcl-x protein in oesophageal adenocarcinoma.

Parameters	No. of cases	Bcl-2 immuno-positive cases	Bax immuno-positive cases	Bcl-x immuno-positive cases
Before-CRX (n = 48)				
CPR & MPR group	17	4	14	16
NPR group	31	9	22	26
***P* value**		0.74 [A]	0.49 [A]	0.42 [A]
After-CRX (n = 39)				
MPR group	8	5	8	8
NPR group	31	7	31	30
***P* value**		0.07 [A]	> 0.99 [A]	> 0.99 [A]

Statistical calculation was performed by Fisher's exact test. [A]

Table 4.3. Expression of bcl-2, bax, bcl-x and clinicopathological parameters.

Parameters	No. of Cases	Bcl-2 immuno-positive cases	Bax immuno-positive cases	Bcl-x immuno-positive cases
Before-CRX				
Gender				
Male	36	9	28	31
Female	12	4	8	11
***P* Value**		0.71 [A]	0.46 [A]	> 0.99 [A]
Age (year)				
< 65	24	6	15	20
≥ 65	24	7	21	22
***P* Value**		> 0.99 [A]	0.093 [A]	0.66 [A]
Type of differentiation				
Well/moderate	27	11	21	25
Poor	21	2	15	17
***P* Value**		0.02 [A]	0.76 [A]	0.38 [A]
After-CRX				
Gender				
Male	30	7	30	29
Female	9	5	9	9
***P* Value**		0.10 [A]	> 0.99 [A]	> 0.99 [A]
Age (year)				
< 65	21	6	21	20
≥ 65	18	6	18	18
***P* Value**		> 0.99 [A]	> 0.99 [A]	> 0.99 [A]
Type of differentiation				
Well/moderate	26	11	26	25
Poor	13	1	13	13
***P* Value**		0.03 [A]	> 0.99 [A]	> 0.99 [A]
Tumours extension				
pT1	8	4	8	7
pT2	5	3	5	5
pT3	26	5	26	26
***P* Value**		0.08 [B]	> 0.99 [B]	0.13 [B]
Lymph node metastasis				
Negative	25	10	25	24
Positive	14	2	14	14
***P* Value**		0.15 [A]	> 0.99 [A]	0.15 [A]

Statistical calculation was performed by Fisher's exact [A] and chi-square [B] test.

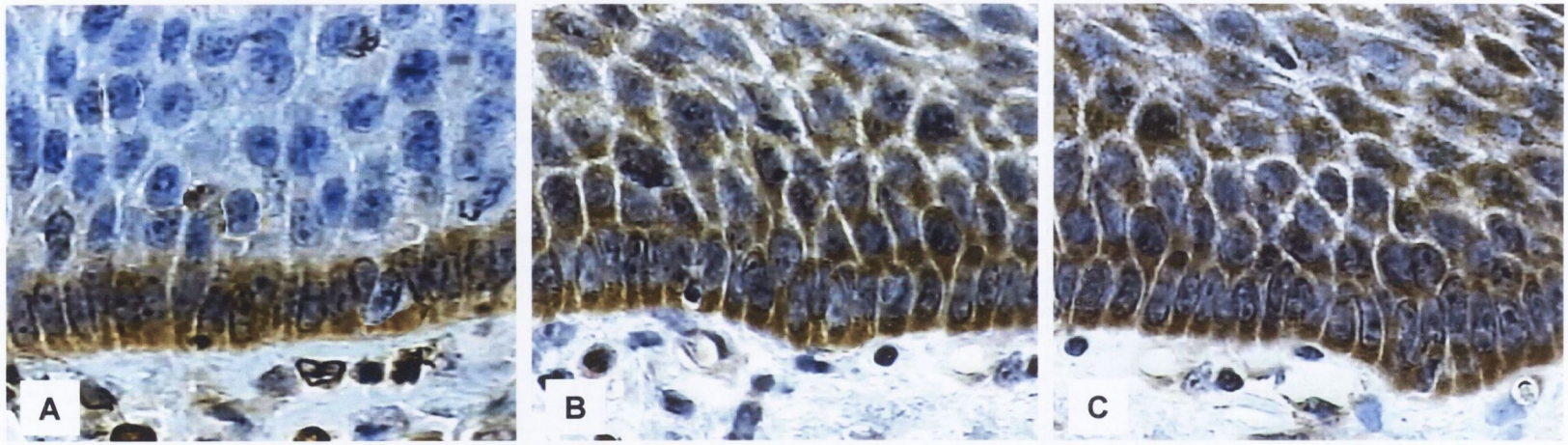

Figure 4.1. Expression of bcl-2, bax and bcl-x proteins in normal oesophageal mucosa. Photomicrographs (A, B and C) represent immunocytoplasmic staining of bcl-2, bax and bcl-x, respectively. Bcl-2 was only detected in the basal-cell layer, while bax and bcl-x were detected in the basal-cell layer and supra-basal layers of normal oesophageal mucosa (magnification x200).

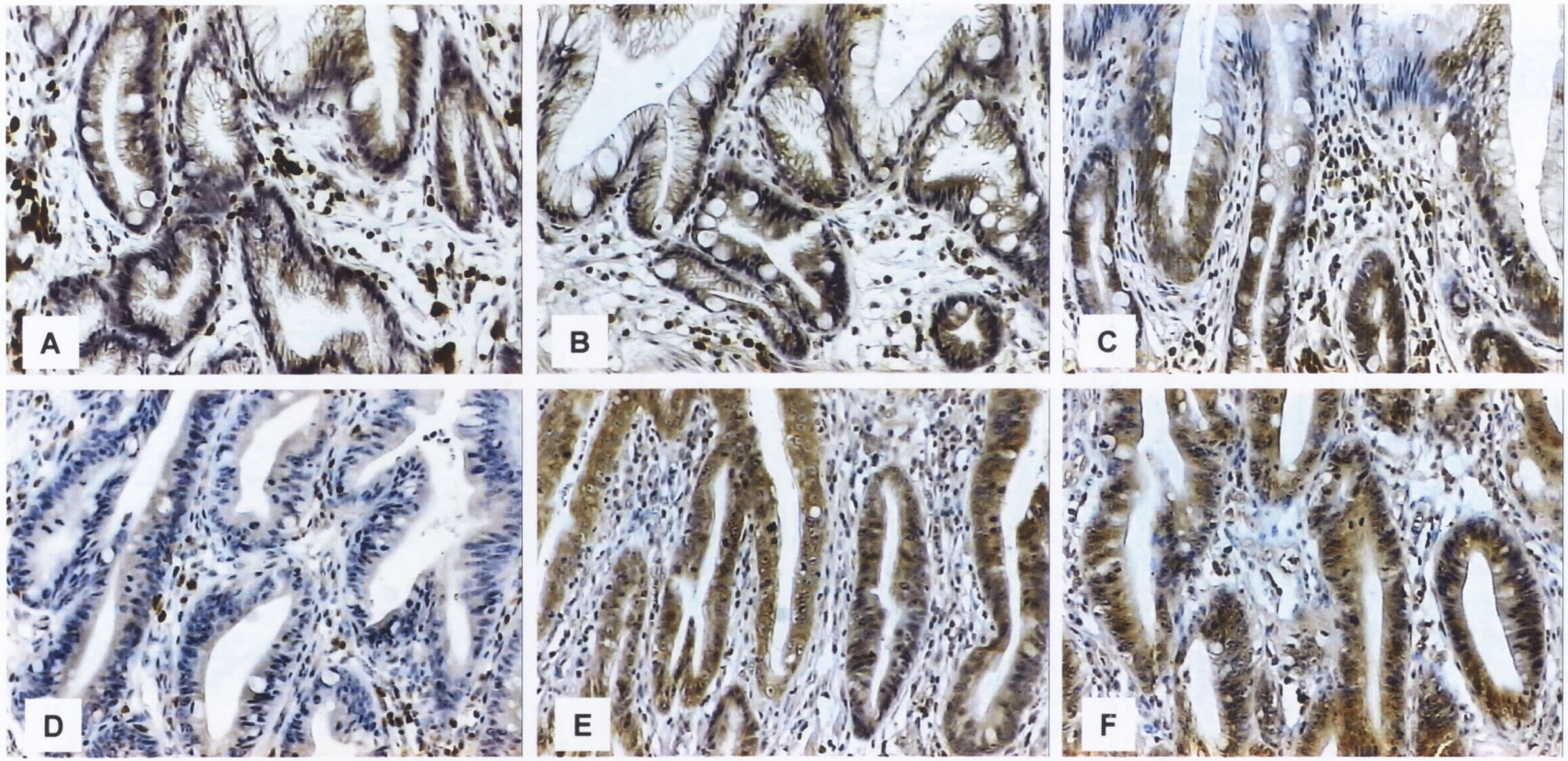

Figure 4.2. Expression of bcl-2, bax and bcl-x proteins in Barrett's mucosa. Photomicrographs (A-C) and (D-F) represent non-dysplastic and severely-dysplastic Barrett's mucosa, respectively. Bcl-2 (A), bax (B) and bcl-x (C) immunocytoplasmic staining was detected in non-dysplastic Barrett's lesions. Severely-dysplastic Barrett's widely expressed bax (E) and bcl-x (F) but not bcl-2 (D) (magnification x100).

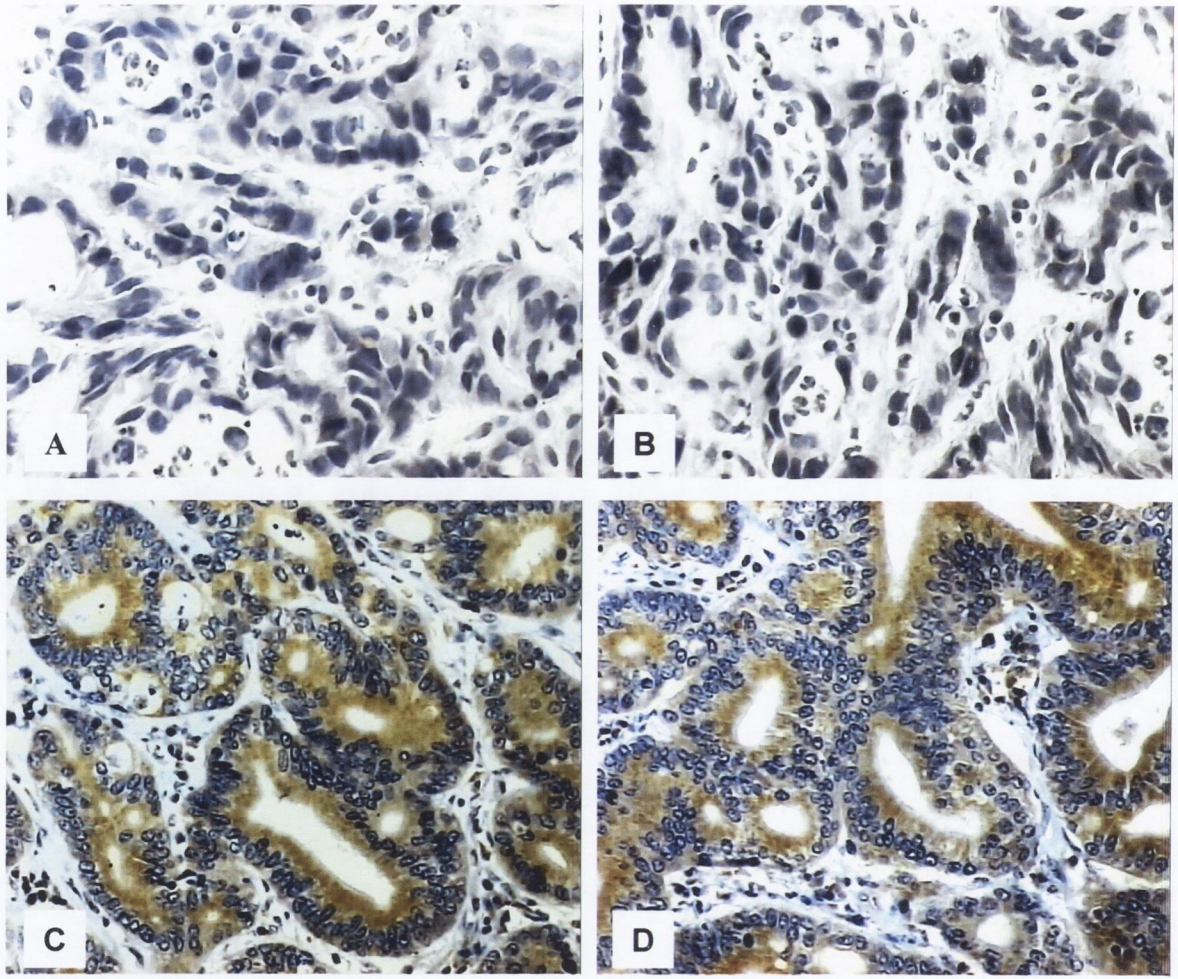

Figure 4.3. Expression of bax and bcl-x proteins in oesophageal adenocarcinoma. Photomicrographs represent pre-treatment bax (A) and bcl-x (B) immunonegative tumour, (C) and (D) represent post-treatment immunopositive bax and bcl-x, respectively (magnification x200).

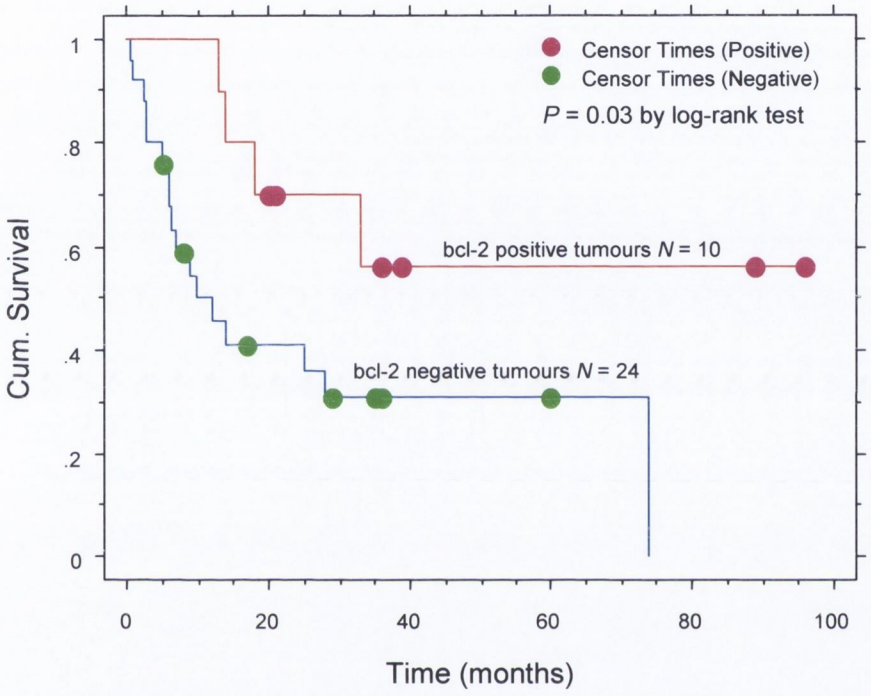

Figure 4.4. Kaplan–Meier plot for disease-free survival of patients with oesophageal adenocarcinoma, classified according to the tumours' expression of bcl-2 in the resected specimens after chemoradiotherapy.

4.5 DISCUSSION

It has been hypothesised that alteration in the activity or expression of bcl-2 family proteins might be an initial step toward malignancy. To determine whether the bcl-2 and its associate proteins bax and bcl-x are involved in oesophageal carcinogenesis, we analysed the expression of these proteins in normal mucosa, pre-cancerous Barrett's mucosa and invasive adenocarcinomas. Our results showed that normal oesophageal squamous mucosa widely expresses bcl-2, bax and bcl-x. Bcl-2 is expressed in the basal-cell layer, while bax and bcl-x were also detected in supra-basal call layers. Cytoplasmic immunopositivity was also detected in the normal oesophageal mucous glands and normal muscle cells. The intensity of bcl-2 staining was weaker in the basal-cell layer of normal squamous epithelium compared to mucosal glands or muscle cells, while the pattern of expression of bax and bcl-x was quite homogeneous and consistent. The presence of apoptotic regulator proteins in normal oesophageal mucosa as reported here and in similar studies (Sarbia *et al*, 1996; Torzewski *et al*, 1998) could be a normal protecting mechanism to regulate cellular life span by modulating apoptosis, particularly in non-proliferating normal cells.

This study indicated a pattern of a progressive reduction of bcl-2 expression through low- to high-grade dysplastic Barrett's epithelium and adenocarcinoma, whereas, expression of bax and bcl-x gene products demonstrated increased levels of expression. These observations suggest that loss of bcl-2 regulation is a factor in the multistep progression of Barrett's- adenocarcinoma. Additionally, this study also implies that elevated levels of bax and bcl-x protein represent a common event in the pathogenesis and progression of epithelial malignancies of the gastrointestinal tract, as similar trends in the expression levels of bax and bcl-x have been reported in gastric (Krajewski *et al*, 1996) and colorectal carcinomas (Krajewski *et al*, 1996).

Although reduced apoptosis, in association with reduced bcl-2 has been reported early in the dysplasia-carcinoma sequence of Barrett's oesophagus (Katada *et al*, 1997). It would be possible to interpret the latter event and our data if the prevalence of the bcl-x gene at a high level during the neoplastic development was associated with the presence of its dominant form (bcl-x_L) which shares homology with bcl-2 by inhibiting apoptosis and possibly antagonizing bax function. Further support for this suggestion is found in the fact that in almost all normal- and malignant- tissues examined throughout the body,

the most abundant form of the bcl-x protein found, has been the anti-apoptotic bcl-x_L protein, and very little or no proapoptotic bcl-x_S protein was detected (Krajewski *et al*, 1994; Broome *et al*, 1995; Krajewski *et al*, 1996; Xerri *et al*, 1998; Marone *et al*, 1998, Mozzetti *et al*, 2000). On the other hand, the absence of immuno-detectable bcl-2, bax or bcl-x may indicate that other apoptotic pathways may be associated with the progression of this disease. It is also conceivable that the ratio of epithelial growth to apoptosis plays an important role in multistep epithelial carcinogenesis.

The association of p53 as a transcription modulator of bcl-2 family proteins has been recently studied. Where immunohistochemistry results were evaluated, an inverse relationship between bcl-2 and p53 protein expression was reported (Tomaszewska *et al*, 1999; Ioachim *et al*, 2000; Chan *et al*, 2000). We, therefore, undertook a detailed comparison of p53 and bcl-2 proteins expression in a selection of the 48 cases examined in this study. Evaluating the data as continuous variables, we confirmed a strong inverse relationship between expression of bcl-2 and p53 immunopositivity. Over expression of bcl-2 protein was exclusively detected in normal oesophageal tissues, while, p53 was absent. In dysplastic Barrett's epithelium, interestingly, the expression pattern of bcl-2 appeared to change in a direction opposite to that of p53 expression. The percentage of bcl-2 immunopositive tumour cells was significantly lower as compared to the p53 immunopositive tumour cells. Furthermore, induction chemoradiotherapy did not alter the association of p53 to bcl-2 in tumours which failed to responsed to therapy. These observations suggest that alteration (over expression or mutations) in p53 is somehow related to regulation of bcl-2 gene expression. However, in some other types of cells, p53 has the opposite effect on bcl-2 expression (Liukkonen *et al*; 1997; Pettaway, 1998), implying a great deal of tissue specificity in the interaction of p53 with bcl-2. Furthermore, it has been shown that wild-type p53 can bind directly to the bax gene promoter and induce its transcriptional activation (Miyashita *et al*, 1995). However, in our samples the expression of p53 and bax was reciprocal throughout the neoplastic development and in tumours, although not in normal tissues, and no correlation between the percentages of bax- and p53-immunopositive tumour cells was observed. Similar observations between p53 and bcl-x have been noted, although their interactions in normal and neoplastic tissues have yet to be defined. Overall, our results are in accord with previous reports, and further support the concept that loss of p53 function, together with loss of the apoptotic regularity function of bcl-2 proteins results in the

accumulation of genetic defects giving rise to uncontrolled cell growth and progressive disease. It can also be expected that bcl-2 family members may or may not have a straightforward impact on clinical outcomes in human cancers as their interactions in the regulation of apoptosis are complex.

In the management of patients with cancer, it would clearly be useful to identify prognostic biofactors in order to optimise therapeutic strategies, to facilitate identification of the most clinically effective drugs and also to obviate the requirement for costly and potentially toxic therapy in non-responders. Anti- or pro- apoptotic genes are purported to influence tumours' response rate to cancer therapies and overall outcome. Studies indicated that the effects of bcl-2 expression on the sensitivity of epithelial cells to different therapies, vary depending on the particular type of tumour. Expression of bcl-2 is associated with resistance to hormone therapy and recurrence in prostate (Keshgegian *et al*, 1998; Paterson *et al*, 1999) and breast carcinomas (Kenny *et al*, 2001), whereas; such expression is associated with improved prognosis in lung cancer (Krajewski *et al*, 1999). Bcl-x_L expression was found to be an independent negative prognostic factor for response to chemotherapy in patients with acute myeloid leukaemia (Schaich *et al*, 2001) and in the resistance of oropharyngeal cancers to ionising radiation (Aebersold *et al*, 2001). Expression of bcl-x protein was also shown to be responsible for modulating resistance to chemotherapy-induced apoptosis in ovarian cancer (Liu *et al*, 1998). In contrast, expression of bax protein, for example, was demonstrated to increase the sensitivity and responsiveness of maxillary sinus squamous cell carcinoma (Bandoh *et al*, 2002) and breast cancer (Kymionis *et al*, 2001) patients to chemotherapy. Patients' response to low-dose 5-fluorouracil and cisplatin treatment in gastric cancer was associated with the induction of apoptotsis and overexpression of bax protein, resulting in good prognosis (Kim *et al*, 2000). The realisation that bcl-2 can retard entry into the cell cycle also has implications for anti-cancer therapy (David *et al*, 1997). Since most chemotherapeutic drugs target dividing cells, malignant cells expressing bcl-2 are 'doubly' protected: they are refractory to apoptosis and more likely to be quiescent. Therefore, any agents that could overcome the inhibitory effects of bcl-2 on cell cycle entry should prove useful adjuncts to currently available chemotherapeutic drugs. Antisense oligonucleotide also deserves attention as a therapeutic compound in many malignancies in which antiapoptotic bcl-2 and or bcl-x_L is overexpressed (Miyake *et al*, 1999, Zangemeister *et al*, 2000).

The superiority of multimodality therapy (chemotherapy and radiation therapy) compared with surgery alone in the treatment of oesophageal adenocrcinoma has been established in randomised and controlled clinical trials (Walsh *et al*, 1996). The present study assessed the relevance of bcl-2, bax and bcl-x gene products to such parameters. We established that the combination of chemotherapy and radiation therapy influenced tumours' expression of bax and bcl-x proteins but not bcl-2. All pre therapeutic bax and 80% bcl-x negative tumours demonstrated marked immunopositivity at resection. In addition, there was a significant increase in the expression level of bax (4cases) and bcl-x (5 cases) positive tumours compared to pre-treatment values. Despite the observed immunotype shifting of tumours bax and bcl-x expression during the treatment we were unable to determine the pathological type of response to preoperative chemoradiotherapy according to the levels of expression of the bcl-2 family members analysed in this study. More importantly, we could not establish the exact tendency of the tumour cells toward different apoptotic stimuli and therapeutic agents.

A correlation between bcl-2 expression and a favourable outcome has been described for breast cancer (Sjostrom *et al*, 2002), non small cell lung cancer (Cox *et al*, 2001), colorectal cancer (Manne *et al*, 2000) and oesophageal squamous cell carcinoma (Ohbu *et al*, 1997). This may reflect a reduction in lymph node metastases in tumours expressing bcl-2. A further possible interpretation of this is that when a tumour progresses, bcl-2 expression decreases and carcinoma cells transform to a more malignant phenotype, capable of metastasis to lymph nodes. In this study, bcl-2 expression was significantly associated with improved survival and with well/moderate differentiation compared to poorly differentiated tumours. Furthermore, there was a trend towards decreased earlier T stage (P = 0.08), improved response rate to chemoràdiotherapy (MPR vs. NPR; P = 0.07) and to a lesser extent toward node-negativity (P = 0.1) in bcl-2-positive cases. The latter observations may suggest that bcl-2 positive tumours could be more sensitive to chemoradiotherapy than are bcl-2 negative tumours. With respect to bax and bcl-x, analysis of the data indicated that neither were factors associated with the above mentioned histopathological parameters, including tumour differentiation or depth of invasion or lymph node metastasis. The latter observation was due to the elevated homogenous expression of bax and bcl-x in the majority of pre-treatment and almost all resected tumours, while, bcl-2 was less frequent with heterogeneous expression.

4.6 SUMMARY OF FINDINGS

In conclusion, this study indicates that bcl-2, bax and bcl-x gene products are widely expressed in normal oesophageal tissue. A significant reduction of bcl-2 expression is associated with the progression of normal oesophageal mucosa to Barrett's and adenocarcinoma. Lack of bcl-2 expression is associated with poorly differentiated tumours. Preoperative chemoradiotherapy induces expression of bax and bcl-x gene products. Neither bcl-2, bax nor bcl-x protein expression influences or predicts the response or resistance of oesophageal adenocarcinoma to neuadjuvant chemoradiotherapy.

CHAPTER 5

SPONTANEOUS AND INDUCIBLE APOPTOSIS IN OESOPHAGEAL ADENOCARCINOMA

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5.1 INTRODUCTION

5.1.1 Introduction to Apoptosis

Apoptosis, or programmed cell death, refers to specific morphological and genetic changes that occur during the orderly self-destruction of cells. Apoptosis was first defined genetically in invertebrates (nematodes), but has also been found to be critical for the development of vertebrates (Kerr *et al*, 1972; Wyllie, 1992; Jacobson *et al*, 1997). Apoptosis is an ancient Greek word used to describe the "falling off" of petals from flowers or leaves from trees. It was first coined by John Kerr and his colleagues in 1972 to describe physiologically occurring programmed cell death. Apoptosis plays an important role in embryonic development and tissue homeostasis. Examples are the morphogenesis of limbs from limb buds, the development of the nervous system and the maturation of the haematopoietic and lymphatic systems (Hammar *et al*, 1971; Kerr *et al*, 1972; white, 1996). Hormonal regulation of cells and tissues is also partly executed through apoptosis (Hockenberry *et al*, 1991). Deregulation of programmed cell death can lead to pathological changes which result in either degenerative disease (excessive apoptosis) or cancer (impaired apoptosis) (Tsujimoto *et al*, 1984; Wyllie, 1992; Cotman *et al*, 1995). Apoptosis also contributes to the maintenance of the immune system by reducing the number of potential self-reactive lymphocytes in the thymus and peripheral lymphoid organs. Failure of apoptosis in this situation effectively results in the accumulation of auto-reactive lymphocytes which can lead to autoimmune disease (Nagata, 1997).

The process of programmed cell death often occurs in response to specific ligands or stress stimuli that either engage certain "death receptors" (e.g. Fas, TNF receptor) or threaten the integrity of the genome (e.g. irradiation, chemotherapeutic agents). A lack of survival signals (e.g. growth factors) can also trigger the apoptosis of a cell. Regulation of apoptosis by cancer associated genes such as c-myc, bcl-2 and p53 has also been elucidated and can be classified into two broad categories: (1) genes that primarily suppress apoptosis, and (2) genes that promote apoptosis. Apoptosis in mammalian cells is controlled by equilibrium between suppressor and promoter genes.

Apoptosis usually affects individual cells rather than cell groups or a whole tissue or organ compartment, as is often characteristic of necrosis. Once initiated, apoptosis proceeds rapidly. Cells undergoing apoptosis may disappear completely within several (1-12) hours (Gavrieli *et al*, 1992; Schwartzman *et al*, 1993, Potten, 1996). Of all the aspects of apoptosis, the defining characteristic is a complete change in the cellular morphology. During apoptosis the cell undergoes shrinkage, chromatin condensation, membrane blebbing, nuclear condensation and then segmentation, and division into apoptotic bodies which are then phagocytosed (Kerr *et al*, 1991; Majno *et al*, 1995; Darzynkiewicz *et al*, 1997). Apoptotic bodies are ingested by nearby cells, which can be monocytes or machrophages, but also epithelial cells, vascular endothelium or tumour cells (Bosman *et al*, 1996). Exposure and disruption of the phosphatidylserine to the outer leaflet of the cytoplasmic membrane early in apoptosis, is thought to be important for macrophage recognition of cells undergoing apoptosis. The execution of apoptosis minimises the leakage of cellular constituents from dying cells. For example, proteases could damage adjacent cells or stimulate an inflammatory response. This cardinal feature of apoptosis distinguishes it from necrosis, which usually results from trauma that causes injured cells to swell and lyse, releasing the cytoplasmic material that stimulates an inflammatory response (Wyllie *et al*, 1980; Steller, 1995). The morphological and biochemical differences between apoptosis and induced cell necrosis are listed in (Table 5.1).

Although the characteristics that initially permitted recognition of apoptosis were morphological, it is now clear that there is a highly conserved biochemistry underlying and responsible for the process. One of the key biochemical hallmarks of apoptosis is the fragmentation of the cell's DNA into nucleosome fragments (Wyllie, 1980; Arends *et al*, 1990). The occurrence of DNA fragmentation during apoptosis is due to activation of specific cations (Ca^{2+}/Mg^{2+}) dependent endonucleases which initially cleaves the DNA into large fragments of 300-50 kilobases and subsequently into smaller fragments that are multimers of 180-200 bases. These multimers appear as the familiar "DNA ladder" seen on standard agarose electrophoresis gels (Arends *et al*, 1990; Walker *et al*, 1994; Hale *et al*, 1996). It has been reported that the endonucleolytic activities responsible for the initial fragmentation of the DNA are distinct from those causing subsequent internucleosomal DNA cleavage. Electron microscopy showed that the degree of cleavage at 300-50 kb is sufficient to cause the chromatin to undergo

Table 5.1. Types of cell death: differential characteristics.

APOPTOSIS	NECROSIS
Morphological Criteria	
Deletion of single cells	Death of cell groups
Membrane blebbing, but not loss of integrity	Loss of membrane integrity
Cells shrink and formation of apoptotic bodies	Cells swell and lyse
No inflammatory response	Significant inflammatory response
Phagocytosis by adjacent normal cells, and some macrophages	Phagocytosis by macrophages
Lysosomes intact	Lysosomal leakage
Compaction of chromatin into uniformly dense masses	Clumpy, ill-defined aggregation of chromatin
Biochemical Criteria	
Induced by physiological stimuli disturbances	Evoked by nonphysiological disturbances
Tightly regulated process with synthetic and activation steps	Loss of regulation of ion homeostasis
Require energy	No requirement of energy
Requires macromolecular synthesis	No requirements for protein or nucleic acid synthesis
De novo gene transcription	No new gene transcription
Non-random oligonucleosomal length fragmentation of DNA	Random digestion of DNA

condensation and for apoptosis to proceed (Walker *et al*, 1994). In rare instances, studies indicated that internucleosomal DNA cleavage may not be required for apoptosis or is incomplete in induced apoptosis (Martikainen *et al*, 1990; Levin *et al*, 1993; Darzynkiewicz *et al*, 1997). Furthermore, some studies support the suggestion that apoptosis and the initial phases of the cell cycle have common mechanisms or may even be coupled (Rubin *et al*, 1994). Some investigators have even suggested that apoptosis might be an aberrant mitosis. Evidence in favour of this concept stems from the finding that apoptosis associated genes, such as p53, c-myc, Rb1 etc, also play a role in the regulation of the cell cycle (Debbas *et al*, 1993). It has been established, however, that apoptosis can occur without activation of the cell cycle (King *et al*, 1995).

5.1.2 Molecular Regulation of Apoptosis

During recent years, much has been learnt about the mechanisms which control apoptosis. Apoptosis is a tissue-specific process and for a given stimulus there are many pathways that lead to apoptosis. The significance of apoptotic regulatory function of the p53 as an example of a tumour suppresser gene and the role of bcl-2 family as a proto-oncogene has been described earlier (Chapters 3 and 4). In this section we briefly reference the role of some other genes and factors that modulate apoptosis such as A) genes responding to DNA damage, B) specific proteases (caspases), C) proteolytic enzymes (granzymes) and D) death signals received at the cell membrane (TNF family).

The process of programmed cell death often occurs in response to DNA strand breaks that threaten the integrity of the genome. Stimuli for apoptosis that originated in DNA injury can be generated in different parts of the cell. Thus, DNA damage is recognized by a variety of proteins such as ATM, DNA-PK, MSH and p53 which can signal to apoptosis as well as to the repair pathway (Wang, 1998). The involvment of c-myc in sensitising cells to apoptosis following exposure to DNA-damaging agents has also been reported (Evan *et al*, 1992, Supino *et al*, 2001). In addition, growth arrest induced by DNA damage in mammalian cells requires the function of the retinoblastoma (Rb) tumor suppressor protein. The antagonism between Rb and c-Abl/p73 may modulate the function of p53 to direct the choice between growth arrest and apoptosis in DNA damaged cells (Wang *et al*, 2001). Poly-ADP-Ribose-Polymerase (PARP) is another target of apoptosis. PARP is a DNA repair enzyme whose expression is triggered by DNA-strand breaks. In cells undergoing apoptosis, PARP is cleaved from a 116 kD

peptide into 24 kD and 89 kD polypeptides. It appears that cleavage of PARP facilitates the degradation of cellular DNA which is a hallmark of apoptosis (Murcia *et al*, 1994; Lippke *et al*, 1996).

At the molecular level, apoptosis is also regulated by the activation of the aspartate-specific cysteine protease (caspase) cascade (Thornberry *et al*, 1998). Caspases are present in healthy cells as inactive pro-enzymes. During apoptosis most caspases are activated by proteolytic cleavage (Slee *et al*, 1999). Caspases are differentiated into two categories based on whether they are at the initiating end of the apoptotic process (cell death signalling events) (i.e. caspase 2, 8, 9 and 10), or at the effector end (cell disassembly) (i.e. caspase 3, 6 and 7). Caspase activity results in the cleavage of cytoskeletal proteins, disruption of the nuclear membrane, disruption of cell-cell contact, and the freeing of the DNA nuclease (CAD, caspase-activated deoxyribonuclease) from its associated protein inhibitor (ICAD) to allow DNA fragmentation (Enari *et al*, 1998). The restricted proteolysis results not in cellular lysis but in membrane-bound sealed apoptotic bodies. These irreversible proteolytic events are responsible for the morphological changes characteristic of apoptotic cells and the DNA ladder pattern that is the signature of apoptosis (Israels *et al*, 1999). The caspases may, in addition, act on mitochondrial membranes which further release cytochrome c and some intramitochondrial procaspases. A family of caspase inhibitors, IAPs (inhibitors of apoptosis), which are over expressed in many malignant cells selectively inhibits effector caspases and blocks the apoptotic process. The IAP family also inhibits apoptosis through non-caspase mechanisms; by modulation of transcription factors and involvement in cell-cycle control (Israels *et al*, 1999). In addition, apoptosis inducing factor (AIF), which is released from the mitochondria during the apoptosis can activate proenzymatic caspases (Tsujimoto, 1998; Susin *et al*, 1999). Some of the targets of activated caspases have already been characterised. They include poly (ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase (DNA-PKcs) both of which are involved in sensing DNA-damage and repair (Alnemri *et al*, 1996; Nicholson *et al*, 1997; Cohen, 1997).

In the removal of pathogen-infected cells from our body, a secretory apoptotic pathway is thought to be operative. Granzymes are proteins contained within the cytoplasmic secretory granules of cytotoxic lymphocytes (CTLs) and natural killer (NK) cells

(Shresta *et al*, 1995). Granzyme B, also known as CTL proteinase-1, fragmentin-2, and RNKP-1, is a serine protease which is released by cytotoxic T-cells as part of the cytolytic granules, upon a specific interaction with a target cell. Delivery of granzyme B to the cytoplasm of a target cell, which is facilitated by the pore-forming protein perforin, is sufficient for the induction of apoptosis, a hallmark of cytotoxic lymphocytes killing (Trapani *et al*, 1996; Pham *et al*, 1999). Interestingly, Granzyme B has the same substrate specificity as the caspases. It is speculated that granzyme B induces apoptosis either by mimicking the action of caspases or by converting caspases from their inactive to the active form (Quan *et al*, 1996; Trapani *et al*, 1998). In addition, it has been shown that granzyme B is capable of inducing cytochrome c release from the mitochondria in a caspase independent way (Heibein *et al*, 1999). A second protease (Granzyme A) also acts synergistically with perforin in the apoptotic process, apparently through a caspase-independent pathway (Israels *et al*, 1999).

Apoptosis can also be induced by activation of death receptors on the cell membrane by the corresponding death ligands (Ashkenazi *et al*, 1998). Death receptors are a subgroup of the tumour necrosis factor receptor (TNFR) family and are characterized by the presence of extracellular cysteine-rich domains (Smith *et al*, 1994; Gruss *et al*, 1995). Intracellularly, however, these recepters contain an area of weak homology which is required to transduce a cell death signal termed the "death domain" (DD) (Tartaglia *et al*, 1993; Nagata *et al*, 1997). The "death receptors" of the TNFR family include Fas (CD95), TNFR-1, DR3, and the TNF-related apoptosis-inducing ligand (TRAIL)/Apo-2L receptors (TRAIL-R1/DR4, TRAIL-R2/DR5) (Peter *et al*, 1997; Ashkenazi *et al*, 1998). When these death receptors are bound by ligand (Fas-ligand (FasL), TNF or lymphotoxin, the ligand for DR3, or TRAIL/Apo-2L, respectively), apoptosis can occur as a consequence. The TNF-R family is expressed in a wide range of cell types, for example, Fas is expressed on several different cell types mainly in the thymus, activated T and B lymphocytes, macrophages, liver, spleen, lung, brain, intestines, heart, etc. Its expression can be augmented by cytokines, such as interferon-γ and TNF, and also by lymphocyte activation (Zimmermann *et al*, 2001). In contrast, expression of Fas-L is more tightly regulated, often only inducible under specific conditions. Fas-L expression is restricted to immune cells, including T and B lymphocytes, macrophages, and natural killer cells, and to non-immune sites, such as the, kidney, testis, lung, intestine, and the eye (Suda *et al*, 1993; Nagata *et al*, 1995). Ligation of death receptors causes the rapid

formation of a death-inducing signalling complex, through the receptors death domain. This domain is responsible for coupling the death receptor either to a cascade of caspases, leading to induction of apoptosis, or to the activation of kinase signalling pathways, resulting in gene expression through nuclear factor-$_{K}$B (NF-$_{K}$B) and or activator protein-1 (AP-1) (Zimmermann *et al*, 2001). Activation of apoptosis via Fas and Fas ligands pathway plays a significant role in the removal of activated T cells at the end of an immune response, deletion of virus-infected target cells, killing of tumour cells, and the destruction of cells in numerous pathological states (Israels *et al*, 1999; Zimmermann *et al*, 2001). In some cell types, tumour necrosis factor (TNF) also induces apoptosis through TNFR. The majority of TNF functions are primarily mediated through TNFR-1, while signalling through TNFR-2 occurs less extensively and is confined to cells of the immune system (Hsu *et al*, 1995). The most important functions of TNFR-1 are induction of apoptosis and NF-$_{K}$B activation (Tartaglia *et al*, 1992). The signalling pathways are triggered by the binding of membrane bound or soluble TNF which leads to trimerization of the receptor. To date, two pathways are particularly well understood. In the first pathway, TNFR1 first associates with TNFR-associated death domain (TRADD) and Fas-associated death domain (FADD) by homotypic interactions between the death domains. In a second step, the proenzymatic form of caspase 8 binds to the complex by interaction between the death effector domains (DED) of the FADD and the caspase 8 molecules. This binding results in the activation of caspase 8 which then triggers the caspase cascade. The second pathway involves the accessory proteins TRADD and receptor interacting protein (RIP). Binding of these proteins to oligomerized TNFR-1 can either lead to apoptosis by induction of the caspase cascade or formation of the second messenger ceramide or to survival by NF-$_{K}$B activation and the subsequent expression of survival factors (Tartaglia *et al*, 1993; Nagata, 1997). Apoptosis signalling by death receptor-3 (DR3) shows close sequence similarity to TNFR1 (Chinnaiyan *et al*, 1996, Kiston *et al*, 1996). Upon overexpression, DR3 triggers responses that resemble those of TNFR1, namely NF-$_{K}$B activation and apoptosis. DR3 binds to Apo3-L, which is related most closely to TNF (Marsters *et al*, 1998). Apo3L activates NF-$_{K}$B through TRADD, TRAF2, RIP, and NIK and triggers apoptosis through TRADD and FADD. There are notable differences, however, in the expression of these ligands and receptors. TNF expression occurs mainly in activated macrophages and lymphocytes (Tartaglia *et al*, 1993), whereas Apo3L is expressed constitutively in many tissues (Chicheportiche *et al*, 1997; Marsters

et al, 1998). Conversely, TNFR1 is expressed ubiquitously (Tartaglia *et al*, 1993), whereas DR3 transcripts are present mainly in spleen, thymus, and peripheral blood and are induced by activation in T cells (Marsters *et al*, 1996). Hence, despite overlapping signalling mechanisms, Apo3L-DR3 and TNF-TNFR1 interactions probably have distinct biological roles. TNF-related apoptosis-inducing ligand (TRAIL) receptor signalling for apoptosis appears to be most similar to FasL (Wiley *et al*, 1995, Pitti *et al*, 1996). TRAIL-R1 and TRAIL-R2 contain a cytoplasmic domain which is similar to the death domains of Fas and TNFR1 while, TRAIL-R3 lacks this domain. Therefore, upon binding of these receptors to their ligand, TRAIL-R1 and TRAIL-R2 induce apoptosis whereas TRAIL-R3 can not (Degli-Esposti *et al*, 1997; Walczak *et al*, 1997). Furthermore, it is speculated that TRAIL-R3 counteracts the activity of TRAIL-R1 and -R2 by competing with these molecules for binding to the ligand. The apoptotic signal transmitted by TRAIL-R1 and R-2 appears to be via a caspase dependent pathway (Walczak *et al*, 1997). However, current evidence suggests that TRAIL-R1 does not bind to the known accessory proteins FADD, RAIDD and RIP whereas TRAIL-R2 seems to transmit its signal by recruiting FADD and caspase-8 (Pan *et al*, 1997).

Apoptosis can also be modulated by certain hormones like glucocorticoids. This pathway appears to be particularly significant in the case of thymocytes and can explain at least in part, the immunosuppressive effect of the glucocorticoids. How these hormones induce apoptosis is not known precisely, but it is assumed that it is the gene regulatory activity of the glucocorticoid receptor complex which is responsible for this effect (Schwartzman *et al*, 1994; Kiefer *et al*, 1995).

5.1.3 Apoptosis and Disease

Deregulation of apoptosis is associated with several diseases, including cancer, AIDS, haematopoietic and neurodegenerative diseases. In the case of cancer, dysregulation of growth is explained largely in terms of increased cell proliferation. It has become clear now, that decreased cell death may also contribute to the pathological cell accumulation in a neoplasm. Enhancing cell survival through inhibition of apoptosis may be one of the mechanisms through which tumours arise. Intensive research into this effect was triggered by the discovery of the bcl-2 gene, which is involved in the t (14;18) translocation, characteristic for follicular lymphoma (Tsujimoto *et al*, 1984). Due to this translocation, the bcl-2 gene is situated adjacent to the Ig heavy chain locus on the

chromosome 14. Driven by the Ig heavy chain promoters, the bcl-2 gene is transcribed at a much higher level. Overexpression of bcl-2 is thought to block apoptosis and thus to contribute to the development of this neoplasm. Inhibition of the proapoptotic function of certain genes has also been regarded as a factor in the development and progression of cancer (Degenhardt *et al.* 2002). Increased apoptosis is also known to be a critical event in tumuorigenesis and a high degree of apoptosis in premalignant lesions is considered to reflect an ardent effort to eliminate genetically damaged cells. This, in fact, has been suggested in studies on apoptosis in gastric premalignant lesions and in dysplasias of the oral cavity. The frequency of apoptosis may be even higher in dysplastic lesions than in the corresponding invasive carcinomas (Birchall *et al*, 1995; shida *et al*, 1996, Hawkins *et al*, 1997). Increased apoptosis has also been reported in association with the progression of the neoplastic lesions of gallbladder epithelium (Turunen *et al*, 2000).

A critical aspect of AIDS pathogenesis that remains unclear is the mechanism by which human immunodeficiency virus type 1 (HIV-1) induces death in CD4 positive T lymphocytes (Meyaard *et al*, 1992, Ho *et al*, 1995). Multiple mechanisms, including viral and immunological processes, have been proposed to explain the death and dysfunction of CD4 positive T cells. Among the virus-mediated mechanisms proposed are toxicity caused by accumulation of unintegrated viral DNA (Shaw *et al*, 1984), membrane permeability changes resulting from viral particles budding at the surface of the infected cell (Fauci, 1988), and terminal differentiation causing a shortened life span of the CD4 positive lymphocytes (Zagury *et al*, 1986). Among the immunological mechanisms that may contribute to the death of CD4 positive lymphocytes during HIV-1 infection are killing by specific cytotoxic T-lymphocytes (CTL) and signalling through the CD molecules leading to apoptosis (Ameisen *et al*, 1991; Finkel *et al*, 1994). In addition, infection with HIV-1 in vitro has been shown to enhance the expression of Fas ligands. Thus, apoptosis may be caused by inappropriate ligation of overexpressed Fas-L with its receptor, Fas, on the surface of CD4 positive lymphocytes (Westendorp *et al*, 1995; Badley *et al*, 1996).

Proliferation and differentiation of haematopoietic precursor cells is regulated through various growth factors, such as stem cell factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin and thrombopoietin. It has been shown

that these factors are not only required for proliferation and maturation of these cell lineages, but are also required for adequate cell survival. In the absence of these growth factors, terminally differentiated cells undergo apoptosis. Thus, chronic hematological disorder, characterized by insufficient production of blood cells such as anemia and myelodysplastic syndromes, might be partly caused by activation of cell death genes through stromal factors from the bone marrow or deficiencies in haematopoietic growth factors essential for cell survival (Yoshida, 1993).

Defective apoptotic death pathways are shown to be etiologically responsible for the neurodegenerative diseases (Solary *et al*, 1996; Honig *et al*, 2000). Acute disorders, occurring over minutes to hours, such as brain trauma, infarction, haemorrhage, or infection, prominently involve cell death, much of which is by necrosis (Honig *et al*, 2000). Chronic disorders, with relatively slow central nervous system degeneration, may occur over years or decades, but may also involve programmed cell losses. Such disorders include motor neuron diseases such as amyotrophic lateral sclerosis (ALS), cerebral dementing disorders such as Alzheimer's disease and frontotemporal dementia, and a variety of degenerative movement disorders including Parkinson's disease, Huntington's disease, and the inherited ataxias (Honig *et al*, 2000). Investigators have indicated that several cysteine proteases (caspase) have been implicated in regulating neuronal cell death in response to amyloid beta (A beta) exposure in vitro, in animal models of neurodegenerative diseases, suggesting that caspase-dependent neuronal death contributes to Alzheimers disease and neuron loss (Roth, 2001). In addition, oxidative stress, excitatory toxicity, calcium toxicity and survival factor deficiencies also have been implicated as responsible for neuronal cell death (Bosman *et al*, 1996; Honig *et al*, 2000).

An additional example of a degenerative disease that is associated with defective apoptotic pathways is retinal degeneration, which includes retinitis pigmentosa, retinal detachment, and age-related macular degeneration. These diseases are characterized by the death of retinal cells by apoptosis which may lead to blindness (Yoshimura, 2001; Chader, 2002). Using retinal ischemia-reperfusion as a modal to study retinal neuronal apoptosis, DNA microarray analysis revealed that approximately 200 messengers were found to be up- or down- regulated (Yoshimura *et al*, 2001). These genes were divided into four groups: (1) transcription factor genes, (2) cell cycle-related genes, (3) reactive

oxygen scavenger genes and (4) molecular chaperone genes. The possible roles of these genes in neuronal apoptosis following retinal ischemia-reperfusion injury were speculated upon. For example, reactive oxygen species produced by reperfusion was found to generate lipid peroxides and induced up-regulation of a transcription factor, c-Jun, that further induced aberrant expression of cell cycle-related genes (cyclin D1) resulting in cell death by a G1 arrest. Furthermore, the possibility that inhibition of a "death message" could induce necrosis rather than apoptosis was also demonstrated. Earlier studies (Chang *et al*, 1993, Wong, 1994) had already shown that retinal degeneration occurs in association with mutation in one of the three specific photoreceptor genes; rhodopsin, peripherin and cGMP phosphodiesterase. Mutation in these genes induced apoptosis in photoreceptor cells.

It seems likely that pro- and anti-apoptotic factors determine either susceptibility or resistance to apoptosis and, consequently, play a crucial role in the evolution, propagation, and chronicity of cancerous, autoimmune and degenerative conditions. Thus, precise identification of the distinct errors in the complex apoptotic machinery holds great promise for elucidating the pathogenesis of various important diseases and for devising more specific and effective treatments. It may also be possible to develop protective therapies based on alleviating the process of cell death. This is particularly likely in degenerative disorders if the cell loss is through apoptosis, a programmed process for which the molecular cascades are increasingly understood.

5.2 OBJECTIVES OF STUDY

Therapeutic agents, primarily drugs and radiation, are typically potent inducers of cell cycle arrest and programmed cell death. Genetic and epigenetic alterations that are important in tumourigenesis often disable these pathways and therefore, affect the way that cancer cells respond to therapeutic insult. These alterations often differ between cancers and sometimes between tumours of the same tissue type. This study was, therefore, designed to assess the significance of apoptosis in relation to tumours' responsiveness to neuadjuvant chemoradiotherapy, clinicopathological parameters, cell proliferation activity, and the expression of apoptotic and cell-cycle related proteins in patients with primary oesophageal adenocarcinoma.

5.3 MATERIALS AND METHODS

5.3.1 Patient Cohort

Archival formalin-fixed paraffin-embedded tissue samples from 42 patients (32 male, 10 female) with primary oesophageal adenocarcinoma were included in this study. Patients' ages ranged from 48 to 77 (64.3 ± 7.6 mean ± SD, 66 median). All patients had oesophagogastro-endoscopy and histological confirmation of diagnosis prior to treatment with chemotherapy (cisplatin and 5-fluorouracil) and radiotherapy (CRX) followed by oesophagectomy. Surgery was undertaken at a median of 4 (range 3-6) weeks following neoadjuvant therapy.

5.3.2 Histopathological Examination

Diagnostic biopsy and resection specimens were fixed in 10% buffered formalin, routinely processed and embedded in paraffin. Haematoxylin and eosin slides were reviewed and sequential 4μm sections were used for in-situ end labelling and immunohistochemistry. Tumours were staged according to the TNM system (chapter 2 section 2). Patients (n = 9) were defined as complete pathological responders (CPR) to CRX. Twenty-nine patients were defined as non-responders (NPR) to CRX. A third intermediate group (n = 4) was defined as having a major pathological response (MPR) to CRX. For the purpose of statistical analysis CPR and MPR data are combined to one group (n = 13) versus NPR (n = 29).

5.3.3 Assessment of Apoptosis

In situ terminal deoxynucleotide transferase (TdT) method was used for identification of apoptotic cells and bodies in paraffin tissue sections using Apoptag®peroxidase in situ apoptosis detection kit (S7100), Intergen Company). The staining procedure is described in detail in (chapter 2 section 14). Briefly, 4μm sections were deparaffinised, rehydrated and placed in 10mM citrate buffer pH at 6.0 and gently boiled for 10 minutes in a microwave oven. Sections were incubated with 20ug/ml proteinase K at room temperature for 10 minutes followed by exposure to 3% hydrogen peroxide in PBS to quench endogenous peroxidase activity. The sections were then treated with terminal transferase enzyme and digoxigenin-labelled nucleotides. Apoptotically fragmented DNA that had been labelled with the digoxigenin-nucleotide was then allowed to bind an anti-digoxigen antibody that is conjugated to peroxidase molecules.

The reaction products of peroxidase were visualized with 3'-3' diaminobenzidine (DAB). After the colour reaction, sections were counterstained with methyl green. Unstained rat mammary glands [Apoptag® positive control slides (S7115), Intergen Company] were used as positive tissue controls. Omission of the TdT during the staining procedure provided negative control for reagents. The number of apoptotic cells and bodies in a total of 2000 tumour cells were counted and the percentage positivity recorded as the apoptotic index (AI).

5.3.4 Immunohistochemistry

Expression of p53, bcl-2, bax and bcl-x protein was detected by the avidin-biotin peroxidase method using microwave antigen retrieval (chapter 2 section 4). Immunostaining was carried out on the 4μm sections. Sections were incubated with mouse anti-human bcl-2, rabbit anti-human bax, and rabbit anti-human bcl-x. Biotinylated anti mouse/rabbit IgG was used as a secondary antibody. The reaction product of peroxidase was visualised by 3`-3`diaminobenzidine-tetrachloride as a chromagen that results in brown staining within the positive cells. Sections of colon adenocarcinoma, follicular lymphoma, prostate carcinoma and intestinal type gastric adenocarcinoma were used as positive tissue controls for p53, bcl-2, bax, and bcl-x, respectively. The presence of lymphocytes within the sections served as an internal positive tissue control. Omission of the primary antibody during the staining procedure provided negative control for reagents. In each section up to 2000 cells were counted and the percentage positivity recorded as labelling index (LI). Sections demonstrating weak immunopositivity with LI <5% were considered to be negative for p53 bcl-2, bax or bcl-x.

5.3.5 Statistical Analysis

The significance of association among variables (AI, p53, bcl-2, bax and bcl-x expression) and the clinicopathological factors including tumours differentiation, depth of invasion and lymph node status were determined by Mann-Whitney or Kruskal-Wallis tests. For paired comparison of apoptotic index pre- to post- CRX values, Wilcoxon test was used. Statistical significance was defined as $P < 0.05$.

5.4 RESULTS

5.4.1 Apoptotic Index and Tumours Response to Chemoradiotherapy

Apoptotic cells and bodies were detected among viable tumour cells in all adenocarcinoma cases examined in this study. The occurrence of spontaneous apoptosis in pre-treatment tumours (n = 42) ranged from 0.16 to 1.60 % (0.68 ± 0.32 mean ± SD; 0.63 median). A significant association (P = 0.01) was observed between the apoptotic index and types of response to chemoradiotherapy. Tumours that responded completely to preoperative therapy and those showing a major pathological response had a higher apoptotic index in pre-treatment tumour samples compared to tumours with no evidence of pathological response. At resection; it was not possible to determine the apoptotic indices in residual carcinomas of patients having a major pathological response to CRX (n = 4) due to an insufficient number of residual tumour cells. In residual carcinomas of patients having no pathological response to chemoradiotherapy (n = 29), however, the apoptotic index was significantly increased ($P < 0.0001$) compared to its pre-treatment value (Table 5.2) (Figure 5.1 and 5.2).

5.4.2 Apoptotic Index, P53, bcl-2, Bax and Bcl-x Expression

Prior to chemoradiotherapy, expression of p53, bcl-2, bax and bcl-x protein was detected in 27/42 (64.2%), 9/42 (21.4%), 32/42 (76.1%), 37/42 (88.1%) of tumours, respectively. At resection, Expression of p53 and bcl-2 protein was detected in 2/4 (50%) of tumours having major pathological response to chemoradiotherapy, while, bax and bcl-x expression was detected in 3/4 (75%) tumours, respectively. Tumours which failed to respond to therapy (NPR group) expressed 23/29 (79.3%), 7/29 (24.1%), 29/29 (100%), and 28/29 (96.5%) p53, bcl-2, bax and bcl-x, respectively. There was no significant association between the apoptotic index and tumour expression of p53, bcl-2, bax or bcl-x proteins (Table 5.3).

5.4.3 Apoptotic Index and Clinicopathological Parameters

Both before and after treatment, no significant association was observed between the apoptotic index and patient age and sex. No significant association was observed between the apoptotic index and tumours' differentiation or metastatic potential toward the lymph nodes. At resection, increasing depth of invasion of residual tumours was significantly associated (P = 0.02) with an increasing apoptotic index (Table 5.4).

Table 5.2. Apoptotic index and tumours response to chemoradiotherapy.

Parameters	No. of cases	Apoptotic index Mean ± SD / Median	*P* value
Response to CRX*			
CPR and MPR	13	0.83 ± 0.31 / 0.75	
NPR	29	0.60 ± 0.29 / 0.55	0.01 [A]
Labelling index**			
Pre-CRX	29	0.60 ± 0.29 / 0.55	
Post-CRX	29	1.36 ± 0.47 / 1.40	< 0.0001 [B]

* data represent pre-treatment values ; ** data represent tumours with no evidence of pathological response (NPR) to CRX. Statistical calculation was performed by Mann-Whitney [A] and Wilcoxon [B] test.

Table 5.3. Apoptotic index: correlation to p53, bcl-2, bax and bcl-x expression in oesophageal adenocarcinoma prior to and after chemoradiotherapy.

Parameters	No. of cases	Apoptotic index Mean ± SD / Median	*P* value
Before-CRX			
P53 expression			
Negative tumours	15	0.57 ± 0.34 / 0.52	
Positive tumours	27	0.73 ± 0.29 / 0.65	0.07 [A]
Bcl-2 expression			
Negative tumours	33	0.67 ± 0.32 / 0.64	
Positive tumours	9	0.68 ± 0.32 / 0.56	0.79 [A]
Bax expression			
Negative tumours	10	0.67 ± 0.23 / 0.58	
Positive tumours	32	0.67 ± 0.34 / 0.64	0.81 [A]
Bcl-x expression			
Negative tumours	5	0.60 ± 0.13 / 0.55	
Positive tumours	37	0.68 ± 0.33 / 0.64	0.83 [A]
After-CRX*			
P53 expression			
Negative tumours	6	1.33 ± 0.50 / 1.20	
Positive tumours	23	1.37 ± 0.47 / 1.48	0.78 [A]
Bcl-2 expression			
Negative tumours	22	1.40 ± 0.48 / 1.44	
Positive tumours	7	1.20 ± 0.44 / 1.31	0.62 [A]
Bax expression			
Negative tumours	0	–	
Positive tumours	29	1.36 ± 0.47 / 1.4	> 0.99 [A]
Bcl-x expression			
Negative tumours	1	1.48 ± – / 1.48	
Positive tumours	28	1.35 ± 0.47 / 1.35	0.81 [A]

*data represent tumours with no evidence of pathological response (NPR) to CRX. Statistical calculation was performed by Mann-Whitney test. [A]

Table 5.4. Apoptotic index and the clinicopathological parameters in oesophageal adenocarcinoma prior to and after chemoradiotherapy.

Parameters	No. of cases	Apoptotic index Mean ± SD / Median	*P* value
Pre-CRX			
Sex			
Male	32	0.69 ± 0.33 / 0.66	
Female	10	0.60 ± 0.25 / 0.51	0.28 [A]
Age (year)			
< 66	19	0.75 ± 0.36 / .67	
≥ 66	23	0.60 ± 0.25 / 0.52	0.13 [A]
Tumours differentiation			
Well/moderate	30	0.64 ± 0.29 / 0.57	
Poor	12	0.74 ± 0.36 / 0.71	0.26 [A]
Post-CRX*			
Sex			
Male	23	0.62 ± 0.31 / 0.62	
Female	6	0.53 ± 0.21 / 0.48	0.43 [A]
Age (year)			
< 66	13	0.66 ± 0.35 / 0.64	
≥ 66	16	0.55 ± 0.24 / 0.48	0.21 [A]
Tumours differentiation			
Well/moderate	25	1.35 ± 0.50 / 1.31	
Poor	4	1.40 ± 0.18 / 1.40	0.75 [A]
Tumours extension			
pT1	5	0.9 ± 0.24 / 0.91	
pT2	3	1.29 ± 0.19 / 1.30	
pT3	21	1.48 ± 0.47 / 1.50	0.02 [B]
Lymph node metastasis			
Negative	18	1.39 ± 0.51 / 1.40	
Positive	11	1.31 ± 0.4 / 1.20	0.70 [A]

* data represent NPR group. Statistical calculation was performed by Mann-Whitney[A] and Kruskal-Wallis [B] test.

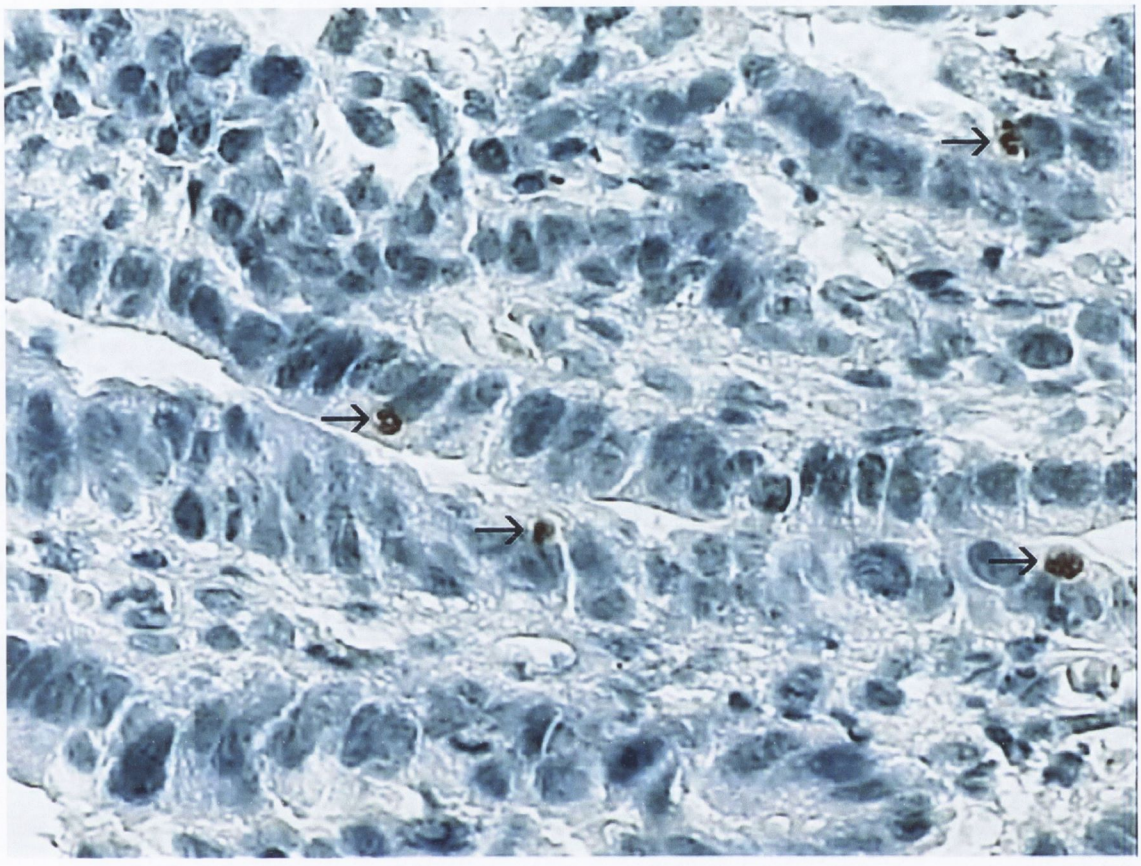

Figure 5.1. In-situ labelling of apoptotic tumour cells of pre-treated oesophageal adenocarcioma. This tumour had a complete pathological response to chemo-radiotherapy. Apoptotic tumour cells are characterised by condensed chromatin and nuclear fragmentation represented by dark brown staining (arrows, magnification X400). The Apoptotic index is significantly higher in chemoradio sensitive compared to resistant tumours.

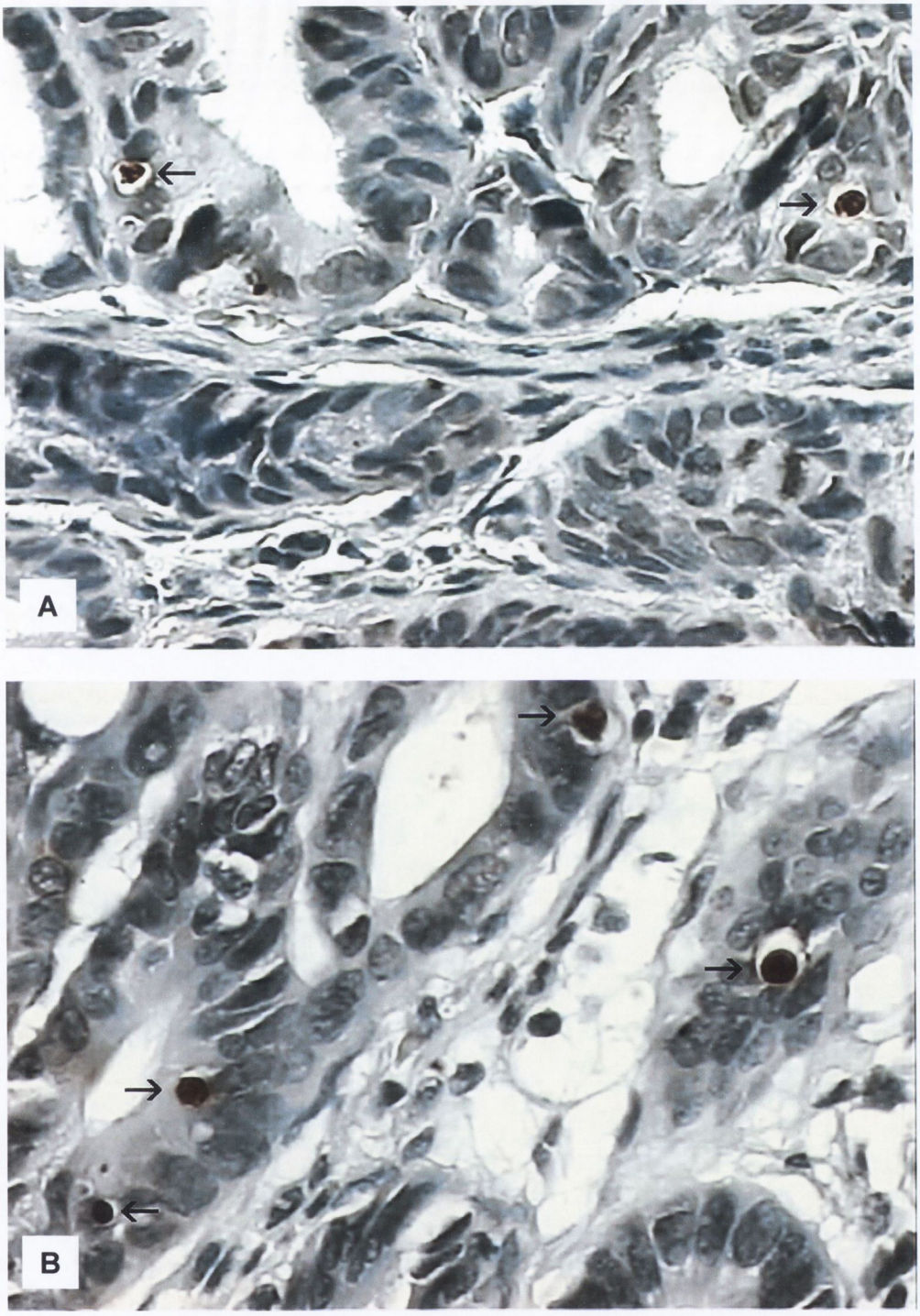

Figure 5.2. In-situ labelling of apoptotic tumour cells of pre-treatment (A) and post-treatment (B) oesophageal adenocarcioma. This tumour showed no pathological response to neoadjuvant therapy. Apoptotic tumour cells are characterised by condensed chromatin and nuclear fragmentation represented by dark brown staining [arrows, magnification (A) X250 and (B) X400]. Apoptotic index is significantly higher in resected tumours following chemoradiotherapy.

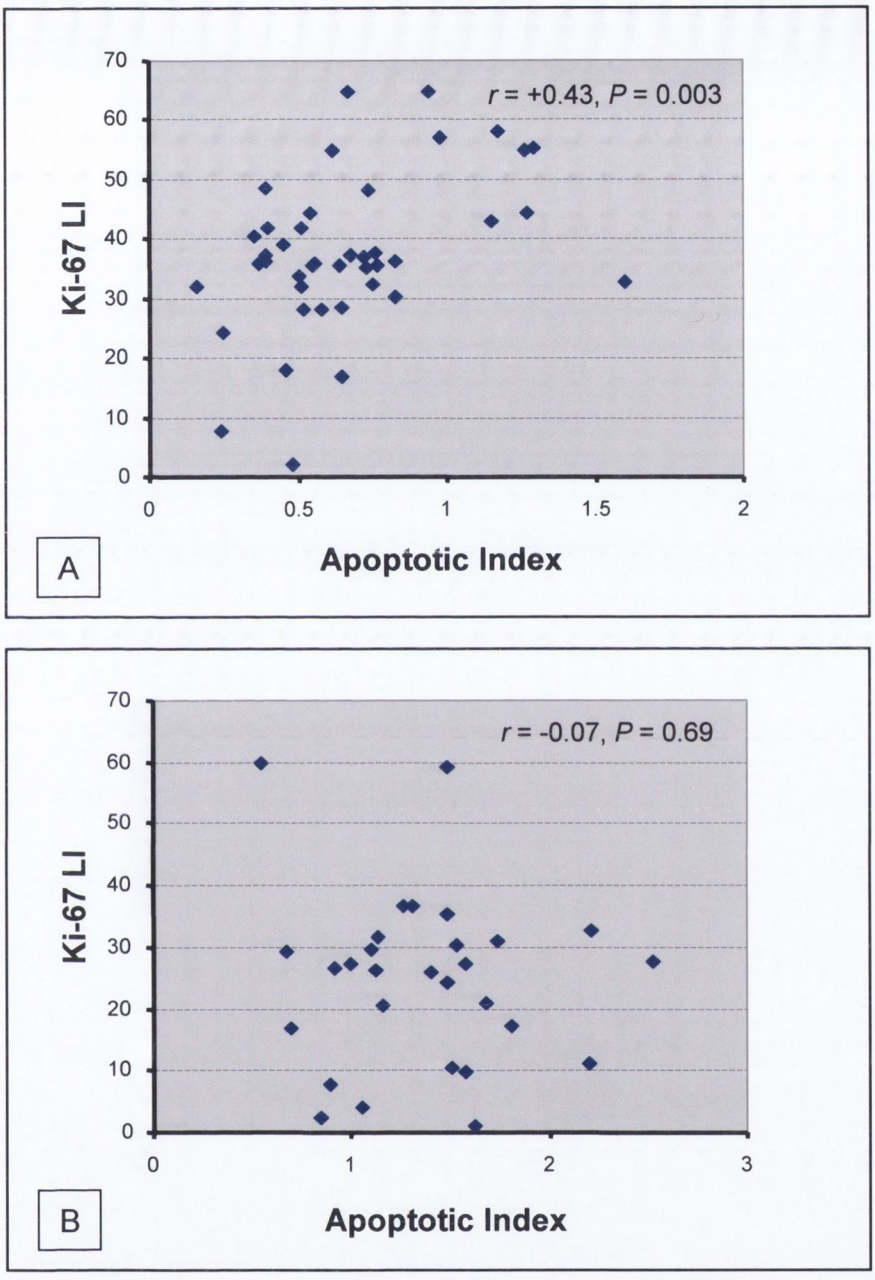

Figure 5.3. Correlation between cell proliferation and apoptosis in oesophageal adenocarcinma before and after chemoradiotherapy. There was a positive correlation between the frequency of spontaneous apoptosis and proliferation in pre-treatment tumours (A). This correlation was not observed in resected tumours due to a significant increase in the apoptotic index which was accompanied by a significant decrease in the proliferation index following chemoradiotherpay (B).

5.5 DISCUSION

Neoadjuvant chemotherapy with and without radiotherapy can result in complete or major pathological responses and improve local control and overall survival of patients with cancer (Walsh *et al*, 1996; Giatromanolaki *et al*, 1999; Kollmannsberger *et al*, 2000). The process of programmed cell death is considered as a mechanism to counter proliferation activity (Katada *et al*, 1997) and is a potential predictor of treatment efficacy (Logsdon *et al*, 1999; Rodel *et al*, 2000; Cameron *et al*, 2000). In the present study, apoptotic tumour cells were visualized by enzymatic labelling of fragmented DNA with a terminal transferase reaction. This methodology facilitates detection of the early and late stages of apoptotic cells in tissue sections. The results from this study indicate that an increased rate of spontaneous apoptosis in oesophageal adenocarcinomas may be a prognostic biomarker for chemoradiotherapy. Tumours with a high apoptotic index were more sensitive to chemotherapy and radiation therapy than tumours with a low apoptotic rate, with more complete and major responders to the regimen in the high apoptosis group. This study further indicates that the combination of chemotherapy and radiation therapy induces apoptosis. This was evident from analysis of resection specimens in patients who failed to respond to neoadjuvant therapy. In these patients, the apoptotic index in 23 of 29 tumours was more than twice its pre-treatment value. The remaining tumours showed less of an increase in the apoptotic index (4 cases) or a slight reduction in the percentage of apoptotic cells (2 cases). The biological and molecular response of tumours to neoadjuvant therapies is, as yet, not well elucidated. Pre-operative chemoradiotherapy may modulate tumor growth via induction of apoptosis and overcome the adverse influence of the biological factors or genes that usually down-regulate apoptosis. Cell death triggered by anticancer agents can also occur as a result of delayed events such as prolonged cell-cycle arrest. A substantial amount of evidence supports the contention that the induction of apoptosis in cancer cells is critical to their successful elimination by therapeutic agents (Reed, 1999; Lowe *et al*, 2000).

In this study, attention has also been given to investigation of the correlation between apoptosis and proliferation (Ki-67 LI) in oesophageal adenocarcinoma. Some studies suggest that apoptosis is a form of cell death resulting from active cell division and proliferation (Arendsn *et al*, 1994). In breast, pancreatic and ovarian cancers, the

apoptotic index shows a strong positive correlation to markers of cell proliferation, such as mitotic index and Ki-67 immunolabelling (Van-Slooten *et al*, 1998; Ellis *et al*, 1998; Sarela *et al*, 2002; Brustmann, 2002). Furthermore, an examination of apoptotic cell death in colorectal carcinomas revealed that the fractions of both apoptotic cells and proliferative cells were significantly higher in metastatic than in primary lesions, implying that the observed increase in the number of apoptotic cells reflects the higher activity of cell division (Tatebe *et al*, 1996; Kim *et al*, 2002). Analysis of our data revealed a similar finding to these studies. A positive correlation (r= +0.43, P=0.003) was observed between the frequency of spontaneous apoptosis and tumour proliferation. This association was not detected in resected tumours which failed to respond to chemotherapy and radiotherapy (r=-0.07, P=0.69) (Figure 5.3). In the case of oesophageal squamous cell carcinoma, an inverse relationship has been observed (Ohbu *et al*, 1995) while, no significant correlation between apoptosis and proliferation index was observed, for example, in hepatocellular (Wu *et al*, 1999) or laryngeal (Hirvikoski *et al*, 1999) carcinoma. Thus, it is apparent that there is a wide variation in the extent of apoptosis not only between different tumours but also within individual tumour types.

It is obvious from the above considerations that apoptosis is increased in some types of cancer, although this is not the case in oesophageal carcinomas. This phenomenon is open to a number of interpretations. Part of the explanation probably lies in the activation of proteins that operate in the cell cycle checkpoints that are also regulators of apoptosis. Examples of such are p53 and Rb proteins, which act on the G1/S checkpoint. Also, overexpression of cyclins, such as cyclins D1, A, and B, can induce apoptosis (Bortner *et al*, 1995; Sofer *et al*, 1996). Another link relates to the fact that apoptosis may be initiated in any phase of the cell cycle and the majority of cells undergo apoptosis primarily in the G1 phase of cycling cells (Thomaidou *et al*, 1997). Increased spontaneous apoptosis in tumour cells is probably associated with the loss of matrix attachment or cell-cell contacts (McGill *et al*, 1996). This could be due, for instance, to loss of expression of cell adhesion molecules such as integrin and cadherin molecules from the surface of the neoplastic cells (Bates *et al*, 1995). Still another factor that is conducive to apoptosis is the hypoxic conditions that prevail in many tumours (Arai *et al*, 1997).

Recent studies indicate that different types of tumour cells undergo programmed cell death via different pathways. Deregulation of the apoptotic pathway due to loss of p53 function probably contributes to treatment failures in cancer patients by making malignant cells more resistant to chemotherapy and radiotherapy (Lowe *et al*, 1993). p53 has been shown to be a key regulator of apoptosis (Levine, 1997; Kagawa *et al*, 1997). However, the occurrence of apoptosis and its modulation via p53-independent pathways has also been reported (Xie *et al*, 1999, Ikeguchi *et al*, 1999; Kupryjanczk *et al*, 2000). Our study has shown that overexpression of p53 protein does not contribute to the attenuation of apoptosis in oesophageal adenocarcinoma. On the contrary, it has a positive influence on this process, mainly by increasing growth fraction (Raouf *et al*, 2001). We found, for instance, that tumours overexpressing p53 (before and after chemoradiotherapy) had similar apoptotic indices to p53 negative tumours. It is possible that immunohistochemically-detected p53 might be associated with an accumulation of functionless protein due to p53 gene mutation (Hamelin *et al*, 1994; Gleeson *et al*, 1998). As one of the normal functions of p53 is to control the cell cycle and apoptosis in response to DNA damage such as that induced by chemotherapeutic agents or irradiation, tumour cells with defective p53 might well be less able to repair DNA damage or to undergo apoptosis.

Accumulated evidence suggests that the proteins of the bcl-2 gene family may interact with each other, and that their relative proportions regulate the process of apoptosis (Oltvai *et al*, 1992; Reed, 1995). Overexpression of bax has been observed to promote apoptosis by increasing susceptibility to anticancer drugs and radiation (Wagener *et al*, 1996; Kitada *et al*, 1996) whereas overexpression of bcl-2 and bcl-x, permitted cells to survive such influences (Reed, 1995; Datta *et al*, 1995). In the current study, we therefore analysed the possible correlation between the level of apoptosis and expression of the apoptosis-related proteins bcl-2, bax, and bcl-x. An absence of any correlation between the occurrence of spontaneous apoptosis and the level of bcl-2, bax and bcl-x expression was observed. In addition, the current study could not confirm that induction of apoptotic regulatory genes bax and bcl-x (8 and 3 cases, respectively) by chemoradiotherapy influences tumours' susceptibility to undergo apoptosis. The apoptotic index was similarly induced by chemoradiotherapy in tumours which sustained or increased expression of bax and bcl-x protein. Niether did the relative ratios of these proteins influence the occurrence of apoptosis and its modulation by chemoradiotherapy.

The extent of apoptosis was also shown to be a factor associated with many clinical variables. Examination of apoptosis in gastric carcinoma (Kasagi *et al*, 1994) and oesophageal squamous carcinoma (Hamada *et al*, 1996) showed that the fraction of apoptotic cells was higher in undifferentiated tumours than in differentiated ones. In colorectal carcinoma, apoptosis was less frequently observed in tumours with higher malignant potential, such as those at advanced stages; in tumours showing evidence of moderate differentiation than in well-differentiated tumours and also in tumours with venous invasion or lymph node metastasis than in those without these features (Sugamura *et al*, 1998). The prognostic significance of apoptotic index (low apoptotic index and poorer survival) has also been reported in gastric (Ikeguchi *et al*, 1999) colorectal (Sugamura *et al*, 1998) and non-small cell lung carcinomas (Tanaka *et al*, 1999). Analysis of our data indicated that the histological differentiation or metastatic potential of the tumour was not a major determinant of apoptosis. At resection, however, increasing depth of invasion (pT) of residual tumours was significantly associated with an increasing apoptotic index, a feature similar to that been found in gallbladder carcinoma (Turunen *et al*, 2000).

5.6. SUMMARY OF FINDINGS

In conclusion; this study demonstrates that apoptosis occurred among viable tumour cells in all adenocarcinoma cases examined. Pre-operative chemoradiotherapy significantly increased apoptotic cell death. Immunohistochemically detected p53, bcl-2, bax and bcl-x gene products had no regulatory role in the apoptotic process in oesophageal adenocarcinoma, nor did they influence tumour susceptibility to neuadjuvant therapy. The evaluation of apoptosis in pre-treatment specimens, however, may have potential application in predicting the efficacy of neuadjuvant approaches for oesophageal adenocarcinoma.

CHAPTER 6

MECHANISMS OF CELL DEATH INDUCED IN HUMAN OESOPHAGEAL CARCINOMA CELL LINES IN RESPONSE TO 5-FLUOROURACIL, CISPLATIN AND TAXOL TREATMENT

CHAPTER 6

MECHANISMS OF CELL DEATH INDUCED IN HUMAN OESOPHAGEAL CARCINOMA CELL LINES IN RESPONSE TO 5-FLUOROURACIL, CISPLATIN AND TAXOL TREATMENT

6.1 INTRODUCTION

6.1.1 Cellular Response to Anti Cancer Agents

Cancer therapy is continually improving but no single type of treatment is likely to benefit all patients, even those with a similar type of tumour. This is because each tumour varies in its biological characteristics, due to diversity in the genetic and micro-environmental factors that determine its evolution. It is, therefore, important to assess the biological features of individual tumours that might influence the success of a particular treatment. In this way, the most appropriate treatment modality or scheduling can be selected, and the potential success of each treatment maximized. Both chemotherapy and radiotherapy are affected by many physical and biological factors including proliferation, hypoxia, vascular supply, intrinsic resistance, multi-drug resistance, and the integrity of cell-cycle control and cell-death mechanisms. An understanding of these molecular events could be, in part, achieved by the study, in vitro, of cells derived from tumours mimicking the in vivo situation. In addition, gene expression studies are complementary. Investigation of how such gene defects affect the response of cells in different cancer tissues, where cytotoxicity may be mediated by different response pathways, may reveal new molecular targets that could be used in modifying tumour response, e.g. in gene therapy protocols.

The rationale for the use of chemotherapy and radiotherapy is to combine an agent that has an effect upon systemic micrometastasis with a modality that enhances local tumour control. In an overall scenario, anticancer treatment is considered to mediate cell death by activating elements of the apoptosis program and the cellular stress response. In radiotherapy, irradiation photons release excited electrons: $H_2O + O_2$ to form O_3-OH free radicals. These cause DNA double-strand breaks and cell death mainly by apoptosis if the DNA damage exceeds the capacity of repair mechanisms. Lack of available substrates (hypoxia) and oxygen free radical scavengers (i.e. glutathione, reactivated by glutathione-S-transferase [GST-π]), however, have been shown to block the effect of

radiotherapy (Harpole *et al*, 2001). Defective apoptotic pathway (i.e. mutation or overexpression of tumour suppressor or oncogenes) could also be related to radioresistance of the tumours (Obata *et al*, 2000; Mukherjee *et al*, 2001). Anti cancer drugs also inhibit proliferation and induce apoptosis in sensitive tumour cells. The cellular targets for different cytotoxic agents are diverse. Thus, anticancer drugs are classified as DNA-damaging agents (cisplatin, cyclophosphamide, doxorubicin), antimetabolites (5-fluorouracil, methotrexate), mitotic inhibitors (vincristine), nucleotide analogs (6-mercaptopurine), or inhibitors of topoisomerases (etoposide). The common underlying mechanism for chemotherapy-induced apoptosis might be damage to DNA, lipid components of the cell membrane and cellular proteins causing an imbalance of the cellular homeostasis commonly designated as cellular stress. This, in turn, initiates a complex cascade of stress-inducible signalling molecules in an attempt to return the cell to its previous equilibrium. The type and dose of stress within the cellular context appears to dictate the outcome of the cellular response. In addition, in vitro assays have convincingly demonstrated that deregulation expression of apoptosis-mediating molecules may confer drug resistance (Zhang *et al*, 2000; Kaufmann *et al*, 2000).

Owing to the presence of advanced disease at the time of diagnosis, oesophageal carcinomas, therefore, have been shown to be refractory to neuadjuvant therapies (Walsh *et al*, 1996; Cooper *et al*, 1999; Rice *et al*, 2001). It is well established that poor prognosis depends more on the biology of the tumour, the stage of the disease and the rate of local and systemic recurrence of the disease, (Ilson *et al*, 1997; Rice *et al*, 2001, Dunne *et al*, 2001). Even though multimodality treatment regimens involving the use of chemotherapy. Chemotherapy has played a minor role in the treatment of oesophageal cancer. Of the hundreds of screened cytotoxic agents, few have been tested in the fight against the disease and, therefore, more effective anti-tumour agents and better combination regimens of new and available cytotoxic drugs are necessary to improve the therapeutic efficacy of multimodality treatment. In addition, further treatment strategies will also require a better understanding of the molecular biology and biochemistry of the disease, including a greater insight into the mechanism of tumour resistance to anticancer agents (Calabresi *et al*, 1990). Various biochemical changes that are usually specific for the selecting agent, characterize resistant tumour cell populations in experimental systems. These changes include: (i) alterations in drug

transport, i.e. decreased drug uptake/enhanced drug transport from the cell; (ii) alterations in drug target, i.e. increased concentrations of a target enzyme or changes in affinity for an inhibitor; (iii) an increased capacity to inactivate the drug, e.g. increased drug detoxification enzymes/increased thiol-group scavengers; (iv) a failure to engage apoptosis; and (v) a capacity to develop simultaneous resistance to more than one type of chemotherapeutic agent (the multidrug resistance (MDR) phenotype).

6.1.2 Anti Tumour Activity of 5-fluorouracil, Cisplatin and Taxol

5-fluorouracil (5-FU) is one of the most widely used chemotherapeutic agents for the treatment of gastrointestinal tumours (Ansfield *et al*, 1979; Gamelin *et al*, 1996; Sugamura *et al*, 1997). Although the precise mechanisms of action of 5-FU have not been fully elucidated, the main mechanism is thought to be the binding of the deoxyribonucleotide of the drug (5-fluoro-dUMP) and the folate cofactor, $N^{5\text{-}10}$–methyl-enetetrahydrofolate, to thymidylate synthase (TS) to form a covalently bound ternary complex, which inhibits the formation of thymidylate from uracil, thereby interfering with DNA synthesis. In addition, FUTP can be incorporated into RNA in place of uridine triphosphate (UTP), producing a fraudulent RNA and interfering with RNA processing and protein synthesis (Pinedo *et al*, 1988; Parker *et al*, 1990). The effect of 5-FU on living cells is limited mainly to those in the proliferative phase, but while cells in the G2 and S phases are most affected there may be effects at any stage of the cell cycle. Treatment of cells with 5-FU has been shown to induce p53-dependent apoptosis (Wadler *et al*, 1998). Apoptosis induced by 5-FU was also found to be mediated by the activation of a caspase cascade involving caspase 1, 3 and 8 (Ohtani *et al*, 2000). Increased expression of c-fos and c-myc proto-oncogenes has been correlated to 5-FU resistance following drug exposure (Kashani *et al*, 1990). The limited therapeutic response of oesophageal carcinomas has encouraged many modifications of the schedule of 5-FU administration, using various modulators including radiotherapy, cisplatin (Walsh *et al*, 1996; Blazeby *et al*, 1997), leucovorin (Feliu *et al*, 1996; Ide *et al*, 1997) and recently, taxol (Ajani *et al*, 2001; Schnirer *et al*, 2001).

Cisplatin is a cycle-phase nonspecific antineoplastic agent that has biochemical properties similar to those of alkylating agents. Platinum-containing antineoplastic agents appear to exert their effect by binding to DNA, thereby inhibiting DNA synthesis. Cisplatin enters cells by diffusion. Intracellularly, in the presence of a low

chloride concentration, the chloride ligands of the complex are displaced by water, resulting in the formation of positively charged platinum complexes that are toxic and which react with the nucleophilic sites on DNA. The drug produces predominantly DNA intrastrand and interstrand cross-links, with intrastrand cross-links resulting from the formation of adducts between activated platinum complexes of the drug and areas of specific base sequence; DNA-protein cross-links are also formed. RNA and Protein synthesis are also inhibited by cisplatin, although less extensively. Cisplatin binds to free sulfhydryl groups in tubulin and causes a partial depolymerization of microtubules (Peyrot *et al*, 1986). It changes microtubule assembly by direct tubulin modification (Boekelheide *et al*, 1992) and causes changes in the cytoskeletal pattern of tumour cells (Kopf-Maier *et al*, 1992). Cisplatin complexes with phosphatidylserine lipid in the membrane (Speelmans *et al*, 1997) and has been reported to induce apoptosis in many human cancer cells (Zaffaroni *et al*, 1998; Qin *et al*, 2001). Furthermore, injection of cisplatin prior to irradiation was shown to increase radioresponse and tumours' cure rate (Raaphorst *et al*, 1996; Sersa *et al*, 2000).

The mechanisms by which cells develop resistance to cisplatin is an area of intense research because it is one of the major impediments to the clinical success of this drug. The invulnerability of cells to cisplatin cytotoxicity has been attributed to several processes including: 1) the inhibition of drug uptake, 2) an increase in the production of cellular thiols i.e. methallothioneins and glutathione, both of which block the formation of DNA adducts, 3) enhanced replicative bypass of the cisplatin-DNA adducts, 4) changes in the concentration of regulatory proteins and 5) increase in the repair of cisplatin-DNA adducts (Dempke *et al*, 2000; Zamble *et al*, 2002). Cellular resistance, to cisplatin, however, is of two types: First - is acquired resistance: This type of resistance develops both in patients undergoing chemotherapy and in cell lines exposed to increasing concentrations of cisplatin until they have reached a high tolerance for the drug. In many cell lines, DNA repair has been implicated as a cause of resistance, but since the extent of repair did not correlate exactly with the degree of acquired resistance, other mechanisms, such as those mentioned above are presumed to have been operative. Second – is intrinsic resistance: This type of resistance is a phenomenon encountered in patient tumours that are naturally unaffected by platinum treatment. Some cell lines cultured from such patients were more proficient at removing cisplatin-DNA adducts than cell lines with normal cisplatin sensitivity. However, experiments

using these cell lines did not examine the possibility that resistance in tumours may be due to mechanisms that are only manifest in vivo (Zamble *et al*, 2002). At the molecular level, the increase in DNA repair that accompanies cisplatin resistance could be caused by enhanced expression of the proteins involved in DNA repair. C-fos and c-myc proto-oncogenes have also been correlated with cisplatin resistance following drug exposure (Kashani *et al*, 1990, Leonetti *et al*, 1999). Activating the transcription of these genes may lead to a cascade of gene expression that, in turn, stimulates the activity of proteins having a direct role in DNA repair. p53 gene product also has been linked to the ability of DNA repair to confer cisplatin sensitivity. Disruption of the p53 function in human breast cancer cells increased their sensitivity to cisplatin, possibly because of a decrease in DNA repair (Fan *et al*, 1995). ERCC1 is one of the essential components of the mammalian Nucleotide Excision Repair (NER) pathway. When relative expression of the gene encoding for ERCC1 was monitored in human ovarian cancer cells, Northern blot analysis revealed that increased expression of ERCC1 was associated with cisplatin resistance (Ferry *et al*, 2000). In addition, analysis of the genes that were consistently and significantly regulated by cisplatin demonstrated significant cisplatin-dependent induction of several subsets of genes with roles in oxidative stress i.e. superoxide dismutase (SOD) and glutathione-linked enzymes, DNA damage/repair (p21, PCNA, DNA polymerase beta) and apoptosis (Fas, BAK) (Burczynski *et al*, 2000). Cisplatin also caused a notable repression of several co-regulated ER stress-response genes (grp78, grp 94 and disulfide isomerase-related protein 72 [Erp72]), which function as molecular chaperones and appear to inhibit Ca^{2+}-dependent cell death (Liu *et al*; 1998; Yoshida *et al*, 1998).

Taxol (paclitaxel), a prototypic taxane compound, is one of the most active new agents currently used for the clinical treatment of oesophageal cancer. Using a taxol-based combination including cisplatin and 5-fluorouracil, several studies have described a response rate of 30% to greater than 45% in patients having advanced adenocarcinoma or squamous cell carcinoma of the oesophagus (Ilson *et al*, 1998; Ajani *et al*, 2001). While the precise mechanism of action is not fully understood, particularly in respect of oesophageal cancer, the drug is a well-known antineoplastic agent that specifically binds to the β-tubulin subunit in microtubulin (Rao *et al*, 1994), promoting polymerization of tubulin and disrupting microtubule dynamics (Carlier *et al*, 1983; Jordan *et al*, 1993). It has been observed that taxol arrests cells at the G2/M phase of the

cell cycle (Jordan *et al*, 1996; Jordan *et al*, 1999) and that defects of spindle assembly or the presence of detached chromosomes activates an internal signalling pathway that probably initiates the induction of taxol-induced apoptosis (Rudner *et al*, 1996). Evidence suggests that taxol may induce cell death by triggering apoptosis (Halder *et al*, 1996; Zaffaroni *et al*, 1998; Luo *et al*, 1999). Taxol has been shown to effect the stimulation of mitogen-activated protein kinase-2 (MAP-2 kinase) activity and to have blocked processes which are important for invasion and metastasis in prostate tumour cells (George *et al*, 1995). Induction of apoptosis by taxol in leukemia cells was associated with Raf-1 and bcl-2 phosphorylation and bcl-2 cleavage, but is independent of the MAPK pathway (Blagosklonny *et al*, 1999). The activation of caspase 3 and 8 has been reported to play a crucial role in taxol-induced apoptosis in lung carcinoma cell line (Oyaizu *et al*, 1999). Furthermore, studies with Hela cells and fibroblasts showed that taxol blocks cells in the G2 and M phase of the cell cycle (Schiff *et al*, 1980, Jordan *et al*, 1996). An increase in cisplatin sensitivity in human ovarian cell lines was induced by taxol (Parker *et al*, 1993). In addition, taxol enhances the effect of ionizing radiation , possibly by blocking cells in the G2/M phase, the phase of the cycle in which the cells are most radiosensitive (Rosenthal *et al*, 1997; Formenti *et al*, 1999).

6.2. OBJECTIVES OF STUDY

The aims of this study were to assess (in-vitro) the effect of the chemotherapeutic agents 5-fluorouracil, cisplatin and taxol on tumour cell growth, apoptosis and the levels of p53, bcl-2, bax and bcl-x proteins in human oesophageal carcinoma cell lines.

6.3. MATERIALS AND METHODS

6.3.1 Source of Cell Lines

The human oesophageal adenocarcinoma JROECL-33 (referred to as OE-33) and squamous cell carcinoma JROECL-21 (referred to as OE-21) were both supplied by the European Collection of Animal Cell Cultures (chapter 2 section 5.1). Cell lines were maintained as a monolayer in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 100units/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine and incubated at 37°C in a 5% CO_2 incubator (chapter 2 section 5.2). Cells were inspected regularly by phase contrast microscopy to determine their condition prior to sub-culturing. Cells growing logarithmically were used for the experiments.

6.3.2 Chemotherapeutic Agents

Antineoplastic drugs; 5-fluorouracil, cisplatin and taxol, were all purchased from Sigma (Sigma-Aldrich, USA). The drugs were dissolved in dimethyle sulphoxide (DMSO) to make a stock solution which was then diluted with a fresh culture medium to obtain the concentrations required for the experiments.

6.3.3 Cell Proliferation and Chemosensitivity Assay

The cytotoxic effect of 5-fluorouracl, cisplatin and taxol on oesophaeal carcinoma cell lines was determined by MTS Assay. The methodology is described in (chapter 2 section 12). In principle the conversion of MTS into the aqueous soluble formazan is accomplished by dehydrogenase enzymes in metabolically active cells (Berridge *et al*, 1993). Thus, the number of viable cells is directly proportional to the production of formazan measured spectrophotometrically. For each antineoplastic agent/cell line the cytotoxicity was measured by plotting the absorbance at 490nm (Y-axis) versus the concentration of the drug (X-axis) (Figure 6.1). In this study each MTS assay was repeated twice and the following range of drug concentrations (0.01-10mM), (0.01-10μM) and (0.01-10μM), respectively, for 5-Fluorouracl, cisplatin and taxol were used.

6.3.4 Polymerase Chain Reaction and DNA Sequencing

Detection of p53 mutation (exons 5-8) in the oesophageal carcinoma cell lines was determined by means of PCR (chapter 2 section 9) and Temperature Modulated Heteroduplex Analysis (TMHA) using the Wave Nucleic Acid Fragment Analysis System (chapter 2 section 10). Automated DNA sequencing with ABI PRIZM 310 Genetic Analyser (Perkin-Elmer-Applied Biosystems, USA) was performed to identify the type of p53 mutations detected by the Wave System (chapter 2 section 10). The Sequencing results in electorpherogrames were compared with the wild type human p53 sequence (GenBank accession number U94788-National Centre for Biotechnology Information) [Appendix 3]. Each variant position was confirmed by amplification and sequencing of the variant site from the two DNA strands (forward and reverse sequence).

6.3.5 Assessment of Apoptosis

All the experiments were performed using the floating and attached cells. The morphological characteristics of apoptotic cells; cell shrinkage, condensed chromatin, reduction in nuclear size and formation of membrane-bound apoptotic bodies were identified by light microscopy (chapter 2 section 13). Ligation-Mediated Polymerase

Chain Reaction (LM-PCR) method was used to characterise the biochemical feature of the apoptotic cells (oligonucleosomal fragmentation) The total genomic DNA from treated and control cells was extracted using QIAamp DNA Mini Kit (chapter 2 section 8) and 0.5μg of DNA samples was used for each ligation reaction using ApoAlert LM-PCR Ladder Assay Kit (Clontech, USA). The principle of the LM-PCR assay and detailed procedures have been described previously (chapter 2 section 13).

6.3.6 Protein Extraction and Western Blotting

Total cell protein was extracted from control and treated cells using ice-cold RIPA buffer with freshly added protease inhibitors (chapter 2 section 6). Protein concentrations were determined by Bradford assay (chapter 2 section 6). Equal amounts (50μg) of total protein were electrophoresed on a 10% SDS-polyacrylamide gel and then electrotransferred to PVDF membranes (chapter 2 section 7). The blots were probed with specific antibodies to p53, bcl-2, bax and bcl-x [Appendix 2]. The immunocomplexes were then visualised by incubation with horse radish peroxides conjugated anti mouse or anti rabbit antibodies, followed by chemiluminescence and radiography.

6.4 RESULTS

6.4.1 Determination of P53 Mutation in OE-33 and OE-21 Cell Lines

TMHA revealed the presence of a mutation in the exon 5 of the p53 gene in oesophageal adenocarcinoma cell line OE-33. Under partial-denaturing conditions used for separating the heteroduplex fragments, exons 5-8 of the OE-21 cells and exons 6-8 of the OE-33 cells have retention patterns, referred to as a peak in the chromatograms, similar to the wild type homodeuplex p53. As the exon 5 of OE-33 cells was analysed at 65°C, the heteroduplex PCR fragment started to denature in the region side of the mismatched bases and began to emerge ahead of the still intact homoduplexes, thereby indicating the presence of a mutation (Figure 6.2). This shift in the retention time and pattern suggested the presence of a homozygous mutation or alternatively, that the normal allele had been lost. The mutation was demonstrated by direct DNA sequencing as a missense mutation (a single base substitution) at codon 135 resulting in the substitution of an amino acid cysteine to tyrosine (T<u>G</u>C → T<u>A</u>C) (Figure 6.3). A wild type DNA sequencing result was observed in the remaining exons 6-8 and 5-8 of the p53 gene in OE-33 and OE-21 cell line, respectively.

6.4.2 Modulation of P53 and Bcl-2 Proteins Following 5-FU, Cisplatin and Taxol Treatment

In untreated cells, western blotting analysis revealed a sustained high level of p53 protein in OE-33 cells, indicating the presence of a mutant protein with prolonged half-life. p53 protein was not detected in OE-21 cells. Neither of the carcinoma cell lines expressed either the death repressor bcl-2 or the death promoter bcl-x_S, whereas, high levels of antiapoptotic bcl-x_L and steady basal levels of proapoptotic bax protein were detected. There was no significant up or down regulation of bcl-2, bax, bcl-x_S or bcl-x_L protein during the apoptotic process induced by cisplatin, taxol or following 5-FU treatment. Cisplatin treated OE-33 cells exhibited a significant reduction in the p53 protein level. p53 protein was not induced in cisplatin-treated OE-21 cells (Figure 6.4).

6.4.3 Cytotoxic Effect of 5-FU, Cisplatin and Taxol on OE-33 and OE-21 Cell Lines

The growth inhibition of OE-33 and OE-21 cells following treatment with the antineoplastic agents is shown in (Figure 6.1). With respect to apoptosis no obvious morphological or biochemical (DNA Laddering) changes were observed in the early stage (1-6 hours) of drug treatment. This preliminary examination indicated that longer exposure time was required to assess the exact cytotoxic effect of these antineoplastic agents. The morphological changes that resulted from the 24 and 48 hours treatment of OE-33 cells with 5-FU were not indicative, except for a few morphologically appeared cells, of cell death by apoptosis. This was further confirmed by the absence of the DNA ladder in treated and control experiments, while, induction of apoptosis in OE-21 cells by 5-FU was found to be both dose ($\geq$1mM) and time dependent (48 hours). At the observation times of 24 and 48 hours; cisplatin and taxol treated OE-33 and OE-21 cells demonstrated marked morphological changes notably: cell shrinkage, condensed chromatin and formation of multiple apoptotic bodies. Internucleosomal DNA fragmentation was also demonstrated by the appearance of DNA ladders. Both of these features are considered typical of apoptosis, indicating that cisplatin and taxol treated OE-33 and OE-21 cells underwent a form of cell death characterised by features common to the apoptotic pathway (Figure 6.5 and 6.6).

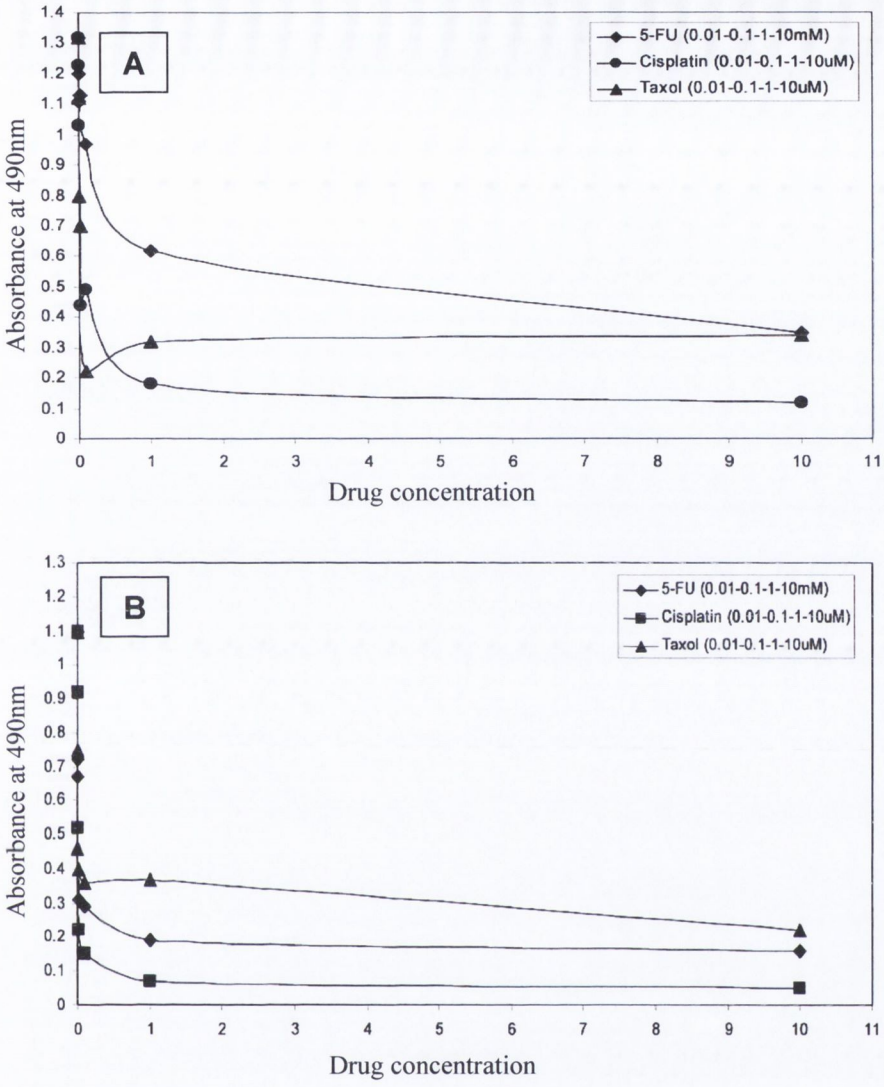

Figure 6.1. Absorbance spectrum of formazan after bioreduction assay by OE-33 (A) and OE-21 (B) following 5-FU, cisplatin and taxol treatment for 48 hours. The cytotoxic effect of the drugs was indicated by the reduction of formazan production. Each point represents the mean value of two assays. The first upper point on the Y axis represents control experiments, the lower points, respectively, represents the range of the drug concentration as indicated in the upper-right corner of the graphs.

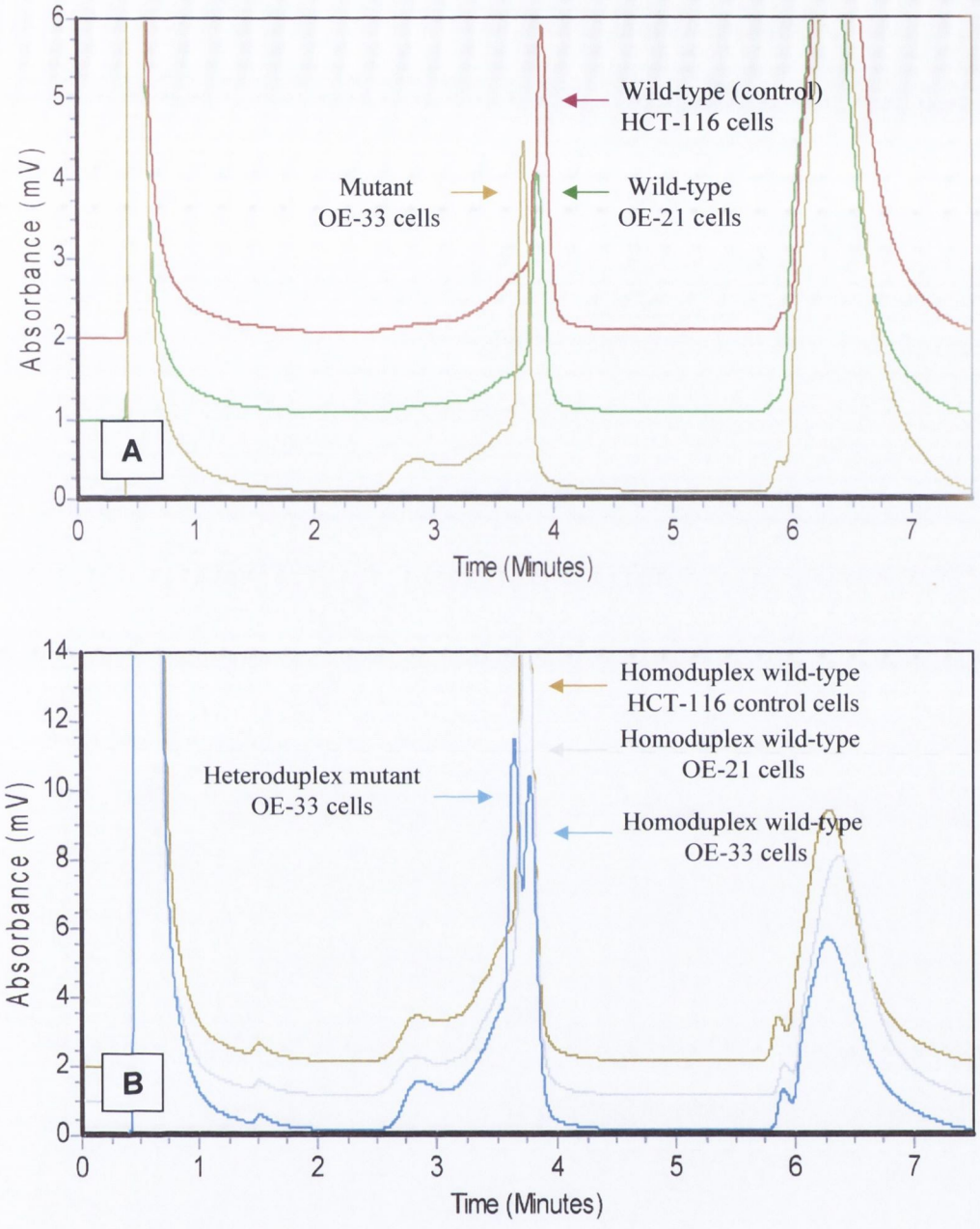

Figure 6.2. Detection of p53 mutation with Wave Nucleic Acid Fragment Analysis System. Chromatograms represents temperature dependent resolution of homoduplexes of the wild-type p53 (HCT-116) and the unknown samples (OE-33 and OE-21). The HPLC elution profile of (A) non-hybridized and (B) hybridized exon 5 of the p53 analysed at 65°C. HCT-116 and OE-21 show a normal elution profile. OE-33 shows elution of a variant (mutant) to the left of the normal tracing.

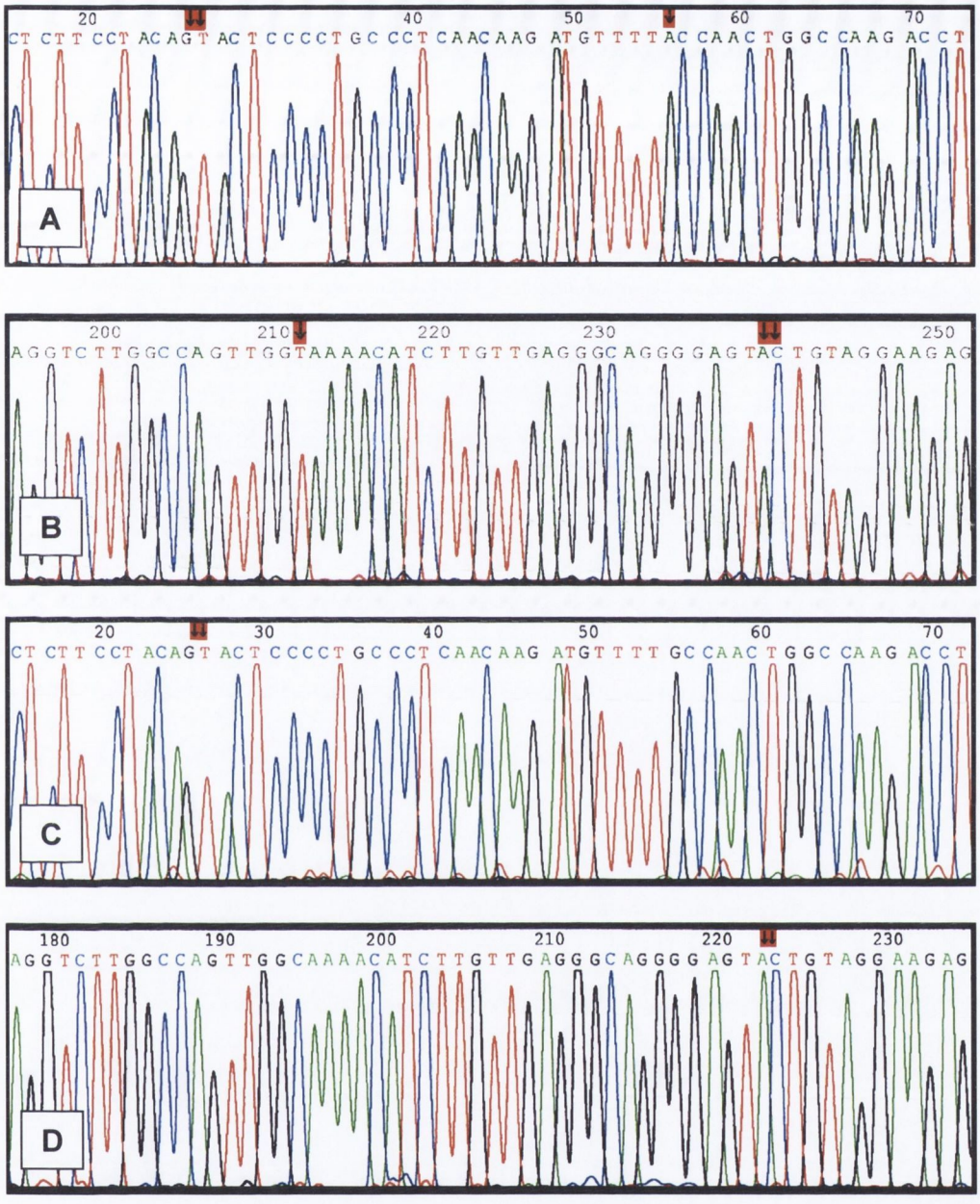

Figure 6.3. Automated DNA sequencing analysis of p53 gene exon 5 in oesophageal carcinoma cell lines OE-33 and OE-21. A base pair substitution (G:C to A:T) resulting in cystine to tyrosine substitution was detected at codon 135 in OE-33 cells (A and B). OE-21 cells show wild type p53 sequence (C and D). The mutant nucleotide is indicated by the single black arrow. The intron-exon junction is indicated by the double black arrows. Electophorograms A&C and B&D represents the forward and the reverse DNA sequence, respectively.

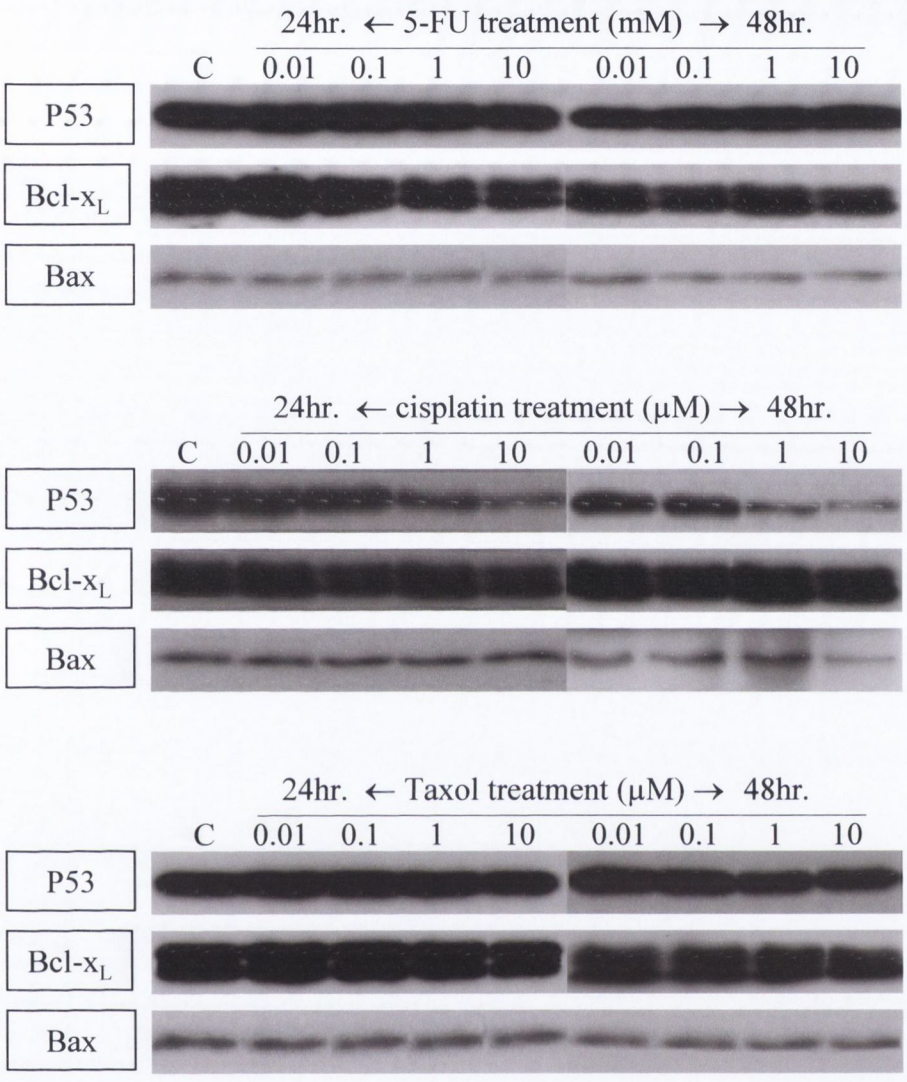

Figure 6.4. Expression patterns of p53 and bcl-2 family proteins in oesophageal adenocarcinoma cell line (OE-33) during 5-FU, cisplatin and taxol treatment analysed by western blotting. Compared to untreated cells, p53 expression was significantly decreased following cisplatin treatment. High levels of bcl-x_L and steady-low levels of bax protein were detected in control and treated cells. Bcl-2 and bcl-x_S protein were not detected in control or treated cells. Regarding the oesophageal squamous cell carcinoma (OE-21) cell line; p53, bcl-2 and bcl-x_S proteins were not detected in either control or treated cells. Bcl-x_L and bax showed a similar expression pattern to OE-33 cells. Each experiment was repeated three times.

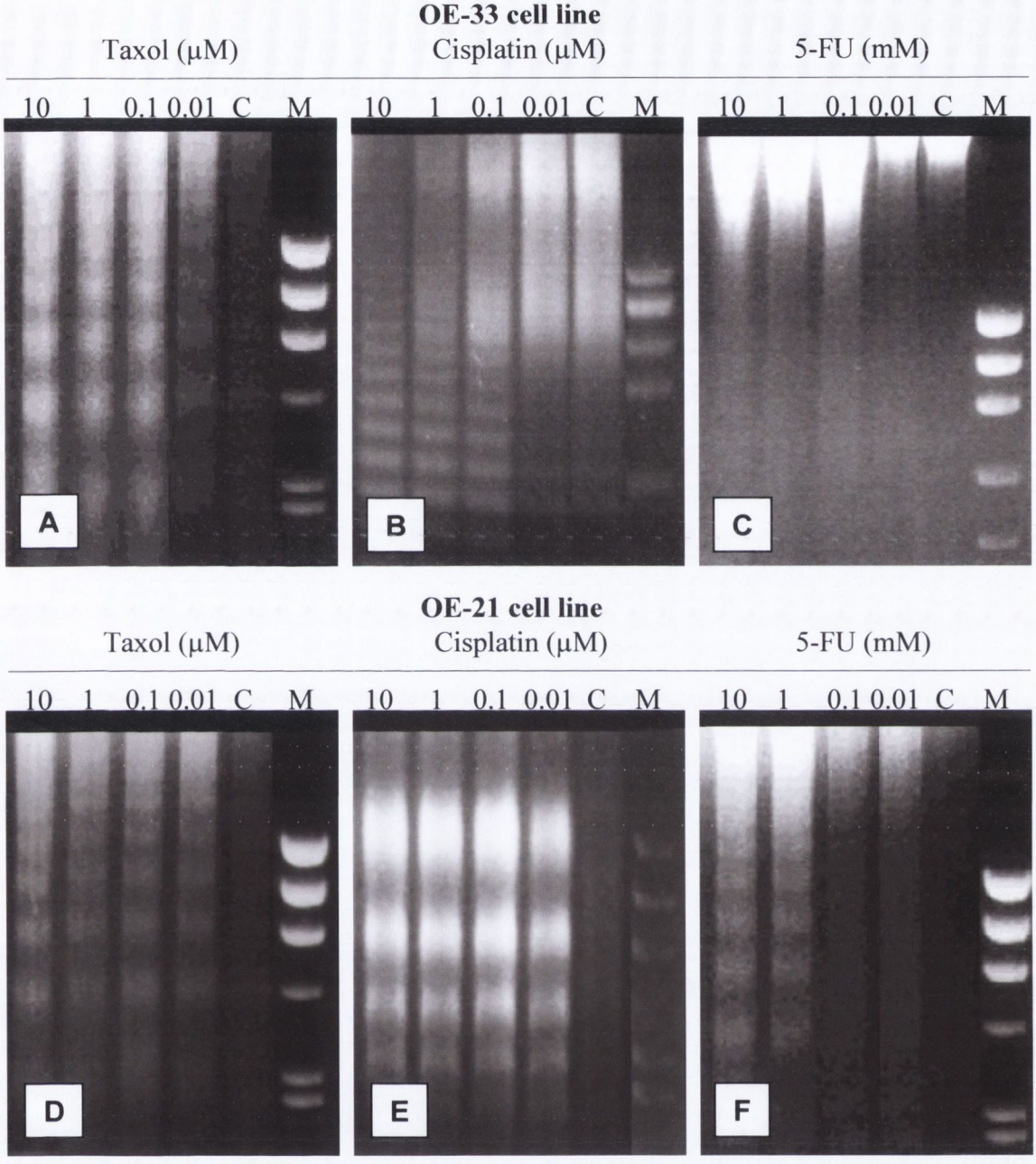

Figure 6.5. Electrophoresis pattern of extracted DNA from oesophageal carcinoma cell lines OE-33 and OE-21 treated for 48 hours with 5-FU, cisplatin and taxol and assayed by LM-PCR for detection of DNA ladder. A typical "Ladder" indicating the presence of DNA internucleosomal fragments is characteristic of apoptosis. Induction of apoptosis by 5-FU, cisplatin and taxol was shown to be dose and time dependent. In OE-33 cells, apoptosis is induced by taxol (A) and cisplatin (B) but not by 5-FU (C). OE-21 cells undergo apoptosis following taxol (D), cisplatin (E) and high dose (≥1mM) 5-FU treatment (F). M = 1kb DNA size marker, sizes of the DNA fragments are given in bp and as follows:- starting from the top of the gels 1353, 1078, 872, 603, 310, 194). C = control cells. Numbers 0.01-10 denote the drug concentration as indicated above. Each experiment was repeated three times.

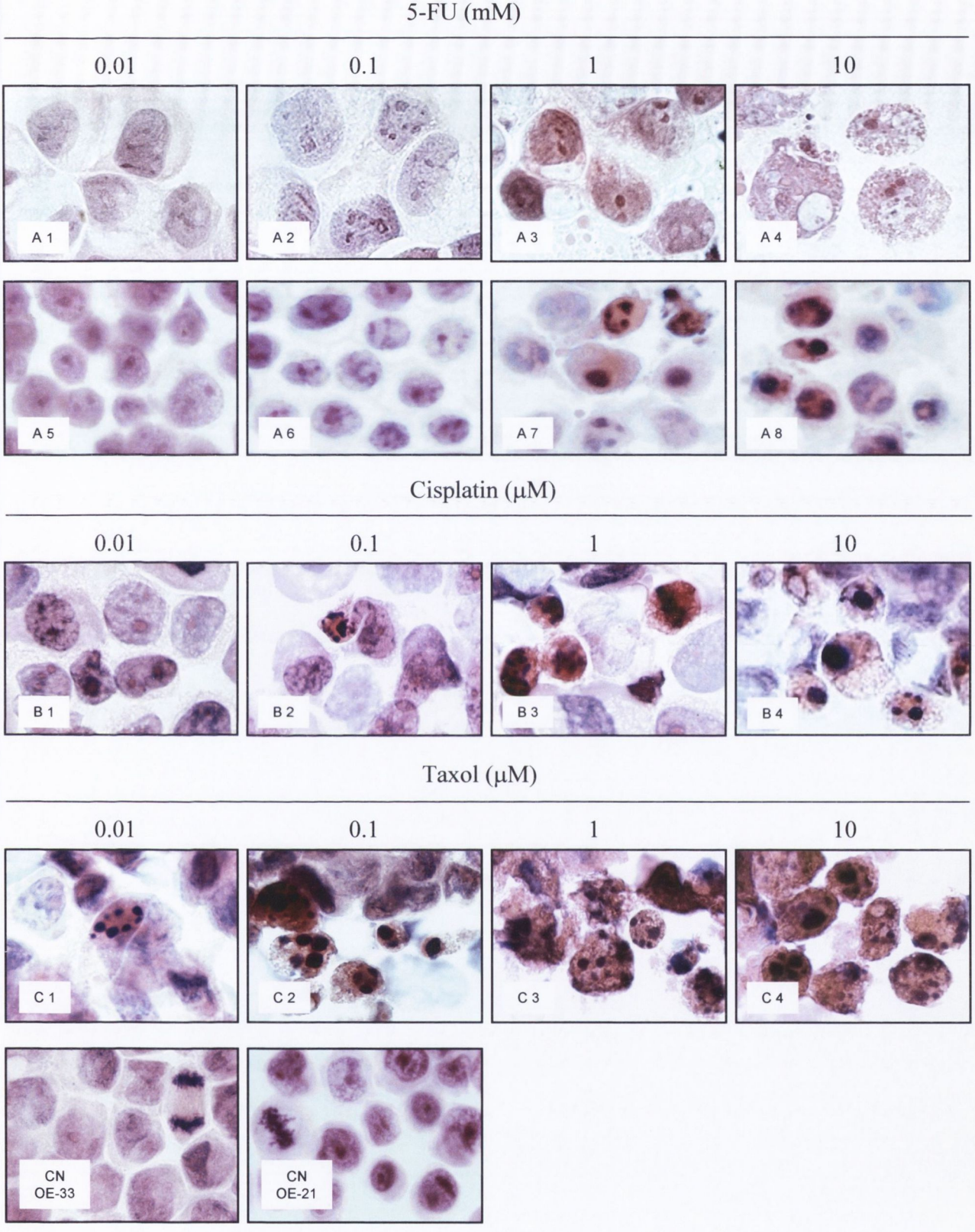

Figure 6.6. Morphological assessment of cell death in OE-33 and OE-21* cells following 5-FU (A1-4) (A5-8)*, cisplatin (B1-4) and taxol (C1-4) treatment for 48 hours. In OE-33 cells, apoptosis was not induced by 5-FU. OE-21 cells undergo apoptosis by 5-FU in a dose dependent manner. Both cisplatin and taxol induce apoptosis. Apoptotic cell death is characterised morphologically, by cell shrinkage, chromatin condensation and the formation of apoptotic bodies. Cells were exposed to a dose range of (0.01-10mM) for 5-FU and (0.01-10μM) for cisplatin and taxol as indicated above. CN denotes untreated control cells showing a mitotic cell. Cells were stained with haematoxylin and eosin and examined using light microscopy (magnification X1000).

6.5 DISCUSSION

In this study, different cytotoxicity results were obtained in the two oesophageal carcinoma cell lines (OE-33 and OE-21) as a function of 5-FU, cisplatin and taxol treatment. Specifically, cisplatin and taxol induced cell death in both cell lines; morphological and biochemical changes were typical of the apoptotic process, but the molecular mechanisms of cells undergoing apoptosis were different. On the other hand, the biochemical mechanisms responsible for cell death by 5-FU in oesophageal carcinoma cell lines OE-33 and OE-21 were not clearly identified. In the latter cell line, induction of apoptosis was sustained by 5-FU treatment but correlated with a high dose and long exposure time. As apoptosis is emerging as a novel mechanism of chemosensitivity, we considered it appropriate, once we had confirmed the occurrence of apoptosis, to study alterations of p53 and the bcl-2 family of proteins because they are reported to be related to apoptosis induction in some cell lines. To the best of our knowledge, this study is the first report demonstrating the mutational status of the p53 gene in OE-33 and OE-21 cell lines. We screened the entire coding region of the exons 5-8 where most mutations (>85%) of the p53 gene have been located (Soussi *et al*, 1990; Greenblatt *et al*, 1994). Our data indicated the presence of a point mutation in the coding region of the exon 5 (codon 135) in OE-33 cells. The presence of mutated sequence only, also suggests that the normal allele has been lost. This mutation resulted in a substitution of cysteine to tyrosine thus, changing the coding sequence and resulting in the production of a mutant protein with prolonged life span. P53 Point mutation at codon 135 has been reported in patients with oesophageal carcinoma (Gao *et al*, 1994), and in other human malignancies (Pollock *et al*, 1996, Park *et al*, 2001). Furthermore, it has been reported that the growth rate of wild-type p53 tumours are relatively slower compared to mutant p53 tumours (Perdomo *et al*, 1998; Piovesan *et al*, 1998) and mutation between codon 100 to 295 is considered a critical region essential for growth suppression (Srinivasan *et al*, 1993). In different cell lines, other types of mutation at codon 135 have also been reported to have transforming ability and have been found to be associated with the presence of mutant p53 protein with altered suppression activity (Slingerland *et al*, 1991; Slingerland *et al*, 1993; Pollock *et al*, 1996).

It has been described in both in-vivo and in-vitro studies, that p53 modulates the 5-FU ability to induce apoptosis. In human solid tumour 5-FU is generally believed to induce G1-S-phase arrest, and its cytotoxic effects are attributed to apoptosis, via a p53-

dependent pathway (Hashimoto *et al*, 2001). In experimental models, for example gastric cancer, cell lines expressing wild-type p53 are more sensitive to 5-FU treatment compared to those cells bearing a mutant protein (Osaki *et al*, 1997). Induction of apoptosis by 5-FU in colon cancer cells has also been shown to be p53 dependent (Yang *et al*, 1996; Tokunaga *et al*, 2000;). It has been demonstrated that, in cells expressing wild-type p53, 5-FU-induced apoptosis was accompanied by increased expression of bax and bak without consistent modulation of other bcl-2 family proteins as opposed to cells containing mutant-type p53 (Nita *et al*, 1998). It has also been shown that overexpression or suppression of bcl-x_L was capable of inducing 5-FU resistance or sensitivity, respectively, in colon cancer cell lines (Liu *et al*, 1999; Nita *et al*, 2000). These results suggest that members of the bcl-2 family of proteins are modulated by 5-FU and / or p53 and may be related to chemosensitivity (Koshiji *et al*, 1997; Violette *et al*, 2002). In the current study, our results address the question of the functionality of p53 protein and its possible role in cell death induction by 5-FU. In OE-33, cell death induced by 5-FU was not confirmed to be via apoptosis even when cells were exposed to chronic doses and prolonged exposure, while, OE-21 cells sustained apoptosis following 5-FU treatment in a dose and time dependent manner. In agreement with the above reports, our finding indicates that 5-FU might be less effective in cancer cells bearing mutated p53 genes. The presence of non-functional or mutant p53 protein in oesophageal cells might be involved in the relative failure of 5-FU-induced apoptosis. However, this study could not establish the presence of an association between the pattern and extent of cell death induced by 5-FU and the bcl-2 proteins. At the transcriptional level, p53 did not modulate the expression of bcl-x_L or bax nor induced bcl-2, suggesting that bcl-2 proteins do not influence oesophageal carcinoma cell lines response to 5-FU treatment and that other apoptotic cascades may, therefore, be at play during apoptosis. Our data provide significant insight into mechanisms that establish oesophageal tumour cell sensitivity to 5-FU, demonstrate clearly the necessity of exercising caution in considering combining novel strategies that target elevated p53 with standard 5FU-based therapy, and suggest alternative therapeutic strategies that target p53 mutations in the treatment of oesophageal cancer.

Another finding of our experiments was that the kinetics of cisplatin-induced apoptosis was similar in both oesophageal cell lines even though the extent of DNA fragmentation was slightly greater in OE-21 cells than in OE-33 at all observation times. We also

concluded that cisplatin can selectively influence (reduce) the expression of p53 protein in OE-33 cells. This inhibition was not related to any modulation of bcl-2 proteins, but it is possibly related to apoptosis. Loss of p53 protein is likely to be a primary effect of cisplatin's addiction to the DNA (DNA platination) and subsequent reduction in the DNA and protein synthesis (Sorenson *et al*, 1990). It is also possible that cisplatin selectively alters the transcription activity of some genes in response to DNA damage. Degradation of p53 protein can occur by ubiquitination involving the proteasome complex. P53 breakdown was also shown to correlate with caspase 3 activities during apoptosis (Wang *et al*, 2000). These results show that there is a link between p53 transcription and apoptosis. Thus, for drugs which react with both DNA and protein, protein damage is worth considering as a factor contributing to apoptosis. Therefore, we may consider that changes in the expression level of p53 could be predictive of cisplatin-induced apoptosis in oesophageal cell lines. As demonstrated in OE-21 cells, we also concluded that cisplatin can induce apoptosis by triggering other pathways independent of p53. Induction of apoptosis by cisplatin has been reported to be more extensive in wild-type p53 tumours than mutant p53 tumours (Perdomo *et al*, 1998; Kanata *et al*, 2000). Interestingly, a study of human lymphoblastoid cell lines indicated that cells expressing mutant p53 at codon 135 were less resistant to cisplatin-induced apoptosis than cells bearing p53 mutation at other sites (Piovesan *et al*, 1998). These observations indicate that genetic alteration in p53 might be associated with tumours' response rate to cisplatin and, therefore, a comparative study of cell lines bearing different types of p53 mutations is required to further elucidate the mechanism of action of cisplatin and its efficacy in respect of particular tumours. The bcl-x_L protein, a functional and structural homologue of bcl-2, in contrast to bax, has also been reported to provide protection against cisplatin-induction of apoptosis (Simonian *et al*, 1996; Simonian *et al*, 1997). Suppression of bcl-x_L levels in a human cancer cell line by use of either bcl-x antisense or overexpression of its antagonist proteins was found to sensitize the cells to apoptosis following treatment with 5-fluorouracil or cisplatin (Kondo *et al*, 1998). Our results show that the differential ability of cisplatin to induce apoptosis is independent of bcl-x_L and bax. We also concluded that the lack of correlations between drug sensitivities and bcl-2 proteins expression was also unaffected by the p53 status of the cell lines, suggesting that other members of the bcl-2 proteins family may be targeted by the drug.

Our experiments also demonstrated that taxol can induce apoptosis in oesophageal carcinoma cell lines. Taxol is latterly utilized for the treatment of oesophageal cancer. However, the mechanisms underlying its induction of apoptosis are still unclear. Generally, taxol exerts its cytotoxic effect by binding and stabilizing microtubules (Diaz *et al*, 2000) and induces apoptosis in a variety of cell types (Moos *et al*, 1998; Shen *et al*, 1998). Taxol-induced apoptosis has frequently been found to be independent of p53 status, despite the fact that some treated cells showed up-regulation of p21 (Lanni *et al*, 1997; Debernardis *et al*, 1997). Taxol was recently demonstrated to release cytochrome c by direct action on the mitochondria (Andre *et al*, 2000), to activate small GTP-binding proteins (Lou *et al*, 2000; Subbaramaiah *et al*, 2000), and activate the NF-$_k$B/I$_k$B-α signaling pathway (Huang *et al*, 2000). These actions were all implicated as the mechanisms through which taxol induces apoptosis. Induction of apoptosis in cells treated with taxol also correlated with the phosphorylation and inactivation of bcl-2 and bcl-x_L (Haldar *et al*, 1996; Poruchynsky *et al*, 1998). In addition, the pro-apoptotic protein bax was found to enhance taxol-induced apoptosis through a p53-independent pathway (Strobel *et al*, 1996). Our results are consistent with these recent observations. We demonstrated that both oesophageal carcinoma cell lines have a similar response to taxol. The extent of apoptosis was independent of p53 status and neither cell line showed evidence of cellular resistance, even to low doses of taxol. Although we found no substantial change in the levels of p53, bcl-x_L or bax protein after treatment with the drug, we cannot exclude the possibility that other bcl-2 family members or other regulatory proteins yet to be discovered, could be altered by taxol and could perhaps differentially modulate apoptosis. It is important to emphasize that as the biochemical phenomena related to apoptosis reported herein were obtained at a taxol concentration that can be reached in vivo, they may well, therefore, be relevant for clinical settings.

6.6. SUMMARY OF FINDINGS

In conclusion, this study demonstrated that (i) the p53 status is sufficient to predict 5-fluorouracil chemosensitivity of oesophageal cancer cells in vitro; (ii) cisplatin and taxol are inducers of apoptosis in oesophageal cell lines, albeit via different molecular mechanisms; (iii) The bax and bcl-x_L proteins, are not relevant markers to predict response to these chemotherapeutic agents. Therefore, oesophageal tumour cell lines appear to be an important tool for analysing the pathway(s) of apoptosis after selective treatment and for defining new markers to predict response to 5-FU or to other drugs used in combination with 5-FU, in the treatment of oesophageal carcinoma.

CHAPTER 7

GENERAL DISCUSSION

CHAPTER 7

GENERAL DISCUSSION

7.1 DISCUSSION

Barrett's oesophagus is an acquired condition in which the squamous epithelium of the distal oesophagus is replaced by metaplastic columnar epithelium of various types. It represents a peculiar form of healing that can occur at any time in patients with chronic acid or bile reflux. Barrett's metaplasia is a premalignant lesion, because it is the initiating phenotype of a metaplasia-to-dysplasia-to-carcinoma sequence. It offers an ideal opportunity for the investigation of the genesis of oesophageal carcinoma. Endoscopic innovations enable easy oesophageal access for biopsy of the premalignant lesion without removal. This also permits follow-up of the premalignant tissue. The progression of Barrett's oesophagus to adenocarcinoma is associated with several changes in gene structure and expression. These alterations render the cell independent of regulated proliferative and cell death pathways, and infuse the cell with an invasive capacity. As a consequence, a malignant tumour composed of cells with an increased proliferative activity, a prolonged life span, and metastasising capacity is generated. Research in this project focused on identifying key molecular changes, which occur during the development of oesophageal adenocarcinoma. This research had three aims; to increase our understanding of the fundamental processes underlying disease development, to develop these molecular changes as clinical biomarkers to detect early disease and finally, to predict the responsiveness of oesophageal adenocarcinoma to therapy. The genes that are believed to be associated with the development of malignancy are broadly categorised as tumour suppressor genes and oncogenes that programme cell proliferation and cell death.

Appreciation of the function of the tumour suppressor gene p53 has given new insight into regulation of the cell cycle, and this gene appears to play an important role in many solid tumours. We concluded that p53 malfunction arises as an early event in the carcinogenic process and accordingly, this has been demonstrated in patients with dysplastic Barrett's oesophagus. Rates of p53 positivity are highest in patients with high-grade dysplasia and cancer. In cases in which tumours showed p53 protein overexpression, the adjacent dysplastic epithelium often overexpresses p53. In agreement with earlier reports, our study identified a pattern of increasing p53

expression and aberrant cell proliferation, assessed by Ki-67 expression, along with the various steps of the dysplasia-carcinoma sequence. In addition, localisation of p53 and epithelial cell growth was shown to correlate with the histological findings in dysplastic Barrett's epithelium. Our findings support a concept whereby proliferation, stimulated by oncogene activation and loss of the tumour-suppressor function, as in other parts of the gastrointestinal tract (i.e. colon), plays a central role in the neoplastic progression. Lack of p53 expression, however, indicates that malignant progression and the loss of proliferative control is possible without the involvement of the p53. Furthermore, this study indicated a pattern of progressive reduction of bcl-2 expression through low- to high-grade dysplastic Barrett's epithelium and adenocarcinoma, whereas, expression of bax and bcl-x gene products demonstrated increased levels of expression. The latter finding suggests that loss of bcl-2 regulation is a factor in the multistep progression of Barrett's oesophagus and that cells acquire other ways of avoiding apoptosis as malignancy appears. Additionally, this study also implies that elevated levels of bax and bcl-x protein represent a common event in the pathogenesis and progression of epithelial malignancies of the gastrointestinal tract, as similar trends in the expression levels of bax and bcl-x have been reported in gastric and colorectal carcinomas. In conclusion, our studies of Barrett's oesophagus and similar studies described in this thesis demonstrate that a higher rate of epithelial cell proliferation with an altered genome is a common theme in Barrett's oesophagus, correlating with progression and can, therefore, be regarded as a biological marker for at risk patients.

The striking increase in the incidence of oesophageal adenocarcinoma during the last 20 years, and the associated mortality, has prompted many clinical centres to search for multimodal therapies that may improve outcome. Molecular studies have also been conducted to search for biological markers associated with tumour response to chemotherapy and / or radiotherapy. The rationale for using neoadjuvant therapy in the treatment of oesophageal cancer is to combine chemotherapeutic agents that have an effect upon systemic disease with neoadjuvant therapy, a modality that enhances local tumour control. Up to 25-35% of patients will have a complete pathological response to neoadjuvant therapy. Many patients, however, will not respond and the molecular mechanisms responsible for this variation in the clinical response are still largely unclear. An understanding of apoptosis and cell proliferation may be relevant in this context and this study has, therefore, focused on apoptotic indices and cell-cycle related

Ki-67, p53 and bcl-2 protein expression in patients with primary oesophageal adenocarcinoma. We documented (Raouf *et al*, 2001) that preoperative chemotherapy and radiation therapy significantly increases apoptotic cell death and significantly decreases cell proliferation. Analysis of our data indicated that the frequency of spontaneous apoptosis and tumour growth activity at resection is a prognostic biomarker associated with tumour response to neoadjuvant therapy and tumours' metastatic potential, respectively. The expression level of p53, bcl-2 associated proteins bax and bcl-x did not, however, influence or predict the response or resistance of oesophageal adenocarcinoma to neoadjuvant chemoradiotherapy.

The impact of the p53 tumour suppressor gene in-vitro on cellular sensitivity to anti cancer drugs has recently been elucidated and a number of studies have suggested that loss of the normal p53 function may be a major determinant underlying a tumour's failure to respond to chemotherapy and radiotherapy. It must be acknowledged, however, that other studies have also produced results disassociating p53 status and chemosensitivity. On the other hand, besides p53 status, response to anticancer treatment is also counteracted (enhanced) by the expression of antiapoptotic (proapoptotic) members of bcl-2 proteins. Exploration of the expression levels of tumour suppressor genes or oncogenes during treatment may identify their roles in sensitising cells to anti cancer agents as well as identifying new biological markers that could be integrated into in-vivo settings. Thus, in the present study an attempt was made to investigate the effect of the chemotherapeutic agents 5-fluorouracil, cisplatin and taxol on cell growth, apoptosis and the levels of p53 and bcl-2 associated proteins in oesophageal carcinoma cell lines. The study was designed to determine whether mutation in the p53 gene could modulate the effect of the chemotherapeutic agents 5-fluorouracil, cisplatin and taxol, and to assess the significance of the pro- (bax) and anti-(bcl-x_L) apoptotic proteins and their relevance to apoptosis and cellular resistance. We demonstrated that the mutational status of p53 is associated with the chemosensitivity of oesophageal carcinoma cells to 5-fluorouracil and that the presence of the mutant p53 protein may provide cells with pathways counteracting apoptosis. Both cisplatin and taxol induced apoptosis in oesophageal carcinomas, but through different molecular mechanisms, with cisplatin selectively reducing the stability of the mutant p53 protein in adenocarcinoma cells. Our study also shows that apoptosis and its modulation by 5-fluorouracil, cisplatin or taxol occur independently of bax and bcl-x_L, the dominant

splice variant of bcl-x, protein. Furthermore, it is important to emphasize that as the biochemical phenomena related to apoptosis reported herein were obtained at a taxol concentration that can be reached in vivo; they may well be relevant for clinical settings and neoadjuvant clinical trials. In conclusion, our study shows that oesophageal carcinoma cell lines appear to be an important tool for analysing the pathway(s) of apoptosis following selective treatment and for defining new molecular markers to predict response to 5-fluorouracil or to other drugs used in the treatment of oesophageal carcinoma.

7.2 FUTURE STUDIES

Overall, the goal of this thesis was to understand oesophageal adenocarcinoma with a view to the appropriate management of patients with Barrett's oesophagus. Barrett's oesophagus and its progression to oesophageal adenocarcinoma is associated with a wide variety of molecular and cellular changes which parallel histological progression from metaplasia through dysplasia to invasive cancer. An improved understanding of these molecular changes may help to target patients in need of regular surveillance and could lead to improved treatment. Future effort will require multidisciplinary, multisite approaches. Research is needed to definitively establish the molecular basis for the observed link between environmental factors and genetic disease. Study of the natural history of Barrett's oesophagus is also a priority. Such studies require standardised biopsy protocols and a common database to collect and analyse results. Study findings need to be rapidly translated back to the medical community and to the public. Thus, advanced molecular based studies should be conducted and the characterisation of Barrett's oesophagus might then be achieved with translational research (e.g. examining biomarkers), new technologies (e.g. cDNA microarrays) and the appropriate application of bioinformatics.

It is clear that bcl-2 and related proteins are important inhibitors or inducers of apoptosis in human cell systems. Many human tumours do clearly over express bcl-2 or other members of the group such as bax or bcl-x. Furthermore, it is highly likely that the expression of bcl-2 proteins is relevant for carcinogenesis in several model systems. However, based on the data presented in this thesis, the value of bcl-2 and similar proteins as markers for tumour progression has been elucidated. Our results clearly help to define future research directions. New studies on prognostic factors should focus on

parallel investigations of several important regulators of epithelial cell proliferation and apoptosis such as the role of tumour suppressor genes p63 and p73, other members of bcl-2 family, specific proteases (i.e. caspases) and death receptors and their corresponding death ligands (TNF family).

Future efforts should develop and employ non-invasive markers for Barrett's oesophagus or oesophageal cancer which would obviate the use of endoscopy for screening and surveillance. Translational studies that examine, for example, the role of Cox-2, Cox-2 inhibitors (NS-398), and the use of the antioxidant agents (e.g. ascorbic acid) in Barrett's oesophagus should bank specimens for prospective use in marker development and design molecular based therapy, ideally, as a cancer prevention approach. Studies such as these are currently in progress in this Department and a preclinical trial using animal models has also been considered.

In addition to the development of new prognostic markers or marker patterns for oesophageal cancer, studies on cell-death and related proteins will guide future research strategies, possibly leading to new drugs or new combination therapies. Our own work on the position and sequence of chemoradiation-induced cell death in oesophageal adenocarcinoma using apoptotic markers, p53 and bcl-2 revealed a high potential value of combined chemoradiation in the treatment of patients with oesophageal adenocarcinoma and highlighted the necessity of conducting further studies focussing on the molecular response of oesophageal cancer to neoadjuvant therapies. It also emphasises the potential integration of biological modifier agents (i.e. growth receptor-tyrosine kinase- inhibitors) as an additional or alternative therapeutic approach. Therefore, it is desirable to broaden our research in the field of apoptosis and its link to tumours' response to chemoradiotherapy. Future studies should include and use multiple tumour biopsies at different time points during the course of the treatment and at surgery. In addition, the use of cDNA microarray and fluorescence in situ hybridisation technologies will greatly define the various cellular and molecular changes that are associated with tumours' response to neoadjuvant therapies.

Future studies, it is suggested, might also focus on determining the metastatic potential of oesophageal carcinomas with particular attention to micrometastasis. Detection of cancer cells in the lymph node, blood or bone marrow might be an indicator of metastasis in cancer patients and may predict widespread disease and a poor prognosis.

Generally, the most frequent metastastatic cancers are adenocarcinomas and a significant proportion of oesophageal cancer is also adenocarcinoma. As a result, the differential diagnosis between primary and metastatic cancers and the precise identification of various metastatic cancer types requires the coordinated use of various morphological (light- and electron- microscopic), immunological and nucleic acid based techniques. Multicentre clinical trials are now warranted to establish the clinical impact of molecular staging in oesophageal cancer. The integration of molecular methods into the pathological protocols is essential to envision significant improvement in clinical staging and assessment of the results of cancer therapy. Studies of the haematogenous dissemination of cancers have indicated that tumour cells from various cancer types can be readily and much more easily detected in the bone marrow, even in the case of those tumours where the bone is not the site for development of metastasis. The bone marrow provides an accessible tissue normally not contaminated with epithelial cells since the native cells are mesenchymal in origin and easily distinguished from malignant epithelial cells by a variety of molecular techniques. Sentinel lymph node biopsy, with serial sectioning and screening for micrometastases, has improved staging accuracy and offered valuable insights into the behaviour of malignant cells. The methods commonly used are immunocytochemistry, flow cytometry and molecular techniques (RT-PCR). The molecular markers are usually cell-specific tumour-associated antigens (e.g. CK, CEA, MGB2, PSA, K-ras). The prognostic significance of nodal and bone marrow micrometastases has been established in several malignancies, but there are many aspects of the micrometastases concept that have yet to be elucidated in oesophageal cancers. The challenge now is to incorporate this knowledge into management strategies that improve our understanding of the molecular biology of this disease and which may allow improved diagnosis, therapy and prognosis.

Researchers and others involved in the treatment of cancer can, I believe, take a justified pride in the fact that the disease, although still serious and often severe indeed, is no longer the killer which until comparatively recently, it very often was. Cure rates today are much higher overall than they were even 40 years ago and happily, this is particularly true in respect of cancers affecting children. The prospects for many adult cancer patients have also improved dramatically with recent advances in treatment but of course, these advances have not been fortuitous. Instead, they are the result of sustained research by dedicated scientists and medical staff who collaborate freely and

generously and without regard to national boundaries or the other "differences" which too often, in the outside world, set people apart from from one another. Cancer research is, indeed, fortunate to be undertaken by professionals whose unified effort at least promises achievement of the full potential which only prolonged fruitful collaboration in a common endeavour can deliver.

As with so many other fields of research, cancer research is extremely expensive and of its nature, it occurs over rather extended time periods. There is, therefore, an on-going necessity for research funding and that is why I would now like to conclude with an appeal for continuing, generous support of this singularly, worthy cause by both Government and public.

APPENDICES

APPENDIX 1

IMMONOHISTOCHEMISTRY

2% APES (3-aminopropyltreithoxysaline) Solution

APES	12ml
Alcohol (ethanol 95%)	600ml

Citric Acid Buffer pH = 6.0

Citric acid powder	2.1gm
Distilled Water	1L

Adjust pH with 2M NaOH.

2M NaOH

NaOH pellets	40gm
Distilled Water	500ml

Tris Buffer pH=7.6

Tris HCL	6.1gm
Tris Base	1.4gm
Distilled Water	1L

Saline

NaCl	52.56gm
Distilled Water	10L

Tris Buffer Saline

Saline	9L
Tris Buffer	1L

DAB (3'-3' diaminobenzidine tetrahydrochloride)

DAB	1gm
TBS	200ml

Aliquot into 10 ml plastic tubes and store at -20 °C.

CELL TISSUE CULTURE

Culture Cocktail

L-Glutamine	100ml
Penicillin/Streptomycin	100ml

Stored in 10 ml aliquots at -20 °C.

RPMI Complete Medium

RPMI-1640	500ml
Heat Inactivated FCS	50ml
Culture Cocktail	10ml

Hanks Balanced Salts Solution (HBSS) Washing Buffer

HBSS	500ml
Hepes Buffer (1M)	10ml

Ethidium Bromide (EB) Stock

EB	100 mg
PBS	20ml

Acridine Orange (AO) Stock

AO	20mg
PBS	20 ml

EB/AO Working Solution

EB Solution	4 ml
AO Solution	4 ml
Distilled Water	1L

Cryoprotective Medium

FCS	9ml
Dimethylsulphoxide (DMSO)	1ml

Phosphate Buffer Saline (1X PBS)

Dibasic sodium phosphate	9.1mM
Monobasic sodium phosphate	1.7mM
NaCl	150mM

Adjust pH with NaOH.

CELL PROTEIN PREPARATION

Lysis Buffer (RIPA Buffer)

Nonidate P-40	1ml
Sodium deoxycholate	100mg
SDS	500mg
PBS	75ml

Add PBS to a final volume of 100 ml. Add protease inhibitors at the time of use.

Protease Inhibitors Stock Solutions

PMSF (10mg/ml isopropanol)	10 μl/ml RIPA
Aprotinin	30 μl/ml RIPA
100 mM sodium orthovanadate	10 μl/ml RIPA

Bovine Serum Albumen (BSA) Stock Solution

BSA	50mg
PBS	50ml

Bradford Reagent

Coomassie Blue G	100mg
95% Ethanol	50ml
0.85% Phosphoric Acid	100 ml

Made up to 1 litter with distilled water.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

2x Electrophoresis Sample Buffer

Glycerol	10ml
Mercaptoethanol	0.5ml
10% SDS	3ml
1M Tris-HCl (pH 6.7)	1.25ml
Bromophenol blue	1mg

Store frozen in small aliquots. Alternatively, make buffer without mercaptoethanol and store at room temperature. Add mercaptoethanol just before using.

1.5M Tris base (Resolving Gel Buffer)

Tris base	36.3gm
Distilled Water	200ml

Add 36.33 gm tris base to 140ml D.W and adjust pH to 8.8 with conc. HCl then add distilled water for final volume of 200 ml.

1M Tris base (Stacking Gel Buffer)

Tris base	24.22gm
Distilled Water	200ml

Add 324.22 gm Tris base to 140 ml D.W adjust pH to 8.8 with conc. HCl then add distilled water for final volume of 200 ml.

Transfer Buffer

Tris base	2.9gm
Glycine	1.45gm
SDS	0.185gm
Methanol	100ml

Make up to 500 ml with distilled water.

10x Electrode Buffer (Running Buffer)

Tris base	15gm
Glycine	57gm
SDS	2.5gm
Distilled Water	500ml

Dilute 1/10 with distilled water before use.

10% APS (Ammonium persulfate)

APS	0.1gm
Distilled Water	1 ml

Water Saturated Butanol

Butanol	10ml
Distilled Water	1ml

Mix The solution well, allowed to settle and pipette aliquots from the top layer.

0.1% Coomassie Blue Solution

Coomassie Blue G	0.1gm
Distilled water	100ml

Keep solution in dark bottle.

Gel Distain Solution

Methanol	400ml
Glacial Acetic Acid	70ml
Distilled Water	530ml

Blotto/Tween Blocking Solution

Low fat skimmed milk	5gm
Tween 20	50μl
PBS	100ml

Developing Solution

Iodophenol	4mg (0.004 gm)
Luminol	12 mg (0.012 gm)
DMSO	0.5 ml
H_2O_2	18µl.
100mM Tris base pH 8.8	50 ml

Procedure

1- Mix iodophenol with 0.5ml DMSO.

2- Mix Luminol with 1ml of 100mM Tris Buffer then add the rest of buffer.

3- Add iodophenol/DMSO solution (step 1) to Buffer (step 2)

4- Add 18µl Hydrogen peroxide.

5- Incubate the PVDF Membrane for 1 minute in the developing buffer in a dark place.

DNA ELECTROPHORESIS SOLUTION

TBE (Tris Borate Buffer)

Tris base	5.4gm
Boric acid	2.8gm
0.5M EDTA	2ml
Redistilled Water	1L

Dissolve 5.4 gm tris base and boric acid in 800 ml redistilled water. Add 2ml of 0.5M EDTA solution. Stir until dissolve, adjust the pH to 8 and fill up to 1 litre with redistilled water.

Gel Loading Buffer

SDS	0.1gm
Bromophenol Blue	25mg
Glycerol	3ml
Redistilled water	7ml

Dissolve 0.1 gm SDS and 25 mg bromophenol blue in 7 ml redistilled water and add 3 ml glycerol.

APPENDIX 2

LIST OF THE PRIMARY AND SECONDARY ANTIBODIES USED FOR IMMUNOHISTOCHEMISTRY AND WESTERN BLOTTING ANALYSIS.

Immunohistochemistry

Type	Dilution	Incubation Time / Temperature
Primary Antibodies		
Mouse anti p53-DO-7* (Monoclonal)	1:50	overnight / 4°C
Mouse anti human bcl-2 (monoclonal)	1:50	overnight / 4°C
Rabbit anti human Ki-67 (polyclonal)	1:50	overnight / 4°C
Rabbit anti human bcl-x** (polyclonal)	1:75	1 hour / room temperature
Rabbit anti human bax (polyclonal)	1:75	1 hour / room temperature
Secondary Antibodies		
Biotinylated rabbit anti mouse	1:300	30 mins. / room temperature
Biotinylated swine anti rabbit	1:300	30 mins. / room temperature

*React with both wild type and mutant form. ; **React with bcl-x_S and bcl-x_L form; antibodies were diluted with Tris Buffer (pH = 7.6). Antibodies obtained for Dako.

Western Blotting

Type	Dilution	Incubation Time / Temperature
Primary Antibodies		
Mouse anti p53-DO-7* (Monoclonal)	1:1000	1 hour / room temperature
Mouse anti human bcl-2 (monoclonal)	1:1000	1 hour / room temperature
Rabbit anti human bcl-x** (polyclonal)	1:1000	1 hour / room temperature
Rabbit anti human bax (polyclonal)	1:1000	1 hour / room temperature
Secondary Antibodies		
HRP conjugate goat anti mouse	1:5000	45 mins. / room temperature
HRP conjugate goat anti rabbit	1:5000	45 mins. / room temperature

*React with both wild type and mutant form ; **React with bcl-x_S and bcl-x_L form. Antibodies were diluted with blotto-tween. HRP denotes horseradish peroxidase conjugated secondary antibody. Antibodies, except p53 as indicated above, were obtained form Santa-Cruz Biotechnology.

APPENDIX 3

GENBANK ENTRY FOR HUMAN P53 GENE ACCESSION NUMBER-U94788.

REFERENCES

Rozemuller EH, Kropveld A, Kreyveld E, Leppers FG, Scheidel KC, Slootweg PJ, Tilanus MG. Sensitive detection of p53 mutation: analysis by direct sequencing and multisequence analysis. Cancer Detect Prev 2001; **25**: 109-16.

mRNA

Join [843-949, 11689-11790, 11906-11927, 12021-12299, 13055-13238, 13320-13432, 14000-14109, 14452-14588, 14681-14754, 17572-17678, 18599-19876].

CODING SEQUENCE (STAR CODON-EXON 2 POSITION 11717→ATG)

Join [11717-11790, 11906-11927, 12021-12299, 13055-13238, 13320-13432, 14000-14109, 14452-14588, 14681-14754, 17572-17678, 18599-18680].

TRANSLATION: (393 AMINO ACIDS)

"MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQAMDDLMLSPDDIEQWF
TEDPGPDEAPRMPEAAPRVAPAPAAPTPAAPAPAPSWPLSSSVPSQKTYQGSYG
FRLGFLHSGTAKSVTCTYSPALNKMFCQLAKTCPVQLWVDSTPPPGTRVRAMAI
YKQSQHMTEVVRRCPHHERCSDSDGLAPPQHLIRVEGNLRVEYLDDRNTFRHS
VVVPYEPPEVGSDCTTIHYNYMCNSSCMGGMNRRPILTIITLEDSSGNLLGRNSF
EVRVCACPGRDRRTEEENLRKKGEPHHELPPGSTKRALPNNTSSSPQPKKKPLD
GEYFTLQIRGRERFEMFRELNEALELKDAQAGKEPGGSRAHSSHLKSKKGQSTS
RHKKLMFKTEGPDSD"

COMPLETE DNA SEQUENCE (20303 BASES)

BASE COUNT 5245 A 4967 C 5107 G 4984 T

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    1 TTCCCATCAA GCCCTAGGGC TCCTCGTGGC TGCTGGGAGT TGTAGTCTGA ACGCTTCTAT
   61 CTTGGCGAGA AGCGCCTACG CTCCCCCTAC CGAGTCCCGC GGTAATTCTT AAAGCACCTG
  121 CACCGCCCCC CCGCCGCCTG CAGAGGGCGC AGCAGGTCTT GCACCTCTTC TGCATCTCAT
  181 TCTCCAGGCT TCAGACCTGT CTCCCTCATT CAAAAAATAT TTATTATCGA GCTCTTACTT
  241 GCTACCCAGC ACTGATATAG GCACTCAGGA ATACAACAAT GAATAAGATA GTAGAAAAAT
  301 TCTATATCCT CATAAGGCTT ACGTTTCCAT GTACTGAAAG CAATGAACAA ATAAATCTTA
  361 TCAGAGTGAT AAGGGTTGTG AAGGAGATTA AATAAGATGG TGTGATATAA AGTATCTGGG
  421 AGAAAACGTT AGGGTGTGAT ATTACGGAAA GCCTTCCTAA AAAATGACAT TTTAACTGAT
  481 GAGAAGAAAG GATCCAGCTG AGAGCAAACG CAAAAGCTTT CTTCCTTCCA CCCTTCATAT
  541 TTGACACAAT GCAGGATTCC TCCAAAATGA TTTCCACCAA TTCTGCCCTC ACAGCTCTGG
  601 CTTGCAGAAT TTTCCACCCC AAAATGTTAG TATCTACGGC ACCAGGTCGG CGAGAATCCT
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 661 GACTCTGCAC CCTCCTCCCC AACTCCATTT CCTTTGCTTC CTCCGGCAGG CGGATTACTT
 721 GCCCTTACTT GTCATGGCGA CTGTCCAGCT TTGTGCCAGG AGCCTCGCAG GGGTTGATGG
 781 GATTGGGGTT TTCCCCTCCC ATGTGCTCAA GACTGGCGCT AAAAGTTTTG AGCTTCTCAA
 841 AAGTCTAGAG CCACCGTCCA GGGAGCAGGT AGCTGCTGGG CTCCGGGGAC ACTTTGCGTT
 901 CGGGCTGGGA GCGTGCTTTC CACGACGGTG ACACGCTTCC CTGGATTGGG TAAGCTCCTG
 961 ACTGAACTTG ATGAGTCCTC TCTGAGTCAC GGGCTCTCGG CTCCGTGTAT TTTCAGCTCG
1021 GGAAAATCGC TGGGGCTGGG GGTGGGGCAG TGGGGACTTA GCGAGTTTGG GGGTGAGTGG
1081 GATGGAAGCT TGGCTAGAGG GATCATCATA GGAGTTGCAT TGTTGGGAGA CCTGGGTGTA
1141 GATGATGGGG ATGTTAGGAC CATCCGAACT CAAAGTTGAA CGCCTAGGCA GAGGAGTGGA
1201 GCTTTGGGGA ACCTTGAGCC GGCCTAAAGC GTACTTCTTT GCACATCCAC CCGGTGCTGG
1261 GCGTAGGGAA TCCCTGAAAT AAAAGATGCA CAAAGCATTG AGGTCTGAGA CTTTTGGATC
1321 TCGAAACATT GAGAACTCAT AGCTGTATAT TTTAGAGCCC ATGGCATCCT AGTGAAAACT
1381 GGGGCTCCAT TCCGAAATGA TCATTTGGGG GTGATCCGGG GAGCCCAAGC TGCTAAGGTC
1441 CCACAACTTC CGGACCTTTG TCCTTCCTGG AGCGATCTTT CCAGGCAGCC CCCGGCTCCG
1501 CTAGATGGAG AAAATCCAAT TGAAGGCTGT CAGTCGTGGA AGTGAGAAGT GCTAAACCAG
1561 GGGTTTGCCC GCCAGGCCGA GGAGGACCGT CGCAATCTGA GAGGCCCGGC AGCCCTGTTA
1621 TTGTTTGGCT CCACATTTAC ATTTCTGCCT CTTGCAGCAG CATTTCCGGT TTCTTTTTGC
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1741 GCCGGTTCCT CTTACTTGGC AGAGGGAGGC TGCTATTCTC CGCCTGCATT TCTTTTTCTG
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16441 AAGTATTGGG GTTACAGGCA TGAGCTACCA CACTCAGCCC TAGCCTACTT GAAACGTGTT
16501 CAGAGCATTT AAGTTACCCT ACAGTTGGGC AAAGTCATCT AACACAAAGC CCTTTTTATA
16561 GTAATAAAAT GTTGTATATC TCATGTGATT TATTAGATAT TGTTACTAAA AGTGAGAAAC
16621 AGCATGGTTG CATGAAAGGA GGCACAGTCG AAGCCAGGCA CAGCCTGGGC GCAGAGCGAG
16681 ACTCAAAAAA AGAAAAGGCC AGGCGCACTC TCACGCCTGT AATCCCAGCA TTTCGGGAGG
16741 CTGAGGCGGG TGGATCACCT GAGGTCAGGA GTTCAAGACC AGCCTAGCCA ACATGGTGAA
16801 ACCCCGTCTC TACTAAAATA CAAAAATTAA CCGGGCGTGA TGGCAGGTGC CTGTAATCCC
16861 AGCTACTTGG GAGGCTGAGG CAGGAGAATC GCTTGAACCA GGAGGCGGAG GTTGCAGGGA
16921 GCCAAGACGG CGCCACTGCA CTCCAGCCTG GGCGATAGAG TGAGACTCCG TCTCAGAAAA
16981 AAAAGAAAAG AAACGAGGCA CAGTCGCATG CACATGTAGT CCCAGTTACT TGAGAGGCTA
17041 AGGCAGGAGG ATCTCTTGAG CCCAAGAGTT TGAGTCCAGC CTGAACAACA TAGCAAGACA
17101 TCATCTCTAA AATTTAAAAA AGGGCCGGGC ACAGTGGCTC ACACCTGTAA TCCCAGCACT
17161 TTGGGAGGTG GAGGTGGGTA GATCACCTGA CGTCAGGAGT TGGAAACCAG CCTGGCTAAC
17221 ATGGTGAAGC CCCATCTCTA CTAAAAACAC AAAAATTAGC CAGTGTGAGA CACGTTGAGT
17281 CCACGTACTC GGAGGCTGAG GCACAAGAAT CACTTGAACC CCAGAGGCGG AGATTCGAAT
17341 CAGCCAAGAT TGCACCATTG CACTCCCGCC TGGGCGACGA GAGTGAGACC CCATCTCAAA
17401 ATAAATAAAT AAATATTTTT AAAAGTCAGC TGTATAGGTA CTTGAAGTGC AGTTTCTACT
17461 AAATCGATGT TGCTTTTGAT CCGTCATAAA GTCAAACAAT TGTAACTTGA ACCATCTTTT
17521 AACTCAGGTA CTGTGTATAT ACTTACTTCT CCCCCTCCTC TGTTGCTGCA GATCCGTGGG
17581 CGTGAGCGCT TCGAGATGTT CCGAGAGCTG AATGAGGCCT TGGAACTCAA GGATGCCCAG
17641 GCTGGGAAGG AGCCAGGGGG GAGCAGGGCT CACTCCAGGT GAGTGACCTC AGCCCCTTCC
17701 TGGCCCTACT CCCCTGCCTT CCTAGGTTGG AAAGCCATAG GATTCCATTC TCATCCTGCC
17761 TTCATGGTCA AAGGCAGCTG ACCCCATCTC ATTGGGTCCC AGCCCTGCAC AGACATTTTT
17821 TTAGTCTTCC TCCGGTTGAA TCCTATAACC ACATTCTTGC CTCCACGTAG TATCCACAGA
17881 ACATCCAAAC CCAGGGACGA GTGTGGATAC TTCTTTGCCA TTCTCCGCCA ACTCCCCAGC
17941 CCAGAGCTGG AGGGTCTCAA GGGGCCTAAT AATTGTGTAA TACTGAATAC AGCCAGAGTT
18001 TCAGGTCATA TACTCAGCCC TGCCATGCAC CGGCAGGTCC TAGGTGACCC CCGTCAAACT
18061 CAGTTTCCTT ATATATAAAA TGGGGTAAGG GGGCCGGGCG CAGTGGCTCA CGAATCCCAC
18121 ACTCTGGGAG GCCAAGGCGA GTGGATCACC TGAGGTCGGG AGTTTGAGCC CAGCCTGACC
18181 AACATGGAGA AACCCCATCT CTACTAAAAA TACAAAAGTA GCCGGGCGTG GTGATGCATG
18241 CCTGTAATCC CAGCTACCTA CTCGGGAGGC TGAGGCAGGA GAATCGCTTG AACCCGGGAG
18301 GCAGAGGTTG CGGTGAGCTG AGATCTCACC ATTACACTCC AGCCTGGGCA ACAAGAGTGA
18361 AACTCCGTCT CAAAAAAGTA TAATAAAGTA AAATGGGGTA AGGGAAGATT ACGAGACTAA
18421 TACACACTAA TACTCTGAGG TGCTCAGTAA ACATATTTGC ATGGGGTGTG GCCACCATCT
18481 TGATTTGAAT TCCCGTTGTC CCAGCCTTAG GCCCTTCAAA GCATTGGTCA GGGAAAAGGG
18541 GCACAGACCC TCTCACTCAT GTGATGTCAT CTCTCCTCCC TGCTTCTGTC TCCTACAGCC
18601 ACCTGAAGTC CAAAAAGGGT CAGTCTACCT CCCGCCATAA AAAACTCATG TTCAAGACAG
18661 AAGGGCCTGA CTCAGACTGA CATTCTCCAC TTCTTGTTCC CCACTGACAG CCTCCCACCC
18721 CCATCTCTCC CTCCCCTGCC ATTTTGGGTT TTGGGTCTTT GAACCCTTGC TTGCAATAGG
18781 TGTGCGTCAG AAGCACCCAG GACTTCCATT TGCTTTGTCC CGGGGCTCCA CTGAACAAGT
18841 TGGCCTGCAC TGGTGTTTTG TTGTGGGGAG GAGGATGGGG AGTAGGACAT ACCAGCTTAG
18901 ATTTTAAGGT TTTTACTGTG AGGGATGTTT GGGAGATGTA AGAAATGTTC TTGCAGTTAA

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18961 GGGTTAGTTT ACAATCAGCC ACATTCTAGG TAGGTAGGGG CCCACTTCAC CGTACTAACC
19021 AGGGAAGCTG TCCCTCATGT TGAATTTTCT CTAACTTCAA GGCCCATATC TGTGAAATGC
19081 TGGCATTTGC ACCTACCTCA CAGAGTGCAT TGTGAGGGTT AATGAAATAA TGTACATCTG
19141 GCCTTGAAAC CACCTTTTAT TACATGGGGT CTAAAACTTG ACCCCCTTGA GGGTGCCTGT
19201 TCCCTCTCCC TCTCCCTGTT GGCTGGTGGG TTGGTAGTTT CTACAGTTGG GCAGCTGGTT
19261 AGGTAGAGGG AGTTGTCAAG TCTTGCTGGC CCAGCCAAAC CCTGTCTGAC AACCTCTTGG
19321 TCGACCTTAG TACCTAAAAG GAAATCTCAC CCCATCCCAC ACCCTGGAGG ATTTCATCTC
19381 TTGTATATGA TGATCTGGAT CCACCAAGAC TTGTTTTATG CTCAGGGTCA ATTTCTTTTT
19441 TCTTTTTTTT TTTTTTTTTT CTTTTTCTTT GAGACTGGGT CTCGCTTTGT TGCCCAGGCT
19501 GGAGTGGAGT GGCGTGATCT TGGCTTACTG CAGCCTTTGC CTCCCCGGCT CGAGCAGTCC
19561 TGCCTCAGCC TCCGGAGTAG CTGGGACCAC AGGTTCATGC CACCATGGCC AGCCAACTTT
19621 TGCATGTTTT GTAGAGATGG GGTCTCACAG TGTTGCCCAG GCTGGTCTCA AACTCCTGGG
19681 CTCAGGCGAT CCACCTGTCT CAGCCTCCCA GAGTGCTGGG ATTACAATTG TGAGCCACCA
19741 CGTGGAGCTG GAAGGGTCAA CATCTTTTAC ATTCTGCAAG CACATCTGCA TTTTCACCCC
19801 ACCCTTCCCC TCCTTCTCCC TTTTTATATC CCATTTTTAT ATCGATCTCT TATTTTACAA
19861 TAAAACTTTG CTGCCACCTG TGTGTCTGAG GGGTGAACGC CAGTGCAGGC TACTGGGGTC
19921 AGCAGGTGCA GGGGTGAGTG AGGAGGTGCT GGGAAGCAGC CACCTGAGTC TGCAATGAGT
19981 GTGGACTGGG GGGCCCAGTG CCCGGGTTCC GGGAGGGGAA CAAAGGCTGG AGACTGGGTC
20041 AGTCTGCGGG CTGCATGACA ACAAGGGAGG GGGTGGCTCC ATTCATAACT CAGGAACCAA
20101 CCGTCCCTCC TCCCCTCCGC CCACGGCTGG CACAAGGTTC TCTGCCTCCC CTGCTTCTAG
20161 GATTGGGCTG CTTCCCCCTC GGCAGCCTCT CACCAAGGAT TACGGGATTT AAATGTCGTG
20221 ATTTACGAAG GCTGAGCCTC CAGGGTGGCC ATCTTCGTCC ATCAGAAGTG GCAGGATACC
20281 TGGGTTCCAA GGGAACAGGG TGG
```

APPENDIX 4

CONTROL DNA SEQUENCE.

Partial sequence of pGEM-3Zf(+). The pGEM-3Zf(+) sequence below is the sequence of the –21 M13 forward primer, followed by the ensuing 1000 bases.

TGTAAAACGACGGCCAGT (–21 M13 primer)

```
   1 GAATTGTAAT ACGACTCACT ATAGGGCGAA TTCGAGCTCG
  41 GTACCCGGGG ATCCTCTAGA GTCGACCTGC AGGCATGCAA
  81 GCTTGAGTAT TCTATAGTGT CACCTAAATA GCTTGGCGTA
 121 ATCATGGTCA TAGCTGTTTC CTGTGTGAAA TTGTTATCCG
 161 CTCACAATTC CACACAACAT ACGAGCCGGA AGCATAAAGT
 201 GTAAAGCCTG GGGTGCCTAA TGAGTGAGCT AACTCACATT
 241 AATTGCGTTG CGCTCACTGC CCGCTTTCCA GTCGGGAAAC
 281 CTGTCGTGCC AGCTGCATTA ATGAATCGGC CAACGCGCGG
 321 GGAGAGGCGG TTTGCGTATT GGGCGCTCTT CCGCTTCCTC
 361 GCTCACTGAC TCGCTGCGCT CGGTCGTTCG GCTGCGGCGA
 401 GCGGTATCAG CTCACTCAAA GGCGGTAATA CGGTTATCCA
 441 CAGAATCAGG GGATAACGCA GGAAAGAACA TGTGAGCAAA
 481 AGGCCAGCAA AAGGCCAGGA ACCGTAAAAA GGCCGCGTTG
 521 CTGGCGTTTT TCCATAGGCT CCGCCCCCCT GACGAGCATC
 561 ACAAAAATCG ACGCTCAAGT CAGAGGTGGC GAAACCCGAC
 601 AGGACTATAA AGATACCAGG CGTTTCCCCC TGGAAGCTCC
 641 CTCGTGCGCT CTCCTGTTCC GACCCTGCCG CTTACCGGAT
 681 ACCTGTCCGC CTTTCTCCCT TCGGGAAGCG TGGCGCTTTC
 721 TCATAGCTCA CGCTGTAGGT ATCTCAGTTC GGTGTAGGTC
 761 GTTCGCTCCA AGCTGGGCTG TGTGCACGAA CCCCCCGTTC
 801 AGCCCGACCG CTGCGCCTTA TCCGGTAACT ATCGTCTTGA
 841 GTCCAACCCG GTAAGACACG ACTTATCGCC ACTGGCAGCA
 881 GCCACTGGTA ACAGGATTAG CAGAGCGAGG TATGTAGGCG
 921 GTGCTACAGA GTTCTTGAAG TGGTGGCCTA ACTACGGCTA
 961 CACTAGAAGG ACAGTATTTG GTATCTGCGC TCTGCTGAAG
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APPENDIX 5

THE STANDARD GENETIC CODE.

1st position (5' end)	2nd position				3rd position (3' end)
	U	**C**	**A**	**G**	
U	Phe	Ser	Tyr	Cys	**U**
	Phe	Ser	Tyr	Cys	**C**
	Leu	Ser	STOP	STOP	**A**
	Leu	Ser	STOP	Trp	**G**
C	Leu	Pro	His	Arg	**U**
	Leu	Pro	His	Arg	**C**
	Leu	Pro	Gln	Arg	**A**
	Leu	Pro	Gln	Arg	**G**
A	Ile	Thr	Asn	Ser	**U**
	Ile	Thr	Asn	Ser	**C**
	Ile	Thr	Lys	Arg	**A**
	Met	Thr	Lys	Arg	**G**
G	Val	Ala	Asp	Gly	**U**
	Val	Ala	Asp	Gly	**C**
	Val	Ala	Glu	Gly	**A**
	Val	Ala	Glu	Gly	**G**

Amino acids and their symbols			**Codons**					
A	Ala	Alanine	GCA	GCC	GCG	GCU		
C	Cys	Cysteine	UGC	UGU				
D	Asp	Aspartic acid	GAC	GAU				
E	Glu	Glutamic acid	GAA	GAG				
F	Phe	Phenylalanine	UUC	UUU				
G	Gly	Glycine	GGA	GGC	GGG	GGU		
H	His	Histidine	CAC	CAU				
I	Ile	Isoleucine	AUA	AUC	AUU			
K	Lys	Lysine	AAA	AAG				
L	Leu	Leucine	UUA	UUG	CUA	CUC	CUG	UCU
M	Met	Methionine	AUG*					
N	Asn	Asparagine	AAC	AAU				
P	Pro	Proline	CCA	CCC	CCG	CCU		
Q	Gln	Glutamine	CAA	CAG				
R	Arg	Arginine	AGA	AGG	CGA	CGC	CGG	CGU
S	Ser	Serine	AGC	AGU	UCA	UCC	UCG	UCU
T	The	Threonine	ACA	ACC	ACG	ACU		
V	Val	Valine	GUA	GUC	GUG	GUU		
W	Trp	Tryptophane	UGG					
Y	Tyr	Tyrosisne	UAC	UAU				

*AUG forms part of the initiation signal as well as coding for internal Met residues. Stop codons are UAA , UAG , UGA.

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