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The Study of Molecular Markers for the Progression of Barrett's Oesophagus to Adenocarcinoma, to Identify Markers that can be used as Diagnostic Tools

Cranfield Postgraduate Medical School

PhD THESIS

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Academic Year 2001 – 2002

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The Study of Molecular Markers for the Progression of Barrett's Oesophagus to Adenocarcinoma, to Identify Markers that can be used as Diagnostic Tools

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May, 2002

This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Summary

Barrett's oesophagus is a complication of gastro-oesophageal reflux disease and is the single most important predisposing factor for the development of adenocarcinoma of the oesophagus. New molecular markers are needed for early diagnosis and to monitor disease progression.

Telomerase is a ribonuclear protein with reverse transcriptase activity, which uses its own RNA component as a template for the addition of telomeric repeats to the end of chromosomes. Telomerase activity has been studied during the neoplastic progression of Barrett's oesophagus using a TRAP based ELISA technique, which found telomerase to be reactivated early during disease progression. A non-isotopic method of *in situ* hybridisation for the detection of the RNA component of telomerase has also been successfully developed.

Plasminogen activation is an inducible extracellular proteolytic system involved in the regulation of cellular interactions and invasion. The components of the urokinase-type Plasminogen Activator system have been fully investigated during the progression of Barrett's oesophagus to adenocarcinoma utilising immunohistochemistry and ELISA techniques. Changes in the expression of this invasive phenotype were found to occur late during disease progression in malignant tissues.

Two-oesophageal cell-lines have been characterised using molecular biological techniques to detect a range of molecular markers to produce *ex vivo* models of oesophageal adenocarcinoma and oesophageal squamous cell carcinoma. In order to assess the effects of bile salts and acidity on oesophageal tissues these cell-lines were then utilised as *ex vivo* models. Exposure to acidic conditions both alone and with bile salts altered the morphological appearance of the cells and disrupted adhesion molecules in the cellular membrane.

Investigations into both telomerase reactivation and the plasminogen activator system have provided new information concerning the nature and timing of molecular changes during the Barrett's metaplasia/dysplasia/ adenocarcinoma sequence.

Acknowledgements

I would like to thank my supervisors, Phil Warner, Neil Shepherd and Hugh Barr for providing me with the opportunity to work on an oesophageal cancer project and for all their support and enthusiasm over the past four years.

Thanks to the staff of Gloucestershire Royal hospital for the collection and preparation of tissues especially Richard Alison in the Histopathology laboratory and also Katherine Kendel for help in the collection and preparation of the fresh frozen samples.

I would like to thank Lizzie Bull for help, support and understanding especially during the final year and writing. I don't think I would have survived without Wednesday lunchtimes and video rentals. I thank Julien for always being much more depressed than me, Nik for entertainment in the final year and Heidi for motherly support and canine chats.

I would like to thank Jonathan Cox for his love and support during the final and most stressful year of this project. Although he missed the research stage of my PhD he was a pillar of support during my viva and while I was completing the corrections. I have to say this really was the worse year of my life so far!

Last but not least, I would like to thank my family especially Mum and Daddy for their support both financially and in every other way during the past five years. I would have quit many times without their Friday evening dinners and chats.

Dedication

For Nanny, Grampy, Mum and Daddy.

Table of Contents

List of Figures and Tables

Abbreviations

A	Absorbance
ABC	Advin-Biotin Complex
AMV	Avian Myoblastosis Virus (reverse transcriptase)
APC	Adenomatous Polyposis Coli gene/gene product
bp	Base pairs
CD44	Cluster of Differation marker 44
cDNA	Copy DNA
CEA	Carcino-embryonic antigen
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
CK	Cytokeratin (either 8 or 18)
c-Ki-ras	A type of oncogene
CLO	Columnar Lined Oesophagus
DAB	3,3-Diaminobenzine
DCA	Deoxycholic acid (Secondary bile acid)
DCC	Deleted in colorectal carcinoma gene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonuceic acid
DNP	Dinitrophenyl
dNTP	deoxynucleotide (5'-) triphosphate
DPC4	Deleted in pancreatic carcinoma 4 gene

E-Cad	Epithelial-Cadherin
EDTA	Ethylene Diaminetetra-acetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-linked immunosorbent assay
F	Forward (primers)
Fas receptor	An apoptosis-signalling surface receptor
FCS	Foetal Calf Serum
G-	Guanine-
GCA	Glycocholic acid (Primary bile acid)
GCDA	Glycochenodeoxycholic acid
GDC	Glycodeoxycholic acid (Secondary bile acid)
GLUT1	Human erythrocyte glucose transporter
GORD	Gastro-oesophageal reflux disease
H & E	Heamatoxylin and Eosin
HCl	Hydrochloric acid
hTERT	The catalytic component of human telomerase
hTR	The human RNA component of telomerase
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IM	Intestinal Metaplasia
ISH	In Situ Hybridisation

JV18-1	Human homologue of the Drosophila "mothers against decapentaplagic" (Mad) gene
kDa	Kilodaltons
Ki-67	Antigen used to indicate proliferation index, also known as MIB-1
mRNA	messenger RNA
MUC1, MUC2, MUC5AC	Types of Mucins
OE21	Oesophageal cell-line
OE33	Oesophageal cell-line
p53	53 kDa tumour suppresser protein
p63	63 kDa tumour suppresser protein also known as p51, p73L and KET
PAI-1	Plasminogen Activator Inhibitor type-1
PAI-2	Plasminogen Activator Inhibitor type-2
PBS	Phosphate Buffered Saline
P-Cad	Placental-Cadherin
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
pGRN83	Plasmid from Geron Corp containing the ISH probe for the RNA component of telomerase
pH	logarithmic measure of hydrogen ion concentration
R	Reverse (primers)
Rb	Retinoblastoma gene

RNA	Ribonucleic acid
RNAse	Ribonuclease
RT	Reverse Transcription/transcriptase
SDS	Sodium Dodecyl Sulphate
Src	Sarcoma oncogene
T25	25ml culture flask
T75	75ml culture flask
TAE	Tris-acetic acid-EDTA buffer
Taq	*T. aquaticus* DNA polymerase
TBS	Tris-buffered saline
TCA	Taurocholic acid (Primary bile acid)
TCDA	Taurochenodeoxycholic acid
TDC	Taurodeoxycholic acid (Secondary bile acid)
TFF1, TFF2	Types of Trefoil Peptides
TGF-α	Transforming Growth Factor – alpha
TGF-β1	Transforming Growth Factor – beta 1
T_M	Melting Temperature
TMB	Tetramethylbenzidine
tPA	tissue-type Plasminogen Activator
TRAP	Telomerase Repeat Amplification Protocol
Tris	Trizma base
TSR8	A synthetic oligonucleotide with 8 telomeric repeats

uPA	urokinase-type Plasminogen Activator
uPAR	urokinase-type Plasminogen Activator Receptor
UV	Ultra-Violet

Units

°C	Degrees centigrade
g	Gram
l	litre
M	molar

Nucleic acid notation

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil

1 Introduction

1.1 What is Cancer

The term cancer refers to more than 100 forms of disease. Almost every tissue throughout the whole body can spawn malignancy; some can even yield several types of tumour. Normal healthy cells within the body live in a complex interdependent way regulating one another's rates of proliferation, thus ensuring each tissue maintains a size and architecture appropriate to the body's needs. Cancer cells in contrast violate these cellular controls resulting in uncontrolled proliferation and the formation of cellular masses. A mass of cancer cells within otherwise normal tissue is called a tumour. If the tumour remains at this original size and does not invade surrounding tissues it is described as benign, whereas a malignant tumour spreads to blood vessels and other parts of the body. Tumours composed of malignant cells become more aggressive as time passes and become lethal as other tissues and organs are disrupted which are essential for the survival of the overall organism. Cancer cells may also possess the ability to migrate from the site where they initially grew, invading nearby tissues and forming masses in distant sites in the body. This spread of cancer cells beyond their original site is called metastasis (Weinberg 1996 and Campbell 1993).

The cancerous cells within a tumour are descended from a common ancestral cell that at one point many years before initiated a program of inappropriate proliferation. The malignant transformation of a cell occurs through the accumulation of mutations within two classes of genes. In normal cells these genes control the sequence of events leading to the proliferation of the cell. Proto-

oncogenes code for protein products that encourage cell growth, division and adhesion in normal cells. Mutated forms of these genes in cancer cells are known as oncogenes. Changes in the genes that inhibit cell division are also involved in the formation of cancer cells. These genes are called tumour suppresser genes, because they code for proteins that normally prevent uncontrolled cell growth. Collectively mutations in these two classes of genes account for much of the uncontrolled cell proliferation in human cancers (Weinberg 1996 and Campbell 1993).

The cells of malignant tumours are abnormal in many ways besides the lack of self-control over cell proliferation. They may have unusual numbers of chromosomes, their metabolism may be deranged, they may cease to function in a constructive way and the surface of the cells may change (Campbell 1993). The formation of a tumour occurs in stages through the accumulation of abnormalities from the single genetically altered cell through hyperplasia where the cells look normal, but proliferate too much. Later in addition to proliferating too much the cells begin to appear abnormal and are termed dysplasic. Cells that have formed a mass, but have yet to break through the tissue boundaries are described as a cancer *in situ.* Once the accumulated genetic abnormalities allow the cells to invade the underlying tissues or blood vessels a malignant tumour is present (Weinberg 1996).

1.2 Barrett's oesophagus

In 1950 Norman Barrett, an influential British surgeon, published a report in which he described the oesophagus as "that part of the foregut, distal to the pharyngeal sphincter, which is lined by squamous epithelium." (Barrett 1950). Today, an oesophagus lined with columnar epithelium rather than the normal squamous epithelium is called Barrett's oesophagus. Barrett's oesophagus is a complication of chronic gastro-oesophageal reflux disease and is the single most important predisposing factor for the development of adenocarcinoma of the oesophagus and gastric cardia. For the rest of this thesis a columnar lined oesophagus (CLO) will be refered to as Barrett's Oesophagus.

1.3 The Oesophagus

The oesophagus is a segment of the alimentary canal that conducts food from the pharynx to the stomach and is approximately 25cm in length. It is lined by stratified squamous epithelium and has a submucosa containing the oesophageal glands (Hopwood 1989). The normal oesophageal mucosa has a basal layer that comprises of proliferating cells and differentiated superficial squamous cells in the upper portion of the stratified squamous epithelium. Peristalsis squeezes a bolus of food along the narrow oesophagus. Swallowing is initially a voluntary skeletal muscle contraction followed by involuntary waves of contraction by smooth muscle (Campbell 1993).

1.4 History

Norman Barrett was not the first to describe the columnar lined oesophagus. Tileston, a pathologist reported several patients in 1906 who had "peptic ulcer of the oesophagus" and noted "the close resemblance of the mucous membrane about the ulcer to that normally found in the stomach." (Tileston 1906). Over the next few decades various other people described patients with similar peptic ulcers and gastric type columnar epithelium. In 1950 Barrett argued that the columnar lined tissue was not the oesophagus, but a tubular segment of the stomach that had been tethered in the chest by a congenitally short squamous lined oesophagus (Barrett 1950).

In 1953 Allison and Johnstone argued convincingly that the columnar lined structure Barrett had called the stomach was in fact the oesophagus. They pointed out that the structure "often contained squamous epithelium islands, had submucosal glands and a muscularis propria characteristic of the oesophagus" (Allison and Johnstone 1953). Barrett finally agreed that the columnar lined structure was in fact oesophagus and suggested the condition be called "lower oesophagus lined by columnar epithelium." (Barrett 1957).

Over the following four decades concepts regarding the criteria for the diagnosis of Barrett's oesophagus have undergone major changes and have therefore lead to confusion for both clinicians and investigators alike (Spechler 1996).

Soon after Norman Barrett's original description Morson and Belcher reported an association of adenocarcinoma with the columnar lined distal oesophagus (Morson 1952). A causal relationship between Barrett's oesophagus and

oesophageal adenocarcinoma was proposed by Alder and is now generally accepted (Alder 1963).

1.5 Epidemiology of Barrett's oesophagus

Adenocarcinoma of the oesophagus is largely a disease of white males of average age 60 years, with ongoing reflux as a risk factor (Skinner 1983 and Cameron 1997). Analysis of cancer incidence data from 1976-1987 in the United States revealed steadily rising rates of adenocarcinomas of the oesophagus and gastric cardia. The increases among men during this period exceeded those of any other type of cancer (Blot 1991). Powell and McConkey carried out a similar study in the UK, which also found the incidence of oesophageal adenocarcinoma was increasing from 0.7 to 2.0 per 100,000 between 1962 and 1981 (Powell 1990). By 1990 adenocarcinomas accounted for nearly half of all oesophageal cancers among white men (Pera 2000).

The prevalence (proportion of existing cases in a specified population at a given point in time) and incidence (number of newly diagnosed cases) of Barrett's oesophagus are hard to quantify. To estimate these values correctly endoscopic surveillance of the total population would need to be carried out. Therefore prevalence and incidence values are usually calculated for patients undergoing endoscopy for gastro-oesophageal reflux symptoms, which are common in patients with Barrett's oesophagus, but some patients will have few or no symptoms of reflux. Therefore the true number of cases could be underestimated (Cameron 1997 and Altorki 1997).

Gastro-oesophageal reflux symptoms are common. It has been reported that 4-9% of adults experience daily heartburn and 10-15% of people have heartburn at least once a week (Cameron 1997). In one study, the prevalence of Barrett's oesophagus was 12% for patients undergoing endoscopy for symptoms of gastro-oesophageal reflux (Winters 1987).

Cameron and Lomboy carried out a study between 1976 and 1989 to investigate the development of Barrett's oesophagus. It found that the prevalence of Barrett's oesophagus increases with age to reach a plateau by the seventh decade with the estimated median age of development of the disease being 40 years. Unlike prevalence it was found that the mean length of columnar epithelium did not increase with age (Cameron 1992). Evidence suggests that Barrett's oesophagus is normally present for more than 20 years before it is diagnosed and sometimes this happens only when adenocarcinoma is already present (Cameron 1997).

Patients with Barrett's oesophagus are predominantly white. The disease is rare in black and Asian populations and the ratio is estimated to be between 10:1-20:1 (Phillips 1991).

1.6 Etiology of Barrett's oesophagus

The rise in oesophageal adenocarcinoma over the short two-decade time frame from 1962 to 1981 (Powell 1990) is a strong argument for environmental factors as etiological agents, which could interact with genetic characteristics that define personal susceptibility. Because of the strong link between Barrett's oesophagus and oesophageal adenocarcinoma and the link between Barrett's oesophagus and gastro-oesophageal reflux disease, the risk factors of reflux disease have been

implemented as possible explanations for the rise in oesophageal adenocarcinomas.

Familial occurrence of Barrett's oesophagus and oesophageal adenocarcinoma occurring in successive generations has been reported. It has even been suggested there is an autosomal dominant inheritance for Barrett's oesophagus in some families (Fahmy 1993). A study of families of patients with Barrett's oesophagus, oesophageal adenocarcinoma and reflux disease was carried out in 1997 and found that there may be a genetic predisposition to the development of reflux in families of patients with Barrett's oesophagus and oesophageal adenocarcinoma. For uncomplicated reflux oesophagitis, environmental factors appear to be more important (Romero 1997).

Substantial clinical and experimental evidence supports the importance of pepsin and acid reflux in causing oesophageal mucosal damage. The oesophageal mucosa is resistant to reflux of acid alone unless it is in high concentrations, but the combination of even a small amount of pepsin with the acid causes macroscopic oesophageal injury (Vaezi 1997a).

Duodenogastro-oesophageal reflux is also suggested as a cause of Barrett's oesophagus. The duodenal contents suspected of causing oesophageal damage include bile acids, lysolecithin secreted in bile and the pancreatic enzyme trypsin. Conjugated bile acids are an important constituent of duodenal refluxate and can cause oesophageal damage at an acidic pH. Also unconjugated bile and trypsin may cause oesophageal mucosal damage in neutral pH values (Vaezi 1997a).

Experimental work using animal models has implicated a role for bile salts in the pathogenesis of Barrett's oesophagus and adenocarcinoma of the oesophagus.

Duodenogastric reflux has been shown to occur in normal individuals, especially at night. Duodenogastro-oesophageal reflux probably occurs infrequently in normal individuals, but evidence is strong that it plays a role in Barrett's oesophagus and adenocarcinoma (Marshall 1997). Mucosal damage by bile salts depends on the conjugation state of the bile acids as well as the pH of the solution. Conjugated bile acids cause damage at an acidic pH, whereas unconjugated bile acids as well as the pancreatic enzyme trypsin cause damage at a pH higher than 7 (Buttar 2001).

Cigarette smoking and alcohol have been established as risk factors for the development of squamous cell carcinoma of the oesophagus, but their role in oesophageal adenocarcinoma is less clear (Kim 1997b). Smoking and alcohol are both known to decrease the lower oesophageal spincter pressure a known cause of reflux and obesity will predispose to reflux by increasing intra-abdominal pressure. Dietary factors, particularly high fat content, can influence reflux disease and has been shown to promote the carcinogenic process in a rat model (Clarke 1994). An inverse association between fruit and vegetable consumption and oesophageal adenocarcinoma has been shown suggesting that the risk of adenocarcinoma maybe reduced through dietary interventions (Pera 2000).

1.7 Gastro-oesophageal reflux disease

Barrett's oesophagus is associated with gastro-oesophageal reflux disease; therefore many patients suffer with heartburn and sour regurgitation. Reflux disease has been defined as the response to the injurious effects of duodenal contents on the oesophageal mucosa. It is basically an inflammatory and reparative phenomenon. The reflux may consist of acid, pepsin, duodenal or

intestinal contents, bile, particularly being extremely dangerous (Thompson 1989, Vaezi 1997a).

Gastro-oesophageal reflux is a normal physiological event, but can develop into Barrett's oesophagus in patients with abnormal reflux. Obesity, cigarette smoking, hiatus hernia, alcohol abuse and some drugs amongst other things can cause this abnormal reflux (Thompson 1989). Reflux disease results from three possible abnormalities:

- incompetent lower oesophageal spincter,
- inefficient oesophageal clearance of refluxed gastric juice,
- delayed gastric emptying (Coiffi 1998).

Patients with gastro-oesophageal reflux disease can present a variety of symptoms, which can be intermittent or continuous and progressive. These symptoms include heartburn and sour regurgitation, retrosternal and epigastric pain, cervical discomfort, dysphagia, belching and hiccups, bleeding, coughing and asthma. These can lead to complications such as oesophagitis, stricture, major bleeding, Barrett's oesophagus and pharyngeal complications (Coiffi 1998).

Oesophagitis (inflammation of the oesophagus) develops when harmful substances in the refluxate come in contact with the oesophageal epithelium for a sufficient amount of time to overcome the intrinsic structural and functional defences. These defences can be categorised into three groups:

- the anti-reflux barrier, for example the high pressure zone at the gastro-oesophageal junction,
- efficient clearing of the refluxate, for example gravity, peristalsis and swallowed salivary secretions,

- epithelial defences, for example scattered submucosal glands that secrete bicarbonate (Ter 1997).

Stricture (narrowing) of the oesophagus is caused by repeated and prolonged exposure to acid reflux. This causes severe inflammatory changes, which extend through the full thickness of the wall of the oesophagus and are even found in the surrounding connective tissue of the mediastinum, causing narrowing of the oesophageal lumen (Cioffi 1998).

1.8 Histology of Barrett's oesophagus

The endoscopic appearance of the oesophageal mucosa in Barrett's oesophagus is typically a pink-red velvety type made up of three distinct types of columnar epithelium: (1) specialised columnar epithelium, (2) gastric fundic-type epithelium, and (3) junctional/cardia-type epithelium (Paull 1976, Rothery 1986, and Phillips 1991). Any one or combination of these epithelia constitutes the mucosa of Barrett's oesophagus. These epithelia are examples of metaplasia i.e. the histologic change where one adult cell is replaced by another.

Specialised columnar epithelium is the most common from of mucosa in Barrett's metaplasia (Paull 1976, Offner 1996). It has a villiform surface and crypts that resemble the intestinal mucosa, with intestinal-like goblet cells (which secrete mucus) and columnar cells (Spechler 1986). Columnar epithelium represents incomplete intestinal metaplasia, because it lacks the intestinal absorptive cells and is functionally immature (Phillips 1991).

Fundic-type epithelium resembles the mucosa of the stomach. The surface is pitted without villi and is lined by secreting cells and glands containing mucus-

secreting cells, parietal cells (which secrete hydrochloric acid) and chief cells. There are no goblet cells present (Spechler 1986, Phillips 1991).

Junctional/cardia-type epithelium resembles the mucosa of the gastric cardia. The surface is pitted and the glands are lined almost exclusively by mucus secreting cells (Spechler 1986, Phillips 1991).

These types of epithelium seem to exist as zones within Barrett's metaplasia. Specialised columnar epithelium usually appears adjacent to the normal squamous mucosa in the proximal segment of the Barrett's oesophagus, whereas junctional epithelium occurs more distally, with the fundic-type mucosa blending in with the normal gastric mucosa of the cardia (Phillips 1991). The type of epithelium and dysplasia cannot currently be identified by its endoscopic appearance.

1.9 Dysplasia

Dysplasia is defined as the abnormal development of tissue. Histologically, dysplasia is a benign neoplastic change characterised by architectural and cytological abnormalities (Antonioli 1997). Architectural changes include glandular crowding, papillary infoldings and the mucosal surface may have an exaggerated villiform conformation. Cytological changes include nuclear enlargement, large and abnormally shaped nucleoli, abnormal mitosis and cellular dedifferentiation (Antonioli 1997). Dysplasia in Barrett's oesophagus can be classified as low or high grade depending on the degree of abnormality. Many observers believe that virtually all oesophageal adenocarcinomas arise from these dysplasias.

1.9.1 Low grade

When viewed histologically under the light microscope low grade dysplasia contains nuclei which are slightly enlarged, elongated, crowded, and stratified. Mucous secretions are markedly decreased and the abnormalities extent to the mucosal surface (Haggitt 1994). Architectural changes in low grade dysplasia are minimal.

1.9.2 High grade

When viewed histologically under the light microscope high grade dysplasia contains nuclei which are more enlarged, and contain prominent nucleoli. They also show a loss of nuclear polarity i.e. the long axis of the nucleus is not oriented perpendicular to the basement membrane (Haggitt 1994). The abnormalities extend to the mucosal surface. There are extensive architectural changes in high grade dysplasia.

Adenocarcinoma in Barrett's oesophagus develops through stages of increasing severity of dysplasia (van Sandick 1998). Dysplasia is found more commonly in specialised columnar epithelium associated with adenocarcinoma compared with benign Barrett's oesophagus (Menke-Pluymers 1994).

1.10 Short-segment Barrett's oesophagus

Traditionally, Barrett's oesophagus is defined as "the replacement of the distal oesophageal lining by three or more centimetres of columnar epithelium in continuity with the gastric mucosa" (Skinner 1983). This definition however does not cover tongues or patches of columnar epithelium, which are often seen at the gastro-oesophageal junction (Stein 1993). In patients that fail to fulfil the

traditional definition, the presence of specialised intestinal epithelium at the gastro-oesophageal junction has been defined as short-segment Barrett's oesophagus (Haggitt 1994 and Nandurkar 1997).

It has been shown that dysplastic gastro-oesophageal junction nodules can be a precursor to junctional adenocarcinoma. Gastro-oesophageal junctional adenocarcinoma may occur through increasing stages of dysplasia in small foci of intestinal metaplasia or in the mucus glands of the distal oesophagus without the presence of Barrett's oesophagus (Gangarosa 1999).

1.11 Barrett's oesophagus in childhood

Barrett's oesophagus is a rare disorder in children. This could be due to the fact that Barrett's oesophagus is an acquired disorder, that results from prolonged exposure to severe gastro-oesophageal reflux. Gastro-oesophageal reflux is a common disorder and the incidence has been estimated to be 18% in the infant population (Faubion 1998). In those relatively few children with Barrett's oesophagus there appears to be a high prevalence of serious coexisting disorders such as neurologic impairment, chronic lung disease, oesophageal atresia and malignancies treated with chemotherapy (Hassall 1997). The presence of a columnar lined oesophagus in childhood is often regarded as a congenital anomaly rather than as a consequence of gastro-oesophageal reflux (Dahms 1984).

Adenocarcinoma of the oesophagus is largely a disease of white males of average age 60 years, with ongoing reflux as a risk factor (Skinner 1983 and Cameron 1997). However, adenocarcinoma of the oesophagus in Barrett's oesophagus does

occur in children and young adults (Hassall 1993). In 1993 Hassall *et al* documented 10 patients aged between 11 to 25 years who had adenocarcinoma of the oesophagus (Hassall 1993).

The clinical expression of Barrett's oesophagus in children is similar to that of adults except that strictures appear to be more common in children and the endoscopic appearance of the mucosa is not the typical pink-red velvety type seen in adults (Hassall 1985). Also there is still an unexplainable dominance of males in childhood cases of Barrett's oesophagus similar to that found in adult cases (Hassall 1985).

1.12 Oesophageal adenocarcinoma

It is estimated that 10-40% of all oesophageal cancers are associated with Barrett's oesophagus (Skinner 1983). Barrett's oesophagus is a strong risk factor for the progression to adenocarcinoma of the oesophagus (Pera 1993, Cameron 1995, 1997). Barrett's oesophagus is associated with a 30- to 125-fold increased risk of developing oesophageal adenocarcinoma (Stein 1993). This risk seems to be related to the presence of specialised intestinal type epithelium, and not to the junctional or cardia types (Skinner 1983, Hamilton 1987 and Pera 1993). In particular patients with specialised columnar metaplasia (Peters 1998), dysplasia (van Sandick 1998) or long stretch (>8cm) Barrett's oesophagus (Iftikhar 1992) are at a higher risk of developing adenocarcinoma of the oesophagus. A study carried out by van Sandick *et al* supports the theory that adenocarcinoma develops through stages of increasing severity of dysplasia (van Sandick 1998).

1.13 Barrett's oesophagus and extra-oesophageal malignancies

An increased risk of extra-oesophageal cancers in patients with Barrett's oesophagus, specifically of the head, neck, respiratory system and colon have been observed associated with smoking and alcohol abuse (Altorki 1997). A study carried out in 1985 reported an association between Barrett's oesophagus and colorectal cancer (Sontag 1985). Since this time conflicting evidence has caused controversy over this proposed link. Howden and Hornung carried out a systematic review of all the published data surrounding the association of Barrett's oesophagus and colon cancer in 1995 and concluded that patients with Barrett's oesophagus have an increased risk of colon cancer. This may be particularly true in those patients with specialised columnar epithelium (Howden and Hornung 1995).

Morgan and Vainio hypothesised that the association between colon cancer and Barrett's oesophagus could be due to the disorders sharing several environmental risk factors such as increasing age, alcohol, smoking and obesity (Morgan and Vainio 1998). Both colon cancer and Barrett's oesophagus are associated with mutations to the APC tumour suppresser gene (Zhuang 1997) and the activation of the oncogene Src (Kumble 1997).

1.14 Treatment

Therapies for Barrett's oesophagus can be classified into those that are intended to cure malignancies arising in Barrett's or those to treat the premalignant Barrett's epithelium in order to slow, stop or reverse the progression to adenocarcinoma. Therapy for Barrett's oesophagus may be divided into medical

(proton pump inhibitor therapy), surgical (oesophagogastrectomy) and endoscopic (ablation of Barrett's metaplasia by potassium-titanyl-phosphate laser) (Sharma 1998).

The impact of high-dose proton pump inhibitor therapy on reducing the length of Barrett's oesophagus has been minimal (Morales 1997). Lansoprazole the proton pump inhibitor has been found to relieve reflux symptoms and heal erosive reflux oesophagitis in Barrett's oesophagus patients (Sontag 1997). Long term treatment of Barrett's oesophagus with omeprazole has been shown to cause the reappearance of squamous epithelium within the oesophagus, but has no significant effect on the length of the columnar lined segment has been seen (Neumann 1995).

Endoscopic surgical ablation can be performed by thermal (electrosurgery or laser coagulation), chemical (photodynamic therapy) or mechanical (endoscopic mucosal resection) methods. All endoscopic surgical techniques used in combination with anti-reflux therapy result in reversal of both high grade dysplasia and Barrett's oesophagus to some extent, but some Barrett's metaplasia may remain with the risk of progressing to cancer (van der Boogert 1999).

Severe dysplasia and thin mucosal cancer of Barrett's oesophagus can be completely ablated by photodynamic therapy. This may therefore offer an alternative to oesophagectomy (Gossner 1998). A study by Berenson *et al* found that after ablation of Barrett's epithelium and suppression of acid reflux the growth of normal squamous epithelium occurs. This shows that a progenitor cell

within the metaplastic tissue has the potential to differentiate normally (Berenson 1993).

1.15 Molecular markers for cancer progression

As mentioned previously Barrett's oesophagus predisposes to malignancy, because it is associated with a risk of oesophageal adenocarcinoma up to 125-fold greater than that in the general population. Due to this risk, better cancer prevention, and early diagnosis are required. There are also ongoing discussions as to whether surveillance and screening are useful in patients with Barrett's oesophagus. Dysplasia in Barrett's oesophagus can be distinguished from adenocarcinoma using a thorough endoscopic biopsy protocol. The detection of dysplasia alone is however difficult, because it often has no distinguishing endoscopic characteristics. Detecting dysplasia in short-segment Barrett's oesophagus and adenocarcinoma is even more challenging (Souza 1997). A greater understanding of Barrett's oesophagus and associated adenocarcinoma at the molecular level could generate more sensitive and accurate molecular markers of early malignant change. For this reason, efforts to characterise the molecular events involved in cancer progression have intensified recently.

A suitable molecular marker for the progression of Barrett's oesophagus to adenocarcinoma needs to:

- have an established correlation with the carcinogenesis process,
- be measurable in all patients with Barrett's oesophagus,
- be related with other characteristics of Barrett's oesophagus,
- be effected by intervention.

Over recent years a wide range of molecular markers have been studied during the progression of Barrett's oesophagus to adenocarcinoma. Summarised below is a small selection of some of the possible molecular markers studied during the progression of Barrett's oesophagus.

1.15.1 Genetic alterations

In Barrett's epithelium a sequence of changes in DNA content have been found during the metaplasia-dysplasia-adenocarcinoma sequence beginning with an increased tetraploid (4N) fraction and followed by aneuploidy in high grade dysplasia and cancer. Since most Barrett's associated tumours have been found to be aneuploid and it is relatively easy to detect with an automated flow cytometric system its detection has been proposed as a method of screening for patients with a high risk for developing adenocarcinoma (Mueller 2000).

The genetic abnormalities studied during the progression of Barrett's oesophagus to adenocarcinoma could arise due to the increased rate of proliferation seen in Barrett's metaplasia (Krishnadath 1995).

Microsatellite analysis of Barrett's metaplasia can be used to identify chromosomal loci involved in the malignant transformation of Barrett's metaplasia to oesophageal adenocarcinoma. Allelic imbalance has been found in >45% of oesophageal adenocarcinoma on chromosome arms 3q, 4q, 5q (the APC tumour suppresser gene), 6q, 9p (p16 gene), 9q, 12p, 12q, 17p (p53 tumour suppresser gene) and 18q (DCC, DPC4 and JV18-1 genes). Allelic imbalance was also found in several cases of premalignant Barrett's oesophagus, namely, 4q, 5q, 9q, 9p, 12q, 17p and 18q. The shared novel microsatellite alleles in premalignant and malignant Barrett's epithelium are consistent with the clonal expansion

involved in the progression from Barrett's oesophagus to adenocarcinoma (Gleeson 1998). Losses of 18q and 17p occur earlier than 5q and allelic loss of both 17p and 18q in oesophageal adenocarcinoma identifies patients with poor prognosis (Wu 1998). Microsatellite analysis and familial studies of Barrett's oesophagus could uncover a genetic predisposition to adenocarcinoma (Keller 1995).

Inter-SSR PCR (Inter-Simple Sequence Repeat Polymerase Chain Reaction) is an established method for studying polymorphism between species and can be used for the detection of genetic abnormalities during cancer progression. The technique is based on the amplification of regions located between microsatellite DNA. This method has been used to study the progression of Barrett's metaplasia to adenocarcinoma by Licchesi *et al.* It is a promising technique, which will be used to enhance our knowledge of the genetics of the progression of Barrett's oesophagus (Licchesi 2002).

Y chromosome deletion is strongly and selectively associated with carcinomas, particularly adenocarcinomas of the oesophagus (Hunter 1993). This could account for the strong dominance of this disorder in males. No X chromosome abnormalities have been found in Barrett's oesophagus (Krishnadath 1995).

1.15.2 Tumour suppressor genes

Tumour suppressor genes code for proteins that inhibit cell division and are involved in apoptosis (programmed cell death). Mutations in these genes can cause the proteins to become inactivated and may therefore deprive cells of the needed restraints on proliferation (Weinberg 1996). Tumour suppresser genes

can be called recessive oncogenes, as both copies of the gene must be inactivated for their tumour suppressive affect to be lost (Souza 1997).

A wide variety of tumour suppresser genes have been implicated in the formation of tumours and many have been studied as possible molecular markers for Barrett's associated adenocarcinoma. Below is a brief summary of some of the studies carried out to analyse tumour suppresser genes in Barrett's oesophagus.

The tumour suppresser gene p53 was originally thought to play an important role in the progression of Barrett's metaplasia to adenocarcinoma and it could be an objective marker of neoplasic progression (Younes 1993). Over expression of p53 has been found to occur early in the transformation of Barrett's oesophagus to adenocarcinoma and increases with histologic progression (Kim 1997a). Recent knowledge suggests that p53 protein over expression does not predict the future development of cancer or determine disease outcome (Kubba 1999). Some patients with Barrett's oesophagus and oesophageal adenocarcinoma can develop p53 antibodies that may predate the clinical diagnosis of cancer (Cawley 1998). This is not predictive as cancer does not always develop from this situation.

Another tumour suppresser gene implicated in Barrett's oesophagus associated adenocarcinoma is retinoblastoma. Altered retinoblastoma mRNA was demonstrated in dysplastic and adenocarcinoma Barrett's biopsies (Huang 1992). It has also been shown that the accumulation of abnormal retinoblastoma protein during the progression of Barrett's metaplasia to adenocarcinoma leads to unsuppressed tumour growth (Coppola 1999). The p16 protein inhibits cell proliferation by preventing inactivation of the retinoblastoma gene. Inactivation

of p16 has been found to be a late event in the progression to Barrett's adenocarcinoma (Meuller 2000).

The adenomatous polyposis coli (APC) gene is found on chromosome 5q and is a key tumour suppresser gene in the early stages of carcinogenesis of colorectal cancer. In Barrett's oesophagus the situation is different most studies show APC changes as a latter event during progression to adenocarcinoma (Mueller 2000).

1.15.3 Cell proliferation

Uncoordinated cell proliferation is a hallmark of neoplasic diseases including adenocarcinoma of the oesophagus. Useful markers of cell proliferation in Barrett's metaplasia and dysplasia are proliferating cell nuclear antigen (PCNA) and Ki-67 (also referred to as MIB-1), that can be detected immunohistochemically with nuclear staining. PCNA is an indicator of cell cycle progression at the G_1/S transition and Ki-67 can detect changes in the G_1/S and the G_2/M cell cycle transitions (Rustgi 1997). Quantitative measuring of the proliferation zone as marked by Ki-67 staining has been advocated as a useful marker for the differentiation between low and high grade dysplasia (Hong 1995).

PCNA is an auxiliary protein in DNA synthase found in cells in the S phase of the cell cycle and is used as a marker of cell proliferation. PCNA (Jankowski 1991) and Ki-67 (Koya 1996) have shown a positive correlation between cell proliferation and increasing degrees of dysplasia in Barrett's oesophagus.

There is a significant increase in apoptotic activity in intestinal-type Barrett's metaplasia when compared with gastric-type. There is also a significant increase

in the glandular proliferation to apoptosis ratio in the progression of metaplasia to dysplasia to adenocarcinoma. Therefore the shift could be a useful and sensitive marker of neoplastic change in Barrett's oesophagus (Whittles 1999).

The Fas receptor is an apoptosis-signaling surface receptor that is able to trigger programmed cell death. Fas over-expression may represent a cellular attempt to balance the uncontrolled tumour proliferation by promoting apoptosis (Coppola 1999). An abnormality in Fas expression was observed in Barrett's metaplasia in which immunohistochemical staining of Fas at the cell surface was reduced or absent despite a high level of Fas mRNA (Hughes 1998).

1.15.4 Oncogenes

Genes known as proto-oncogenes code for proteins that stimulate cell division; mutated forms called oncogenes can cause the stimulatory proteins to be over-expressed and result in cells proliferating excessively (Weinberg 1996). Oncogenes are genes whose product may be involved in the processes leading to the transformation of a normal cell into a malignant state. Oncogene products maybe growth factors, growth factor receptors or molecules that transmit the receptor signals within the cell. They are usually activated during embryogenesis and are suppressed in mature cells, if this suppression is damaged reactivation of the genes can occur stimulating increased proliferation.

Relatively few studies of oncogene abnormalities have been carried out in Barrett's metaplasia. Cyclin-D1 has oncogenic properties i.e. once it is over expressed it results in excessive cell proliferation. There is regular over expression of cyclin-D1 in oesophageal squamous cell carcinomas, and this has

also been shown in Barrett's oesophagus and oesophageal adenocarcinoma (Rustgi 1997).

The oncogene Src is activated in Barrett's oesophagus before the development of dysplasia or adenocarcinoma (Kumble 1997). Mutations of the oncogene c-Ki-ras have not been found in Barrett's oesophagus, therefore the c-Ki-ras gene is not thought to be involved in the progression of Barrett's oesophagus to adenocarcinoma (Lagorce-Pages 1995).

1.15.5 Growth factors

Generally chronic inflammation is assumed to predispose to cancer development. Oesophageal adenocarcinoma is no exception due to the strong links with chronic gastro-oesophageal reflux disease. When this inflammation occurs there is increased production of growth stimuli (Souza 1997). There are various different families of growth factors, each of which has many members. An important family of growth factors is the one that binds to the epidermal growth factor receptor (EGFR). This includes epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin, heregulin and cripto. The binding of these substances to EGFR induces a signal transduction pathway within the cell. Expression of EGFR has been shown to increase in some patients with Barrett's oesophagus and oesophageal adenocarcinoma (Rustgi 1997). In addition both TGF-α and EGF are over expressed when measured by immunohistochemistry in Barrett's metaplasia (Souza 1997).

1.15.6 Regulatory proteins

Telomerase is a ribonuclear protein reverse transcriptase that uses its own RNA component as a template for the addition of telomeric sequences to the end of chromosomes, therefore maintaining telomere length. Telomerase activity is present in most primary human cancers, but not in normal somatic tissue except for proliferative cells of renewal tissue (e.g. crypts of the intestine) (Shay 1997b).

Relatively few studies have assessed telomerase activity in Barrett's oesophagus. *In situ* hybridisation has been used to look at telomerase RNA expression in Barrett's oesophagus. This showed low levels in basal cells of squamous mucosa and low to moderate levels in the basal crypts of intestinal type Barrett's metaplasia. This concluded expression of telomerase increased with the progression of Barrett's oesophagus to adenocarcinoma in most cases (Morales 1998). This study was limited as very few samples were assesed. Telomerase and cancer will be covered in detail in section 1.16.

The specialised columnar cells found in Barrett's oesophagus have been found to express the intestinal disaccharide sucrase-isomaltase and are therefore phenotypically similar to intestinal epithelium (Wu 1993). The brush border-associated hydrolyase, aminopeptidase-N has been detected by immunohistochemistry in 84% of Barrett's biopsy specimens (Moore 1994).

The extracellular matrix and basement membrane of cells are physical barriers to migration and invasion. Cancer cells must overcome these barriers in order to invade and metastasis. Localised proteolysis is an important mechanism regulating cellular interactions. Plasminogen activation is a significant inducible extracellular proteolytic system involved in the regulation of cellular

interactions. So far no systematic study of plasminogen activation in metaplastic or dysplastic Barrett's epithelium has been carried out. A study into oesophageal adenocarcinomas that have been completely resected has shown that a high level of urokinase-type Plasminogen Activator was found to be an independent prognostic indicator of subsequent poor survival (Nekarda 1998). The Plasminogen Activator system and cancer are covered fully in section 1.18.

1.15.7 Adhesion molecules

Adhesion molecules are known to be involved in the attachment of epithelial cells to each other and to the extracellular matrix. The processes of invasion and metastasis are central to the development of cancer and are closely associated with the reduction or loss of cell adhesion molecules.

The expression of cell surface glycoproteins encoded by the CD44 variant exons have been shown to be associated with poor prognosis in malignancies. CD44 and v6 are frequently expressed in Barrett's oesophagus. It has been shown by the pattern of expression that CD44 and CD44v6 may be involved in the carcinogenesis of Barrett's. Expression of CD44v6 in Barrett's oesophagus associated adenocarcinoma is correlated to the aggressive pathological features of the disorder (Lagorce-Pages 1998). A study by Bottger *et al* found CD44v4 was an independent prognostic parameter for adenocarcinoma of the oesophagus that can be determined preoperatively by biopsy (Bottger 1998). The draw back of this study was that only a small number of patient samples were analysed.

Epithelial cadherin (E-cad) is the primary regulator of cell adhesion in epithelial tissues. Cadherin function is regulated in part by interactions with cytoplasmic proteins called catenins. There is a reduction in the expression of the E-

cadherin/catenin complexes in Barrett's oesophagus biopsy specimens during the progression to adenocarcinoma, therefore loss or reduced E-cadherin expression may play a role in the progression of Barrett's oesophagus to adenocarcinoma (Swami 1995, Washington 1998). A significant reduction in the expression of E-cadherin was shown by Bailey *et al* to occur during the metaplasia-dysplasia-adenocarcinoma sequence. P-cadherin expression was absent in Barrett's oesophagus, but was present in some adenocarcinomas. There was also reduced expression of catenins (Bailey 1998).

Carcinoembyonic antigen (CEA) is another adhesion molecule. It is a complex glycoprotein comprising of 60% carbohydrate. It is found in adenocarcinomas of endodermally derived epithelia of the digestive system and in foetal colon. CEA immunoassay is useful in the diagnosis and monitoring of cancer patients for recurrent disease or response to therapy, particularly in colonic cancer (Oikawa 1987). The presence of CEA has been established by histochemistry by Ferrando *et al* and was found in 7 of 9 Barrett's cases and 100% of dysplasia and adenocarcinoma samples tested (Ferrando 1998).

The preliminary results of a study by Bottger *et al* did not show that the expression of the adhesion molecule β1 integrin was of prognostic value in patients with adenocarcinoma of Barrett's oesophagus, although further work needs to be carried out (Bottger 1999).

1.15.8 Mucins and peptides

Mucin is a glycoprotein and the main constituent of mucus. The expression of three types of mucins (MUC1, MUC2, and MUC5AC) have been analysed in Barrett's metaplasia and oesophageal adenocarcinoma along with two trefoil

peptides (TFF1 and TFF2). Trefoil peptides are a group of small secretory peptides bearing one or more trefoil structural motifs. Barrett's metaplasia was found to be negative for MUC2 and TFF2. TFF1 and MUC5AC were found in all the Barrett's samples and MUC1 in 90% of the Barrett's samples (Labouvie 1999).

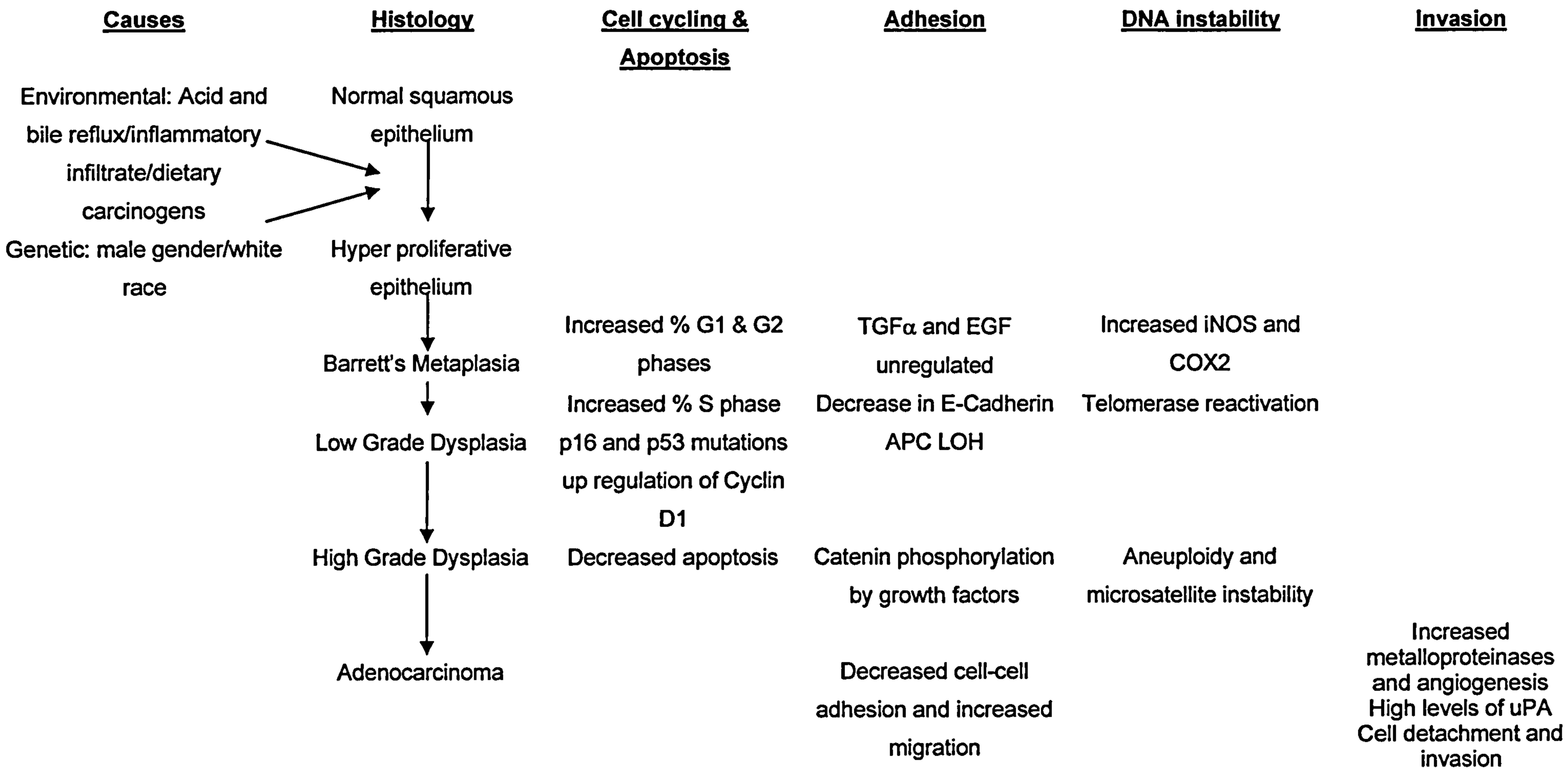
Villin is an actin binding cytoskeletal protein found only in the small intestine and renal cell lineages. Recent studies have shown villin to be highly expressed in 100% of intestinal type Barrett's metaplasia (MacLennan 1999).

Cytokeratins (CK) are a group of cytoskeletal structural proteins present in all epithelial tissue. In human epithelial cells 20 cytokeratins have been identified to date (Salo 1996). Various studies have been carried out to analyse the cytokeratins present in Barrett's metaplasia (Salo 1996, Boch 1997, and Ormsby 1999). CK13 is characteristic of squamous epithelium including oesophagus. Simple columnar epithelium, such as Barrett's epithelium, express mainly CK8, 18 and 19. The presence of CK 13 in Barrett's metaplasia indicates its origin is from squamous epithelium and is not caused by the migration of columnar cells of the gastric cardia (Salo 1996). CK7 is a marker of ductal differentiation and is not expressed in the normal epithelium of the oesophagus. CK20 is a marker of intestinal differentiation and is expressed in the surface and crypt epithelium of the colon and small intestine. Ormsby *et al* found that cytokeratin 7 and 20 could reliably identify intestinal metaplasia in the oesophagus and stomach (Ormsby 1999). Barrett's adenocarcinoma has been found to express CK8, 18 and 19 in high amounts (Moll 1982).

The human erythrocyte glucose transporter (GLUT1) is expressed in adenocarcinoma of Barrett's oesophagus, but not in dysplasia or metaplasia. GLUT1 is expressed as a late event in the neoplastic change (Younes 1997).

A wide variety of molecular changes have been studied increasing our understanding of the molecular biology during the metaplasia-dysplasia-adenocarcinoma sequence of Barrett's Oesophagus. Although a few of these changes appear quite early along the pathway the majority are late events and are only found when high grade dysplasia or adenocarcinoma are evident (See figure 1.1). This means the systematic endoscopic biopsy program for the detection of dysplasia remains the "gold standard" to identify patients at high risk for development of adenocarcinoma. In the future identification of early molecular changes will lead to a more accurate risk assessment for the patient and may also provide targets for intervention to prevent development of dysplasia and adenocarcinoma.

Figure 1.1. Pathway of events in the progression of Barrett's oesophagus to adenocarcinoma in relation to molecular changes within the cells.



1.16 Telomerase

1.16.1 Telomeres

Telomeres are specialised structures found at the ends of chromosomes. They appear to function in chromosome protection, positioning and replication. In vertebrates telomeres consist of hundreds to thousands of tandem repeats of the sequence TTAGGG (Feng 1995). In humans telomeres are made up of an average of 5000-15,000 base pairs of telomeric repeats and telomere binding proteins. The exact number and combination of telomere repeats varies slightly from species to species, but their function is the same,

- they form specific complexes with telomere-binding proteins,
- they protect chromosome ends from exonuclease digestion,
- they prevent aberrant recombination, and
- they prevent chromosome ends from activating checkpoint controls that sense DNA damage (McKenzie 1999).

Chromosomes lacking telomeres can suffer fusion, degradation and extremely high loss rates (Blackburn 1997). Despite the importance of telomeres there is little information of the mechanism by which these tandem repeated DNA base pairs ensure short-term chromosome stability or even at what stage in the cell cycle their job is performed (Blackburn 1997).

1.16.2 The chromosome end-replication problem

DNA replication can be defined as the process by which one parent DNA strand is copied to produce two identical daughter strands. The so-called end-replication problem arises from the inability of standard chromosome replication

mechanisms to do a complete job. When DNA polymerase copies the original DNA strands in a chromosome they leave each of the new daughter strands with a shortened tip at the 5' end. The replication process causes the loss of 50-100 base pairs (see figure 1.2.). If cells did not compensate for this problem in the replicative mechanism chromosomes would shrink and there would be loss of important pieces of coding DNA (Greider 1996 and Cech 1997). Cells have evolved a number of systems to overcome this loss including complex recombination and retrotransposition schemes. The problem is overcome initially by the presence of tandem repeats, telomeres, at the end of chromosomes. Part of the telomere will be missed during the DNA replication mechanism and lost in the daughter strands without being detrimental to the cell. Therefore the cell can divide a certain predisposed number of times before the telomeres are completely lost and the cell reaches crisis (Lange 1995 and Cech 1997). In higher eukaryotes this end-replication problem is overcome by a specialised reverse-transcriptase enzyme, telomerase (see figure 1.3.).

The existence of telomerase was proposed in the early 1980's as a possible solution to this end-replication problem (Greider 1996). One scheme for how telomerase solves the end-replication problem proposes that the enzyme adds DNA to chromosomes before replication begins. The added DNA consists of one or more telomeric subunits. This addition ensures the daughter strand will be at least as long as its parent strand (Greider 1996). The telomerase process maintains a dynamic equilibrium and prevents chromosomes from shortening to a critical length and prevents cells from receiving the signal to stop dividing.

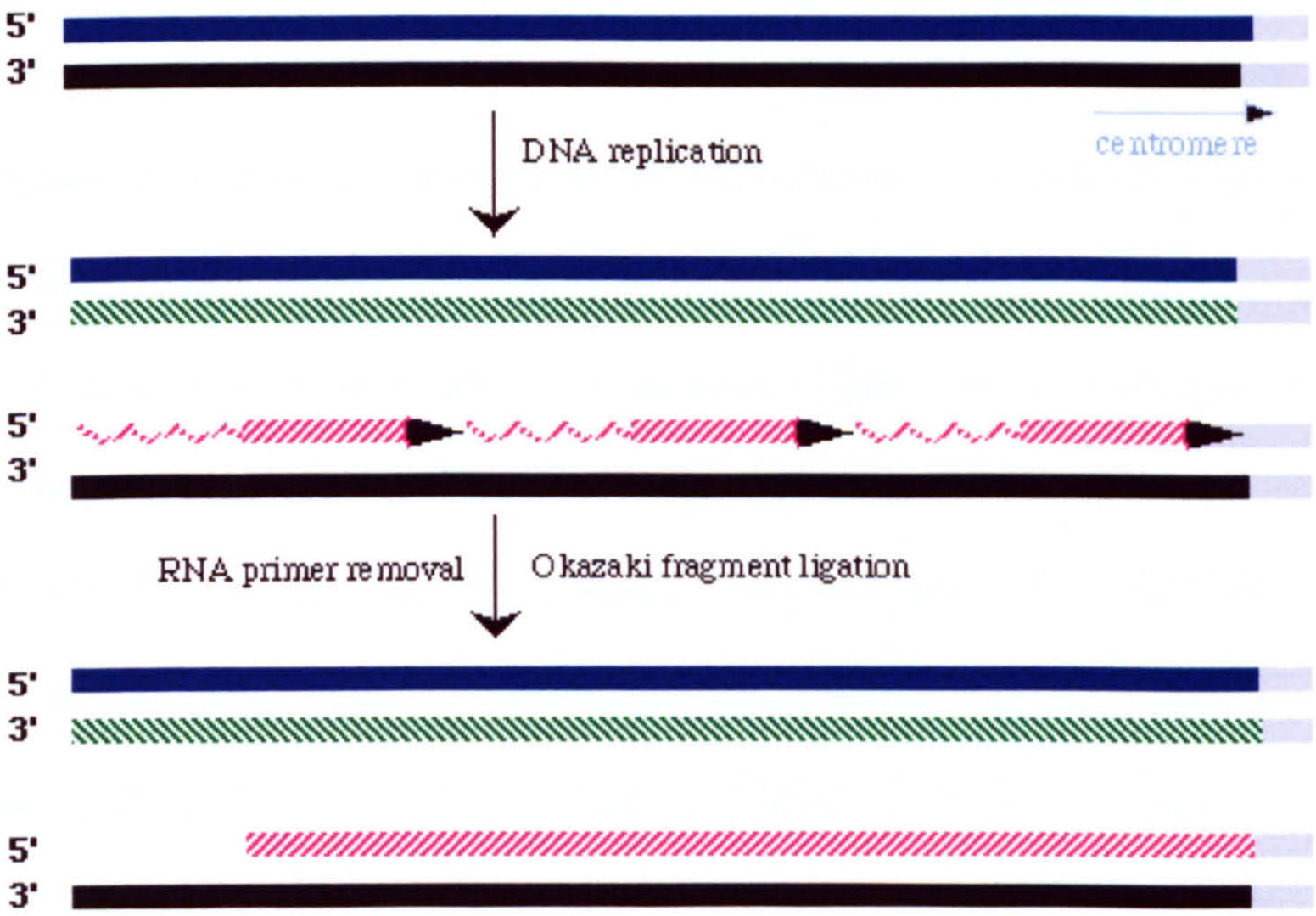


Figure 1.2. The end replication problem.

During DNA replication the 5' section of DNA under the RNA primer is not copied and this short section of DNA is lost. This is over come by the telomeres at the end of chromosomes (Grieder 1996).

1.16.3 Telomerase

Telomerase is a ribonuclear protein complex with a reverse transcriptase activity that uses its own RNA component as a template for the addition of the tandem telomeric sequences to the end of chromosomes, therefore maintaining telomere length. The telomerase protein complex contains several protein subunits including, the human RNA component (hTR) which contains a domain that is complementary to the one hexameric unit of the DNA telomeric repeat sequence, TTAGGG, and the catalytic subunit of human telomerase (hTERT) is homologous to reverse trancriptases. Therefore the enzyme complex contains both template and polymerase activity (McKenzie 1999). Telomerase binds to the 3' ends of DNA strands and extends them by copying it's own RNA template in multiples of the hexamer repeat sequence (see figure 1.3).

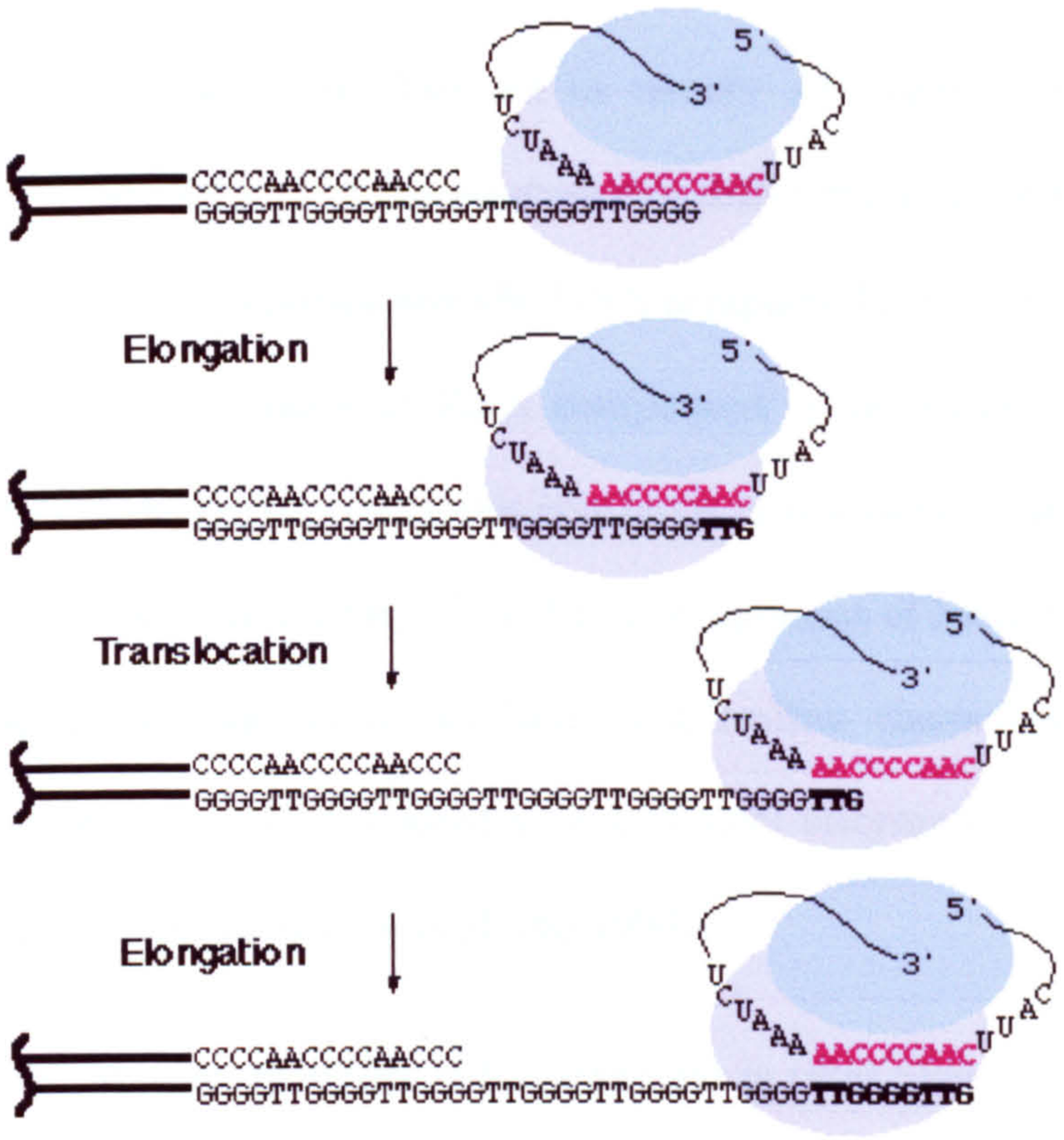


Figure 1.3. How telomerase overcomes the end replication problem by transcribing telomeres at the end of chromosomes.

Initially telomerase binds to the telomere and elongates the DNA adding telomeric repeats. Next the telomerase translocates, binds again to the newly transcribed telomeric repeat and elongates again (Greider 1996).

Telomerase is synthesised in nearly all organisms with nucleated cells. The precise makeup of the enzyme can differ from species to species, but each version possesses a species-specific RNA template for building telomeric repeats (Greider 1996). The essential RNA component of telomerase contains a 9-30 nucleotide template that dictates the synthesis of telomeric repeats onto DNA *in vivo* and *in vitro* (Autexier 1996). The RNA component of human telomerase has been cloned and the expression studied in germline tissues and tumour cell lines. This showed human telomerase is a critical enzyme for the long-term proliferation of immortal tumour cells (Feng 1995).

Telomerase in single cell organisms is vital because the organisms are immortal and barring accidents and genetic upsets they can divide indefinitely and the species can survive (Greider 1996).

From experiments analysing telomere length and telomerase activity in immortalised and cancer cells in culture, a simple model has emerged to describe the role of telomerase in mammalian cells:

1) Telomerase is present in germline tissues where telomere length is maintained,
2) Telomerase is not present in normal somatic cells where telomere shortening occurs,
3) During the extended lifespan, telomeres continue to shorten and the cells enter crisis,
4) After crisis, cells with activated telomerase are selected and telomere length is stabilised in the resulting culture (Autexier 1996).

See figure 1.4 for a graphical representation.

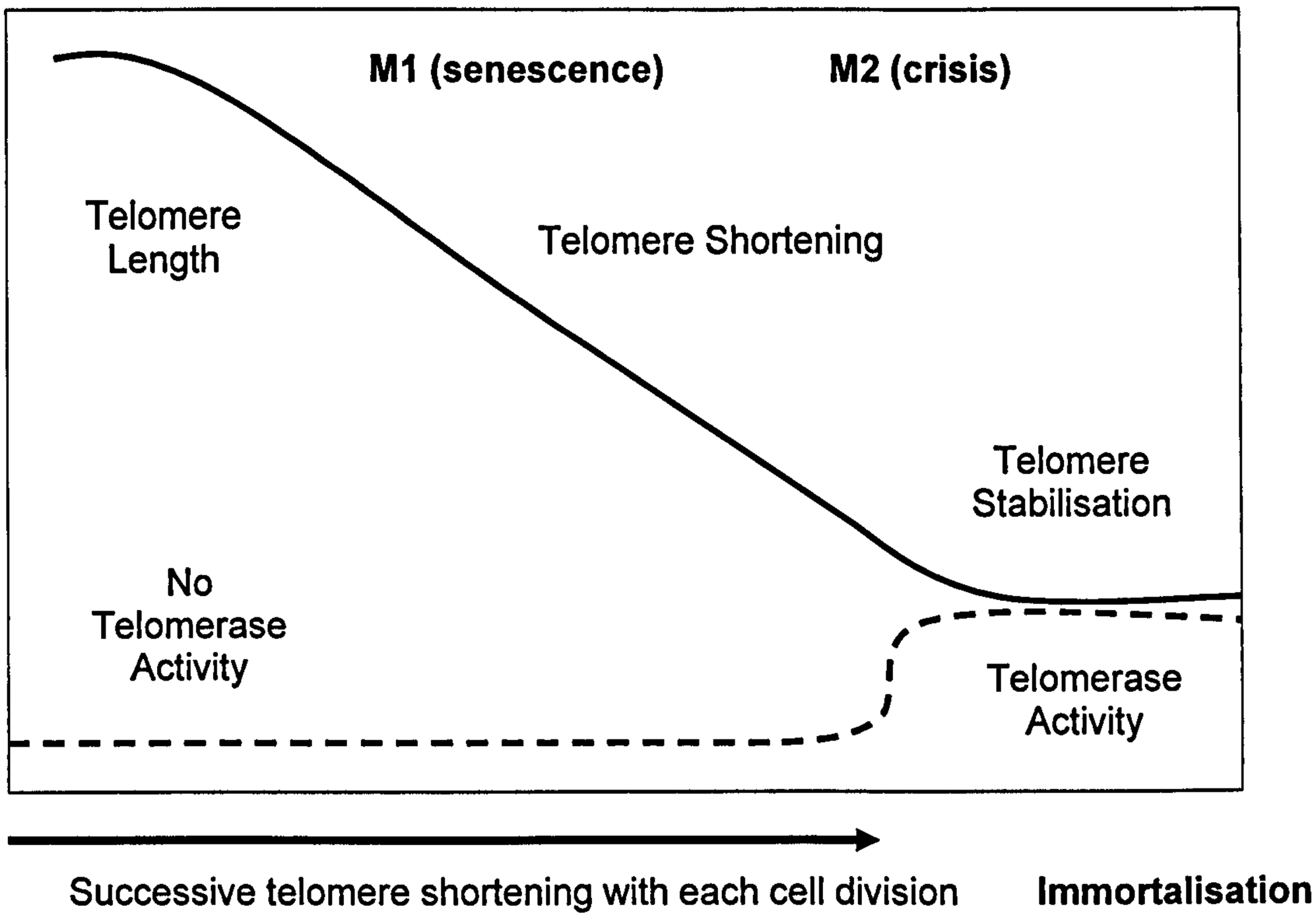


Figure 1.4. Activation of Telomerase Stabilises Telomere Length and leads to immortality in cells.

If telomerase regulation in human tumours is similar to this cell culture model, telomerase could be an important new target for anti-cancer therapy.

1.16.4 Telomerase and Ageing

Telomerase expression is repressed early in development in all somatic tissues (Harley 1997). Telomerase is active in germline cells, maintaining long stable telomeres, but is repressed in most somatic cells resulting in telomere loss during division.

The shortening and loss of telomeres may explain the limited reproductive lifespan of normal diploid cells in culture. The hypothesis is that one or more of the shortened telomeres will generate a specific signal or evoke a DNA damage checkpoint, in either case causing the cell to leave the cell cycle irreversibly (Shall 1997). Somatic cells from human new-borns will usually divide 80-90 times in culture, whereas cells from a 70-year old are only likely to divide 20-30 times (Autexier 1996).

1.16.5 Telomerase Detection

Telomerase can be detected in various ways. Northern blot and reverse transcripase PCR (RT-PCR) allow the expression of mRNA to be studied but require the degradation of the tissues. *In Situ* Hybridisation has the advantage of detecting mRNA within the cells. The protein can be detected using specific antibodies either by immunoblotting or immunohistochemistry. The most popular method developed to date to assay for telomerase activity in small samples of human tissues is a sensitive PCR-based assay (Kim 1994). This assay is called the Telomeric Repeat Amplification Protocol (TRAP). TRAP relies on the fact that telomerase will elongate a G-rich primer non-telomeric sequence. The

method comprises of two steps initially a G-rich oligonucleotide is added to cell lysates as a substrate primer and if telomerase is present it adds TTAGGG repeats to the primer. After the initial telomerase reaction the telomerase products are specifically amplified using a PCR step with non-telomeric sequence and $(CCCTTA)_3$ primers (Kim 1994, Greider 1995 and McKenzie 1999). This method allows for the detection of telomerase activity and can be measured in as little as 12 cells. The simplicity and increased sensitivity of this assay has resulted in an increase in the study of telomerase expression and activity in many cancers and normal tissues as long as they are fresh or freshly frozen samples. Using the TRAP method telomerase activity can be measured in various clinical specimens such as: frozen sections and fine needle aspirates, normal secretions, pathological fluids, aspirates and brushes/washes. Fixing tissues inactivates the enzyme making many banked cancer and tissue samples unavailable for TRAP analysis.

The TRAP method has been further developed and combined with an ELISA detection system instead of a gel for a rapid, easy and reliable telomerase detection. Two kits are currently available commercially, the TRAPEZE™ ELISA Telomerase Detection Kit (Intergen Company, Oxford, UK) and the Telomerase PCR ELISA (Roche Molecular Biochemicals, Lewes, UK). The principles of the TRAPEZE™ kit are described below, as that is the method used for the telomerase detection in this study.

The method combines a one-step TRAP assay with direct non-isotopic, chromogenic detection of the TRAP product through ELISA. In the first step telomerase adds a varied number of telomeric repeats onto the 3' end of a

biotinylated oligonucleotide substrate (See figure 1.5). In step two these extension products are amplified using PCR with a deoxynucleotide mix containing dCTP labelled with dinitrophenyl (DNP). The ELISA step involves the immobilisation of the biotinylated; DNP-labelled TRAP products to a streptavidin coated microtitre plate followed by detection using anti-DNP-peroxidase antibody and tetramethylbenzidine (TMB) substrate.

More recently the cloning of the RNA component and the catalytic subunit of telomerase have opened up new avenues in the analysis and the detection of telomerase-RNA and telomerase–protein components. This will allow for the analysis of fixed tissues for the hTERT protein immunohistochemically, but the results may not mirror those for detectable telomerase activity (McKenzie 1999). The protein may be present in the cells, but not in the active form. hTERT has also been detected using RT-PCR in a variety of tissues. Analysis of hTERT by *in situ* hybridisation has been applied to both normal and tumour tissues and correlates well with telomerase activity detected with the TRAP assay.

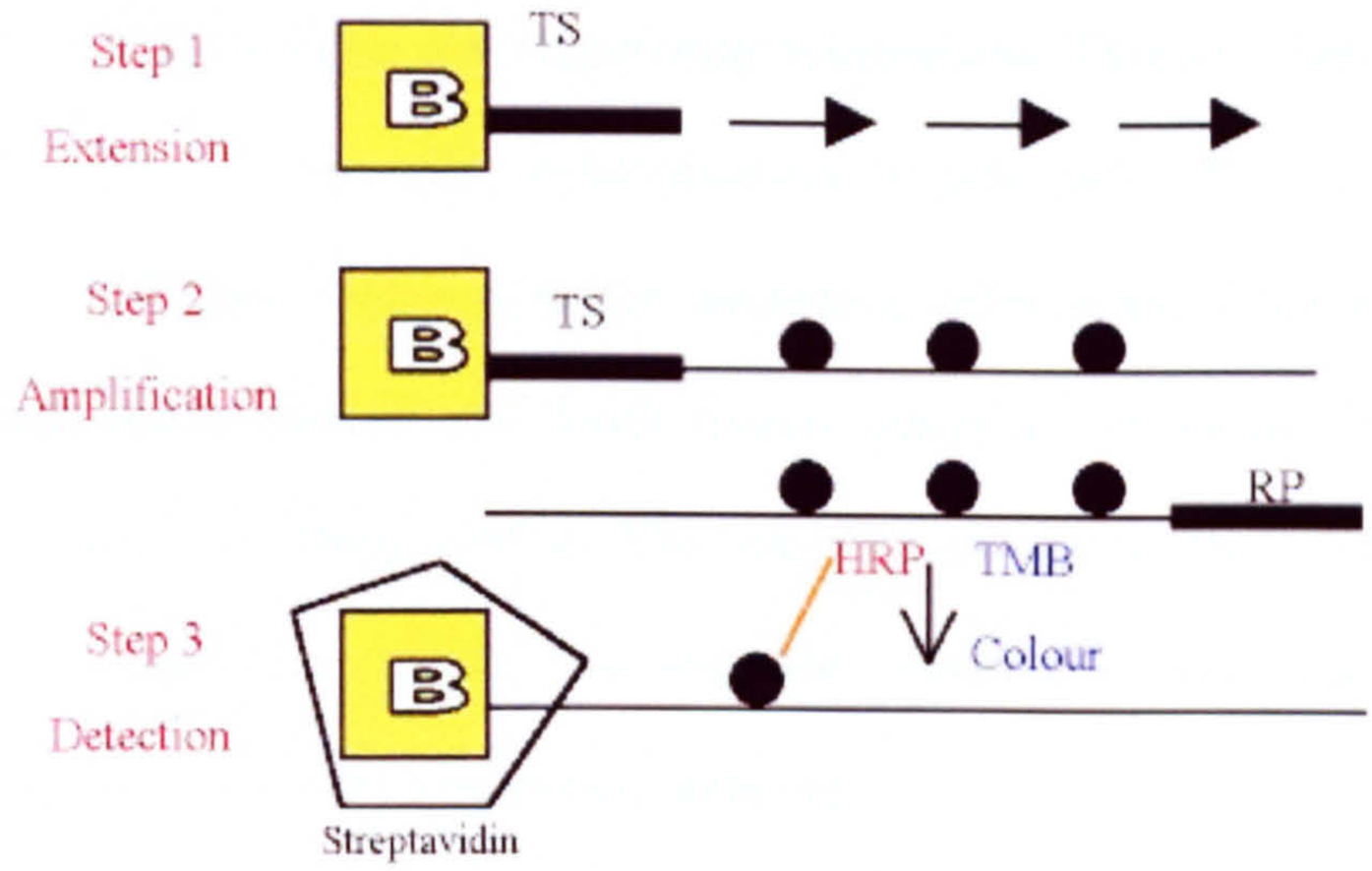


Figure 1.5. Principles of the TRAPEZE™ ELISA Telomerase Detection Kit.

Abbreviations: B – Biotinylated label, TS – oligonucleotide substrate, RP – Reverse PCR primer, HRP – anti-DNP-peroxidase antibody and TMB – tetramethylbenzidine substrate (colour).

The main disadvantage of using the TRAP method for analysing telomerase activity in clinical tissue samples is there is no way of telling exactly which cells within the sample are expressing telomerase. This problem can be overcome with the use of *in situ* hybridisation techniques. The application of *in situ* hybridisation techniques for detecting telomerase RNA expression in archival paraffin-embedded and fresh frozen material can easily distinguish cancer from normal cells (Shay 1997a). The use of *in situ* hybridisation for the detection of the RNA template within the enzyme complex should allow the localisation of specific cells with telomerase activity.

The RNA component of telomerase can be detected with a variety of different methods. Initially studies using northern blotting of mRNA were disappointing as these indicated that expression levels did not correlate with telomerase activity. When RT-PCR was used the molecule was detected in tumour tissues as well as normal tissues although higher levels were found in tumour tissues. RT-PCR detects telomerase mRNA and so does not give information on whether the active molecule is in the tissues. It only gives information about gene expression. In some circumstances analysis of the RNA component of telomerase in paraffin embedded sections correlates with telomerase activity (Matthews 2001).

1.16.6 Telomeres, Telomerase and Cancer

During the progression from normal tissue to cancer telomeres pass through four distinct phases:

1) Telomeres are in decline, but still sufficiently long to protect chromosome ends

2) Loss of telomere function occurs on one or a few chromosome ends. The resulting ends arrest cell proliferation and/or induce apoptosis.

3) Cells continue to lose telomeric DNA and enter a period of increased genome instability with high rates of loss of heterozygosity and gene amplification.

4) The harmful effects of generalised telomere loss may eventually provide a selective advantage to cells that have restored telomere function through the increased expression of telomerase. In the resulting growth phase tumour telomeres are functionally restored, although not necessarily long.

Most tumours may not be clinically detectable until they reach phase 4 (Kipling 1995).

Telomerase activity is present in most (approximately 85%) of primary human cancers, but not in normal somatic tissue, except for the proliferative cells of renewal tissue (e.g. crypts of the intestine) (Shay 1997b and McKenzie 1999). Telomerase activity and expression has been studied in various human cancers including bladder, prostate, breast, neck, lung, colon, pancreas, stomach, liver and ovary. The time of activation of telomerase during the progression of cancer varies from cancer to cancer; for example, telomerase is activated at an early stage in the pathogenesis of breast, neck, lung and skin cancers and late in the pathogenesis of colon, pancreas and thyroid cancers. There is insufficient or conflicting data for the time of activation of telomerase in renal, ovary, prostate, stomach and oesophagus cancers (Shay 1997a). In patients with oesophageal carcinoma the measurement of telomerase in normal epithelium has been found to be highly sensitive method of detecting the microinvasion of cancer cells (Koyanagi 1999).

Although tumours have different expression compared to normal tissues of the same type the results can be confused by the presence of telomerase activity in certain benign lesions. Usually measuring the presence or absence of telomerase is enough, but in some cancers, such as lymphomas a more quantitative analysis is needed.

A number of studies have compared the length of telomeres in human tumours to those in non-tumour tissue from the same organ. It has been shown that some tumours such as colorectal and renal cell carcinoma have significantly shortened telomeres compared to normal colon and renal tissue and the shortened length is maintained during further divisions (Kipling 1995).

Telomerase activity may prove to have another advantage over other histological techniques in the diagnosis of cancer. Telomerase activity can be assayed in cells that are extracted by less invasive methods than surgery. For example oral rinses in head and neck cancer, urine in bladder cancer, pap smear in cervical cancer and pancreatic juice in pancreas cancer (McKenzie 1999). The disadvantage could be that the tumours are made up of many cell types and only a small proportion of them may be expressing telomerase.

The analysis of telomerase may provide insights into two general stages of cancer progression. Firstly telomerase might be useful in identifying pre-cancerous lesions that might progress to cancer, and secondly telomerase analysis may be useful for predicting the clinical course of the patient once cancer has been diagnosed. Both these areas are under intense investigation at the moment in breast and prostate cancer (McKenzie 1999).

Once the mechanism and requirements of telomerase activation and activity are understood fully telomerase should be a viable tool in the diagnosis and treatment of cancer.

1.16.7 Telomerase and Barrett's oesophagus

As mentioned previously in section 1.15.6 telomerase activity has been measured in Barrett's oesophagus using the TRAP assay and it was suggested that Barrett's metaplasia has an immortal potential similar to that found in cancer cells (Ozawa 1997). *In situ* hybridisation has been used to study the expression of the RNA component of telomerase in Barrett's oesophagus. It was found that 100% of oesophageal adenocarcinomas and high-grade dysplasia were strongly positive for telomerase RNA. Basal cells of Barrett's metaplasia demonstrated weak to moderate telomerase RNA in 70% of cases and 90% of low-grade dysplasia had moderate levels of telomerase RNA. The study suggested that the expression of telomerase increased with the progression of Barrett's oesophagus to adenocarcinoma (Morales 1998).

Telomerase activity has been detected in both oesophageal squamous cell carcinoma and adenocarcinoma, squamous carcinoma *in situ,* squamous and glandular dysplasia and Barrett's metaplasia. Telomerase activity has been measured in Barrett's oesophagus using the telomeric repeat amplification protocol (TRAP) and found 50% of Barrett's oesophagus samples had telomerase activity, suggesting that Barrett's epithelia has an immortal potential similar to that of cancer cells (Ozawa 1997).

Quantitative RT-PCR for the detection of the RNA component of telomerase shows an increase from normal through intestinal metaplasia and dysplasia to adenocarcinoma in Barrett's oesophagus (Lord 2000).

1.17 Proteases in Cancer

Tumour progression is a step-wise process beginning with multiple alterations in a normal cell, which lead to a localised tumour. This finally progresses to a tumour that has the ability to invade and metastasise. Tumour cell invasion involves the attachment of tumour cells to the underlying basement membrane, local proteolysis and migration of tumour cells through the degraded region. Proteases are involved throughout the metastatic process. In order for a cell to metastasise it must be able to move into blood vessels (intravasate), survive in circulation, arrest, move out of the blood vessels (extravasate), invade surrounding tissues and grow. All these steps involve interactions between tumour cells, stromal cells, invading lymphocytic cells, endothelial cells and the extracellular matrix. Proteases which are expressed in these cells (including Cathepsin B and D, Urokinase-type Plasminogen Activator and receptor, and interstitial collagenase), are believed to participate in many of these steps (Figure 1.6) (Kobinski 2000).

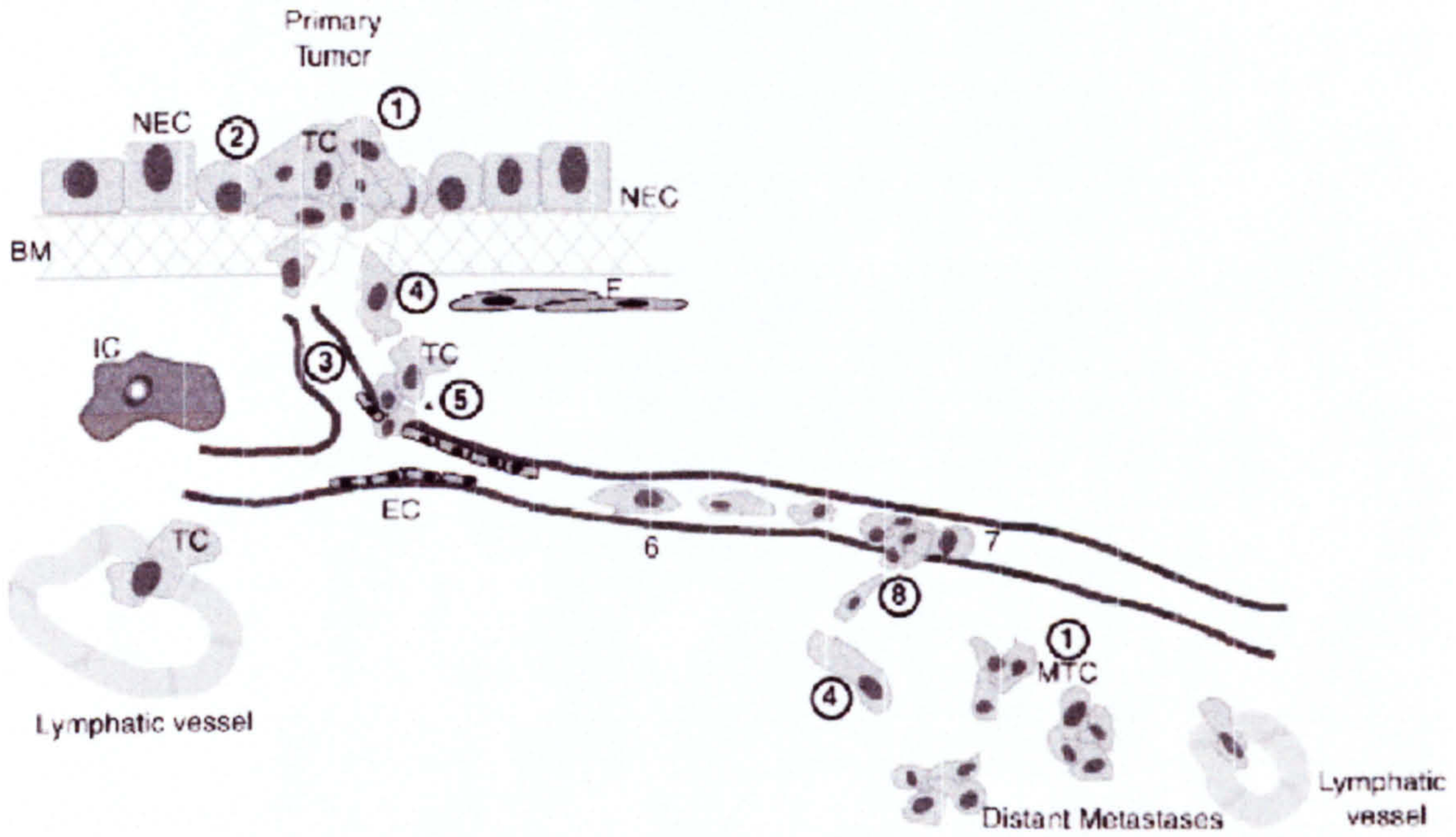


Figure 1.6. The role of proteases in the metastatic process.

Multiple changes occur in the normal epithelial cells (NEC) leading to tumour formation and growth **(1)**. The tumour cells (TC) also undergo epithelial-mesenchymal transformation **(2)** during this time. Angiogenesis is stimulated where endothelial cells (EC) proliferate and invade towards the tumour site **(3)**. TC also invades the connective tissue **(4)** and then the blood vessels **(5)**. The TC must survive in circulation (6), arrest (7), leave the blood vessels **(8)**, invade the local environment **(4)**, and grow **(1)** to set up distant metastases (MTC: metastatic tumour cell). These metastatic steps occur through the interaction of TC, EC, fibroblasts (F), Invading inflammatory cells (IC), such as macrophages, and the extracellular matrix. Steps where proteases are thought to be involved are shown in bold (Kobinski 2000).

The extracellular matrix and basement membrane of cells are physical barriers to migration and invasion. They need to be disrupted and reassembled by migrating cells such as those involved in embryonic developmental processes, inflammatory responses and cancer metastasis. Trophoblast (placental) invasion is analogous to tumour invasion and the same proteolytic mechanisms are involved (Strickland 1976 and Lala 1990). Migration and invasion are properties of many cells, but need to be controlled. Cells which need to overcome barriers need to be equipped with a inducible system that allows them to overcome the extracellular barriers (Blasi 1993b). Tumour cells must overcome extracellular matrix barriers consisting of the basement membrane and interstitial stroma in order to penetrate lymphatic and blood systems (Liotta 1984). In order for tumour invasion and metastasis to occur cancer cells need to co-ordinate the mechanisms involved in the formation and degradation of structural matrix proteins (Reuning 1998). Several processes cause cancer metastasis. These include:

- detachment of cancer cells from their original location
- cancer cell migration
- invasion of cancer cells into the surrounding tissue, requiring adhesion to and subsequent degradation of the extracellular matrix components
- access of cancer cells to blood and lymphatic vessels
- adhesion to and invasion through the endothelium, allowing colonisation at distant sites (Andreasen 1997).

Localised proteolysis is an important mechanism regulating cellular interactions. Fibrinogen, fibronectin, vitronectin, alpha-2-antiplasmin, plasminogen activators and plasminogen activator inhibitors are components of the extracellular matrix

or the basement membrane and have a role in cell adhesion and migration. They are themselves components of the extracellular proteolytic processes (Blasi 1993b).

1.18 Plasminogen

Plasminogen expression has also been linked as a late event in the progression of Barrett's oesophagus to adenocarcinoma. It is a sign of the cells becoming invasive and invading the extracellular matrix of surrounding cells. The study of plasminogen and telomerase as late events in Barrett's cancer progression should help build up a clearer picture of the late events in cancer progression within this disease.

1.18.1 The Plasminogen System

Plasminogen activation is a significant inducible extracellular proteolytic system involved in the regulation of cellular interactions. Plasminogen activators convert inactive plasminogen into the trypsin-like serine protease plasmin. Plasmin can degrade circulating and tissue proteins as well as activate zymogen or growth factor precursors (Plow 1986, Blasi 1993b, and Plesner 1997). There are two types of plasminogen activator urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) (Blasi 1993b). uPA is a 55kD protein produced by a variety of cell types including fibroblasts, various epithelia, monocytes and neutrophils. uPA is produced as a single chain inactive precursor (pro-uPA) which is activated when it binds to the receptor, uPAR (Plesner 1997 and Herszenyi 2000).

uPAR was first detected on monocytes and monocyte-like cells and has since been observed on a variety of cultured cells derived from both cancerous and normal cells. It is a single chain highly glycosylated protein of Mr 55-60,000, which is attached to the plasma membrane by a glycosyl-phophatidyl-inositol lipid anchor (Ronne 1991 and Blasi 1993b). Most cells bearing uPAR have also been found to secrete uPA. The attachment of uPA to its receptor increases the ability of the cell to convert plasminogen to plasmin and degrade the basement membrane and extracellular matrix proteins (Herszenyi 2000). uPAR is often expressed together with uPA at the foci of cancer invasion.

The activity of uPA and uPAR is regulated by plasminogen activator inhibitors known as PAI-1 and PAI-2 (Bacharach 1992 and Andersen 1997, Blasi 1993b). The plasminogen inhibitors are members of the serpin family of proteins. Plasminogen activator inhibitor-type 1 is produced by vascular endothelial cells and inhibits both uPA and tPA, whereas plasminogen activator inhibitor-type 2 inhibits only uPA (Herszenyi 2000). They are present in general in the extracellular matrix and are capable of forming a covalent complex that inactivates the protease. The formation of a receptor bound uPA:PAI-1 complex is an essential component of the regulation of the extracellular proteolytic pathway. It triggers the internalisation and degradation of the complex (Blasi 1993b). The inhibitors are less active against uPA bound to receptor than soluble uPA.

The gene map locations of the components of the Plasminogen Activator system are:

- uPA 10q24 (Tripputi 1985),
- uPAR 19q13 (Borglum 1991),

- PAI-1 7q21.3-q22 (Ginsburg 1986),
- PAI-2 18q21.3 (Antalis 1988).

1.18.2 Plasminogen and cancer

The role of the plasminogen activator system in cancer invasion and metastasis has been covered in many reviews including Blasi 1993a, Mignatti 1993, Andereasen 1997 and Reuning 1998.

In breast cancer uPA has been found to be a possible new and independent prognostic marker. It was found that patients with high levels of uPA had increased risk of recurrence and a shorter survival rate than patients with low levels (Duffy 1991). Carcinogenesis of the human colon is associated with an increase in the tissue content of uPA (Sier 1991). uPA levels have been found to be higher in human lung tumours than the adjacent non-involved tissue (Markus 1980).

The role of the plasminogen activator system in invasive gastro-oesophageal carcinoma has been studied. Hewin *et al* has shown that there is increased expression of uPA and decreased expression of PAI-2 in oesophageal carcinoma compared to normal mucosa (Hewin 1994) and uPA and uPAR are at elevated levels in gastric carcinoma (Hewin 1995b and Hewin 1996). Plasminogen was also shown to significantly increase basement membrane degradation in oesophageal and gastric cell lines (Hewin 1995a). Immunohistological staining has shown the presence of uPA in gastric tumours (Takai 1991), but no studies have been done to look at the distribution of these proteins at the invasion front in gastric or oesophageal tumours.

Nekarda *et al* carried out a study in 1998 to assess the level of expression and significance of uPA and PAI-1 in resected adenocarcinoma of the oesophagus using ELISA techniques. Tumour samples were compared to matched normal gastric mucosa samples. It was found that uPA and PAI-1 levels were significantly elevated when compared with the normal mucosa. Overall it was concluded that tumour uPA content appears to be a powerful independent prognostic factor for survival in adenocarcinoma of the oesophagus (Nekarda 1998). No studies were made of the PA system in the early stages of the progression of Barrett's oesophagus to adenocarcinoma.

1.19 Molecular Biological Techniques

In order to unravel the molecular changes within tissues during the progression of Barrett's oesophagus to adenocarcinoma various molecular biological techniques have been utilised in numerous studies, some of which are described below.

1.19.1 Immunochemical Techniques

All immunochemical techniques rely on the ability of antibodies to recognise and bind to specific antigens. The effectiveness of antibodies is dependent on the quality of the antibody employed. It is possible to make antibodies to proteins, peptides, carbohydrates and nucleic acids, but in general immunochemical methods are devised for use with antibodies that recognise proteins or peptides. Antibodies can be either monoclonal (derived from a single B cell clone and show identical amino acid sequence) or polyclonal (recognises several different epitopes and shows heterogeneity in the amino acid sequences). Monoclonal antibodies can be advantageous for immunochemical purposes.

In most immunochemical assays binding of antibody to antigen can only be visualised by labelling the antibody either with a radioactive isotope, an enzyme or a fluorochrome. Antibodies labelled with a fluorochrome or enzyme are commonly referred to as conjugates.

Immunobinding assays such as ELISA (enzyme-linked immunosorbent assay) utilise either imobilised antigen or antibody to assess the concentration of a molecule in solution. They are very quick, easy, cheap and simple to carry out. Antigen containing solution is simply incubated in the wells of a plastic microtitre plate allowing the imobilised antibody to bind to the antigen. The antigen is then detected either directly or indirectly by an enzyme conjugated antibody. The enzyme then reacts with an added substrate to allow visualisation of the antigen.

In order to understand cell structure and organisation in disease it is often necessary to be able to determine the distribution of a protein *in situ*. Immunohisto/cytochemical (IHC/ICC) techniques exploit the specific interaction of an antibody with its antigen to locate or determine the distribution of the antigen in tissues or cells respectively. The principle of IHC is analagous to ELISA except the antibody is incubated with thin tissue sections mounted on microscope slides or cell preparations containing the antigen. The antibody must be conjugated with a fluorescent or enzyme label that gives a signal to allow visualisation under the microscope.

All IHC procedures require stringent positive and negative controls for comparison to ensure the immunostaining is specific. The main advantage of enzyme labels (usually horse radish peroxidase [HRP] or Alkaline Phosphatase

[AP]) is that an ordinary white light microscope can be used for viewing the slides and the use of a chemical counterstain such as heamatoxylin aids the identification of morphology. The main disadvantage is the presence of endogenous tissue enzymes, which can give a high background staining. This can be overcome with blocking steps during the procedure.

Immunohisto/cytochemistry techniques give information on the cellular location of a protein or peptide detected with an antibody. It does not give information on the expression or activity of the protein. You can measure the expression of proteins within the cell by the detection of mRNA using either reverse transcriptase-PCR or *in situ* hybridisation techniques.

1.19.2 Reverse Transcriptase-PCR

Polymerase Chain Reaction (PCR) is used to amplify a precise fragment of DNA. It requires some knowledge of the DNA sequence, which flanks the target DNA. From this information two oligonucleotide primers can be chemically synthesised each complementary to a stretch of DNA to the 3' side of the target DNA, one oligonucleotide for each of the two strands of DNA. The technique is analogous to the DNA replication process within the cell in that it results in the generation of new complimentary DNA strands based upon existing ones.

The process of PCR consists of three defined sets of time and temperatures: i) denaturation, ii) annealing and iii) extension. Each of these steps is repeated for 30 or 40 cycles. In the first step the double stranded DNA fragment is denatured by heating to above 90°C. Next the temperature is cooled to allow the oligonucleotide primers to anneal to their complimentary sites either side of the target DNA strand to be replicated. The final step, extension is carried out by a

thermostable DNA polymerase (in most cases *Taq* DNA polymerase). The time and temperatures of these steps are critical, but vary and have to be optimised and defined for each reaction.

The method of PCR has been utilised to analyse mRNA through the development of a technique called Reverse Transcriptase-PCR (RT-PCR). Here the RNA is isolated and a first strand cDNA synthesis is carried out using reverse transcriptase. The resulting cDNA is then used as target DNA in conventional PCR. One of the main benefits of RT-PCR is the ability to identify rare or low levels of mRNA transcripts with great sensitivity. RT-PCR also gives information on the gene expression of possible protein molecular markers.

The method of PCR has also been modified to detected active telomerase within protein tissue extracts. TRAP (Telomerase Repeat Amplification Protocol) is covered in section 1.16.5.

1.19.3 In Situ *Hybridisation*

In Situ Hybridisation (ISH) makes it possible to determine the chromosomal location of a particular gene fragment or mutation, or the cellular location of mRNA within a tissue section. ISH is carried out by preparing either a radiolabelled or non-isotopic labelled RNA or DNA probe and applying it to a tissue or chromosomal preparation fixed to a microscope slide. Any probe that does not completely hybridise to complementary sequences is washed off and an image of the distribution or location of the bound probe is viewed by autoradiography or under the light microscope after the reaction of an enzyme

similar to IHC. *In situ* hybrdisation is covered in detail in sections 1.16.5 and 4.3.

2 Aims and Objectives

2.1 Aim

The aim of this thesis is the study of molecular markers for the progression of Barrett's oesophagus to adenocarcinoma, to identify markers that can be used as diagnostic tools.

Currently there are no molecular markers available to distinguish between patients with Barrett's metaplasia who are at high risk of developing adenocarcinoma and those that are not. Current screening programmes for Barrett's oesophagus are not cost effective and therefore a marker for dysplasia and adenocarcinoma has the potential to improve surveillance for patients at a high risk of developing adenocarcinoma.

2.2 Objectives

1. **To investigate the timing and significance of telomerase reactivation during the metaplastic-dysplastic-neoplastic progression of Barrett's oesophagus.**

Both immortalisation and invasion are important factors in the development of malignant tumours and therefore could be significant markers during the progression of Barrett's oesophagus. So far little work has been performed to assess the development of an immortal phenotype, i.e. telomerase reactivation, during the progression of Barrett's oesophagus to adenocarcinoma. This study discusses the timing and significance of telomerase reactivation during the metaplastic-dysplastic-neoplastic progression of Barrett's oesophagus. The

Telomerase Repeat Amplification Protocol (TRAP Assay) and *in situ* hybridisation techniques will be utilised for this purpose.

2. To investigate the progression of Barrett's oesophagus to adenocarcinoma with reference to the components of the urokinase-type Plasminogen Activator system.

The invasive potential of the non-neoplastic stages of Barrett's oesophagus has also been largely neglected from previous investigation. This study therefore involves a full assessment of the levels of the components of the urokinase-type Plasminogen Activator system, by means of immunohistochemistry, enzyme-linked immunosorbant assay (ELISA) and cell culture techniques. One objective of this project is to investigate the progression of Barrett's oesophagus to adenocarcinoma with reference to the components of the Plasminogen Activator system.

3. The characterisation of oesophageal cell lines as potential *in vitro* models of oesophageal tumours and the effects of exposure to bile salts and acidic conditions on molecular markers of disease progression.

The potential role of bile salts and exposure of the oesophageal mucosa to acidic conditions in the development of Barrett's metaplasia and its neoplastic progression are discussed. Oesophageal cell-lines, which have been fully characterised as *in vitro* models of oesophageal tumours during this study, are utilised for this purpose along with various molecular biological techniques including reverse transcriptase-PCR and immunocytochemistry.

In this thesis Chapter 3 details the materials and methodology used during this study. In Chapter 4 the Telomeric Repeat Amplification Protocol has been utilised to assess the timing and significance of telomerase reactivation in tissue from patients suffering from various stages of the Barrett's oesophagus adenocarcinoma sequence. Also a method of non-isotopic *in situ* hybridisation for the detection of the RNA component of telomerase has been developed to assess the cellular localisation of telomerase within paraffin embedded sections of neoplastic tissues.

Chapter 5 describes the components of the urokinase-type Plasminogen Activator system during the metaplastic:neoplastic progression of Barrett's oesophagus. Urokinase-type Plasminogen Activator, its receptor and two inhibitors have been examined by immunohistochemistry on paraffin embedded sections of tissue and on the same protein lysates from the telomerase study using ELISA techniques.

In Chapter 6 cell-culture methods are utilised to assess two oesophageal cell-lines as *in vitro* models of Barrett's associated adenocarcinoma and oesophageal squamous cell carcinoma. Molecular markers are assessed using Reverse Transcripase-PCR, immunocytochemistry, and ELISA techniques. Chapter 7 discusses the use of these cell-lines to determine the effects of bile salts and acidity on cellular adhesion, invasiveness and immortality during Barrett's oesophagus, as refluxate is thought to be a causal factor in the formation of oesophageal neoplasia.

Chapter 8 discusses the data obtained in the previous chapters and suggests areas of further study. Finally Chapter 9 draws together the conclusions of this

study into molecular markers for the progression of Barrett's oesophagus to adenocarcinoma.

3 Materials and Methods

3.1 Chemicals, Reagents and Other Materials

A full list of suppliers and equipment are listed in Appendix 2.

3.2 Cell culture

Human oesophageal cell-lines OE21 and OE33 (also known as JROECL21 and JROECL33) (Rockett 1997) were cultured in RPM1-1640 modified medium supplemented with 10% foetal bovine serum, 5ml stabilised penicillin-streptomycin solution and 1ml amphotericin B solution per 500ml (all regents from Sigma cell culture, Poole, Dorset, England). OE21 is a human oesophageal squamous cell carcinoma cell-line established in 1993 from a mid oesophageal carcinoma of a 74 year-old male patient. OE33 is a human oesophageal adenocarcinoma cell-line also established in 1993 from an adenocarcinoma of the lower oesophagus (with Barrett's metaplasia) of a 73 year-old female patient (Rockett 1997).

Cells were grown in various sized culture flasks ranging from T25cm^2 to T100cm^2. Once established the cells were checked daily for possible infections and to assess growth. The media was replaced every 2 days. Once the cells were 75-80% confluent they were detached from the culture flask surface using 1x trypsin-EDTA solution (Sigma cell culture, Dorset, England) and split into further flasks, frozen down or treated for protein extraction. The growth medium was removed and the cells washed once in sterile phosphate-buffer saline (PBS), then 5ml of trypsin solution was added and the flask left to stand for 1 minute.

Gentile tapping on the side of the flask helped to detach the cells. Trypsin was inhibited using 5ml of growth medium and the cells were pelleted by centrifugation at 100 x g for 5 minutes at 4°C. The pellet of cells could then be split into further flasks, frozen down in 5% Dimethyl sulfoxide (DMSO) in foetal bovine serum, used for Tri Reagent extraction or TRAP Assay/IMUBIND ELISA (see sections 4.2 and 5.3).

Cell-lines were also grown in 0.1mM concentrations of different conjugated bile acids or acidified growth media (pH5) to analyse if this altered telomerase activity or the of components of the Plasminogen Activator system (see section 7.2).

3.3 Clinical Samples

All the clinical samples, both fresh frozen and paraffin embedded were obtained from the histopathology department of Gloucestershire Royal Hospital. Ethical approval for this work was arranged on 11th June 1999 and copies of all the relevant letters and forms can be found in Appendix 1 at the back of this report. All fresh frozen samples were collected with patient written consent during routine surveillance endoscopy and from resected tissue during routine pathological analysis. The samples were placed on card and into cryovials before they were snap frozen in liquid nitrogen.

Tissues were fixed in 10% neutral buffer formalin for a minimum of 24 hours and then embedded in Polywax (Difco Laboratories, Surrey, UK). Sections approximately 4μm thick were taken and placed on vectabond coated slides

(Vector Laboratories, Burlingame, USA) and dried for 24 hours. Full details of the protocol for sample donation can be found in Appendix 1 as mentioned above.

3.4 Haematoxylin and Eosin staining

Haematoxylin and Eosin staining is the most commonly used staining method. This stain differentiates tissues by colouring the nuclei and basic parts of cells blue/black with haematoxylin and connective tissues and acidic parts of cells shades of red and pink with eosin.

The fresh frozen sections of tissue were initially fixed in formal acetic alcohol for 1 minute. They were then washed in tap water and placed in Mayers haematoxylin for 1 minute and rinsed in tap water. The slides were then placed in saturated lithium carbonate to increase the blue colouration and rinsed in tap water. Next the slides were placed in 1% eosin for 15 seconds and rinsed once more in tap water. The slides were then dehydrated in ethanol, cleared in xylene and finally mounted in pertex and cover slipped.

3.5 Tri Reagent™ RNA, DNA and protein extraction

Tri Reagent™ (Sigma-Aldrich Company Ltd. Poole, Dorset, England) is a quick and convenient reagent for the simultaneous isolation of RNA, DNA and protein. Isolation was carried out according to the manufacture's protocol and is detailed below.

3.5.1 Sample preparation

A monolayer of cells approximately 75-80% confluent which had been grown for 5 to 6 days was lysed directly on the surface of the culture dish. (For a T25 culture

flask 200µl of Tri-reagent [Sigma-Aldrich Company Ltd. Poole, Dorset, England] was used. This increased proportionally for the larger flasks.) This was left to stand for 5 minutes to allow complete dissociation of nucleoprotein complexes. The resulting solution was transferred to a 2ml sterile eppendorf tube and 200µl of chloroform was added. The tube was shaken vigorously for 15 seconds and left to stand for 10 minutes at room temperature. The tube was then centrifuged for 15 minutes at 12,000 x g at 4°C. This resulted in the separation of the mixture into three phases: a red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA).

3.5.2 RNA Isolation

The upper aqueous phase containing RNA was completely removed and transferred to a new tube and 500µl of isopropanol was added and the sample was left to stand at room temperature for 10 minutes. This was then centrifuged for at 4°C for 10 minutes at 12,000 x g. The RNA precipitate formed a pellet on the side and bottom of the tube. The supernatant was removed and the pellet washed in 1ml of 75% ethanol. This was centrifuged at 7,500 x g for 5 minutes at 4°C to re-pellet the RNA. The pellet was briefly dried in the air for 10 minutes and redissolved in 300µl of nuclease free water.

3.5.3 DNA Isolation

All the remaining aqueous phase was removed and 300µl of 100% ethanol was added. This was mixed by inversion and left to stand for 3 minutes at room temperature. It was then centrifuged at 2,000 x g for 5 minutes at 4°C and the pink supernatant removed and saved for protein extraction. The pellet was washed twice in 0.1M sodium citrate, 10% ethanol solution for 30 minutes and

centrifuged at 2,000 x g for 5 minutes at 4°C each time. The resulting pellet was resuspended in 75% ethanol and left to stand for 20 minutes at room temperature, before it was dried and dissolved in 300µl 8mM NaOH. This was then centrifuged at 12,000 x g for 10 minutes to remove any insoluble material and the supernatant was transferred to a fresh tube.

3.5.4 Protein Isolation

To the pink supernatant saved from the DNA isolation step for protein isolation 1.5ml of isopropanol was added and incubated at room temperature for 10 minutes. This was centrifuged at 12,000 x g for 10 minutes at 4°C and the supernatant was discarded. The resulting pellet was washed 3 times for 20 minutes in 2ml of 0.3M guanidine hydrochloride/95% ethanol solution and centrifuged at 7,500 x g for 5 minutes at 4°C each time. The supernatant was removed and 2 ml of 100% ethanol was added and left to stand at room temperature for 20 minutes. This was centrifuged for 7,500 x g for 5 minutes at 4°C. The pellet was dried and redissolved in 300µl 1% SDS. Any insoluble material was removed by centrifugation at 10,000 x g for 10 minutes at 4°C and the supernatant transferred to a fresh tube.

3.6 Quantification of RNA and DNA

To estimate the concentration of DNA and RNA UV spectrophotometery was utilised with the M350 double beam UV-visible spectrophotometer (Camspec, Cambridge UK). By measuring the absorbance at 260 and 280 nm the purity and the concentration of the sample could be assessed. Each sample was diluted in water and readings made at each wavelength, blanked against water alone. The resulting readings were used in the following calculations:

Purity = A_{260} / A_{280} (where a value of 1.8 means pure DNA and 2.0 means pure RNA)

Yield (ug/ml) = A_{260} x dilution factor x 50 (for genomic DNA) or x 40 (for RNA)

3.7 Measurement of Total Protein Concentration

The protein lysates were diluted 1:5 in distilled water and mixed with 1.5ml of BioRad Protein Assay dye. This was mixed and incubated for at least 5 minutes at room temperature. The A_{595} was measured for each sample and the concentration estimated from a standard curve of six IgG standards at concentrations between 0 and 1000μg/ml.

3.8 Gel Electrophoresis for the analysis of DNA and RNA

To assess the quality and quantity of extracted DNA and RNA, agarose gel electrophoresis was utilised using the Embitech RunOne system. Agarose gel electrophoresis was also carried out to visualise PCR and *in vitro* transcription products. The percentage of agarose in the gels varied according to the size of product as shown in table 3.1.

Table 3.1 Agarose gel selection chart.

Gel (%)	Optimum Separation Range (Kb)
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

Gels were stained using ethidium bromide solution (0.5μg/ml) for 15 minutes and then destained in dH_2O for 10 minutes. The DNA was visualised using a shortwave (254nm) UV transilluminator within the Syngene Gene Genius Bio-imaging System (Synoptics Ltd.) and images archived on the computer.

3.9 Immunohistochemistry

Immunohistochemistry was used on both cell-lines cultured in slide flasks and paraffin embedded sections of tissue. The overall method was the same although there were differences in the preparation and mounting of the slides.

Non-specific reactions were blocked using the appropriate serum (1:5) (v/v) in PBS. The serum used was from the same species as the secondary antibody was raised in, usually rabbit, goat or sheep. The diluted serum was added to the slides completely covering the tissue section and incubated at room temperature in a humid chamber for 1 hour. The blocking serum was removed without washing and the primary antibody added diluted with PBS containing 1% serum (as used for blocking) again completely covering the tissue section and left in a humid atmosphere at 4°C overnight.

The next day the slides were removed from the fridge and allowed to reach room temperature. The primary antibody was removed and the slides washed three times for 5 minutes each in tris-buffered saline (TBS). Next endogenous hydrogen peroxidase activity was blocked by incubation with 3% hydrogen peroxide in 100% methanol for 10 minutes and the slides washed 3 times for 5 minutes each in TBS. The secondary antibody was then added diluted in PBS and 1% serum and left to incubate for 1-2 hours in a humid chamber. The secondary antibody again varied depending on the animal in which the primary antibody was raised in. The avidin-biotinylated horseradish peroxidase conjugate (ABComplex) (Dako Ltd. Cambridge UK) was prepared according to the manufacturers instructions 20 minutes before it was needed and stored in the dark. The secondary antibody was removed and the slides washed as previously described and the ABComplex added. This was left to incubate for approximately 1 hour and then washed. The slides were then incubated with 3,3'-Diaminobenzine (DAB) (Sigma-Aldrich Company Ltd. Poole, Dorset, England) (50mg DAB in 100ml TBS and 100µl Hydrogen peroxide) and the reaction monitored until staining was seen. The slides were then washed in tap water and counter stained with haematoxylin and mounted. Slides were then viewed under the microscope and archived using AxioVision Version 3 (Imaging Associates, Thame, UK).

3.10 Western Blot Analysis

Immunodetection of proteins extracted from both cell-lines and fresh frozen tissue samples was carried out using the ECL chemiluminecent detection system (Amersham Pharmacia Biotech). Glass plates, plastic spacers and combs from the BioRad Mini Protein II were cleaned and assembled according to the

manufacturers instructions. A 6% main gel and a 4% stacking gel were made according to the recipes shown in table 3.2.

Table 3.2 Acrylamide Gel Composition

Gel	30% Bis/ Acylamide	Tris Buffer	10% SDS	TEMED	Ammonium Persulphate	RO Water	Total
6%	2ml	2.5ml (1.5M pH8.8)	100µl	10µl	50µl	5.35ml	10ml
4%	650µl	1.25ml (0.5M pH7.5	50µl	10µl	25µl	3.05	5ml

Once the gels were poured into the frame (main gel first then stacking gel) and had dried a 1 in 3 dilution of the samples were prepared with 4x loading dye. The samples were boiled at 95°C for five minutes and placed on ice before a volume was loaded into a well of the gel. One well was loaded with 10µl of pre-stained molecular weight marker (SeeBlue™Pre-stained Standard, Novel Experimental Technology). The gel was then run at 75V for 15 minutes to allow the samples to move through the stacking gel and then at 100V for about 1 hour (or until the sample buffer reached the bottom of the gel).

The gel was then removed from the glass plates and blotted onto an Immobilon-P Transfer Membrane, (Millipore) using an electroblotter (Hoefer SemiPhor™ Amersham Pharmacia Biotech) run for 55 minutes at 40-44mA. The pre-stained marker could be seen on the membrane once transfer was completed.

The membrane was blocked in 5% Marvel/TSB for 1 hour. The primary antibody was diluted 1 in 1000 in 5% Marvel/TBS and the membrane incubated with the antibody overnight at 4°C.

The following day the membrane was removed from the fridge and left for 30 minutes to reach room temperature. Next it was washed 5 times for 5 minutes in 0.1% Tween 20/TBS. The membrane was then placed in the peroxidase conjugated secondary antibody again diluted 1 in 1000 in 5% Marvel/TBS and incubated for 1 hour at room temperature. It was then washed 5 times for 5 minutes in 0.1% Tween 20/TBS. The proteins were detected using the ECL detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Once the reagents were added the membranes were incubated for 1 minute wrapped in cling film and placed in a film cassette on a sheet of autoradiography film (Kodak X-OMAT). Exposure was carried out for at least two hours before developing and fixing of the film with solutions from Sigma.

3.11 Production of cDNA (RT-PCR)

β-Actin primers were used to check the mRNA content of the total RNA samples extracted from the cell-lines using Tri-reagent. The primers (as desribed in appendix 4) amplified a product of approximately 500 base pairs from the β-actin gene in cDNA produced from the RNA samples using reverse transcription from both the cell-lines. This proved the RNA samples were viable and therefore were suitable for further RT-PCR analysis.

Using total RNA samples produced by Tri-reagent extraction (section 3.5) a reverse transcription reaction (RT-PCR) was made. The Promega RT System (Promega Corporation, Southampton, UK) was utilised using AMV Reverse Transcriptase to synthesise single-stranded cDNA. Each RT reaction was carried out in a 20μl reaction containing 5mM $MgCl_2$, 1x Reverse Transcription Buffer

(10mM Tris-HCl [pH 9.0 at 25°C], 50mM KCl, 0.1% Triton® X-100), 1mM each dNTP, 1U/μl Recombinant RNasin® Ribonuclease Inhibitor, 15U/μl Oligo $(dT)_{15}$ Primer, 1μg substrate total RNA and nuclease-free water to volume. The reverse transcription reaction was then incubated at 42°C for 15 minutes followed by 99°C for 5 minutes to denature the AMV Reverse Transcriptase. The samples were then placed on ice for 5 minutes.

The RT reaction was then diluted to 100μl with nuclease-free water and 20μl used in the PCR mix with 2mM $MgCl_2$, 1x Reverse Transcription Buffer (10mM Tris-HCl [pH9.0 at 25°C], 50mM KCl, 0.1% Triton® X-100), 50pmol of the upstream and down stream primers and 2.5U Red Hot DNA polymerase (Advanced Biotechnologies Ltd. Epsom Surrey, UK) was added after a "Hot start". No extra dNTP needed to be added as enough was left in the RT mix. The cycling was carried out using the Hybaid Touchdown machine (Hybaid Ltd. Basingstoke, UK). Cycling conditions were one cycle of 94°C for 5 minutes when the DNA polymerase was added. This was followed by 30 cycles of 94°C for 1 minute, 1 minute at the appropriate annealing temperature calculated by the T_M of each primer and a 72°C extension step varying between 30 seconds and 2 minutes. The annealing temperature was 55°C for 1 minute and extension of 2 minutes for the β-actin primers R and F (see appendix 4).

4 Telomerase During the Metaplastic:Neoplastic Progression of Barrett's Oesophagus

4.1 Introduction

As mentioned in section 1.16 telomerase is a ribonuclear protein complex with reverse transcriptase activity that uses its own RNA component as a template for the addition of telomeric repeat sequences to the end of chromosomes, therefore maintaining telomere length. Telomerase activity is present in approximately 85% of primary human cancers, but not in most normal somatic tissues.

Telomerase activity has been studied in a wide range of cancers using various methods of detection. To assay for telomerase activity in small samples of human tissues, a sensitive PCR-based assay has been developed (Kim 1994), called the telomeric repeat amplification protocol (TRAP). The TRAP method has been further developed and combined with an ELISA detection system instead of a gel for a rapid, easy and reliable telomerase detection in human tissue samples. The TRAPEZE™ ELISA Telomerase Detection Kit (Intergen) is the method utilised here to study telomerase activity in the progression of Barrett's Oesophagus to adenocarcinoma in fresh frozen clinical samples.

The main disadvantage of using the TRAP method for analysing telomerase activity in clinical tissue samples is there is no way of telling exactly which cells within the samples are expressing telomerase. This problem can be overcome with the use of *in situ* hybridisation techniques. The application of *in situ* hybridisation techniques for detecting telomerase RNA expression in archival paraffin-embedded and fresh frozen material can easily distinguish cancer from

normal cells (Shay 1997a). The use of *in situ* hybridisation for the detection of the RNA template within the enzyme complex should allow the localisation of specific cells with telomerase activity. Previously a method of *in situ* hybridisation for the detection of the RNA component of telomerase in tissue samples from the metaplastic:neoplastic progression of Barrett's Oesophagus using radiolabeled RNA probes has been developed (Morales 1998). The main problem with this study was the small number of samples analysed. Therefore this chapter describes the development of a method of *in situ* hybridisation for the detection of telomerase using a non-isotopic method.

4.2 The TRAPEZE™ ELISA Telomerase Detection Kit

For non-quantitative detection of telomerase activity in cells and tissues the Oncor TRAPEZE™ ELISA telomerase detection kit (Intergen Company, Oxford, UK) was utilised. The kit contains all the reagents necessary to perform the Telomeric Repeat Amplification Protocol (TRAP) and Enzyme-Linked Immunosorbent Assay (ELISA). The assay was carried out according to the manufacture's protocol and is detailed below. The overall protocol is shown in the form of a flow diagram in figure 4.1.

4.2.1 Extract Preparation

Sections of fresh frozen oesophageal tissue, which had been stored in liquid nitrogen (-146°C) for up to 4 years (mainly resected samples) were removed from cryovials and frozen onto metal chucks. The top of the sample was flattened and then using a cryostat a section 10nm thick was cut and placed on a vectabond-coated slide. The next 10 sections were placed in a cold 1.5ml nuclease-free micro-centrifuge tube. The next section was placed on the slide and the following

10 sections placed in the cold tube. A final section was cut and placed on the slide ready for heamatoxylin and eosin staining, section 3.4. The three sections on the slides were used to check the tissue remained the same through the whole block and all the sections in the tube had the same diagnosis. The sections were lysed in 200µl of CHAPS lysis buffer containing 120U/ml RNasin® Ribonuclease inhibitors (Promega Corporation, Southampton, UK).

The sample suspensions were incubated on ice for 30 minutes and then spun at 12,000 x g for 20 minutes at 4°C. The supernatant (160µl) was transferred to a fresh tube and 6µl set aside for determination of protein concentration (see section 3.7). The kit recommended diluting the sample extracts with CHAPS lysis buffer prior to use tissue extracts (0.01µg/µl - 0.50µg/µl) using less than 1.0µg per assay. Titre experiments were also carried out to find the optimum concentration of protein required for the assay.

4.2.2 Controls

Telomerase is a heat-sensitive enzyme and therefore as a negative control a heat-inactivated sample was produced for each protein extract. Telomerase was inactivated by heat-treating 10µl of each tissue extract at 85°C for 10 minutes prior to the TRAP assay.

For each set of TRAP assays two positive telomerase extract controls, two PCR/ELISA positive controls and two primer-dimer/PCR contamination controls were included. A telomerase containing cell extract was included in the kit as a positive control. This was prepared in the same way as the cell samples and diluted 1/10 with CHAPS lysis buffer. TSR8 was used as a PCR/ELISA positive control. TSR8 is a synthetic oligonucleotide with 8 telomeric repeats and

therefore acted as a template for the PCR reaction. The TRAP assay was also performed with CHAPS lysis buffer instead of the tissue extract. This acted as a PCR contamination control.

4.2.3 TRAP Assay

The TRAP assay was set up for each sample containing 10µl 5x TRAP reaction mix (containing Tris buffer; primers: Biotinylated Telomerase Substrate primer [AATCCGTCGAGCAGAGTT] and Reverse primers [For the PCR step]; dNTPs: dCTP labelled with dinitrophenyl), dGTP, dATP, and dTTP), 2U *Taq* polymerase and dH_20 up to 48µl. 2µl of the test extracts, heat activated extracts or controls were then added to each mix.

Firstly the samples were incubated for 30 minutes at 30°C to allow extension of the telomeric repeats on the biotinylated telomerase substrate oligonucleotide. In the second step the extended products were amplified by PCR. The cycling conditions were 33 cycles of 94°C for 30 seconds followed by 55°C for 30 seconds with no extension step.

A streptavidin-coated microtiter plate was pre-treated with Blocking/Dilution Buffer. Each well was filled with 250µl Blocking/Dilution Buffer, covered in parafilm™ and incubated at 37°C for 30 minutes. This was then removed and each well washed once with 400µl 1x Wash Solution. Into each well 100µl of Blocking/Dilution Buffer was added along with 5µl of the TRAP reaction product. This was mixed thoroughly by pipetting 10 times. The wells were covered in parafilm™ and incubated at 37°C for 1 hour. Table 4.1 shows the sample arrangement for the assay on the microtitre plate.

A. Sample Preparation

1. Cell pellet or tissue in a 1.6ml micro-centrifuge tube
2. Add CHAPS lysis buffer 200µl
3. Incubate on ice for 30 minutes
4. Centrifuge at 12,000 x g for 20 minutes
5. Transfer supernatant to a fresh tube
6. Determine protein concentration

B. TRAP Assay Set Up

1. Prepare master mix:

5x TRAP reaction mix	10µl
Taq DNA polymerase	2 units
DH_2O	up to 48µl

2. Aliquot into PCR tube (48µl/tube)
3. Add sample extract (2µl)

C. TRAP Reaction

1. Extension/amplification step 30°C for 30 minutes
2. PCR Cycling 94°C for 30 seconds
   55°C for 30 seconds
   for 33 cycles

D. ELISA Detection

1. Pipette blocking/dilution buffer into microtitre plate (250µl/well) incubate 37°C for 30 minutes
2. Wash once with 1x wash solution (400µl/well)
3. Add 100µl of blocking/dilution buffer and 5µl TRAP reaction products per well.
4. Incubate at 37°C for 1 hour
5. Wash 5 times with 1x wash buffer
6. Add anti-DNP antibody (100µl/well)
7. Incubate at room temperature for 30 minutes
8. Wash 5 times with 1x wash buffer
9. Add TMB substrate (100µl/well) for 3-10 minutes
10. Add Stop solution (100µl/well)
11. Measure absorbance at 450nm and 650nm

Figure 4.1 TRAPEZE™ ELISA protocol flow chart.

Table 4.1. Sample arrangement for the TRAPEZE™ ELISA on the microtitre plate.

	1	2	3
A	Positive Control	Sample 1	Sample 1
B	Positive Control	Heat-treated sample 1	Heat-treated sample 1
C	TSR8	Sample 2	Sample 2
D	TSR8	Heat-treated sample 2	Heat treated sample 2
E	Buffer	Sample 3	Sample 3
F	Buffer	Heat-treated sample 3	Heat-treated sample 3
G		Sample 4	Sample 4
H		Heat-treated sample 4	Heat-treated sample 4

The solution was removed and each well was rinsed 5 times with 1x Wash Solution. Anti-dinitrophenyl antibody conjugated to horseradish peroxidase was diluted 1:250 with Blocking/Dilution Buffer and 100µl added to each well. This was incubated at room temperature for 30 minutes and then each well rinsed 5 times with 1x Wash Solution. Next 100µl of the horseradish peroxidase substrate tetramethylbenzidine (TMB) was added to each well and incubated for between 3 and 10 minutes at room temperature. To achieve uniform mixing of reagent the microtitre plate was tapped every 1-2 minutes. To change the peroxidase product from blue to yellow 100µl of Stop Solution was added to each well. The plate was then read on a MRX Microplate reader (Dynex Technologies (UK) Ltd. Billingshurst, West Sussex, UK) and the absorbance of the samples at 450nm and 690nm calculated.

The value for each sample was calculated as:

$$\text{Absorbance (units)} = A_{450} - A_{690}$$

For the assay to give valid results the controls must have had values of:

- Primer dimer/PCR contamination control: Absorbance units must be below 0.200.
- TSR8 PCR/ELISA control: Absorbance units must be greater than 0.800.
- Heat-treated sample extract: Absorbance units must be less than 0.250.
- If the extract was telomerase positive: $\Delta A > 0.150$.

4.3 In Situ Hybridisation for the Detection Telomerase

In situ hybridisation for the detection of telomerase was carried out using an RNA anti-sense probe to the RNA component of telomerase. During this study three main methods of *in situ* hybridisation were tested. The DNA template used for producing RNA labelled probes was the same for all methods tested. The first method was carried out using biotin labelled RNA probes and commercially available kits from Ambion (Ambion International, Texas, USA) according to the manufacturer's instructions. The second method was carried out using DIG labelled RNA probes and anti DIG-POD conjugated antibodies with DAB detection (Chang 1999). The final method was a further development of the Chang *et al* method using anti DIG–AP antibodies and BCIP/NBT Alkaline Phosphatase substrate for detection.

4.3.1 Production of the RNA probe

The plasmid pGRN83 was acquired from Geron corp. This construct was made by the PCR of the exact 5' end and approximate 3' end of the RNA component of human telomerase from the genomic clone of hTR. The PCR product was blunted and cloned into the ECL3611 site of pGEM5Zf+ (Geron corp. details). The total size of the plasmid pGRN83 was 3558bp.

The plasmid was transformed into both "home-made" and commercially available competent E. coli cells.

"In House" competent cells

JM83 bacterial cells were grown in solution of LB broth overnight and the following day 100ml of LB broth was inoculated with 1ml of this stock. This was

incubated with shaking at 37°C until the A_{550} was between 0.4 and 0.5. The culture was then placed on ice for 10 minutes. Cells were harvested from 40ml of culture by centrifugation and resuspended in 10ml of cold 0.1M $MgCl_2$. The cells were then pelleted and resuspended in 1ml of cold 0.1M $CaCl_2$ and left on ice for 2 hours.

To 200µl of competent cells 25ng of plasmid was added and incubated on ice for 30 minutes. The cells were then heat-shocked for 2 minutes at 42°C and then placed immediately on ice. The cells were diluted 1:10, 1:100, 1:1000 and 1:10,000 and 100µl was plated out on LB agar containing 1mg/ml amphicillin and incubated overnight at 37°C.

Commercial competent cells

The commercially available competent cells were JM109 (Promega, UK). The frozen competent cells were placed on ice to defrost. Once defrosted 100µl of the cells were aliquoted into pre-chilled thin-walled PCR tubes. To these 25ng of plasmid DNA was added and the tubes placed in ice for 10 minutes. The cells were then heat-shocked for 2 minutes at 42°C and 900µl of cold LB broth was added. This was then incubated at 37°C for 30 minutes with shaking. Dilutions were made of 1:10 and 1:100 and 100µl plated onto antibiotic plates as above and incubated overnight.

4.3.2 Plasmid DNA isolation

The plasmid DNA was extracted from the transformed cells using the SNAP MidiPrep Kit available from Invitrogen (Invitrogen Corporation, USA). The procedure required the following steps:

- Lyse cells with Lysis buffer
- Precipitate protein and genomic DNA
- Filter the Lysate/precipitate through Column A
- Add Binding buffer to the flow through and apply to column B
- Wash the bound plasmid and dry the resin by centrifugation
- Elute the Plasmid DNA with sterile water.

A single colony of transformed bacteria cells was grown for 12 hours at 37°C in 100ml of LB containing amphicillin. The cells were then pelleted by centrifugation at 400 x g. The cells were resuspended in 4ml of Cell Resuspension Buffer by vortexing. To this 4ml of Lysis Solution was added, mixed by inversion and incubated for 3 minutes at room temperature. Next 4ml of Precipitation Salt was added, mixed again by inversion and incubated for 5 minutes in ice. Two 50ml centrifuge tubes were labelled A and B. The Filtering column A was placed in tube A and the Binding column B was placed in the tube labelled B. The precipitated solution was added to column A and centrifuged for 5 minutes at 3,000 x g.

The filtrate was saved, 12ml of Binding buffer added and mixed by inversion. The solution was transferred to column B and centrifuged for 2 minutes at 1,000 x g. The bound plasmid was washed in 5ml of Washing Buffer and centrifuged for 1 minute at 2,000 x g, then washed in 5ml Final Wash Buffer and centrifuged for 2 minutes at 2,000 x g. A final wash of 10ml Final Wash Buffer was added and centrifuged for 2 minutes at 2,000 x g. The column was spun for 5 minutes at 4,000 x g to dry the resin. Column B was then transferred to a clean tube and 750µl of sterile water was added and left to incubate in the column for 3 minutes at room temperature. The column was then centrifuged for 5 minutes at 4,000 x g

to elute the plasmid DNA. To analyse the quality, quanity and size of the resulting plasmid DNA gel elctrophoresis was carried out as described in section 3.8.

4.3.3 Restriction digest of plasmid DNA

In order to produce an RNA probe the template DNA sequence had to be removed from the plasmid DNA including the 19-23 base promoter regions for the phage RNA polymerases, T7 and SP6. From the plasmid map it was decided that the restriction enzyme *Bal 1* (Promega, UK) would be used to obtain the DNA template for the T7 promoter producing a fragment of DNA approximately 200bp with blunt ends. Various restriction enzymes were tried to obtain the SP6 promoter, including *Nar 1* (Promega, UK), *Sma I* (Promega, UK) and *Eco RV* (Roche). It was decided *Eco RV* was the most successful restriction enzyme producing an approximately 200bp DNA fragment containing the template region with blunt ends. All restriction digests were carried out in the same way.

In a sterile thin-walled PCR tube the following reaction mix was compiled; 2µl of 10x Restriction Buffer (500mM Tris-HCl [pH 8.2] and 50mM $MgCl_2$), 0.2µl Acetylated BSA (10µg/µl) and 1µg of plasmid DNA made up to 20µl with nuclease-free water. The resulting solution was mixed by pipetting and then 5U of the restriction enzyme was added and incubated at 37°C for 3 hours. The enzyme was inactivated by heating the reaction to 65°C for 15 minutes. To check the digest was successful 2µl of the resulting mixture was run on a 1% agarose gel and viewed under UV after staining with ethidium bromide solution. See section 3.8 for further details of gel electrophoresis.

4.4 *In Situ* Hybridisation Techniques - Method 1

All glassware and bench areas used during this procedure were cleaned thoroughly with RNA Zap (Ambion) prior to any *in situ* hybridisation work in order to protect the RNA probes from degradation. All solutions were made with nuclease-free water (BDH Merck) and all plastic consumables were brought sterile and RNase free.

4.4.1 In Vitro Transcription

In Vitro Transcription uses phage RNA polymerases to produce RNA from DNA. This was initially carried out using the MAXIscript™ *in vitro* transcription kit (Ambion International, Texas, USA) to produce biotin labelled RNA probes. The polymerase T7 was used to produce sense RNA probes and SP6 was used to produce anti-sense RNA probes. Label was incorporated into the probes during the transcription process using a biotin labelled nucleotide (Biotin-126-uradine-5'-trophosphate) (Roche Biochemicals, East Sussex, UK). Both the sense and anti-sense probe reactions were carried out in the same way.

Into a sterile thin-walled PCR tube the following reaction mix was compiled; 2µl 10x Buffer (containing DTT and Spermidine), 1µl of 10mM ATP, CTP and GTP, 0.6µl of unlabelled 10mM UTP and 0.4µl of 10mM biotin-16-UTP, 1µg of template DNA and 2µl RNA polymerase (10U/µl polymerase and 5U/µl Ribonuclease inhibitor) made up to 20µl with nuclease-free water. This was mixed thoroughly and incubated at 37°C for 1 hour. The DNA template was removed by the addition of 2U of DNase 1 followed by incubation at 37°C for 15 minutes. The reaction was stopped by the addition of 1µl 0.5M EDTA. The resulting probes

were stored at -20°C until use. Gel electrophoresis was carried out as described in section 3.8 to assess the quality and size of the RNA probes produced.

4.4.2 Hybridisation

Initially *in situ* hybridisation for the detection of the RNA component of telomerase was carried out using the commercially available kit mRNA*locator*™-Hyb in combination with mRNA*locator*™-biotin both available from Ambion (Ambion International, Texas). The method was carried out according to the manufacturer's instructions as described below.

Paraffin embedded sections of tissue were deparaffinised using xylene and rehydrated using decreasing concentrations of ethanol from 100% to 50%, before being placed in Tris buffer. The sections were then digested in Proteinase K solution (4μg/ml in Tris buffer). The proteinase K solution was added to the slide completely covering the tissue section and a clean coverslip placed on top. The slide was then placed in a humid chamber and incubated for 30 minutes at 37°C. The slides were then washed 3 times for 4 minutes in tris buffer.

The probes were diluted 1 in 50 in *in situ* hybridisation buffer and 50μl applied to each slide. The slides were then covered with a clean coverslip, placed in a humid chamber and hybridised at temperatures between 40 and 55°C for either 4 hours or overnight.

Wash solutions of 1x and 2x were prepared and warmed to 50°C. One wash was carried out for 4 minutes in 2x wash buffer. Then sections were washed twice for 4 minutes in 1x wash buffer at 50°C.

The Biotin labelled probe was detected using the mRNAlocator™-Biotin kit (Ambion) again according to the manufacturer's instructions. Slides were incubated with a 1/300 dilution of Streptavidin-Alkaline Phosphatase Conjugate for 30 minutes at 37°C in a humid chamber. The slides were then washed twice in tris buffer for 4 minutes each. Next 50µl of NBT/BCIP substrate solution was added to each slide and left to incubate for 60 minutes to overnight at 37°C. Slides were then washed in water, dehydrated in ethanol, cleared in xylene and mounted with pertex.

4.5 *In Situ* Hybridisation Techniques - Method 2

4.5.1 In Vitro Transcription

DIG labelled RNA probes were produced using the DIG RNA Labelling Kit (SP6/T7) from Roche Diagnostics Ltd. East Sussex. RNA probes were produced containing DIG-11-UTP. The reactions were set up according to the manufacturer's instructions; the purified template DNA and nuclease-free water were added to a clean tube up to a volume of 13µl. Next 2µl of 10x NTP-labelling mix, 2µl 10x Transcription buffer, 1µl RNase Inhibitor and 2µl of either SP6 or T7 RNA polymerase were added to each tube. The reaction was mixed gently and incubated for 2 hours at 37°C. The DNA template was removed by the addition of 2µl of DNase I and the reaction incubated for 15 minutes at 37°C. The reaction was stopped by the addition of 1µl 0.5M EDTA (pH8.0). The probes were stored at -20°C until needed.

4.5.2 Hybridisation

The second *in situ* hybridisation method was carried out according to the method described by Chang *et al* (1999). Tissue sections were dewaxed and rehydrated in Histoclear and ethanol. The sections were then digested with proteinase K (20μg/ml) for 30 minutes at 37°C. Followed by post-fixation in 4% paraformaldehyde/PBS and rinsed in PBS. Acetylation was carried out in freshly prepared 0.1M triethanolamine/0.25% acetic anhydride twice for 5 minutes with rocking. The DIG labelled probes were diluted 1/50 in DIG Easy Hyb (Roche), applied to the sections and covered with a clean plastic coverslip. The slides were then incubated for 10 minutes at 65°C in a humid chamber to denature the probes and hybridised overnight at 45°C.

The following day the plastic coverslips were removed by placing the slides in 4x SSC for 5 minutes. The slides were then washed in 4x SSC for 5 minutes, 2x SSC/0.1% SDS twice for 5 minutes at room temperature, 0.2x SSC/0.1% SDS for 15 minutes at 55°C, 1x Washing buffer (Roche) for 5 minutes and 1x Blocking buffer (Roche) for 2 hours at room temperature with rocking. Next 50μl 0.5 unit/ml anti-DIG POD conjugate (Roche) was added to each and incubated for 1 hour in a humid chamber at room temperature. The slides were then washed 3 times for 5 minutes in 1x Wash buffer at room temperature and 0.1% Tween 20/PBS for 5 minutes all with rocking. Colourimetric detection was then carried out using DAB solution and the slides incubated for 10 minutes. The slides were washed in water, counterstained in heamatoxylin, dehydrated and mounted using PDX. A DIG Easy Hyb alone control was carried out with each experiment.

4.6 *In Situ* Hybridisation Techniques - Method 3

4.6.1 In Vitro Transcription

In vitro transcription was carried out as previously described in section 4.5.1. The probes were cleaned to see if unincorporated-labelled nucleotides were effecting the results. Unincorporated nucleotides, salts and labels were removed from the RNA probes using the NucAway™ Spin Columns (Ambion). The columns were prepared according to the manufacturer's instructions. The 20µl transcription reaction was added to the top of the column and spun at 750 x g for 2 minutes. A small amount of the resulting solution was analysed using gel electrophoresis (section 3.8) and the rest was stored at -20°C until needed.

4.6.2 Hybridisation

Hybridisation was carried out as previously described in section 4.5.2 with modifications. The probes were denatured at 95°C for 5 minutes and placed on ice before addition to the tissue sections. Hybridisation was carried out overnight at 42°C.

The following day washes included: 4x SSC for 5 minutes, 2x SSC/0.1% SDS twice for 5 minutes at room temperature, 0.2x SSC/0.1% SDS for 15 minutes at 55°C, 0.1X SSC/0.1% SDS for 15 minutes at room temperature, 1x Washing buffer (Roche) for 5 minutes and 1x Blocking buffer (Roche) for 2 hours at room temperature with rocking. Next 50µl 0.5 unit/ml anti-DIG Alkaline Phosphatase (AP) conjugate (Roche) was added to each and incubated for 1 hour in a humid chamber at room temperature. The slides were then washed 3 times in 1x Wash buffer for 5 minutes at room temperature and 0.1% Tween 20/PBS for 5 minutes

all with rocking. Colourimetric detection was then carried out using Alkaline Phosphatase Substrate kit IV (Vector Laboratories) made according to the manufacturer's instructions. The slides were washed in water, counterstained in Nuclear Fast Red (Vector Laboratories), dehydrated and mounted using VectaMount™ (Vector Laboratories). A DIG Easy Hyb alone control was carried out with each experiment.

4.7 Methods for checking probes were labelled and hybridising to the correct target.

4.7.1 Dot blots

In order to check the sense and anti-sense RNA probes were labelled and the detection antibodies were working correctly a dot blot was carried out with each experiment. Probe (2µl) was pipetted onto a small piece of Hybond N^+ membrane optimised for nucleic acid transfer (Amersham Pharmacia Biotech) and left to dry. Once the dots were dry the membrane was placed in 1x wash buffer (Roche) for 5 minutes then incubated for 2 hours in Block buffer (Roche) with rocking. The membrane was treated in exactly the same way as the slides. All the washes and detection steps were exactly the same as in the hybridisation methods explained above.

4.7.2 North Western Blot

A north-western blot was carried out to see whether the probes to the RNA component of telomerase bound to telomerase once extracted proteins were run on a gel and transferred to a membrane. Protein samples from the oesophageal cell-lines OE21 and OE33 were used. The western blot method was carried out as

previously described in section 3.10, but instead of immunodetection the RNA probes were utilised to detect the telomerase protein band on the membrane.

Glass plates, plastic spacers and combs from the BioRad Mini Protein II were cleaned and assembled according to the manufacturers instructions. A 6% main gel and a 4% stacking gel were made. Once the gels were poured into the frame and had dried a 1 in 3 dilution of the samples was prepared with 4x loading dye. The samples were boiled at 95°C for five minutes and placed on ice before a volume was loaded into a well of the gel. One well was loaded with 10µl of pre-stained molecular weight marker. The gel was then run at 75V for 15 minutes to allow the samples to move through the stacking gel and then at 100V for about 1 hour.

The gel was then removed from the glass plates and blotted onto an Immobilon-P Transfer Membrane, (Millipore) using an electroblotter (Hoefer SemiPhor™ Amersham Pharmacia Biotech) run for 55 minutes at 40-44mA. The pre-stained marker could be seen on the membrane once transfer was completed.

Once the protein bands had been blotted across onto the membrane hybridisation method 3 (section 4.6.2) was used to detect the telomerase band on the membrane. The probes were denatured at 95°C for 5 minutes and placed on ice before addition to 15ml DIG Easy Hyb buffer in a dilution of 1/1000. Hybridisation was carried out overnight at 42°C.

On the following day these washes were carried out: 4x SSC for 5 minutes, 2x SSC/0.1% SDS twice for 5 minutes at room temperature, 0.2x SSC/0.1% SDS for 15 minutes at 55°C, 0.1x SSC/0.1% SDS for 15 minutes at room temperature, 1x

Washing buffer (Roche) for 5 minutes and 1x Blocking buffer (Roche) for 2 hours at room temperature with rocking. Next 15ml 0.5 unit/ml anti-DIG AP conjugate (Roche) was added and incubated for 1 hour at room temperature. The membrane was then washed 3 times in 1x Wash buffer for 5 minutes at room temperature and 0.1% Tween 20/PBS for 5 minutes all with rocking. Colourimetric detection was then carried out using BM Purple AP Substrate (Roche) used according to the manufacturer's instructions. The membrane was washed in water and dried between filter paper.

4.8 Results

4.8.1 Telomerase Repeat Amplification Protocol (TRAP) results from tissue samples.

The TRAP assay was carried out on 156 different fresh frozen tissue types (81 from resected tissues and 75 from biopsy tissues) from 64 patients. They can be broken down into the following:

- 29 Cancer cases
- 25 Dysplasia cases
- 23 Barrett's Metaplasia cases
- 33 Non-involved Oesophageal Epithelium cases.

The details of the patients and sections are explained more in table 4.2.

The TRAP assay was optimised using four resected cancer samples. Titre experiments were carried out to find the optimum protein concentration for the assay. It was found to be about 0.0124μg/μl and to have a similar sensitivity to the cell-line extracts. All the tissue extracts were diluted to a similar concentration before use in the assay.

The results of the TRAP assay on tissue sections from individual patients are shown in table 4.2. A and B. The complete results from the tissue extracts are shown in table 4.2. C.

Table 4.2. TRAP tissue results

Abbreviations: Sample Diagnosis N Oes = non involved squamous epithelium, N Stom = Non-involved gastric epithelium with or without Helicobactor pylori (h. P.) infection, Barrett's IM = Barrett's oesophagus with intestinal metaplasia, C/F Barrett's = Barrett's oesophagus with cardiac or fundic metaplasia, Low Dys = Low grade Dysplasia, High Dys = High grade Dysplasia, and Cancer = adenocarcinoma either well diff., mod diff. or poorly diff. (well, moderately or poorly differenciated).
Telomerase positive either yes no or unsure where a sample gave both a positive and negative result.

A. Trap assay results from resected tissue sections from the individual patients tested.

Patient	Sample diagnosis	Telomerase Positive
1	N Oes N Stom(h. P infection) Cancer (well diff.)	No No Yes
2	N Oes N Stom Low Dys	No No No
3	N Oes N Stom Barrett's IM Cancer (well diff.)	Yes No No Yes
4	N Stom C Barrett's Low Dys Cancer (mod diff.)	No No No Yes
5	N Oes N Stom Cancer (Poorly diff.)	No No Yes
6	N Oes N Stom N Oes/Cancer Cancer (mod diff. With mucin)	No No No Unsure
7	N Oes N Stom (h. P infection) Cancer (mod diff.)	No No Yes
8	N Oes N Stom Barrett's IM Cancer	No No Yes Yes
9	N Stom	No
10	N Stom N Stom (h. P infection) Cancer (well diff.)	No No Yes
11	N Oes N Stom Cancer (poorly diff.)	No No Yes

12	N Oes N Stom (h. P infection) Cancer (mod diff.)	No No Yes
13	N Oes N Stom Cancer (mod diff. with mucin)	No No No
14	N Oes N Stom Cancer (poorly diff. diffuse)	No No No
15	N Stom Cancer (well diff.)	No Yes
16	N Stom (h. P infection) N Oes Cancer (mod diff.)	No Yes Yes
17	N Oes/Cancer N Stom Cancer (poorly diff.)	Yes No Yes
18	N Stom	No
19	N Stom Cancer (poorly diff. with mucin)	No No
20	N Stom (h. P infection) Low Dys	No No
21	N Stom Cancer (poorly diff.)	No No
22	N Oes N Stom Cancer (sqamous carcinoma)	No No Yes
23	Cancer	Yes
24	N Oes	No
25	C/F Barrett's	No
26	N Oes N Stom Cancer (mod. diff.)	No No Yes
27	N Stom (h. P infection) N Oes/Cancer Cancer (poorly diff.)	No No No
28	N Stom Barrett's IM Cancer (poorly diff.)	No Yes Yes
29	N Stom (h. P infection)	No
30	N Oes	No
31	N Oes	No
32	High Dys	No
33	N Stom Barrett's IM Cancer (mod diff.)	No No Yes

B. Trap assay results from biopsy tissue sections from the individual patients tested.

Patient	Sample Diagnosis	Telomerase Positive
34	N Oes	No
	C/F Barrett's	No
35	N Oes	No
	Low Dys	No
	High Dys	Yes
	High Dys (different surgery)	Yes
36	N Oes	No
	High Dys	No
	Intramucosal carcinoma	No
37	N Oes	No
	C Barrett's	No
	High Dys	No
38	N Oes	No
	N Stom	No
	High Dys	Yes
39	Low Dys	No
	High Dys	No
	Cancer in situ	Yes
40	Low Dys	No
	High Dys	No
41	N Stom	No
	High Dys	Yes
42	N Oes	No
	C Barrett's	No
	Barrett's IM	No
	Low Dys	No
43	N Oes	No
	C Barrett's	No
	Barrett's IM	No
	High Dys	No
	Cancer (well diff.)	Yes
44	Squamous Dys	No
	High Dys	No
45	N Oes	No
	N Stom	No
46	N Oes	No
	C/F Barrett's	No
	High Dys	Yes
	Cancer (well diff.)	Yes
47	Barrett's IM	No
	C Barrett's	No
48	Low Dys	No
	Cancer (mod diff.)	No
49	N Oes	No
	N stom	No
	Barrett's IM	Yes
	Low Dys	Yes

50	N Oes Cancer (mod diff.)	No Yes
51	N Stom	No
52	N Oes F Barrett's	No No
53	N Oes High Dys	No No
54	N Oes Barrett's IM Low Dys	Unsure Yes Yes
55	N Oes Low Dys	No Yes
56	N Oes C/F Barrett's	No No
57	N Oes High Dys	No Yes
58	N Oes	No
59	N Oes Barrett's IM	No Unsure
60	High Dys	No
61	C Barrett's High Dys	No No
62	Barrett's IM	No
63	N Oes C Barrett's Low Dys	No No Yes
64	N Oes Cancer (mod diff.)	No Yes

C. Summary of all the tissue section results. As each sample was assayed twice the unsure samples are those where one test was telomerase positive and the other was telomerase negative.

Diagnosis	Number of cases	Number of cases Telomerase positive	Percentage of cases telomerase positive	Unsure
Non-involved oesophagus	38	2	5.2%	1
Non-involved stomach	32	0	-	0
Barrett's Non-intestinal Metaplasia	11	0	-	0
Barrett's Intestinal Metaplasia	11	4	36.4%	1
Low-grade Dysplasia	12	4	33.3%	0
High-grade Dysplasia	15	6	46.2%	0
Adenocarcinoma	29	22	75.9%	0
Total	156	38	-	2

During this study telomerase activity was assayed in 156 fresh frozen samples (81 from resected tissue and 75 biopsy samples) from the 64 patients, using the TRAPEZE™ ELISA assay. Of the 38 non-involved oesophagus cases assayed, 2 (5.2%) were telomerase positive and one was undefinable for telomerase activity. All of the non-involved stomach samples showed no telomerase activity, as did all the non-intestinal Barrett's metaplasia cases. Of the 11 intestinal metaplasia cases assayed, 4 (36.4%) were positive for telomerase activity and one case was undefinable. Of the 12 low-grade dysplasia cases assayed, 4 (33.3%) were telomerase positive. Of the 15 high-grade dysplasia cases assayed, 6 (42.6%) were telomerase positive. Of the 29 adenocarcinoma cases assayed, 22 (75.9%) were telomerase positive.

4.8.2 Results of In Situ Hybridisation Techniques for the Detection of the RNA Component of Telomerase

The application of *in situ* hybridisation techniques for detecting telomerase RNA expression in archival paraffin-embedded and fresh frozen material can easily distinguish cancer from normal cells (Shay 1997a). The use of *in situ* hybridisation for the detection of the RNA template within the enzyme complex should allow the localisation of specific cells with telomerase activity. Previously *in situ* hybridisation has been used by Morales *et al* to study telomerase activity in the progression of Barrett's Oesophagus using a radioactive based method (Morales 1998). Here a method of *in situ* hybridisation for the detection of telomerase using a non-radioactive method has been developed. Figures 4.2, 4.3 and 4.4 contain images showing examples of the resulting staining from each of the *in situ* hybridisation methods developed.

In ISH method 1 the RNA probes were biotin labelled and detected using the enzyme alkaline phosphatase (AP). ISH Method 1 did not work successfully to detect the RNA component of telomerase. From figure 4.2 you can see there was no positive dark grainy staining in either the sense (A) or anti-sense (B) experiments. The kit contained a positive control experiment which resulted in strong dark black/purple staining (results not shown) implying there was a problem with the RNA probes or labelling of the probes. The dot blots and north-western blots for these experiments did work successfully showing the probes were labelled correctly so a new method was devised (results not shown).

In ISH method 2 the RNA probes were labelled with DIG and detected using the enzyme horse radish peroxidase (HRP). As the HRP substrate DAB results in red/brown staining a heamatoxylin counterstain was used in these experiments as shown in figure 4.3. Again this method was not successful as no red/brown staining can be seen in either the sense (A) or anti-sense (B) results. Again there did not seem to be a problem with the RNA probes or labels as the dot blots and north-western blots gave positive results (results not shown).

ISH Method 3 was the most successful and utilised DIG labelled sense and anti-sense RNA probes, an anti-DIG-AP antibody and BCIP/NBT AP substrate. From figures 4.4 and 4.5 it can been seen that dark black purple staining was present in sections hybridised with the anti-sense probe (A and B figure 4.4 and B figure 4.5). The sense probes were used as a negative control and in these experiments resulted in much weaker to negative staining. The staining was grainy and

present in the nuclei of tumour and dysplastic cells (results not shown).

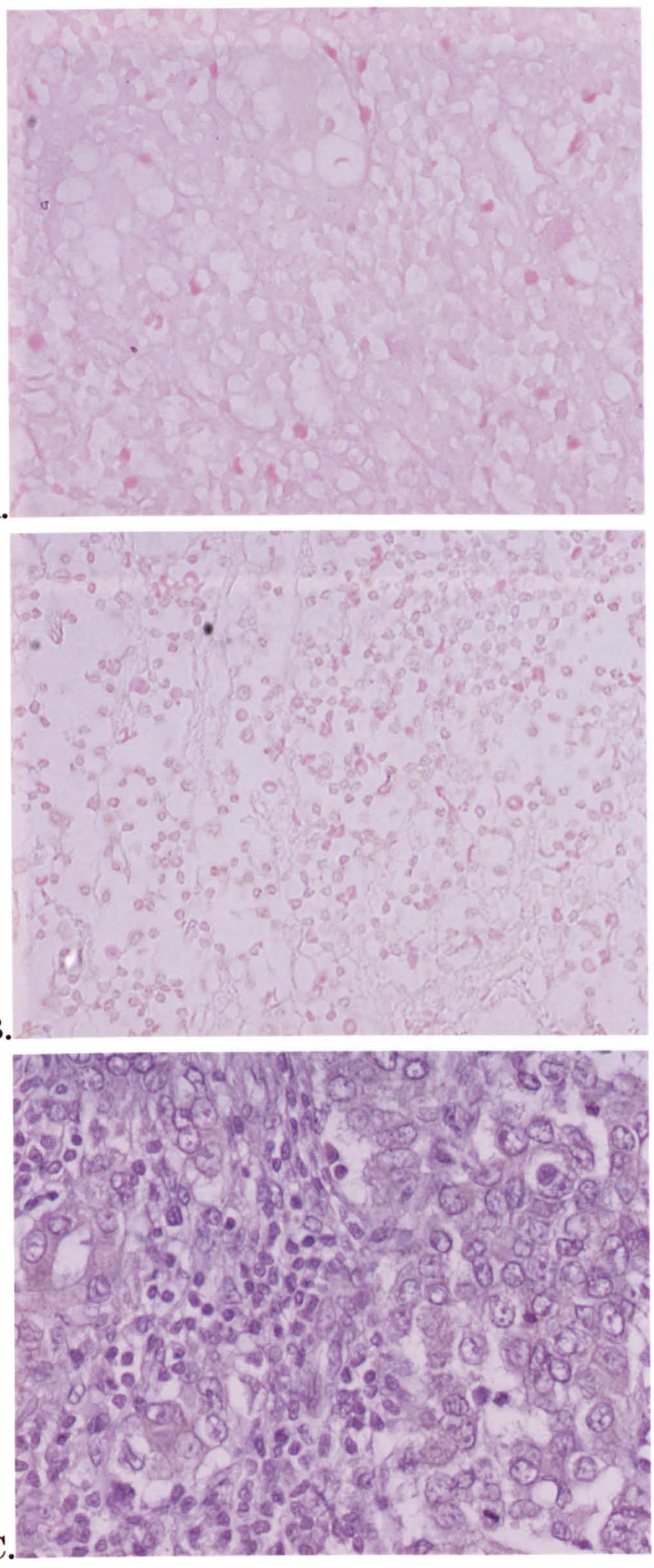
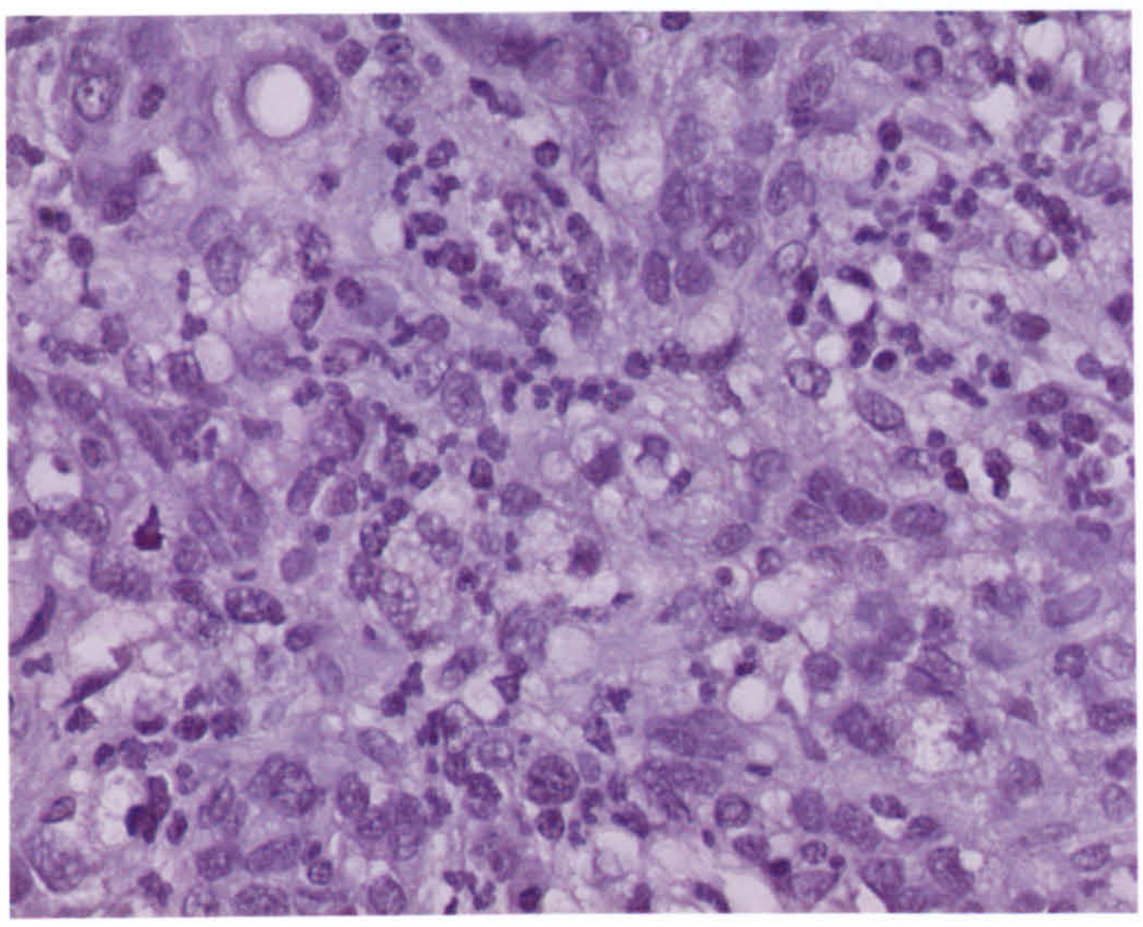


Figure 4.2. Examples of the results obtained on sections of oesophageal adenocarcinoma using ISH Method 1.

A. Sample 102 Sense ISH Method 1 (x10) **B.** Sample 102 Anti-sense ISH Method 1 (x10) **C.** Sample 102 stained for E-Cad IHC (x10).

A.



B.

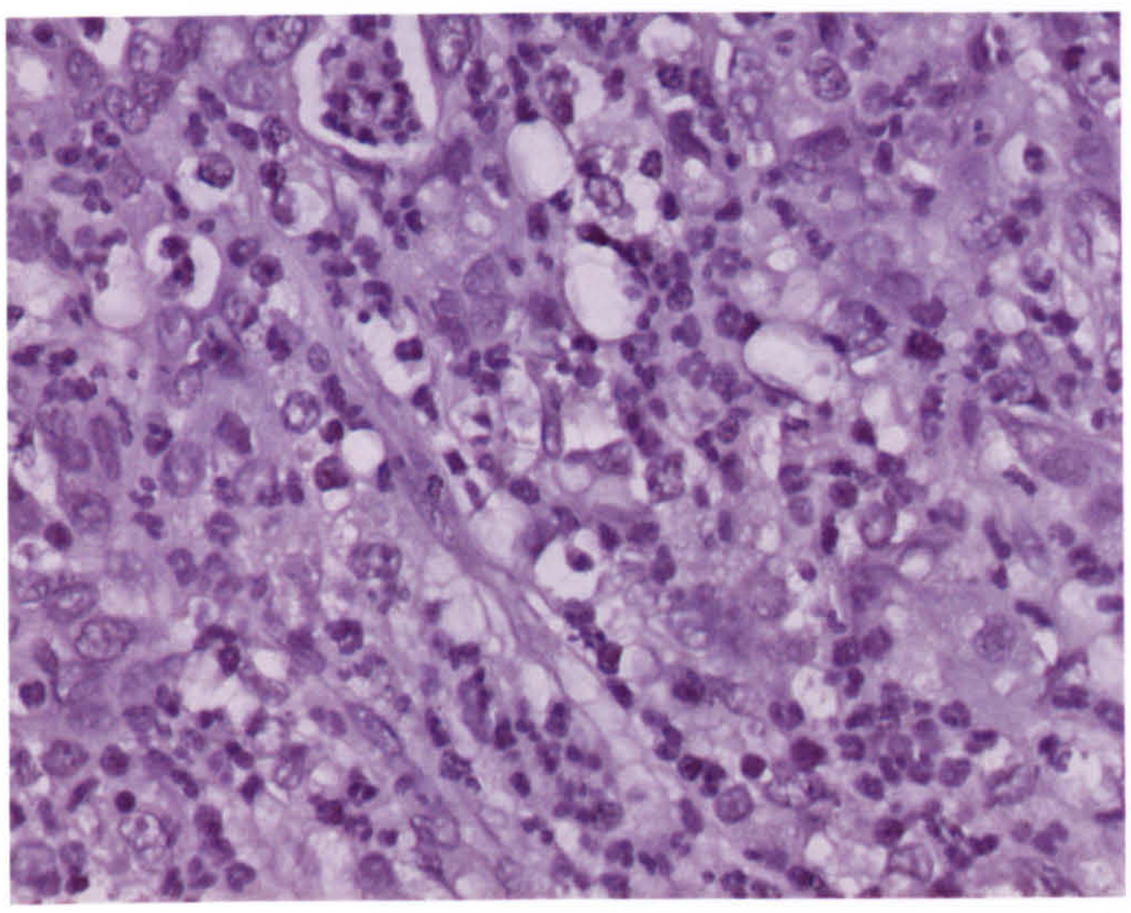
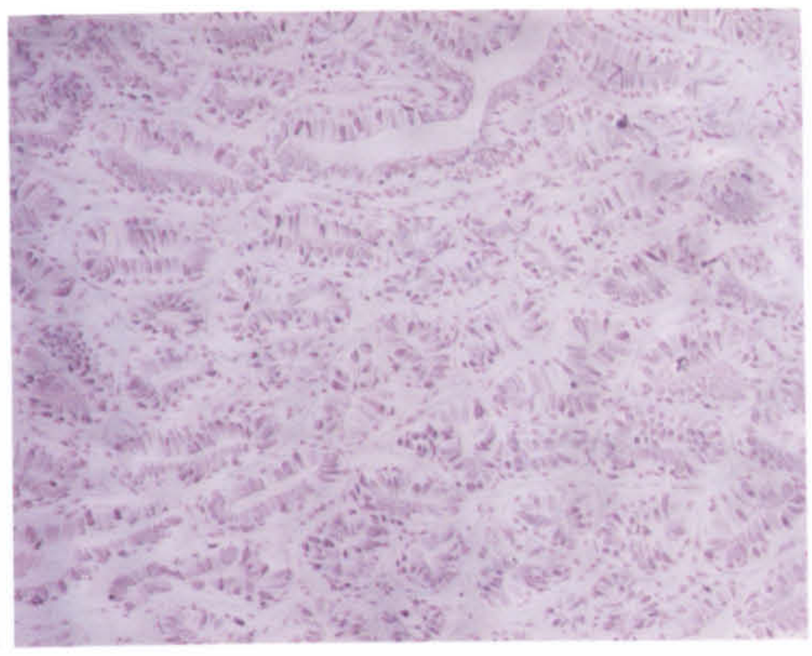
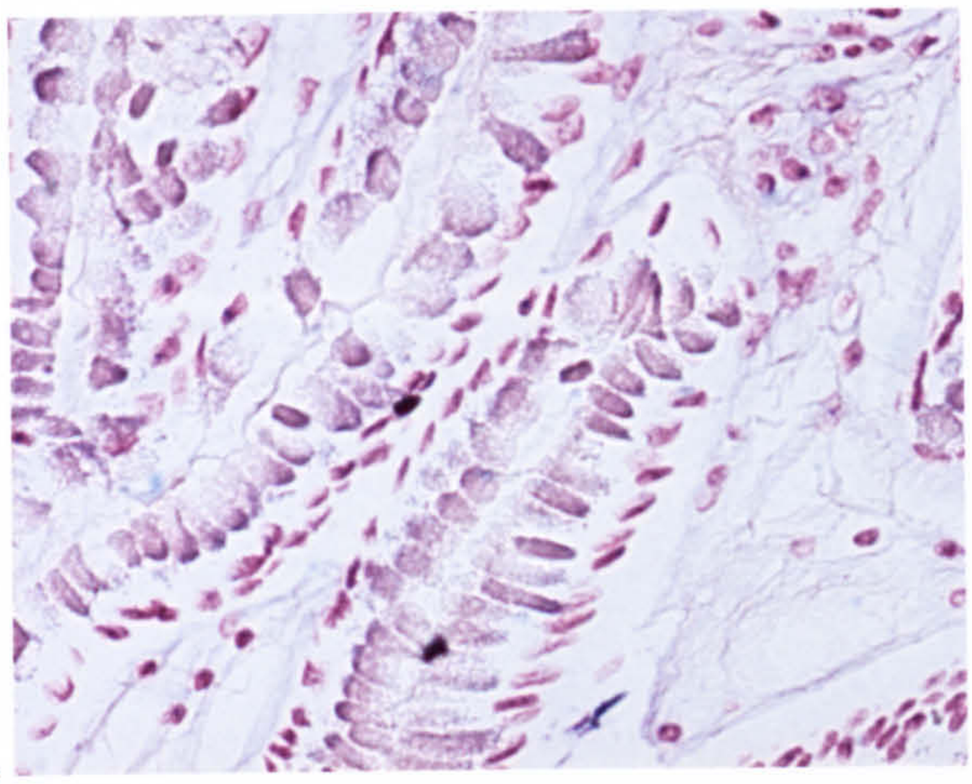


Figure 4.3. Examples of the results obtained on sections of Oesophageal adenocarcinoma using ISH Method 2.

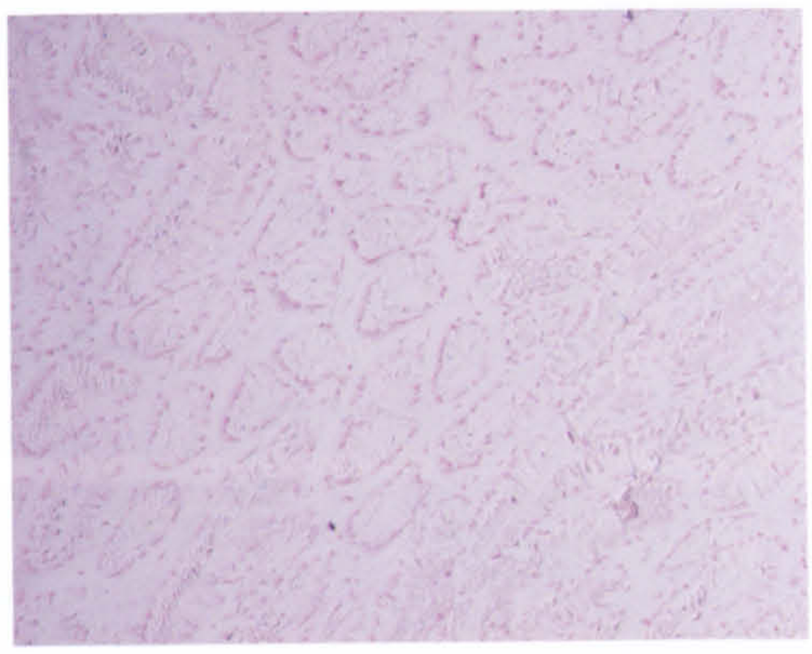
A. Sample 111 Sense ISH Method 2 **B.** Sample 111 Anti-sense ISH Method 2.



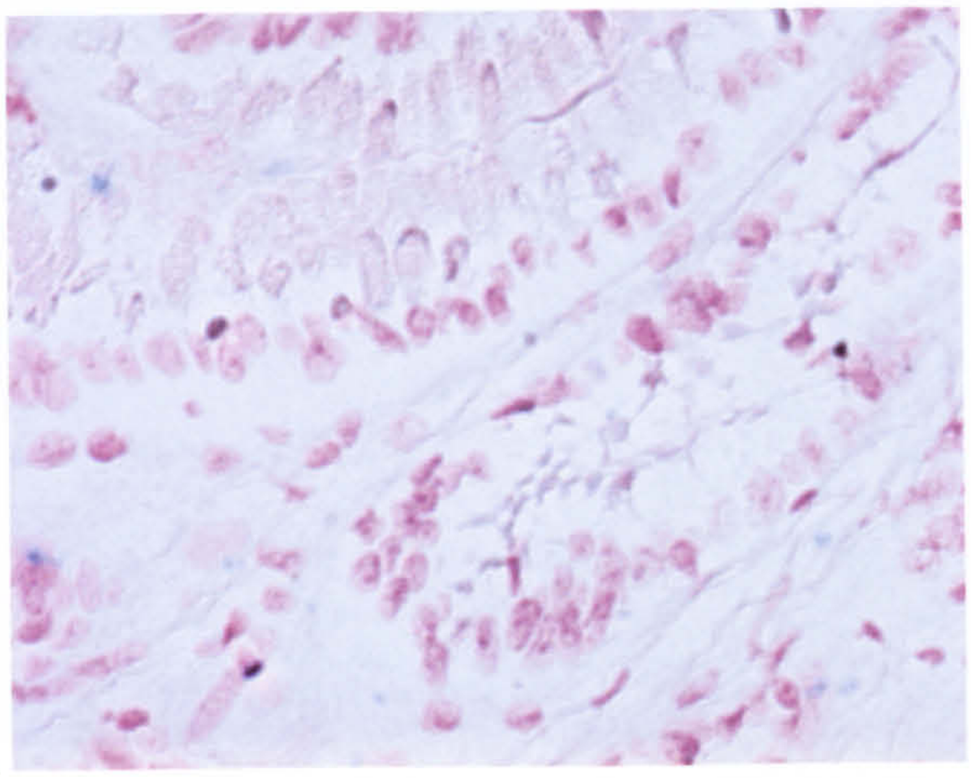
A.



B.



C.

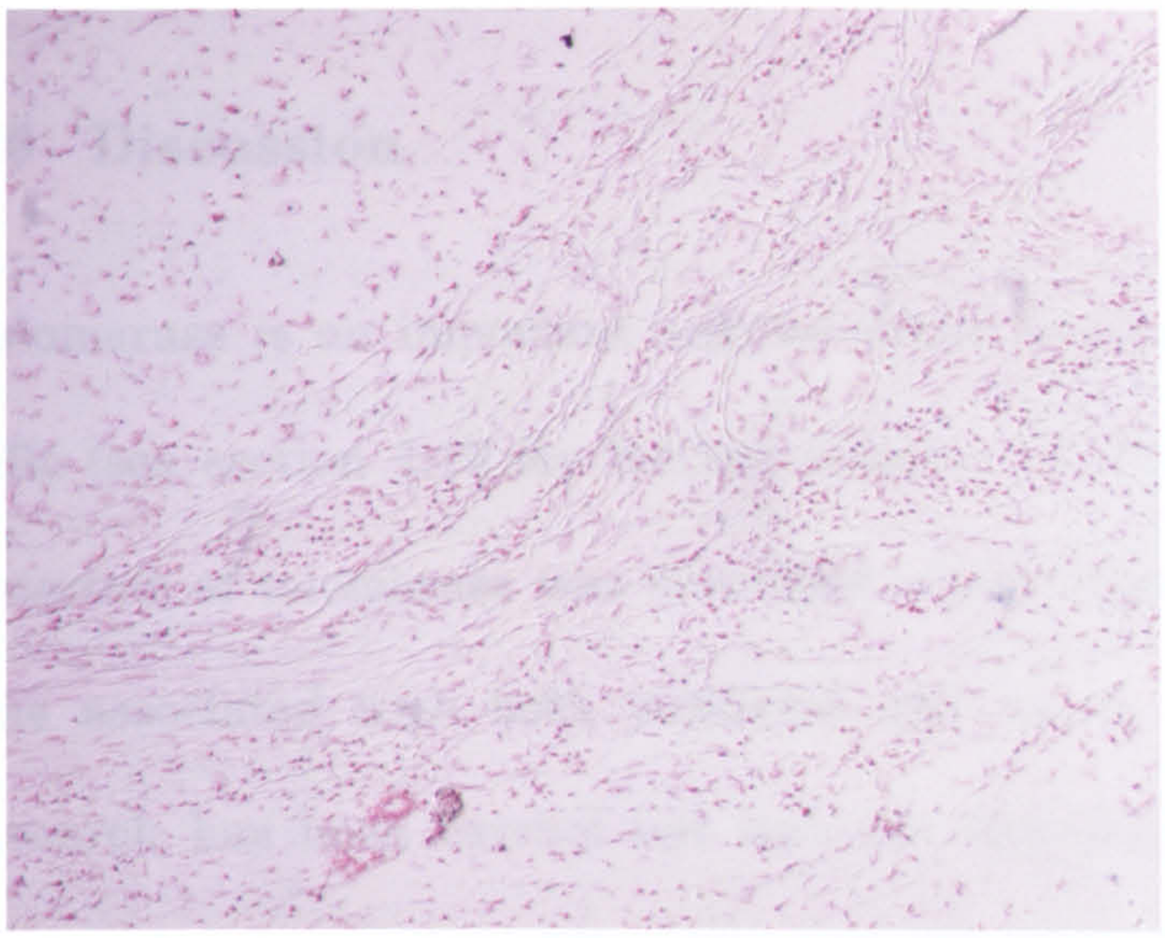


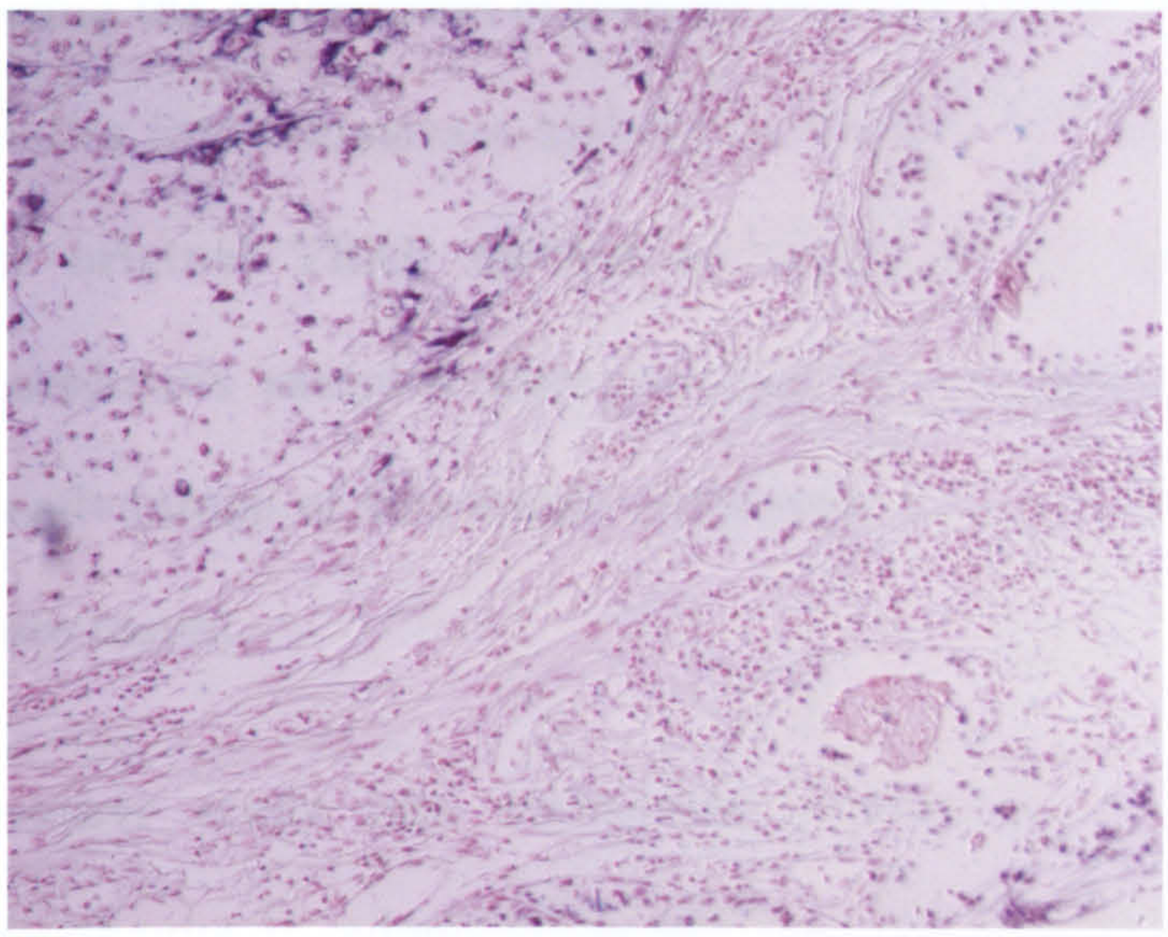
D.

Figure 4.4. Examples of the results obtained on sections of Oesophageal metaplasia using ISH Method 3.

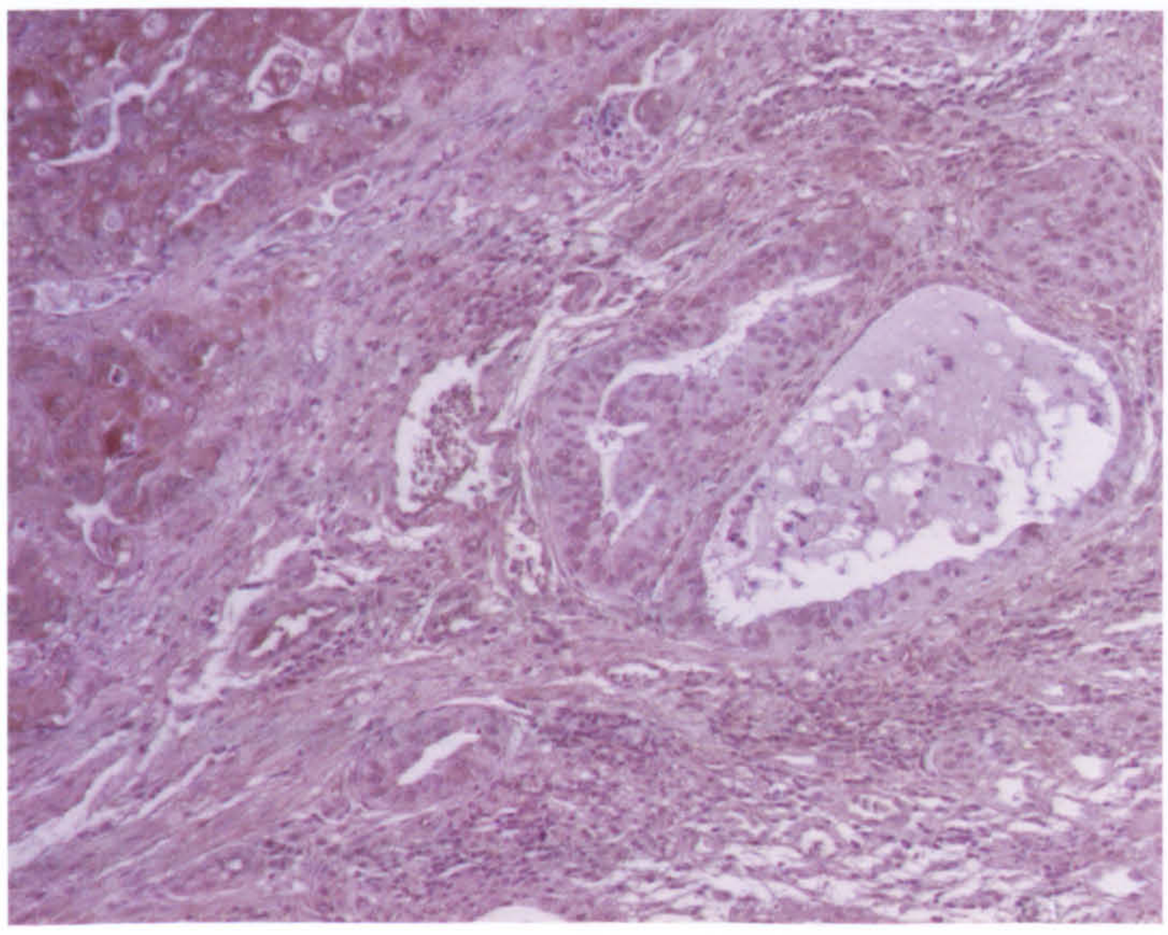
A. Sample 105 Anti-Sense ISH Method 3 (x10) **B.** Sample 105 Anti-Sense ISH (x40) **C.** Sample 105 Sense ISH (x10) **D.** Sample 105 Sense ISH (x40).

A.





B.



C.

Figure 4.5. Examples of the results obtained on sections of Oesophageal adenocarcinoma using ISH Method 3.

A. Sample 94 Sense ISH Method 3 (x10) **B.** Sample 94 Anti-sense ISH (x10) **C.** Sample 94 Stained for PAI-2 IHC (x10).

4.9 Discussion

Telomerase is an important enzyme found in renewal tissues and most cancer cells (see section 1.16 for further details). In 1994 a sensitive method for the detection of telomerase was described which suggested that telomerase activity was associated with both immortal and cancer cells (Kim 1994). Very little research has been carried out into the expression and activity of telomerase during the progression of Barrett's metaplasia to adenocarcinoma. From previous studies into telomerase activity in Barrett's metaplasia it has been shown using the original TRAP method on fresh frozen samples that 50% of Barrett's oesophagus samples were positive for telomerase activity (Ozawa 1997). The disadvantage of this small study was that only 8 Barrett's metaplasia samples were tested.

Another study into telomerase expression in the progression of Barrett's oesophagus to adenocarcinoma used *in situ* hybridisation to detect the RNA component of telomerase in paraffin embedded samples. It was found that 100% of high grade dysplasia and adenocarcinomas were telomerase positive, 90% of low grade dysplasia showed moderate telomerase activity and 70% of Barrett's metaplasia showed weak to moderate telomerase activity (Morales 1998). The results of this study are misleading, as the case numbers were very low (10 Barrett's metaplasia samples were tested and no details were given as to what type of metaplasia they were).

4.9.1 Detection of Telomerase Activity Utilising the TRAP Method

Overall 64 patients have been studied using the TRAP assay, 33 patients provided samples from resected tissues and 31 patients provided biopsy tissue samples. The resected tissues were considerably larger than biopsy samples. Resected tissues come from patients who have undergone surgery to remove large oesophageal adenocarcinomas from their oesophagus, whereas the biopsy samples come from patients who had been diagnosed with Barrett's oesophagus and were undergoing surveillance at the hospital on a regular basis.

There was a large difference in the amount of protein extracted from biopsy samples and resected samples. The biopsy sections were approximately 3-5mm across, whereas the resected samples were between 1-2cm across, therefore from the twenty sections cut from each block there was a larger amount of tissue in the tube of resected sample than biopsy sample. This was overcome by the dilution of the samples before the assay was carried out, but there were more likely to be other types of cells and inhibitors in the resected samples than the smaller biopsy samples.

A study by Bachor *et al* looking at telomerase activity in normal gastrointestinal mucosa and pre-cancerous lesions using the conventional TRAP assay found that all normal gastrointestinal mucosa contained active telomerase and it was not up regulated in pre-cancerous lesions (Bachor 1999). In Bachor's study only superficial biopsy samples were assayed. Here both biopsy and resected tissue samples have been assayed. It has been suggested that the main difference in the two types of tissues is in the amount of connective tissue. This is minimal in superficial biopsy samples, but substantial in resected samples. Connective

tissues are known to be telomerase negative (Harle-Bachor 1996) and may therefore dilute the activity of the telomerase expressing cells. Also proteins maybe present in the connective tissues that inhibit *Taq* polymerase, which is used in the PCR step of the TRAP assay reducing the amplification of the telomerase products.

Confirmatory histological diagnosis was carried out independently using three heamatoxylin and eosin (H and E) stained sections for each sample. These were accessed to check the sample diagnosis remained the same throughout the sections cut and placed in the tube. The tissue type was found to remain the same throughout all the cases used during this study. Although some of the resected samples contained a varied selection of cell types the diagnosis was given for the majority of cell type in the sample.

Of the 38 non-involved oesophagus cases 21 were from patients undergoing biopsy surveillance and 17 were from patients who had undergone oesophageal resection. The two non-involved oesophagus samples, which were telomerase positive, were resected tissue from patients #3 and #16. Both these patients have undergone oesophageal resection to remove oesophageal adenocarcinomas. The H and E sections for both these patients have been thoroughly examined and no cancer cells can be seen. Both patients had strongly positive cancer samples although each was of a different type (patient #6 had a well-differentiated cancer and patient #16 had a moderately differentiated cancer). It could be that the cancer cells in the tumour had influenced the surrounding cells in the non-involved oesophageal epithelium to express telomerase. The undefinable non-involved oesophageal sample came from patient #54. This is a biopsy sample. The

other samples from this patient an intestinal metaplasia sample and a low grade dysplasia sample were both telomerase positive. Each sample in this study was assayed twice and was defined as telomerase positive if both samples had an absorbance >0.150. This sample gave one positive and one negative result and was therefore undefinable.

In skin, telomerase activity has been detected in the basal level of the epidermis, i.e. the proliferative compartment and is switched off during differentiation (Harle-Bachor 1996). All normal telomerase positive tissues are permanently renewing epithelia or tissues that proliferate in regular cycles. Therefore it would be expected that oesophageal epithelium would contain a population of telomerase positive cells. For gastrointestinal tissues it has been found that telomerase activity is detectable in all epithelia of the gastrointestinal tract using the conventional TRAP assay on biopsy samples (Bachor 1999). This could explain the telomerase positive non-involved oesophagus samples.

Of the 32 non-involved stomach samples 5 were biopsy samples coming from patients undergoing surveillance for Barrett's oesophagus and 27 came from patients who had undergone oesophageal resection to remove a large oesophageal adenocarcinoma. All of the samples assayed were telomerase negative.

The non-intestinal Barrett's metaplasia samples include both cardiac and fundic type metaplastic tissues. Of the 11 samples 9 are biopsy samples from patients undergoing surveillance for Barrett's oesophagus and 4 are samples from patients who had undergone oesophageal resection. All of these samples were telomerase negative.

Of the 13 intestinal metaplasia samples 7 were biopsy samples from patients undergoing surveillance for Barrett's oesophagus and 4 were from patients who had undergone oesophageal resection. The telomerase positive samples come from 4 different patients, 2 were biopsy samples (patient #49 and #54) and 2 were from resected tissue (patients #8 and #28). The undefinable sample came from patient #59 and was a biopsy sample.

It has been suggested that the amount of telomerase activity in Barrett's metaplasia samples maybe difficult to detect (Bachor 1999). In Barrett's oesophagus the stratified epithelium is replaced by a single-layered epithelium (resembling stomach and intestine). This will result in a larger amount of connective tissues in the samples tested, as only the thin surface layer of the tissue will contain the metaplastic cells. This problem could be over come with the use of laser capture micro-dissection apparatus where only the metaplastic cells could be selected and lysed for use in the TRAP assay. This was not available for this study.

Of the 12 low-grade dysplasia samples 9 were biopsy samples from patients undergoing surveillance for Barrett's oesophagus and 3 were from resected tissues. All 4 telomerase positive samples were biopsy samples (patients #49, #54, #55, and #63).

Of the 15 high grade dysplasia samples 14 were biopsy samples from patients undergoing surveillance for Barrett's oesophagus and one sample was from resected tissue. All 6 of the telomerase positive samples were biopsy samples (patients #35 [twice on two different surgeries], #38, #41, #46, and #57).

There were significantly more dysplastic samples from patients undergoing surveillance for Barrett's metaplasia than oesophageal resection. This is because it is virtually impossible to differentiate between dysplasia and Barrett's metaplasia with the naked eye. Therefore it is impossible to select a dysplastic area within a piece of resected tissue. It is much easier to collect a biopsy sample from a patient with known dysplasia who is undergoing surveillance. Also there were more fresh frozen biopsy samples than resected tissues available for analysis.

Of the 29 adenocarcinoma samples 6 were biopsy samples from patients undergoing surveillance for Barrett's oesophagus, these can also be defined as cancer *in situ* samples and 23 were from patients undergoing oesophageal resection. One cancer *in situ* sample was telomerase negative (patient #48) and 5 resected samples were telomerase negative (patients #13, #14, #19, #21, and #27). The telomerase negative cancer samples were mainly from poorly differentiated adenocarcinoma samples (patients # 14, #19, #21, and #27). The samples also contained a large amount of mucin, which would act to dilute the amount of telomerase containing cells in the sample (patient #13 and #19). The biopsy sample and one of the resected samples were moderately differentiated adenocarcinomas (patients #48 and #13). These samples also only contained just over 50% of the diagnosed tissue type (adenocarcinoma) the rest of the section was connective tissue. This could act to dilute the amount of telomerase expressing cells present.

The TRAPeze™ ELISA kit used here is not as sensitive for telomerase activity as the conventional TRAP assay. It only gives a positive/negative answer there is no

way of quantifying the telomerase activity and no information about which cells in the samples have telomerase activity. This problem can only be overcome by using a method for detecting telomerase within a section of tissue on a slide. *In situ* hybridisation can be used to detect the RNA component of telomerase in specific cells (Morales 1998). The *in situ* hybridisation technique should be more specific than the TRAPEZE™ kit and therefore should locate small amounts of telomerase in individual cells within the tissue sections.

The main disadvantage of using the TRAP method for analysing telomerase activity in clinical tissue samples is there is no way of telling exactly which cells within the samples are expressing telomerase. As mentioned some of the resected tissue samples studied contained a range of tissue types. In order to get a complete picture of telomerase activity within the progression of Barrett's metaplasia to adenocarcinoma a method of *in situ* hybridisation for the detection of the RNA component of telomerase has been developed.

4.9.2 Detection of Telomerase Utilising in situ Hybridisation

As the active telomerase molecule contains an integral RNA template telomerase can be detected by northern blot, RT-PCR or *in situ* hybridisation. Initial studies into Northern blotting for the detection of the RNA component of telomerase were disappointing. The RNA component appeared to be ubiquitously present in both normal and tumour tissues and expression levels did not correlate with telomerase activity. When RT-PCR was used for detection the molecule was found in tumour as well as normal tissues, although quantitatively higher levels were seen in tumour tissues. Interestingly, in some circumstances analysis of the RNA component of telomerase in paraffin embedded tissues using *in situ*

hybridisation correlates with telomerase activity (Matthews 2001). *In situ* hybridisation for the detection of the RNA component of telomerase has become the most widely used alternative marker for telomerase detection, which allows preservation of tissue morphology.

Two studies have looked at telomerase in oesophageal tumours using *in situ* hybridisation, Morales *et al* detected the RNA component of telomerase during the progression of Barrett's oesophagus to adenocarcinoma (Morales 1998) and Hiyama *et al* detected telomerase mRNA in squamous cell carcinoma and dysplasia (Hiyama 1999). Hiyama found low levels of telomerase the in basal cells of oesophageal squamous mucosa and high levels in cases of oesophageal carcinoma and dysplasia. This is in keeping with other epithelial tissues such as the cervix (Yashima 1998). In the study by Morales *et al* there was a marked increase in expression between low and high grade dysplasia of the oesophagus as well as in other areas of the intestinal tract (Morales 1998).

In Morales study, *in situ* hybridisation for the detection of the RNA component of telomerase has been carried out on tissue samples during the progression of Barrett's Oesophagus using radiolabelled RNA probes (Morales 1998). It was found that 100% of high grade dysplasia and adenocarcinomas were telomerase positive, 90% of low grade dysplasia showed moderate telomerase activity and 70% of Barrett's metaplasia showed weak to moderate telomerase activity. The main problem with Morale's study was that only a small number of samples were analysed. As radioactive labelling was unavailable here a method of non-isotopic *in situ* hybridisation, for the detection of the RNA component of telomerase,

during the progression of Barrett's oesophagus to adenocarcinoma has been developed.

In situ hybridisation for the detection of telomerase was carried out using an RNA anti-sense probe to the RNA component of telomerase. Three main methods of *in situ* hybridisation were tested. The first method was carried out using biotin labelled RNA probes and commercially available kits from Ambion International, Texas, USA according to the manufacturer's instructions. The second method was carried out using DIG labelled RNA probes and anti DIG-POD conjugated antibodies with DAB Detection (Chang 1999). The final method was a further development of Chang's method using anti DIG-AP antibodies and BCIP/NBT Alkaline Phosphatase substrate for detection.

Dot blots were used throughout the development of the *in situ* hybridisation methods to check the labelling of the probes and that the detection steps worked successfully in each experiment. The north-western blot described here also worked well as a method of checking that the RNA probes used were specific and did in fact hybridise to the RNA component of the telomerase protein (results not shown).

In ISH method 1 the RNA probes were biotin labelled and detected using AP. This method did not work successfully there was no positive dark grainy staining in either the sense or anti-sense experiments. This commercially available kit was developed to detected mRNA in tissues. A positive control experiment was included in the kit which detected c-myb mRNA in sections of mouse brain. When tested the positive control experiments resulted in strong dark black/purple staining, implying there was a problem with the RNA probes or labelling of the

probes. The dot blots and north-western blots for these experiments did work successfully showing the probes were labelled correctly. The problem with this method could be that the kit was unsuitable for the detected of the small RNA component of telomerase.

In ISH method 2 the RNA probes were labelled with DIG and detected using HRP. As the HRP substrate DAB resulted in red/brown staining a heamatoxylin counterstain was used in these experiments. Again this method was not successful as no red/brown staining was seen in either the sense or anti-sense experiments. Again there did not seem to be a problem with the RNA probes or labels as the dot blots and north-western blots gave positive results.

This method was altered, as an AP based detection system resulting in dark black/purple staining with a red counterstain would give clearer visualisation of weak staining. In method 2 the heamatoxylin counterstain was too strong for clear visualisation of possible weak red/brown staining in the nucleus of cancer cells.

The most successful of the *in situ* hybridisation methods tested here was the final method developed. This method was loosely based on that described by Chang *et al* 1999 and utilised DIG labelled RNA sense and anti-sense probes detected by the reaction of the enzyme alkaline phosphatase with BCIP/NBT. Dark black purple staining was present in the sections hybridised with the anti-sense probe. The sense probe was used as a negative control and in these experiments resulted in much weaker to negative staining. The staining was grainy and present in the nuclei of cancer and dysplasia cells in the cases analysed.

The results of the few tumour samples analysed with this method looked promising. Unfortunately only eight cases were fully analysed with this method. All eight cases contained telomerase positive cells within dysplasia, adenocarcinoma and lymph node metastasis tissue sections. In the future method 3 could very easily be utilised to carry out a full assessment of the RNA component of telomerase in the metaplastic:neoplastic progression of Barrett's oesophagus and its correlation with telomerase activity detected with the TRAP assay.

5 The Components of the urokinase-type Plasminogen Activator System During the Metaplastic:Neoplastic Progression of Barrett's Oesophagus

5.1 Introduction

Tumour cell invasion involves the attachment of tumour cells to the underlying basement membrane, local proteolysis and migration of tumour cells through this degraded region. Proteases are involved throughout the metastatic process. In order for a cell to metastasise it must be able to move into blood vessels, survive in circulation, arrest, move out of the blood vessels, invade surrounding tissues and grow. All these steps involve interactions between tumour cells, stromal cells, invading lymphocytic cells, endothelial cells and the extracellular matrix. Proteases, which are expressed in these cells (including Cathepsin B and D, urokinase-type Plasminogen Activator and receptor, and interstitial collagenase), are believed to participate in many of these steps.

The progression of Barrett's oesophagus through increasing degrees of dysplasia to adenocarcinoma is characterised by the ability of epithelial cells to invade the basement membrane. Changes in the expression of components of the plasminogen activation system, which allows increased production of plasmin, are thought to have an important role in malignant cell invasion, but little is known about the expression of these components in the premalignant stages of Barrett's oesophagus. Here the components of the plasminogen activation system have been studied during the metaplastic:neoplastic progression of Barrett's Oesophagus using both immunohistochemistry and ELISA techniques.

5.2 Immunohistochemistry on paraffin embedded sections using antibodies to the components of the urokinase-type Plasminogen Activator System

Immunohistochemistry was carried out as described in section 3.9 with the following modifications. Paraffin embedded sections of tissue were dewaxed using Histoclear® (Fisher Scientific) and rehydrated using decreasing concentrations of ethanol from 100% to 50% before being placed in PBS to equilibrate the slides. Non-specific reactions were then blocked using rabbit serum (1:5 [v/v] in PBS).

The blocking serum was removed without washing and the primary antibody added diluted with PBS containing 1% rabbit serum and left in a humid atmosphere at 4°C overnight. Details of primary antibodies and working concentration are given in table 5.1.

Each case was analysed using five antibodies to the components of the plasminogen system (polyclonal uPA, monoclonal uPA, uPAR, PAI-1 and PAI-2). The same cases were analysed using E-Cad antibodies (see table 5.1). This was to ensure the results of the plasminogen components could be comparable to other well-documented molecular markers for disease progression in Barrett's oesophagus. Western blot analysis, as described in section 3.10, was carried out using protein samples from the oesophageal cell-lines OE21 and OE33 to assess the specificity of the primary antibodies.

Once counterstained with heamatoxylin the slides were viewed under a light microscope and archived using AxioVision Version 3 (Imaging Associates, Thame, UK).

Table 5.1 Antibodies used for immunohistochemistry on slide flasks.

Antibody	Type	Working Concentration	Company
uPA	Polyclonal goat anti-human	4μg/ml	American Diagnostica Inc. Greenwich USA
uPA	Monoclonal mouse anti-human	4μg/ml	American Diagnostica Inc. Greenwich USA
uPAR	Monoclonal mouse anti-human	4μg/ml	American Diagnostica Inc. Greenwich USA
PAI-1	Monoclonal mouse anti-human	4μg/ml	American Diagnostica Inc. Greenwich USA
PAI-2	Polyclonal goat anti-human	4μg/ml	American Diagnostica Inc. Greenwich USA
E-Cadherin	Monoclonal mouse anti-human	2μg/ml	Alexis Biochemicals, Nottingham, UK

5.3 Detection of the components of the urokinase Plasminogen Activator System using ELISA techniques

The quantitative analysis of the components of the urokinase Plasminogen Activator system was carried out on the same cell extracts as were analysed using the TRAPEZE™ ELISA using commercially available kits. These kits were the IMUBIND® uPA ELISA kit, the IMUBIND® Total uPAR ELISA kit, the IMUBIND®Tissue PAI-1 ELISA kit and the IMUBIND® PAI-2 ELISA kit (American Diagnostica Inc. Greenwich, USA). These kits contained all the reagents necessary to perform enzyme-linked quantitative analysis of protein extracts from fresh-frozen tissue samples. Analysis was carried out according to the manufacturer's protocol and is detailed below. The principles of ELISA detection are shown in Figure 5.1.

5.3.1 uPA, uPAR and PAI-1

Each kit contained lyophilised standards of varying concentrations which were rehydrated at the start (uPA 0.00, 0.10, 0.25, 0.50, 0.75, 1.0ng/ml, uPAR 0.00, 0.25, 0.75, 1.5, 2.0, 3.0ng/ml and PAI-1 0.00, 1.0, 2.5, 5.0, 7.5, 10.0ng/ml). Protein extracts were selected from those used in the TRAP assay that had a concentration >0.5μg/ml. These were then diluted 1/20 with Sample buffer (1% BSA in Wash buffer [PBS with 0.025% Triton-X 100]). To the microtitre plate 100μl of the standard or diluted protein extract was added to each well as shown in figure 5.2. The plate was then covered and incubated overnight at 4°C in a humid chamber.

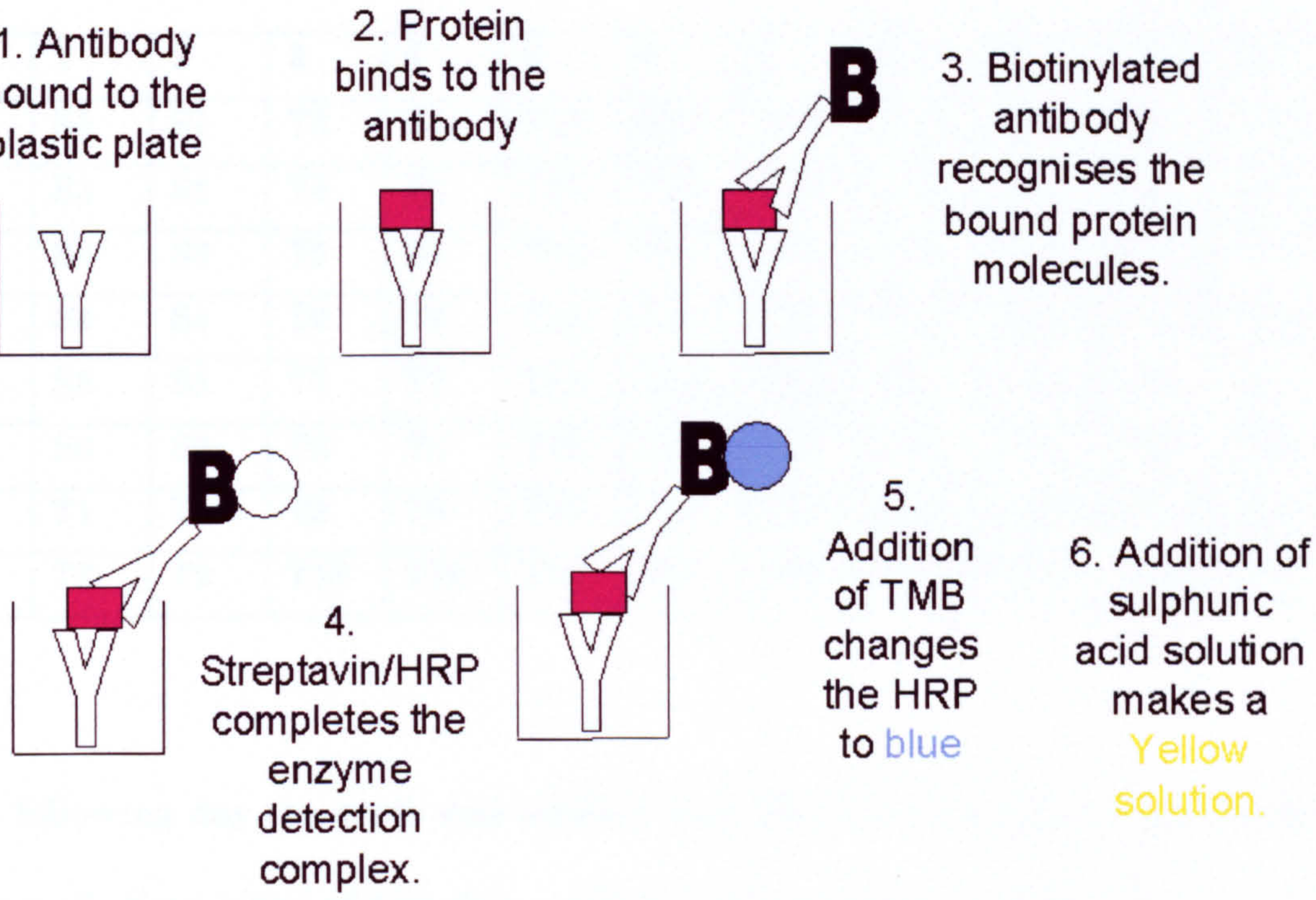


Figure 5.1. The Principles of ELISA detection of the components of the urokinase-type Plasminogen Activator system.

Abbreviations: B = biotin label, HRP = Horseradish Peroxidase and TMB 3,3', 5,5' Tetramethylbenzidine.

Table 5.2. Sample arrangement for the IMUBIND® ELISA on the microtitre plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	T3	T3	T11	T11	T19	T19	T27	T27	T35	T35
B	S2	S2	T4	T4	T12	T12	T20	T20	T28	T28	T36	T36
C	S3	S3	T5	T5	T13	T13	T21	T21	T29	Y29	T37	T37
D	S4	S4	T6	T6	T14	T14	T22	T22	T30	T30	T38	T38
E	S5	S5	T7	T7	T15	T15	T23	T23	T31	T31	T39	T39
F	S6	S6	T8	T8	T16	T16	T24	T24	T32	T32	T40	T40
G	T1	T1	T9	T9	T17	T17	T25	T25	T33	T33	T41	T41
H	T2	T2	T10	T10	T18	T18	T26	T26	T34	T34	T42	T42

The following day the plate was washed four times with 200µl of wash buffer in each well. Next 100µl of detection antibody (biotinylated antibody that recognises the bound molecule; either uPA, uPAR or PAI-1) was added to each well. Each standard and sample was tested in duplicate. The plate was covered and incubated for 1 hour at room temperature. The plate was again washed four times with wash buffer before the enzyme conjugate (streptavidin conjugated to the enzyme horseradish peroxidase) was added (diluted 1/1000). The plate was covered once more and incubated for 1 hour at room temperature. Next the plate was washed four times using wash buffer and 100µl of enzyme substrate was added (perborate/3.3',3,5'-tetramethylbenzidine [TMB]). This complex was incubated at room temperature for 20 minutes while the enzymatic reaction took place producing a blue colour. The reaction was stopped with the addition of 100µl 0.5N H_2SO_4 that caused the solution to turn yellow. The absorbance was then measured at a wavelength of 450nm within 30 minutes. The background

blank value for 0.0ng.ml standard was deducted from all the standard and sample readings.

5.3.2 PAI-2

The PAI-2 ELISA was carried out in the same way as those for uPA, uPAR and PAI-1, with the exception of the overnight incubation of the sample at 4°C in a humid chamber. For the detection of PAI-2 the sample or standard (0.0, 0.5, 1.0, 2.5, 5.0, 10.0ng/ml) was added to the wells and left to incubate for 2 hours at room temperature. Then the plate was washed 4 times in wash buffer and the detection antibody added as for the detection of the other components. The rest of the method was completed as described above.

5.3.3 Calculation of Results

A standard curve was constructed for each experiment by plotting the mean absorbance value for each standard versus the corresponding concentration. A new standard curve was generated every time the assay was performed. The mean absorbance value for each of the diluted samples was then used to determine the concentration in ng/ml from the standard curve. The sample value was then multiplied by 20 and divided by the protein concentration of the tissue extract (mg/ml) to convert ng/ml sample to ng/mg protein.

5.3.4 Statistical Analysis

Statistical analysis of the data was carried out using the computer programme GenStat for Windows. The results of this are detailed in appendix 7.

5.4 Results

In order to assess the usefulness of the components of the urokinase-type Plasminogen Activator system as molecular markers for the progression of Barrett's oesophagus to adenocarcinoma, immunohistochemistry and enzyme linked immuno-sorbent assays (ELISA) techniques were utilised.

5.4.1 Results of Immunohistochemistry on paraffin embedded sections using antibodies to the components of the urokinase Plasminogen Activator System

Overall 847 archived paraffin-embedded sections from 121 clinical cases have been analysed using immunohistochemistry for the detection of the components of the plasminogen activator system, and E-Cadherin. These cases ranged from non-dysplastic Barrett's (40 cases), through low and high grade dysplasia (8 and 18 cases respectively) to adenocarcinoma (41 cases) and lymph node metastasis (14 cases). The results are summarised in table 5.3. Examples of the immunoreactivity for each of the components of the plasminogen activator system are shown in figures 5.2, 5.3, 5.4, and 5.5.

Table 5.3. Overall immunohistochemistry results.

A. Non-Dysplastic Barrett's Metaplasia, **B.** Low Grade Dysplasia, **C.** High grade dysplasia, **D.** Barrett's Associated Adenocarcinoma, **E.** Cancer with no metastasis, **F.** Cancer with Lymph node metastasis and **G.** Lymph Node Metastasis.

Grading of staining: - = Negative, +/- = Weak, + = Moderate, ++ = Strong and +++ = Very Strong.

A. Non-dysplastic Barrett's (n=40)

PuPA			MuPA			uPAR			PAI-1			PAI-2			E-Cad		
	No.	%		No.	%		No.	%		No.	%		No.	%		No.	%
-	14	35	-	6	15	-	12	30	-	3	7.5	-	8	20	-	20	50
+/-	9	22.5	+/-	7	17.5	+/-	13	32.5	+/-	11	27.5	+/-	18	45	+/-	14	35
+	15	37.5	+	23	57.5	+	11	27.5	+	22	55	+	13	32.5	+	4	10
++	2	5	++	4	10	++	4	10	++	3	7.5	++	0	/	++	2	5
+++	0	/	+++	0	/	+++	0	/	+++	1	2.5	+++	1	2.5	+++	0	/
Weak to moderate			Moderate			Weak to moderate			Moderate			Weak to moderate			Weak to negative		

B. Low Grade Dysplasia (n=8)

PuPA			MuPA			uPAR			PAI-1			PAI-2			E-Cad		
	No.	%		No.	%		No.	%		No.	%		No.	%		No.	%
-	2	25	-	0	/	-	4	50	-	1	12.5	-	1	12.5	-	3	37.5
+/-	4	50	+/-	5	62.5	+/-	2	25	+/-	1	12.5	+/-	4	50	+/-	3	37.5
+	12	25	+	3	37.5	+	2	25	+	6	75	+	3	37.5	+	2	25
++	0	/	++	0	/	++	0	/	++	0	/	++	0	/	++	0	/
+++	0	/	+++	0	/	+++	0	/	+++	0	/	+++		/	+++	0	/
Weak to moderate			Weak to moderate			Weak to negative			Moderate			Weak to moderate			Weak to negative		

C. High Grade Dysplasia (n=18)

PuPA			MuPA			uPAR			PAI-1			PAI-2			E-Cad		
	No.	%		No.	%		No.	%		No.	%		No.	%		No.	%
-	7	39	-	4	22	-	5	28	-	0	/	-	2	11	-	5	28
+/-	4	22	+/-	5	28	+/-	6	33	+/-	4	22	+/-	7	39	+/-	6	33
+	5	28	+	4	22	+	4	22	+	11	61	+	6	33	+	3	17
++	2	11	++	5	28	++	3	17	++	2	11	++	3	17	++	4	22
+++	0	/	+++	0	/	+++	0	/	+++	1	6	+++	0	2/	+++	0	/
Weak to moderate			Weak to strong			Weak to moderate			Moderate to strong			Weak to moderate			Weak to moderate		

D. Barrett's associated adenocarcinoma (n=41 both metastatic and non metastatic)

PuPA			MuPA			uPAR			PAI-1			PAI-2			E-Cad		
	No.	%		No.	%		No.	%		No.	%		No.	%		No.	%
-	3	7	-	5	12	-	4	10	-	0	/	-	0	/	-	26	63
+/-	4	10	+/-	4	10	+/-	4	10	+/-	1	2	+/-	0	/	+/-	8	20
+	13	32	+	7	17	+	11	27	+	3	7	+	4	10	+	4	10
++	16	39	++	19	46	++	17	41	++	15	37	++	26	63	++	3	7
+++	5	12	+++	6	15	+++	5	12	+++	22	54	+++	11	27	+++	0	/
Moderate to strong			Weak to strong			Strong			Very strong			Strong			Weak to negative		

E. Cancer with no metastasis (n=28)

PuPA			MuPA			uPAR			PAI-1			PAI-2			E-Cad		
	No.	%		No.	%		No.	%		No.	%		No.	%		No.	%
-	1	4	-	2	7	-	3	11	-	0	/	-	0	/	-	16	58
+/-	2	7	+/-	3	11	+/-	3	11	+/-	0	/	+/-	0	/	+/-	6	21
+	11	39	+	7	25	+	8	28	+	2	7	+	2	7	+	4	14
++	10	36	++	13	46	++	12	43	++	9	32	++	18	65	++	2	7
+++	4	14	+++	3	11	+++	2	7	+++	17	61	+++	8	28	+++	0	/
Moderate to Strong			Moderate to Strong			Moderate			Strong			Strong			Moderate to Negative		

F. Cancer with Lymph node metastasis (n=13)

PuPA			MuPA			uPAR			PAI-1			PAI-2			E-Cad		
	No.	%		No.	%		No.	%		No.	%		No.	%		No.	%
-	2	15	-	3	23	-	1	8	-	0	/	-	0	/	-	10	77
+/-	2	15	+/-	1	8	+/-	1	8	+/-	1	8	+/-	0	/	+/-	2	15
+	2	15	+	0	/	+	3	23	+	1	8	+	2	15	+	0	/
++	6	46	++	6	46	++	5	28	++	6	46	++	8	62	++	1	8
+++	1	8	+++	3	23	+++	3	23	+++	5	38	+++	3	23	+++	0	/
Moderate to Strong			Moderate to Strong			Moderate to Strong			Strong			Strong			Moderate to Negative		

G. Lymph node metastasis (n=14)

PuPA			MuPA			uPAR			PAI-1			PAI-2			E-Cad		
	No.	%		No.	%		No.	%		No.	%		No.	%		No.	%
-	2	14	-	1	7	-	1	7	-	0	/	-	1	7	-	8	57
+/-	0	/	+/-	2	14	+/-	1	7	+/-	1	7	+/-	0	/	+/-	2	14
+	6	43	+	5	36	+	5	36	+	1	7	+	2	14	+	4	29
++	5	36	++	4	29	++	6	43	++	8	57	++	6	43	++	0	/
+++	1	7	+++	2	14	+++	1	7	+++	4	29	+++	5	36	+++	0	/
Moderate to strong			Weak to strong			Moderate to strong			Strong			Strong			Moderate to negative		

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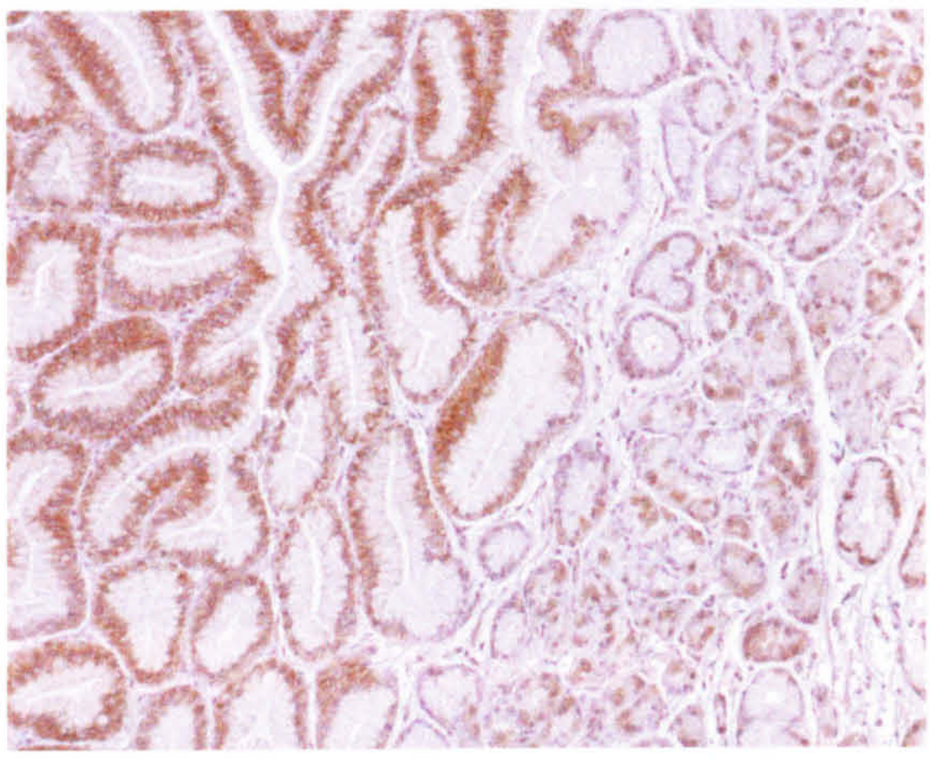

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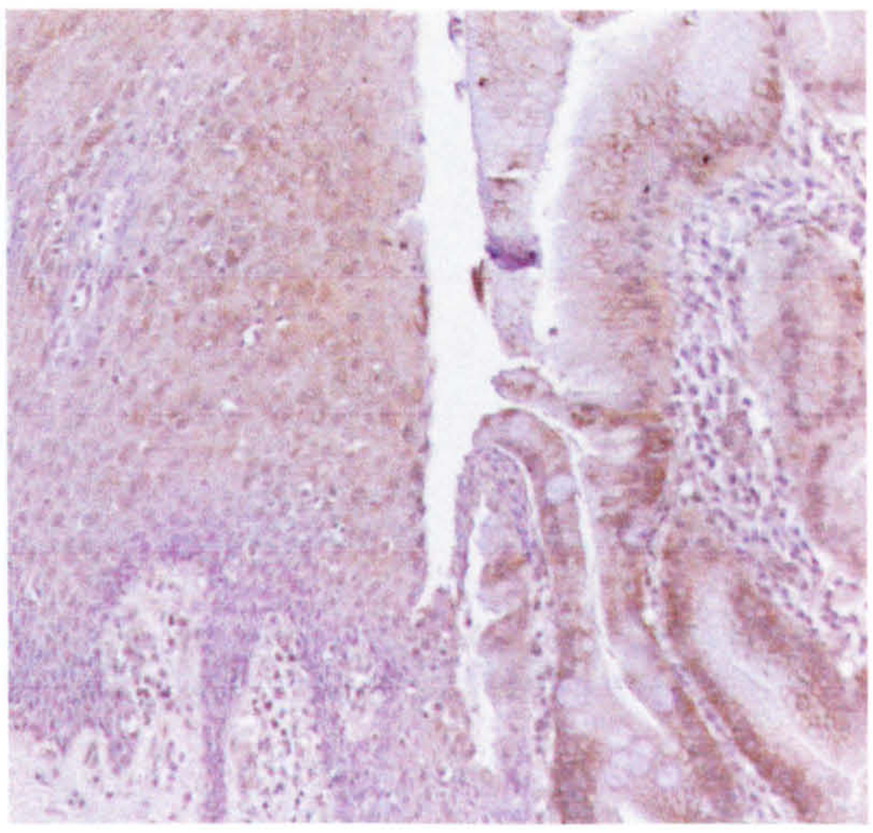

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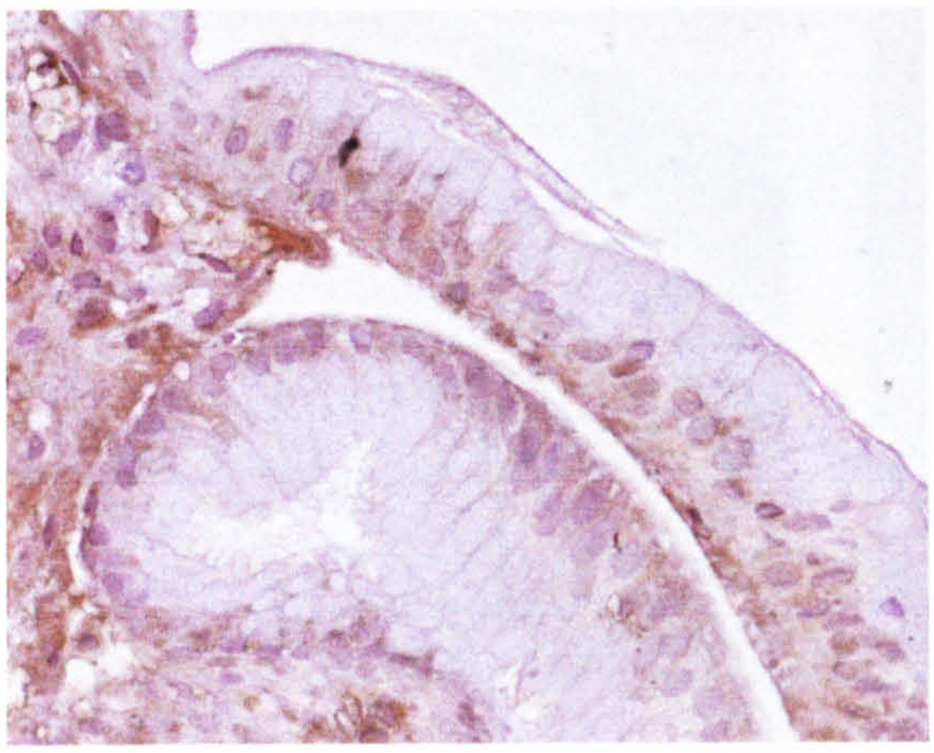

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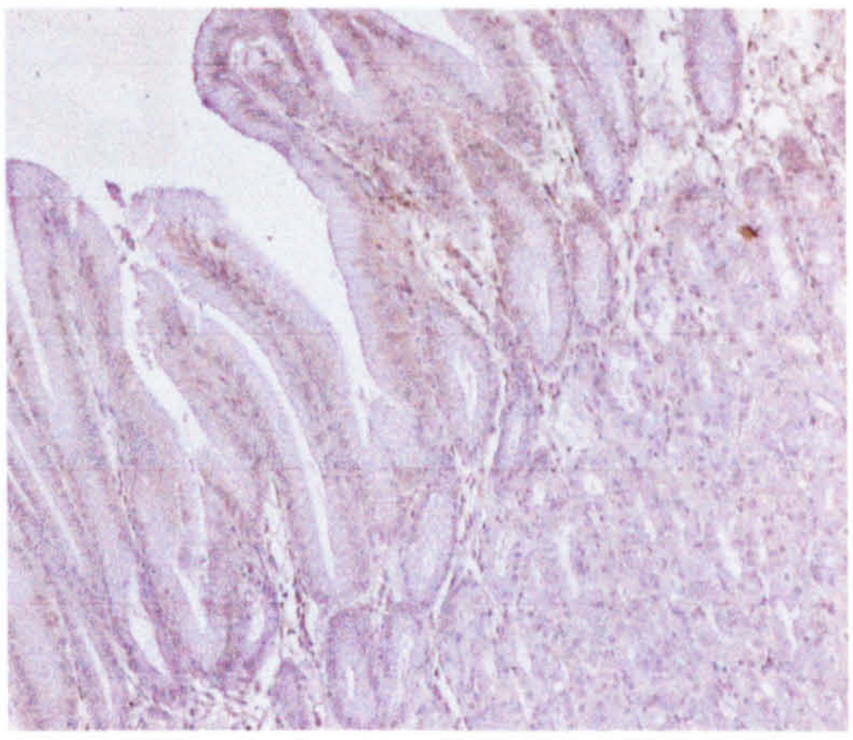

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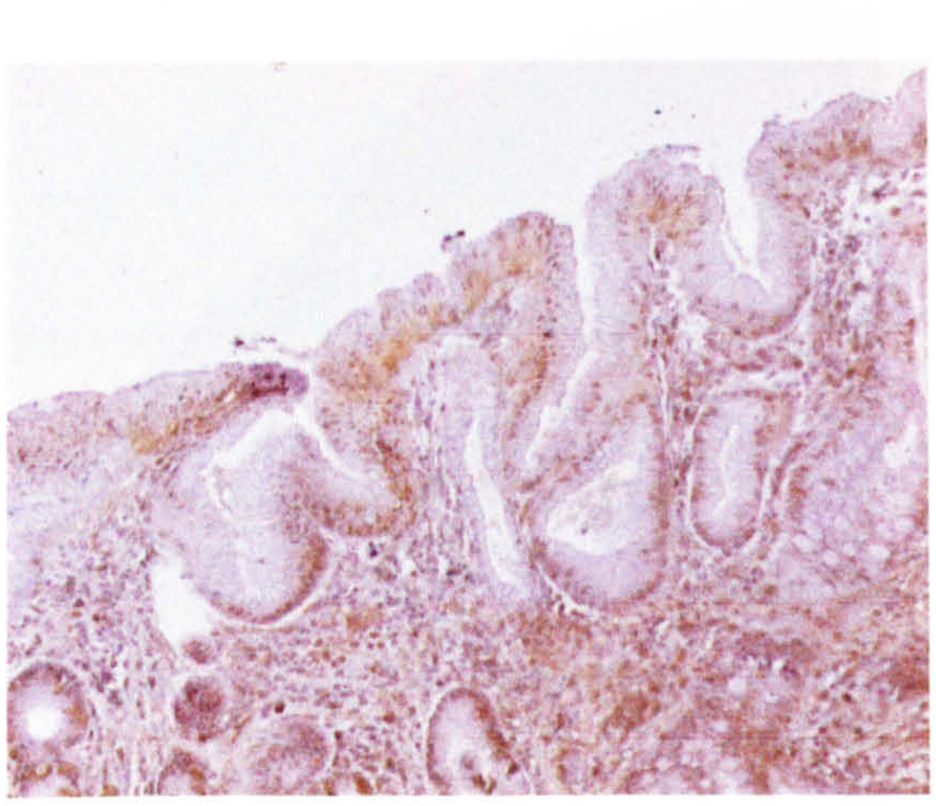

Figure 5.2 uPA immunoreactivity in: A. Fundic-type metaplasia (x10). **B**. Non-involved oesophageal mucosa on left and cardiac-type metaplasia on right (x10). **C**. Cardiac type metaplasia (x10). **D**. Fundic-type metaplasia (x10). **E**. Cardiac metaplasia on left and intestinal metaplasia on the right (x10).

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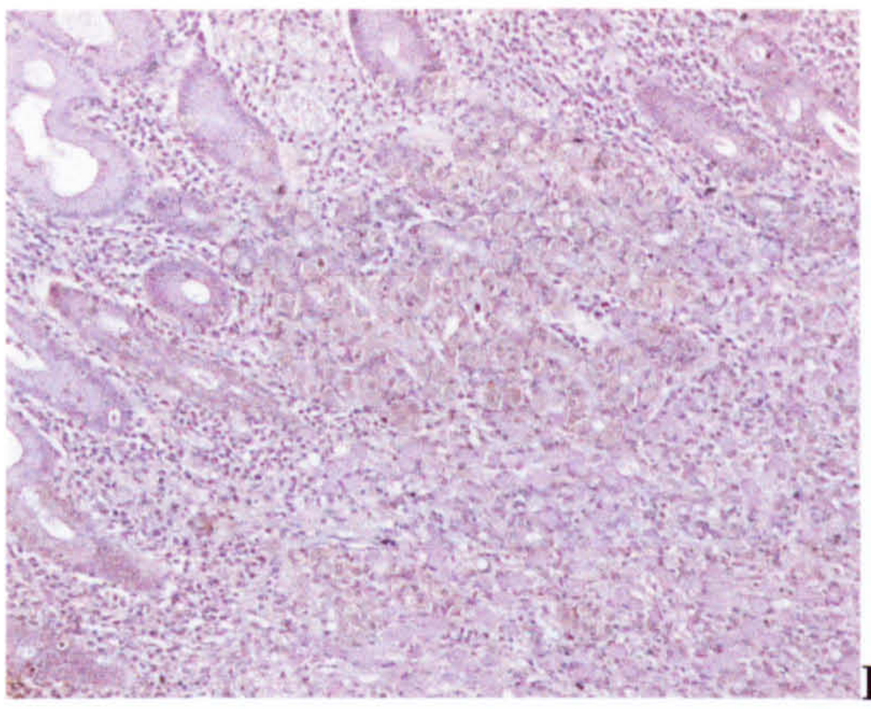
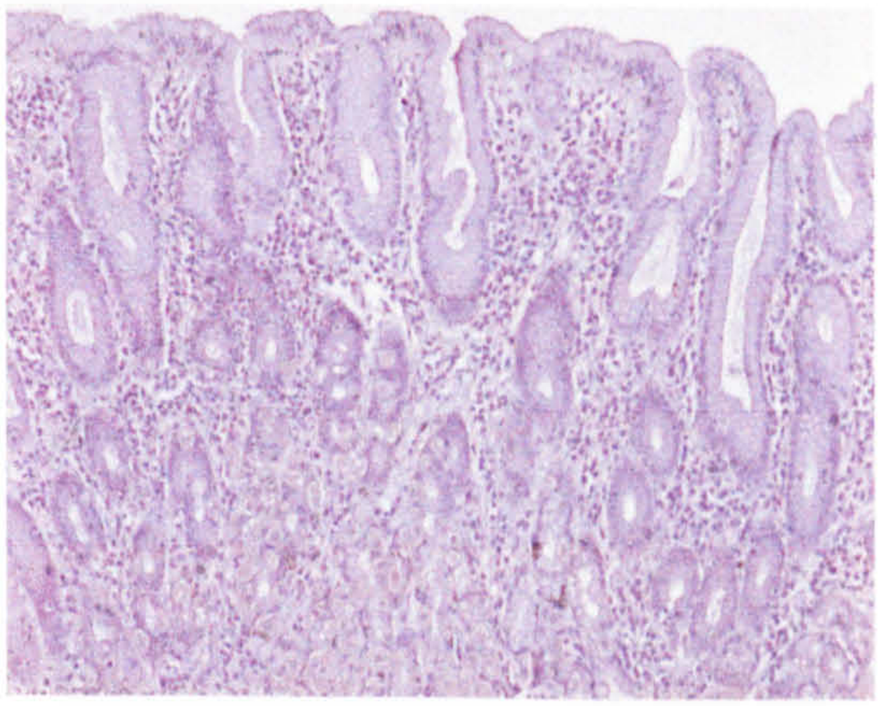


B.



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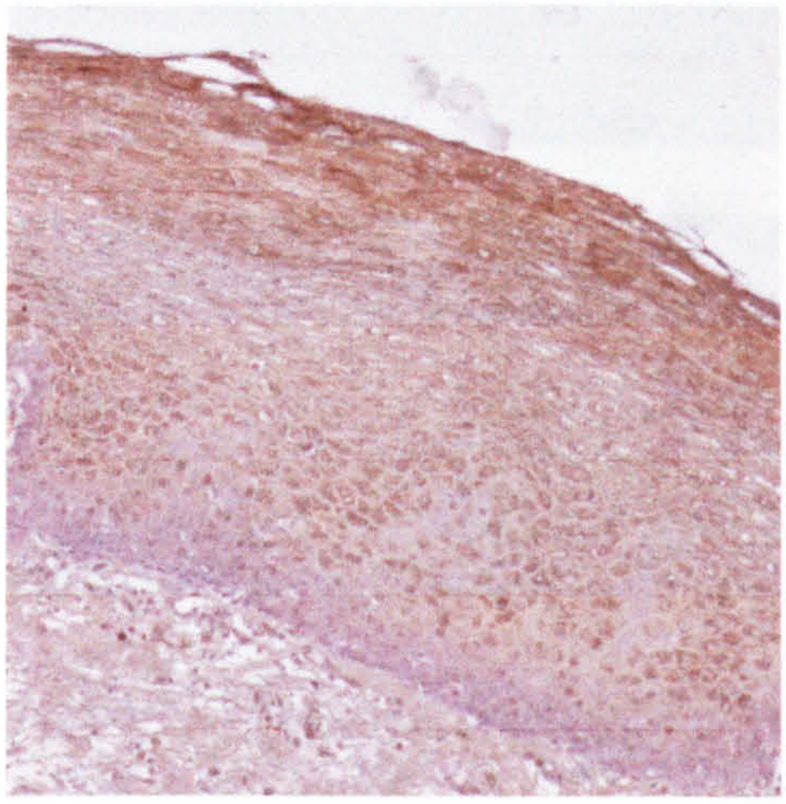

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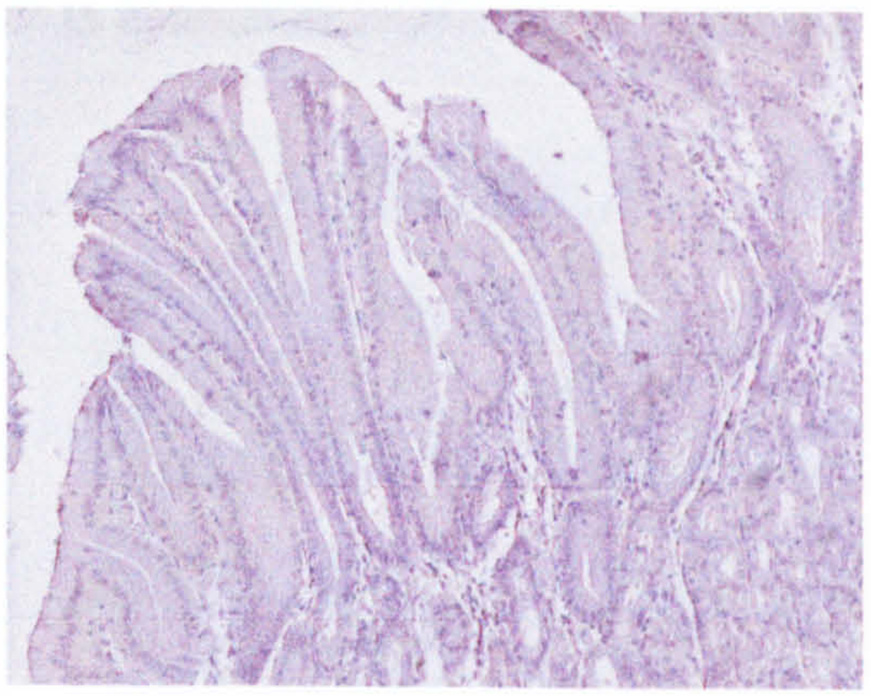

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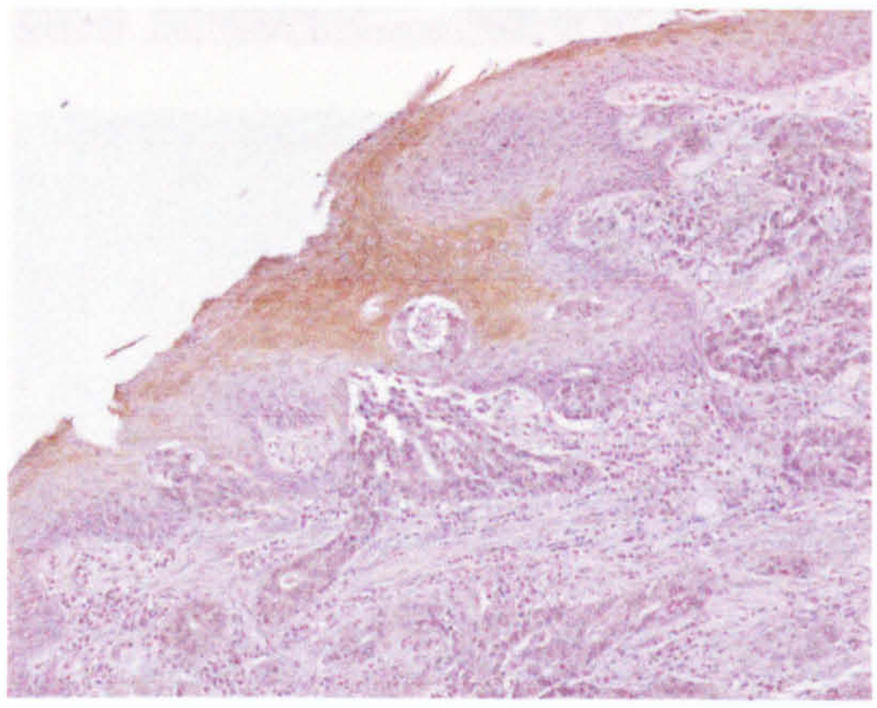
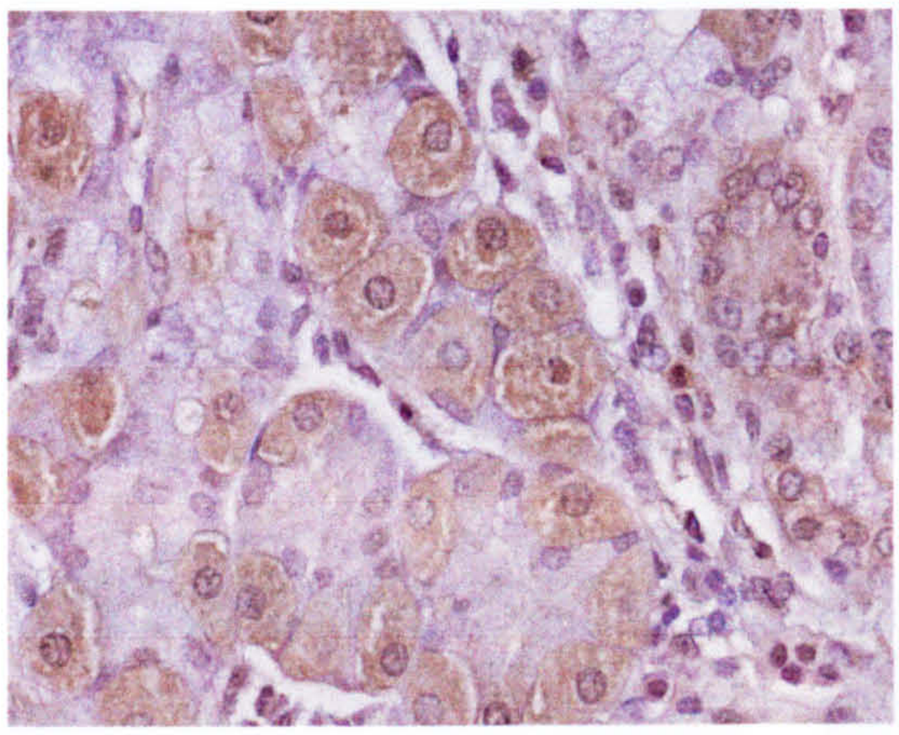


Figure 5.3 uPAR immunoreactivity in: A. Poorly Differentiated Adenocarcinoma (x10). **B**. Cardiac-type metaplasia (x10). **C**. Non-involved oesophageal mucosa (x10). **D**. Adenocarcinoma (x10). **E**. Adenocarcinoma with a small amount of squamous mucosa at surface (x10).



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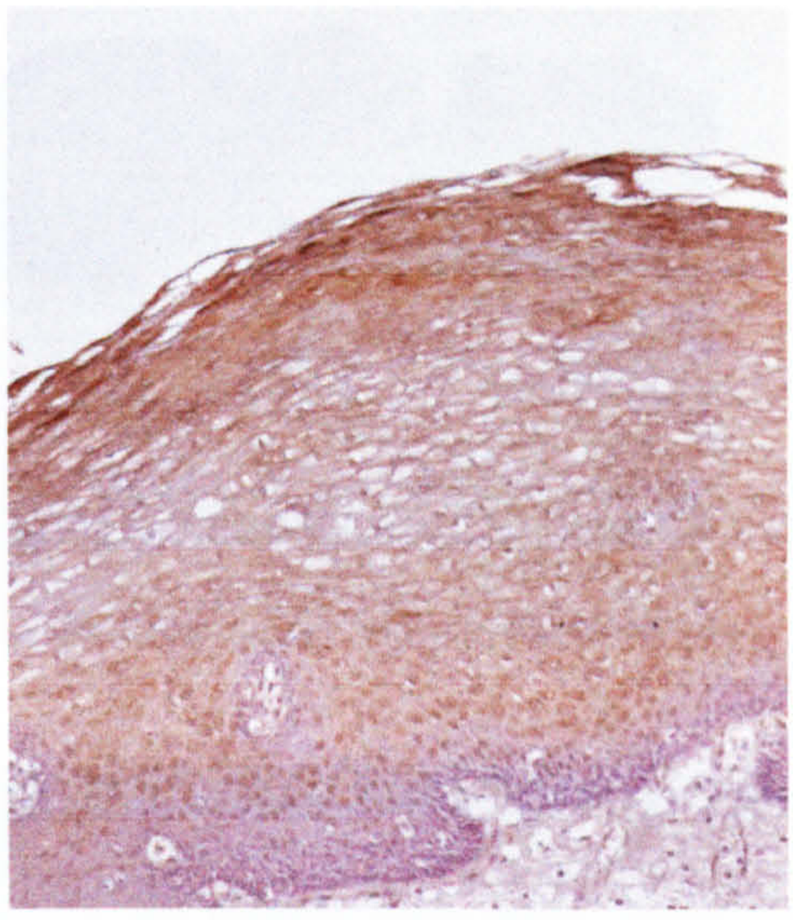
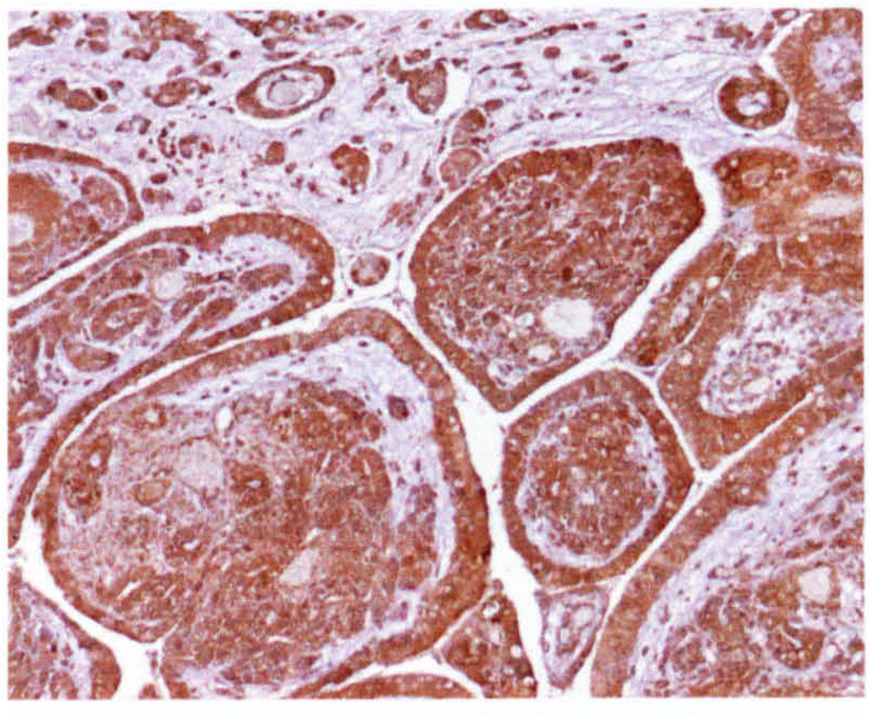
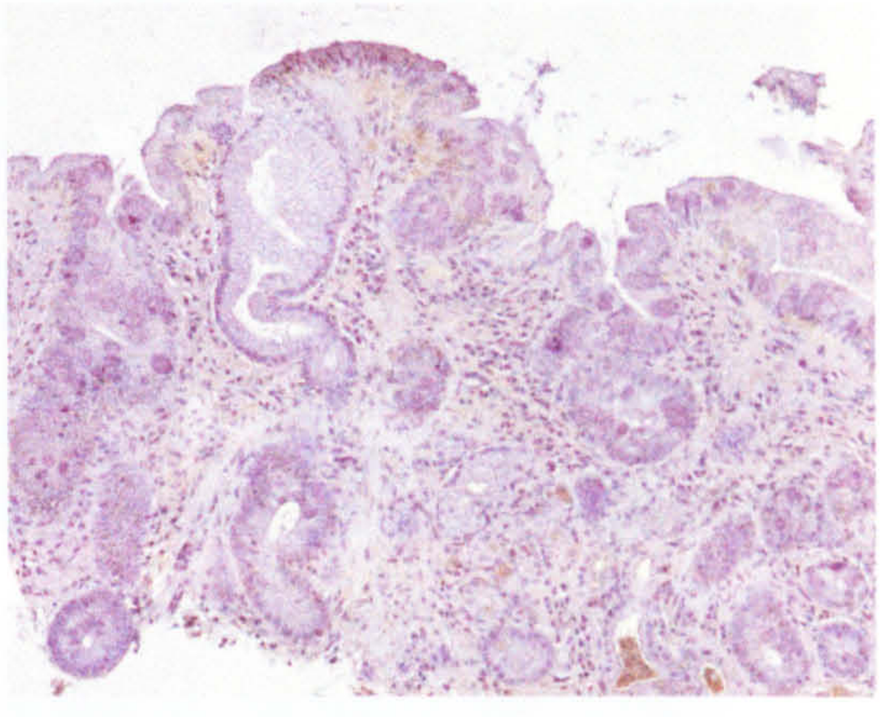


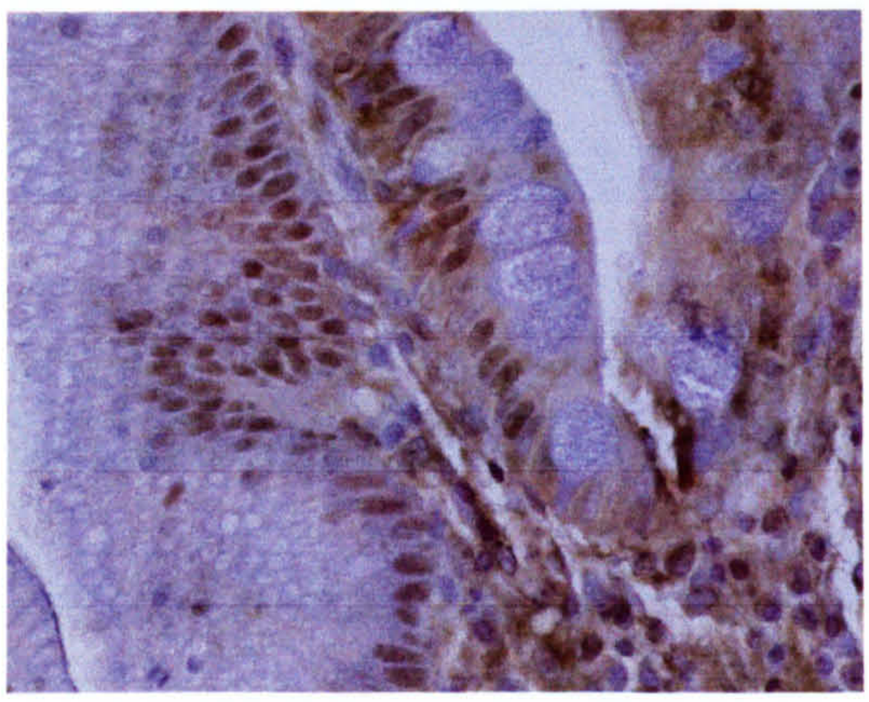
B.



C.

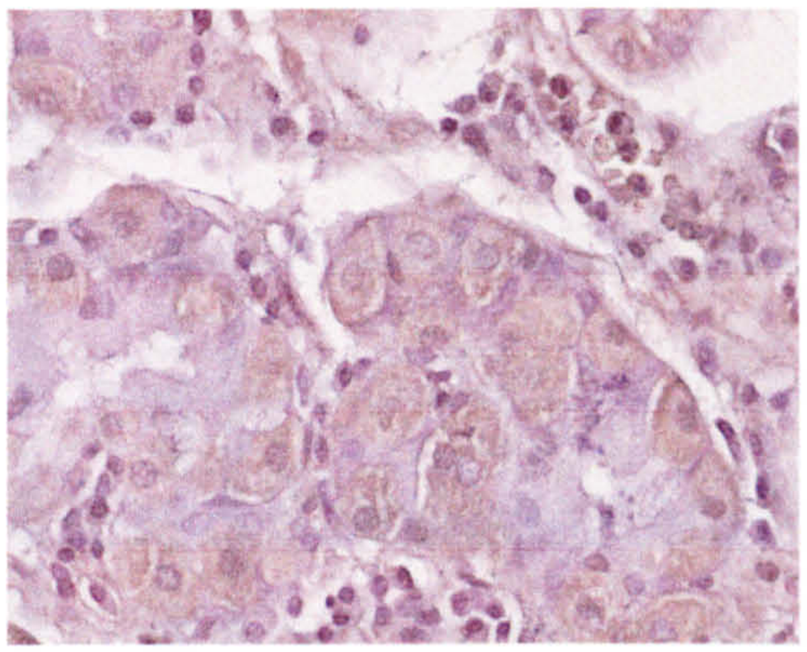


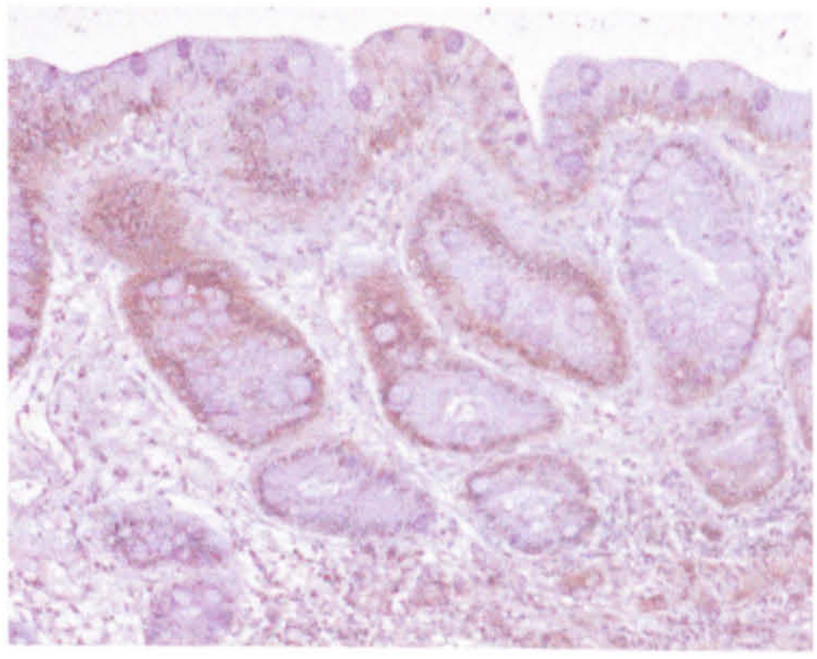
D.

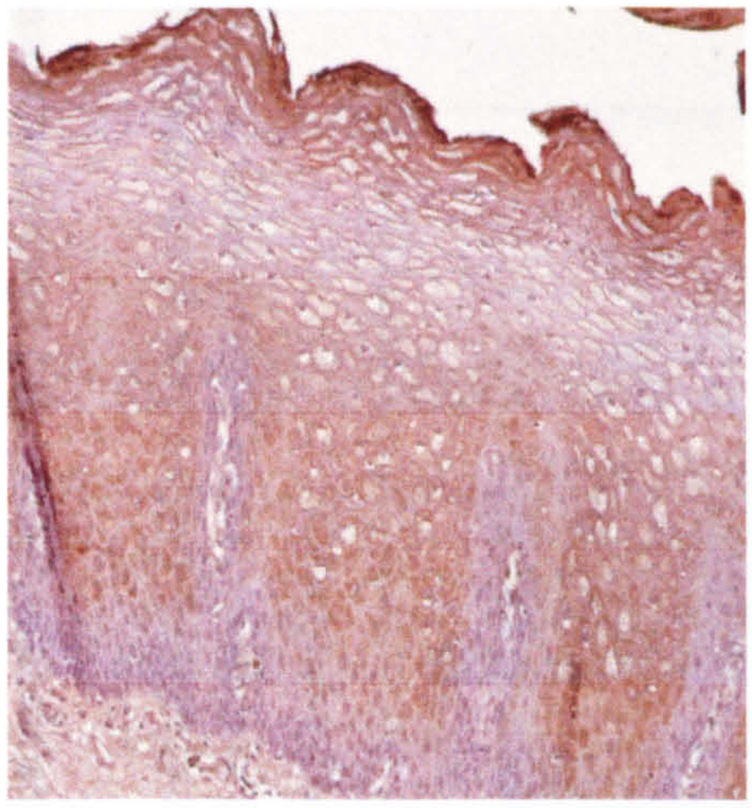


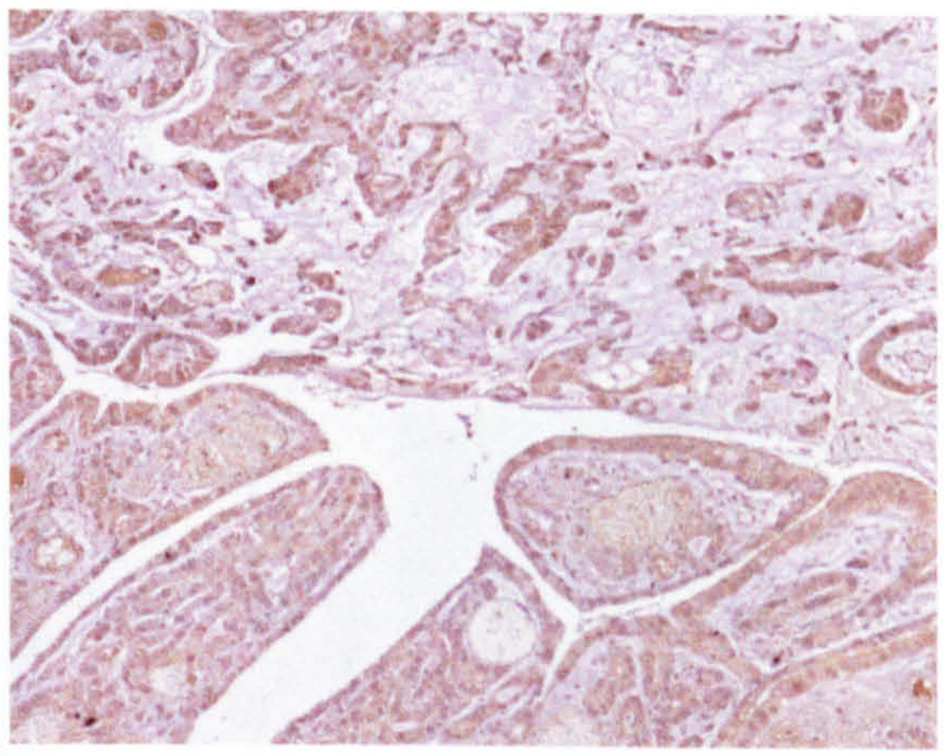
E.

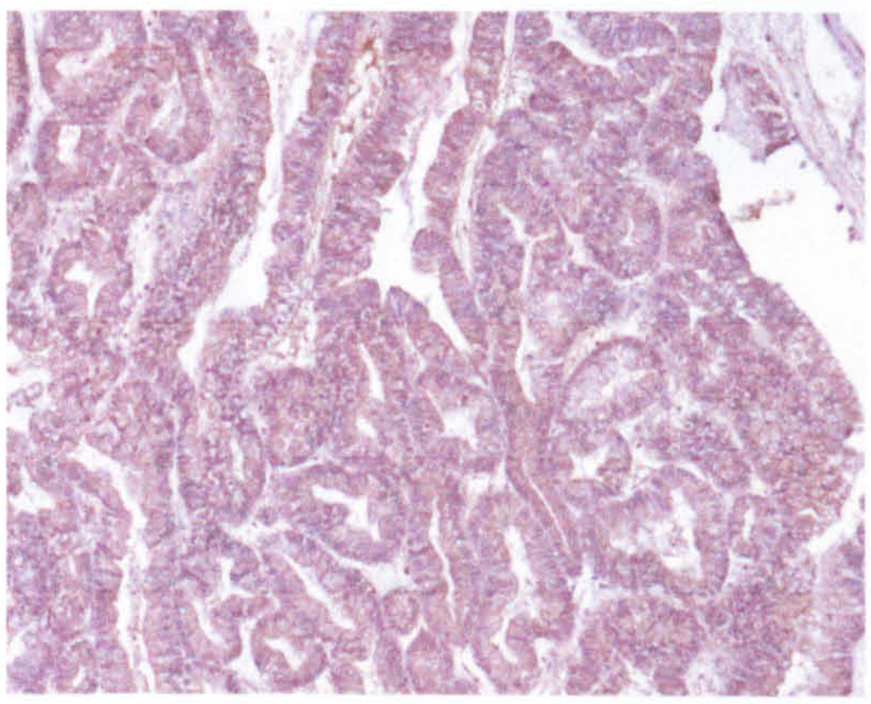
Figure 5.4 PAI-1 immunoreactivity in: A. Fundic-type metaplasia with positive staining for parietal cells (x40). **B**. Non-involved oesophageal mucosa (x10). **C**. Adenocarcinoma (x10). **D**. Intestinal and cardiac type Barrett's metaplasia with minimal staining of glandular areas and staining in stromal cells (x10). **E**. Intestinal metaplasia with nuclear staining (x10).

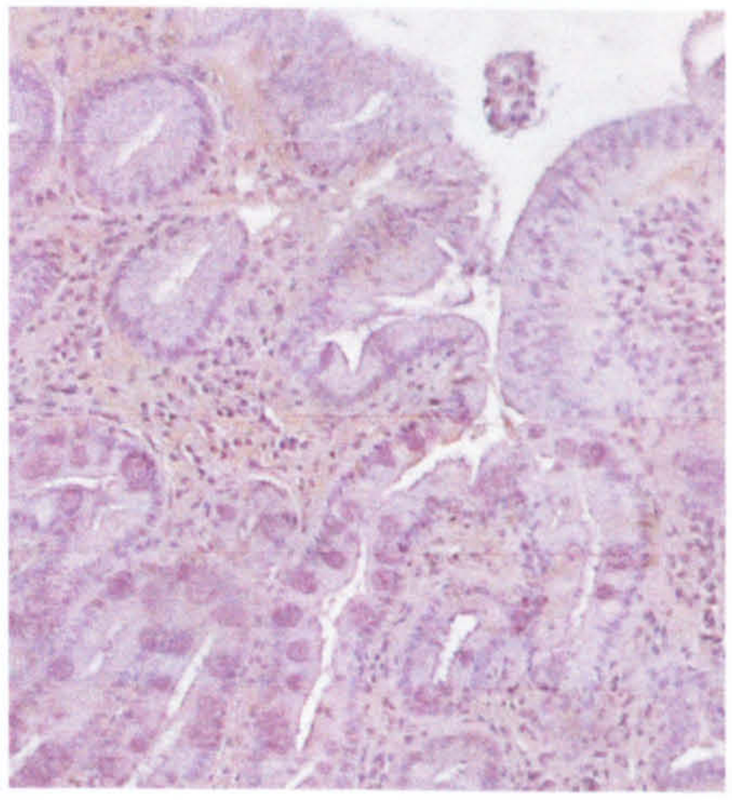
A. 

B. 

C. 

D. 

E. 

F. 

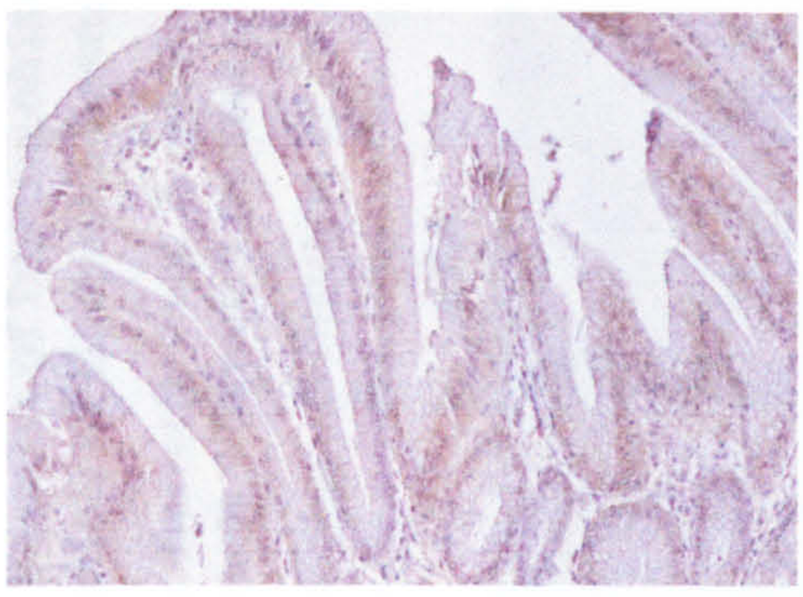
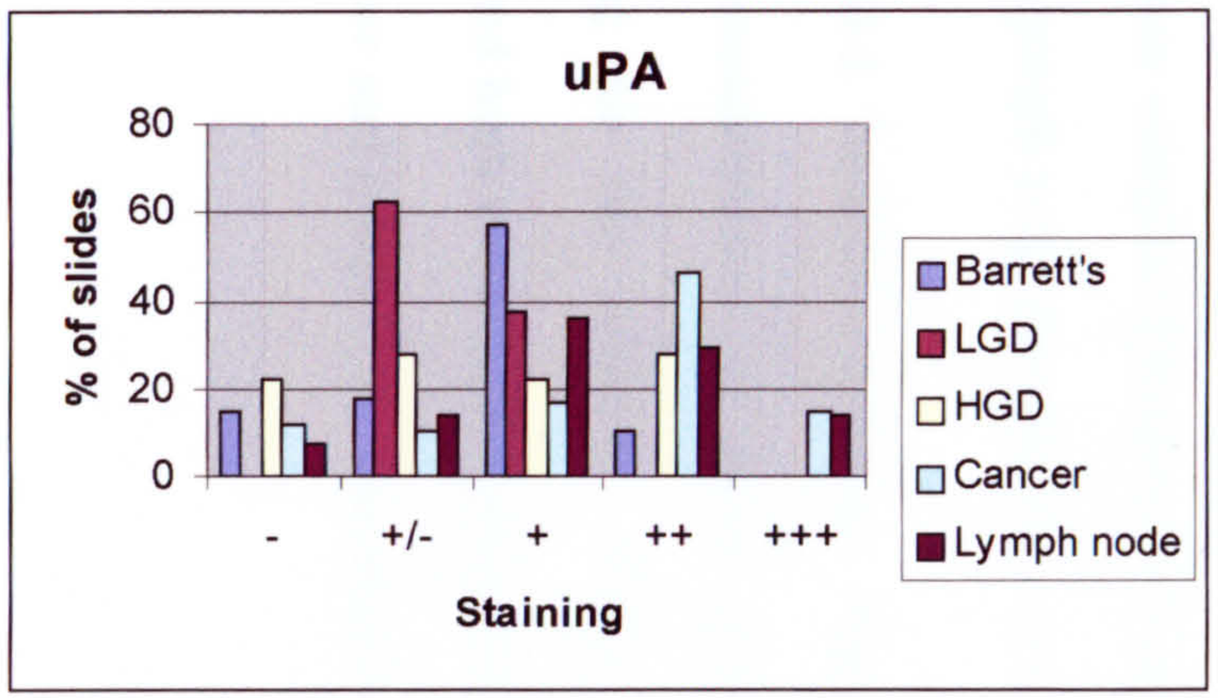
G. 

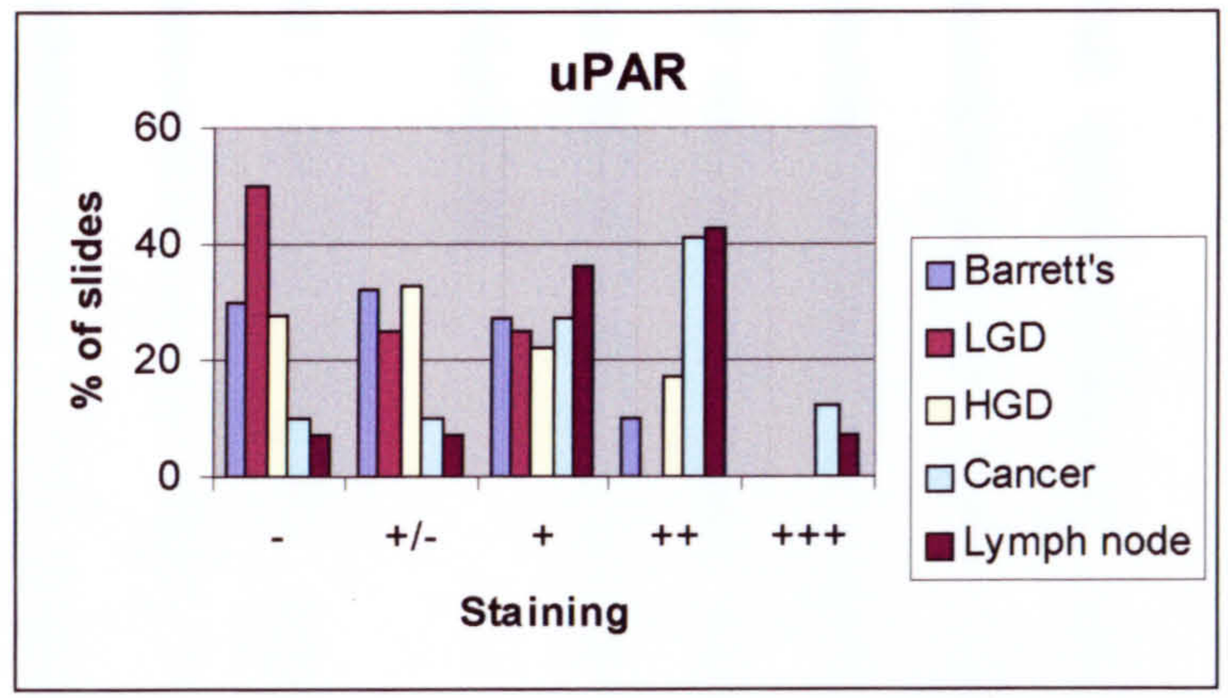
Figure 5.5 PAI-2 immunoreactivity in: A. Gastric/fundic-type metaplasia (x40). **B**. Intestinal metaplasia with strong nuclear staining (x10). C. Non-involved oesophageal mucosa (x10). **D**. Adenocarcinoma (x10). **E**. High grade dysplasia (x10). **F**. Intestinal-type metaplasia (x10). **G**. Fundic-type metaplasia (x10).

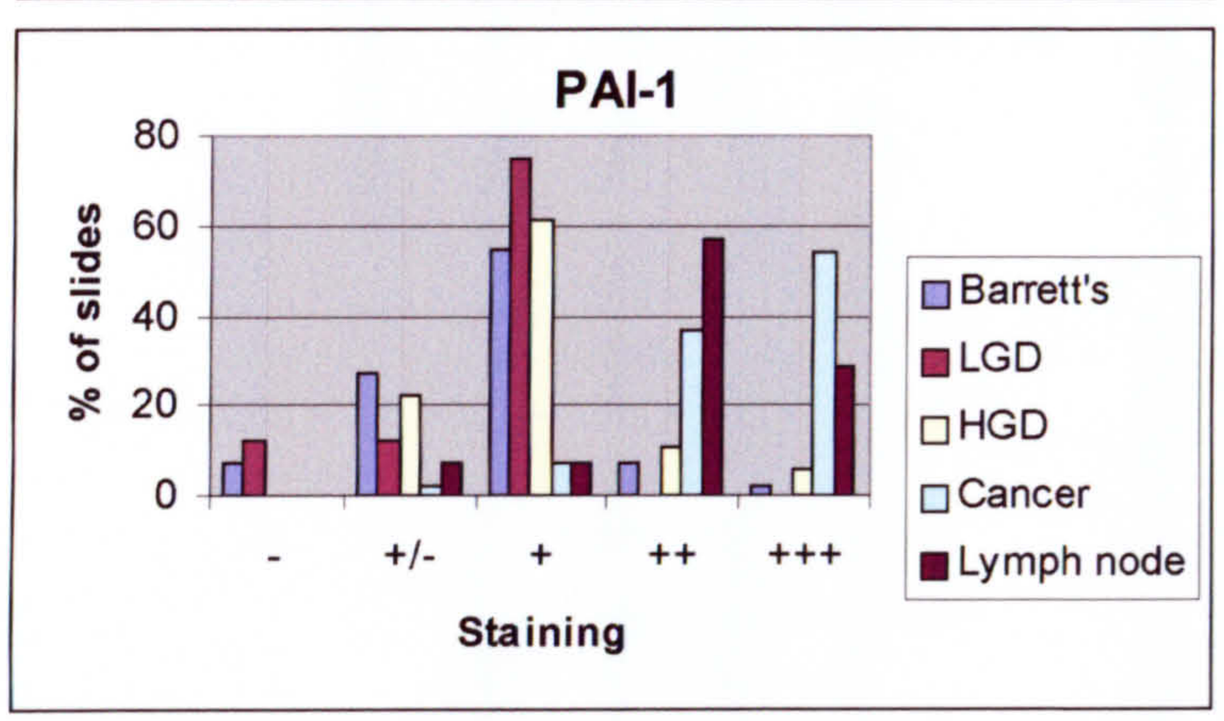
Figure 5.6. Results of Immunohistochemistry for the detection of components of the plasminogen activator system during the progression of Barrett's oesophagus.

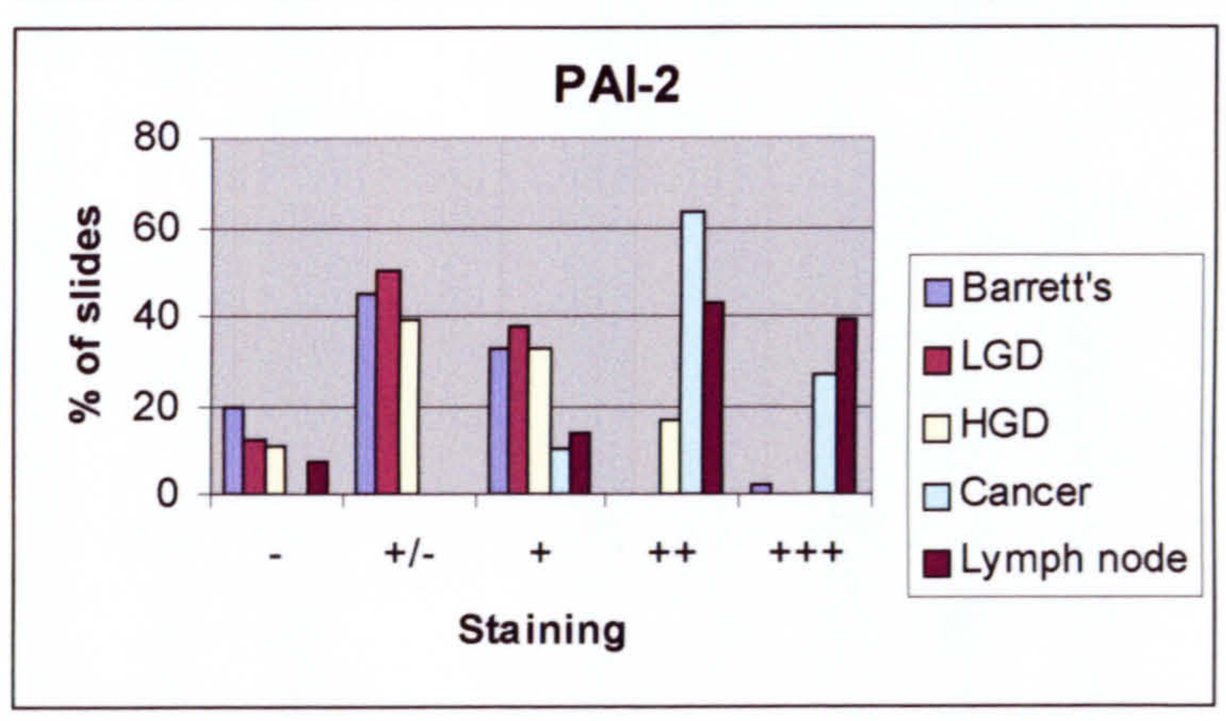
Abbreviations: Barrett's = non dysplastic Barrett's metaplasia (intestinal, cardiac or fundic), LGD = Low grade dysplasia, HGD = High Grade Dysplasia, Cancer = Barrett's associated adenocarcinoma (both metastatic and non metastatic) and Lymph node = Lymph node metastasis.

Grading of staining: - = Negative, +/- = Weak, + = Moderate, ++ = Strong and +++ = Very Strong.









Examples of uPA immunoreactivity during the progression of Barrett's oesophagus to adenocarcinoma are shown in figure 5.2. The level of staining for uPA increased during the metaplastic:neoplastic progression of Barrett's oesophagus. The strongest staining for uPA was seen in both adenocarcinoma and lymph node metastasis samples. Barrett's metaplasia and low grade dysplasia samples showed moderate staining for uPA.

Examples of uPAR immunoreactivity during the progression of Barrett's oesophagus to adenocarcinoma are shown in figure 5.3. The level of staining for uPAR increased during the neoplastic progression of Barrett's oesophagus. The strongest levels of staining were again seen in the cancer and lymph node metastasis samples. Barrett's metaplasia and dysplastic tissues showed weak to moderate staining for uPAR.

Examples of PAI-1 immunoreactivity during the progression of Barrett's oesophagus to adenocarcinoma are shown in figure 5.4. There was moderate staining for the inhibitor PAI-1 in non-dysplastic Barrett's metaplasia, low and high grade dysplasia samples. The level of staining for PAI-1 was again strongest in the cancer and lymph node metastasis samples.

Examples of PAI-2 immunoreactivity during the progression of Barrett's oesophagus to adenocarcinoma are shown in figure 5.5. There was also moderate to weak staining for the inhibitor PAI-2 in the non-dysplastic Barrett's metaplasia, low and high grade dysplasia samples. The level of staining was very strong in the cancer and lymph node metastasis samples.

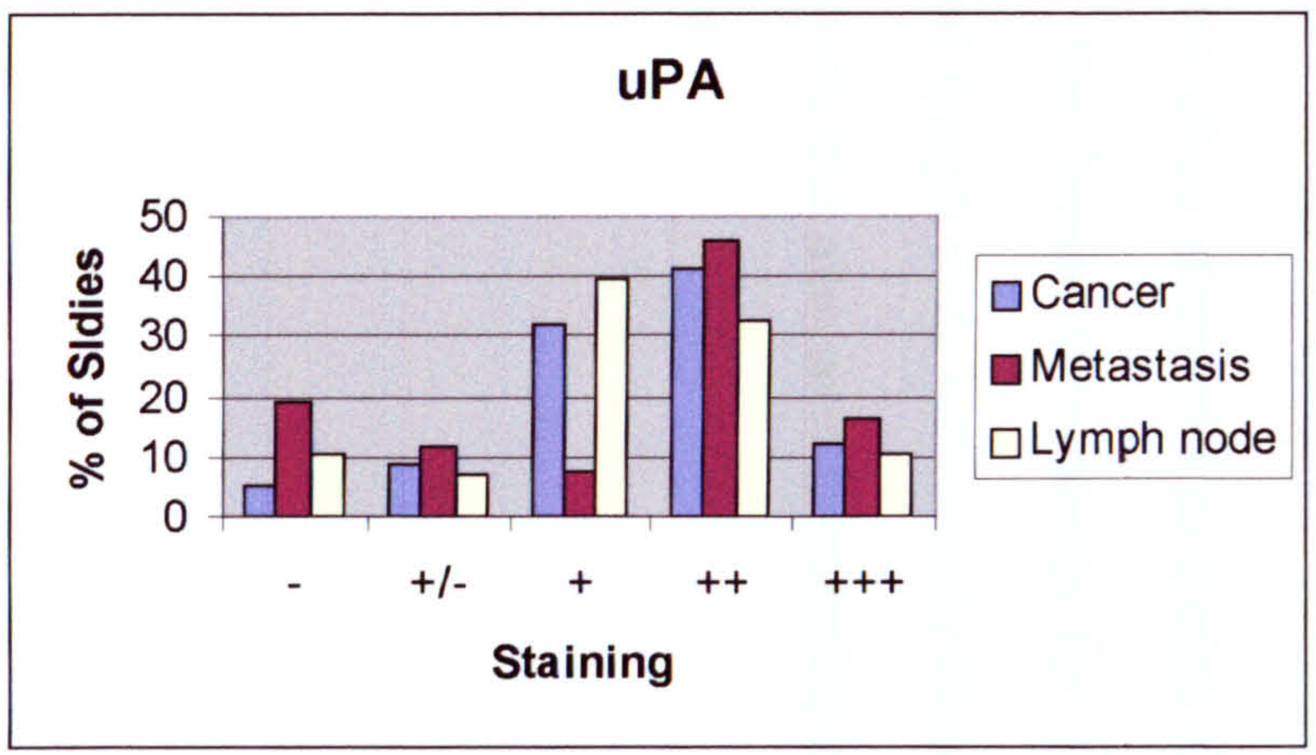
The components of the plasminogen activator system were detectable by IHC at all stages of the progression in Barrett's oesophagus. The levels appear to stay relatively low in non-dysplastic Barrett's metaplasia, low grade and high grade dysplasia, but are increased in Barrett's associated adenocarcinoma. Surprisingly, levels are lower in lymph node metastasis than adenocarcinoma samples, as the components of the plasminogen activator system are associated with cancer invasiveness.

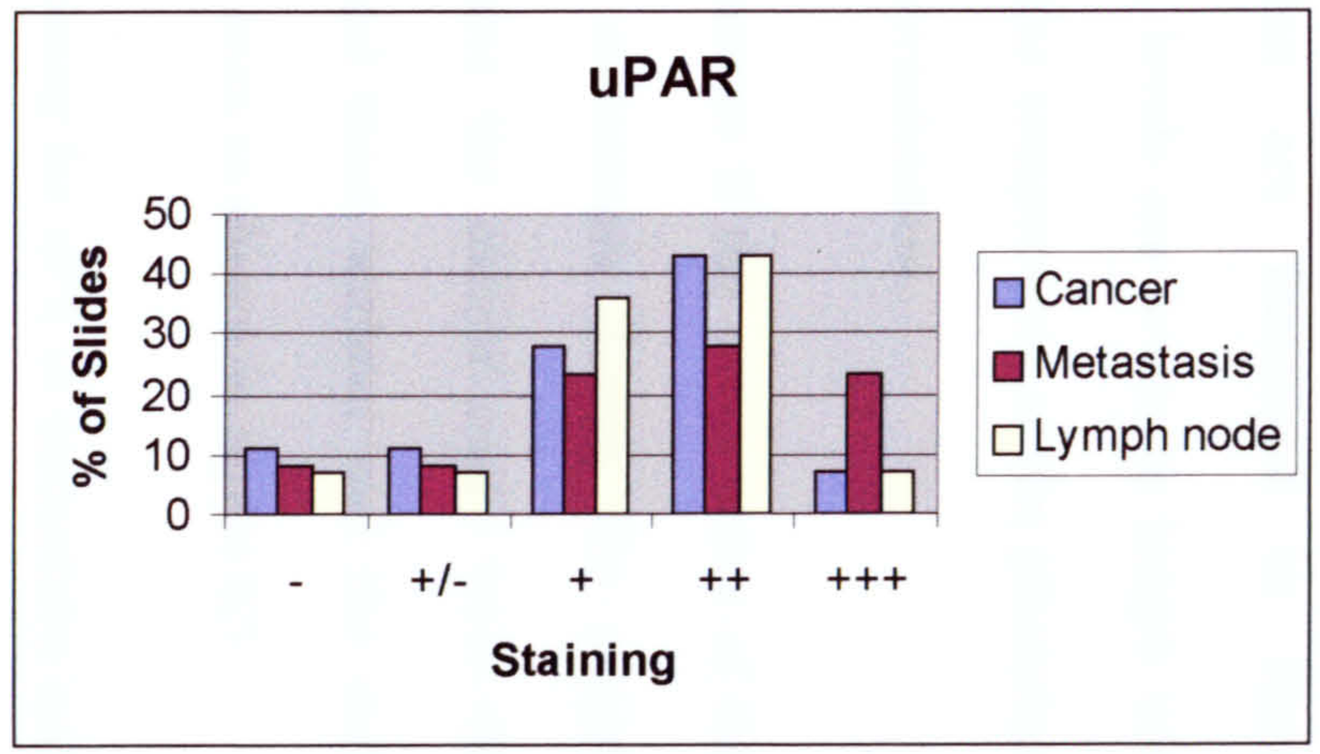
In order to assess if the components of the plasminogen activator system are involved in metastasis during the progression of Barrett's oesophagus to adenocarcinoma the cancer cases were divided into those with lymph node metastasis and those without and the staining levels compared. A pattern of staining was seen as shown in figure 5.7.

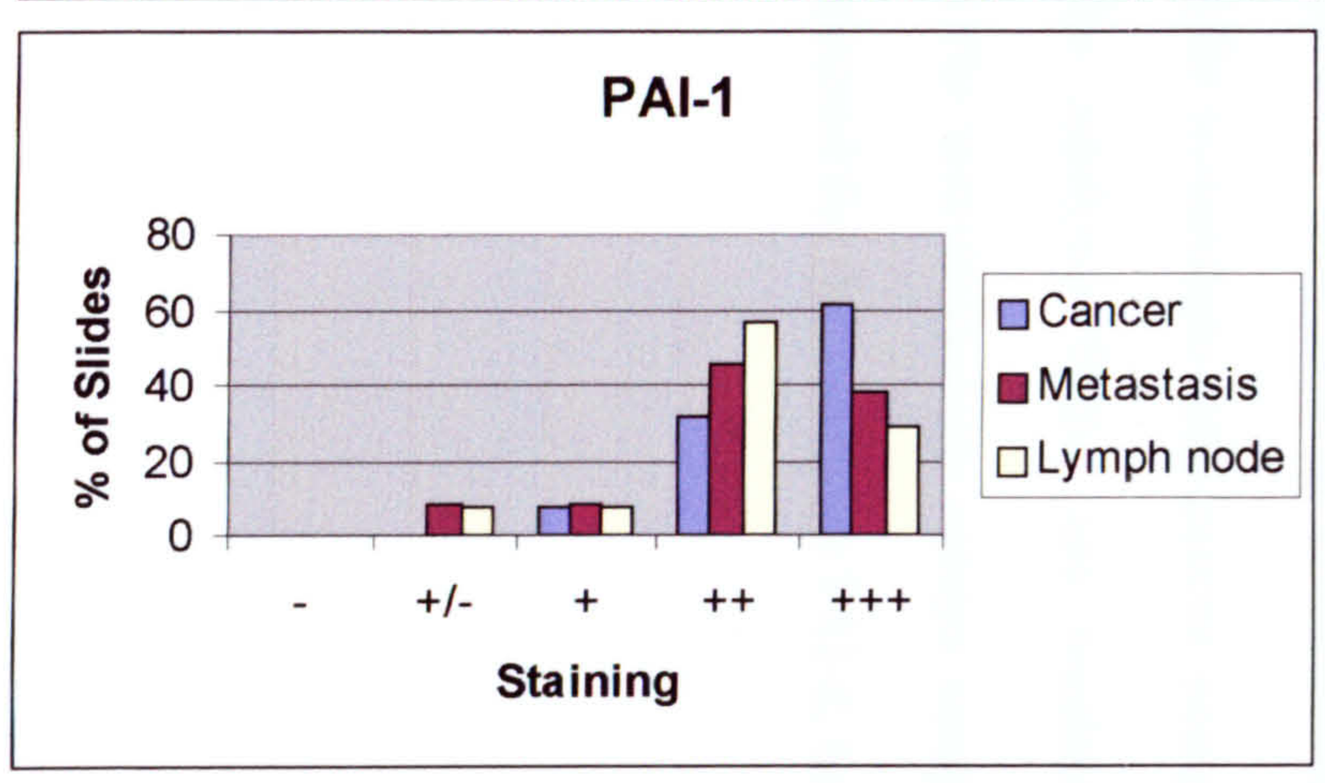
Levels of staining for uPA in metastatic cancer samples appeared stronger than those from non-metastatic cancers, but levels were weaker in lymph node metastasis. The levels of staining for uPAR were strongest in non-metastatic cancer samples when compared to lymph node metastasis and cancer samples. Interestingly the levels of staining for the inhibitor PAI-1 are weaker in metastatic and lymph node metastasis than the non-metastatic cancer samples. A similar pattern was also seen in samples stained for the inhibitor PAI-2, but to a lesser extent.

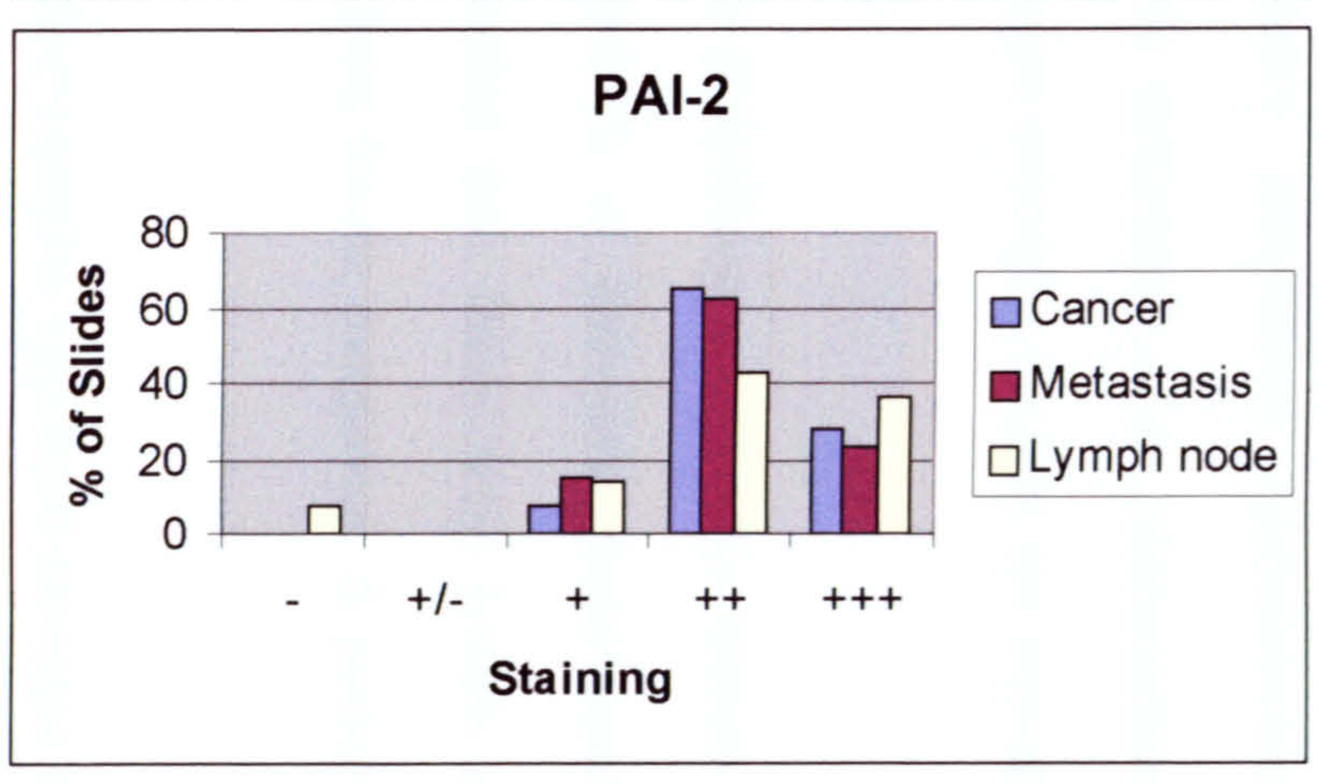
Figure 5.7. Comparison of the levels of the Components of the urokinase type Plasminogen System in adenocarcinoma samples with and without metastasis.

Grading of staining: - = Negative, +/- = Weak, + = Moderate, ++ = Strong and +++ = Very Strong.









5.4.2 Results of the Detection of the components of the urokinase Plasminogen Activator System using ELISA techniques

The protein concentrations of the components of the plasminogen system were measured using ELISA techniques in the samples that had previously been analysed using the TRAP assay for the detection of telomerase (Chapter 4). The samples analysed were as follows; non-involved oesophageal mucosa (n=23), non-involved gastric mucosa (n=31), non-dysplastic Barrett's metaplasia (n=14), Barrett's oesophagus with low grade dysplasia (n=10), Barrett's oesophagus with high grade dysplasia (n=10) and oesophageal adenocarcinoma (n=30) the results of which are shown in table 5.4.

Table 5.4. Results of samples analysed using ELISA techniques to detect both telomerase and the components of the Plasminogen Activator system.

A = Resected Patients and **B** = Biopsy patients (* Same patient, but different surgery) **C.** Median antigen levels in ng/mg protein.

Abbreviations: N = no, Y = Yes, uPA = urokinase-type Plasminogen Activator, uPAR = urokinase-type Plasminogen Receptor, PAI-1 = Plasminogen Inhibitor type-1 and PAI-2 = Plasminogen Inhibitor type-2.

Antigen levels given in ng/mg protein.

A. Resected Patients

Patient	Diagnosis	Telomerase Result	Protein concentration ng/mg protein			
			uPA	uPAR	PAI-1	PAI-2
1R	N Stom	N	0.066	<0.025	1.967	22.295
	N Oes	N	0.240	1.867	1.333	3.467
2R	N Stom	N	0.105	<0.025	3.158	<0.5
	LGD	N	1.364	<0.025	3.030	<0.5
	LGD	N	0.231	<0.025	1.538	<0.5
3R	N Stom	N	0.101	0.633	1.519	<0.5
	N Oes	Y	0.074	0.741	0.741	>200
	IM Barr	N	0.101	0.336	1.681	<0.5
	Cancer	Y	0.280	0.9	0.296	3.4
4R	N Stom	N	0.164	0.164	3.217	0.656
	C Barr	N	0.167	<0.025	1.667	<0.5
	LGD	N	0.170	0.532	1.277	0.851
	Cancer	Y	1.548	4.783	13.217	3.652
5R	N Stom	N	0.087	0.611	2.795	<0.5
	N Oes	N	0.054	1.119	0.805	>200
	Cancer	Y	1.212	3.232	12.929	5.253
6R	N Stom	N	0.071	0.118	2.353	0.471
	N Oes	N	0.054	0.268	0.805	>200
	Cancer	N	1.187	5.167	4.785	>200
	Cancer	N	0.6	3.0	7.0	130.0
7R	N Stom	N	0.049	0.366	0.894	0.650
	N Oes	N	0.070	<0.025	1.033	39.649
	Cancer	Y	2.369	5.049	380447	33.010
8R	N Stom	N	0.111	0.062	4.198	0.864
	N Oes	N	0.103	1.379	1.7724	62.069
	IM Barr	Y	0.207	<0.025	3.448	1.379
	Cancer	Y	0.963	0.012	3.951	2.469
9R	N Stom	N	0.151	<0.025	2.642	3.774
10R	N Stom	N	0.315	2.526	1.789	3.371
	Cancer	Y	0.868	6.509	6.792	2.830
	Cancer	Y	0.580	2.839	6.562	6.183
11R	N Stom	N	0.101	0.404	1.818	<0.5
	N Oes	N	0.036	0.264	0.714	>200
	Cancer	Y	0.590	5.082	2.295	1.639

12R	N Stom	N	0.174	1.375	2.899	10.725
	N Oes	N	0.317	4.024	5.366	>200
	Cancer	Y	0.560	2.0	3.6	8.8
	Cancer	Y	1.028	5.587	89.39	4.693
13R	N Stom	N	0.082	0.619	0.928	2.165
	N Oes	N	0.107	0.982	1.429	>200
	Cancer	N	2.202	10.674	12.135	>200
14R	N Stom	N	0.051	0.380	2.278	>200
	N Oes	N	0.163	9.0	10.0	34.0
15R	N Stom	N	0.165	0.722	2.268	0.412
	Cancer	Y	0.264	1.069	2.421	3.176
16R	N Stom	N	0.189	0.863	1.890	3.780
	N Oes	N	0.108	0.753	10.968	>200
	Cancer	Y	0.8	3.2	17.6	13.6
17R	N Stom	N	0.133	0.623	2.0	<0.5
	Cancer	Y	0.258	0.748	4.731	68.387
	Cancer	Y	1.277	3.987	11.277	>200
18R	N Stom	N	0.184	2.212	2.212	1.475
19R	N Stom	N	0.079	0.984	3.684	0.789
	Cancer	N	1.965	12.281	11.930	14.035
20R	N Stom	N	0.249	1.891	2.189	3.582
	LGD	N	0.216	1.802	1.712	0.631
21R	N Stom	N	0.134	1.033	0.486	1.216
	Cancer	N	0.933	4.513	5.128	10.769
22R	N Stom	N	0.149	2.299	0.805	1.839
	N Oes	N	0.206	1.031	2.887	>200
	Cancer	Y	2.346	4.935	44.557	3.207
23R	Cancer	Y	1.030	7.345	6.667	8.485
24R	N Oes	N	0.275	1.875	1.5	>200
25R	N Stom	N	0.212	0.909	1.667	2.424
	F Barr	N	0.690	2.414	4.138	50.345
26R	N Stom	N	0.304	1.772	1.688	4.895
	N Oes	N	0.217	1.356	0.814	>200
	Cancer	Y	0.786	5.556	21.709	4.615
27R	N Stom	N	0.135	1.422	2.751	2.284
	C Barr	N	0.271	1.711	1.416	1.652
	Cancer	N	0.936	8.511	20.426	1.702
28R	N Stom	N	0.154	0.988	2.037	1.790
	IM Barr	N	0.385	2.404	1.154	2.308
	Cancer	Y	0.545	4.848	3.636	6.061
30R	N Oes	N	0.078	0.340	1.165	>200
31R	N Oes	N	0.308	1.385	1.538	75.692
32R*	HGD	N	0.462	1.536	3.077	4.615

B. Biopsy patients

Patient	Diagnosis	Telomerase Result	Protein concentration ng/mg protein			
			uPA	uPAR	PAI-1	PAI-2
35B	HGD	Y	0.509	2.182	2.909	>200
	HGD	N	0.714	2.857	7.143	>200
36B*	Cancer	N	1.00	3.750	6.667	10.833
38B	N Stom	N	0.093	0.577	1.860	0.930
41B	N Stom	N	0.571	18.095	16.190	22.857
	HGD	Y	0.276	0.345	2.759	2.069
42B	C Barr	N	0.2	<0.025	3.0	4.0
	IM Barr	N	0.6	<0.025	3.0	2.0
	LGD	N	0.167	<0.025	2.5	<0.5
43B	HGD	N	0.766	1.702	2.979	5.957
	HGD	N	0.480	<0.025	2.4	9.6
	Cancer	Y	1.538	<0.025	6.154	7.692
44B	HGD	N	0.4	<0.025	8.0	112.0
45B	N Stom	N	0.125	6.875	2.5	2.5
	N Oes	N	0.054	0.941	1.622	>200
46B	N Oes	N	0.111	<0.025	3.333	>200
	HGD	Y	0.385	0.385	3.077	9.231
	Cancer	Y	0.638	2.029	5.797	5.797
47B	F Barr	N	0.162	1.351	1.081	1.622
	C Barr	N	0.833	5.0	6.667	1.667
48B	LGD	N	0.267	0.333	0.667	14.0
	Cancer	N	0.571	1.42	3.265	17.143
	Cancer	N	1.391	2.383	6.957	17.391
49B	N Stom	N	0.078	0.649	2.338	0.779
	N Oes	N	0.118	<0.025	1.176	>200
	LGD	Y	0.25	1.667	3.333	5.833
50B	N Oes	N	0.174	0.643	1.739	>200
	Cancer	Y	0.171	1.429	4.0	12.571
52B	F Barr	N	0.048	1.190	0.959	<0.5
54B	N Oes	N	0.457	1.566	2.289	>200
	N Oes	Y	0.464	<0.025	2.319	>200
	IM Barr	Y	0.593	5.185	5.926	<0.5
	LGD	Y	0.303	4.545	3.636	<0.5
55B	N Stom	N	0.197	0.984	1.311	>200
	IM Barr	N	0.615	3.077	6.154	35.385
	LGD	Y	0.818	0.909	3.636	6.364
57B	HGD	Y	0.857	2.571	5.143	>200
61B	HGD	N	0.748	0.514	2.286	67.429
63B	N Oes	N	0.465	2.326	1.860	4.651
	C Barr	N	1.125	3.750	157.5	95.00
	LGD	Y	0.632	2.102	4.211	>200

C. Median antigen levels in ng/mg protein.

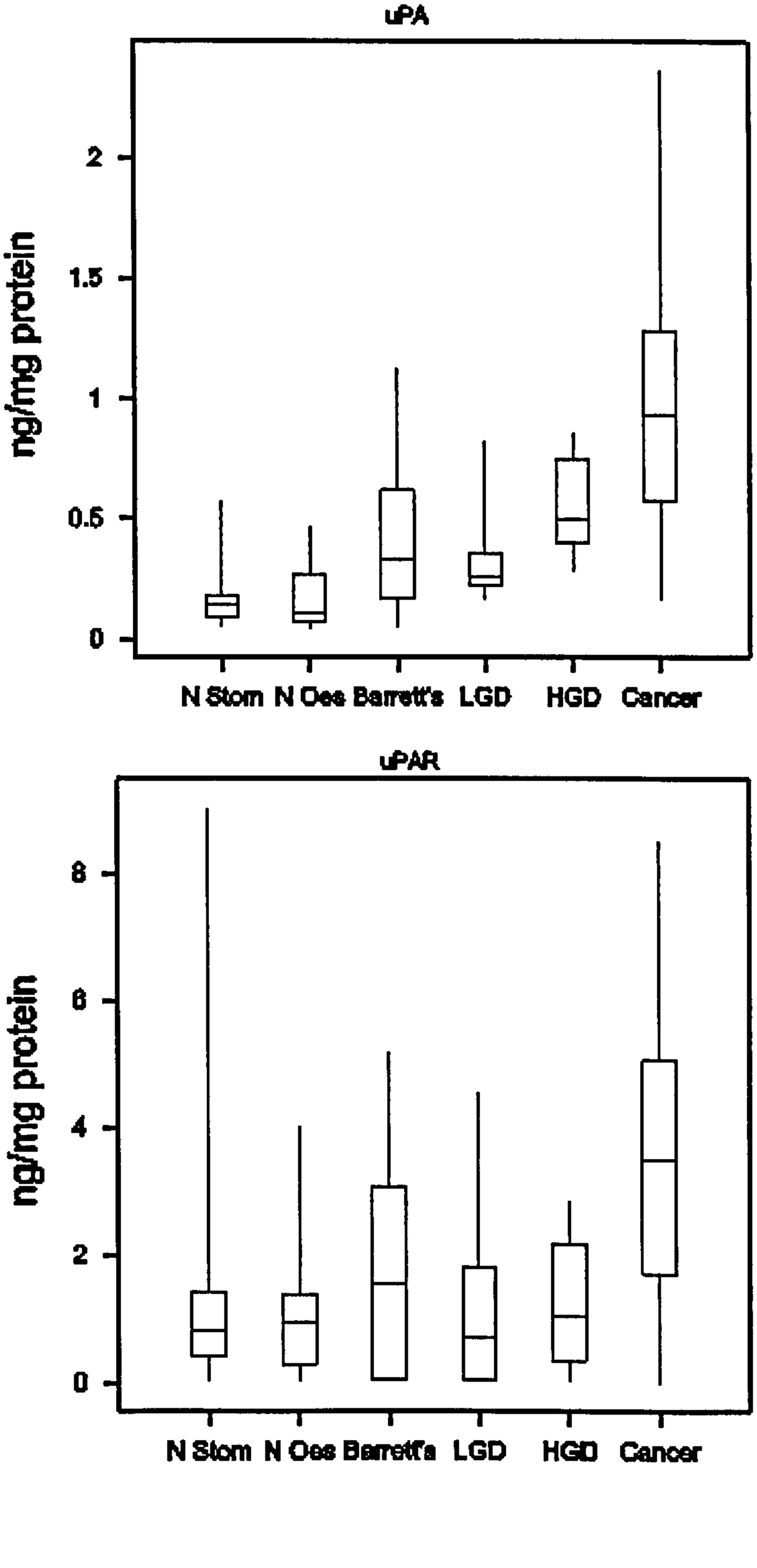
Diagnosis	uPA	uPAR	PAI-1	PAI-2
Non-involved Stomach	0.14	0.86	2.19	1.79
Non-involved Oesophagus	0.11	0.94	1.54	200.0
Non-Dysplastic Barrett's	0.33	1.53	3.00	1.66
Low Grade Dysplasia	0.26	0.72	2.77	0.74
High Grade Dysplasia	0.49	1.03	3.03	38.51
Adenocarcinoma	0.93	3.87	6.67	8.09

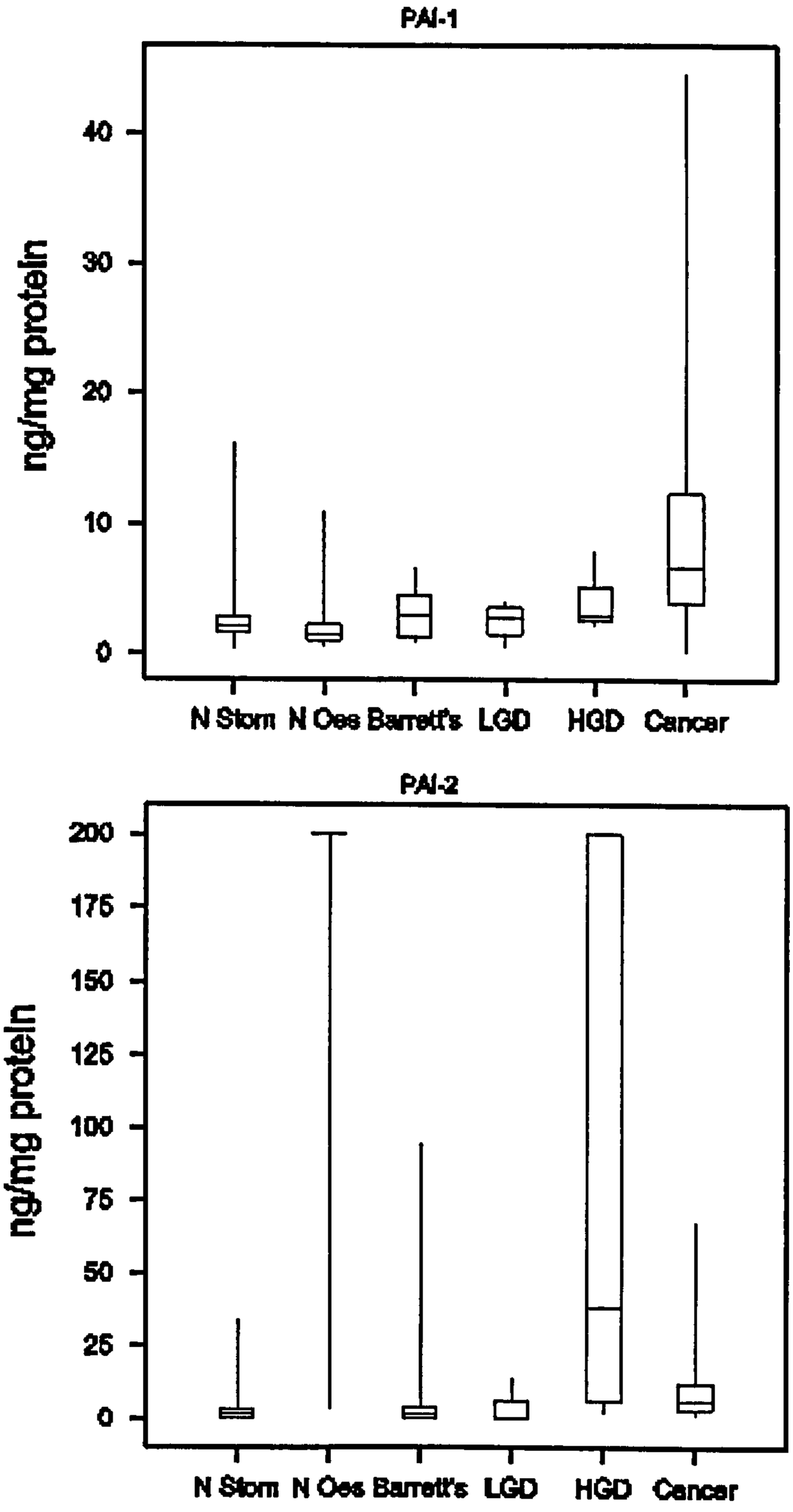
Levels of urokinase-type Plasminogen Activator increased with the progression from non-involved mucosa (median 0.13ng/mg protein) through non-dysplastic Barrett's metaplasia (0.33ng/mg protein), Barrett's with low grade dysplasia (0.26ng/mg protein), and high grade dysplasia (0.49ng/mg protein) to high levels in adenocarcinoma (0.93ng/mg protein). There were similar changes in the levels of Plasminogen Activator Receptor (non-involved 0.89 ng/mg protein; Barrett's metaplasia 1.53ng/mg protein; low grade dysplasia 0.72ng/mg protein; high grade dysplasia 1.03ng/mg protein, adenocarcinoma 3.87ng/mg protein). Plasminogen Activator Inhibitor type-1 levels were higher in non-dysplastic Barrett's (median 3.0ng/mg protein) and dysplastic Barrett's (low grade 2.77ng/mg protein, and high grade dysplasia 3.03ng/mg protein) than in non-involved oesophagus (1.88ng/mg protein), but less than in adenocarcinoma of the oesophagus (6.67ng/mg protein). Plasminogen Activator Inhibitor type-2 levels were very high in high grade dysplasia (38.51ng/mg protein) when compared to non-involved epithelium (4.77ng/mg protein), Barrett's metaplasia (1.66ng/mg protein), low grade dysplasia (0.74ng/mg protein) and adenocarcinoma (8.09ng/mg protein).

Statistical analysis of the data was carried out using GenStat for windows. The results are shown in Figure 5.8 and appendix 7.

Figure 5.8. Statistical representation of the plasminogen ELSIA results.

Box Plots of the results where the rectangles represent the upper and lower quartiles with the median marked in the middle and the tails represent the range of the results.




PAI-1
40
30
20
10
0
ng/mg protein
N Stom
N Oes
Barrett's
LGD
HGD
Cancer
PAI-2
200
175
150
125
100
75
50
25
0
ng/mg protein
N Stom
N Oes
Barrett's
LGD
HGD
Cancer

From the Box Plots (figure 5.8) it can be seen the detectable concentration of uPA increased during the progression of Barrett's oesophagus. Most of the boxes are small showing the majority of values were similar within the upper and lower quartiles. The ranges of values for the detection of uPA were reasonably small, with the exception of the adenocarcinoma samples, which had both high and low extremes. These results are reliable as all the values were within the range of the standards within the kit.

The ranges of values for uPAR were not as good as those for uPA. Samples of non-involved stomach, non-involved oesophagus, low grade dysplasia and adenocarcinoma had values below the standards within the kit (<0.025ng/ml protein). There were large ranges of values for non-involved oesophagus and adenocarcinoma with very high extremes. The boxes for non-involved stomach and oesophagus, and low and high grade dysplasia were reasonably small showing the majority of values were similar.

The results for the detection of PAI-1 were reliable, again all the values were within the range of standards within the kit and the boxes were small showing the majority of values between the upper and lower quartiles were similar. The overall ranges of values are quite small except for the adenocarcinoma samples where high extreme values were detected. The levels of PAI-1 remain quite constant during the progression of Barrett's oesophagus, but were increased in adenocarcinoma.

The least reliable results were from the detection of PAI-2. This kit used a polyclonal antibody to detect PAI-2, whereas all the other kits utilised monoclonal antibodies. A large proportion of samples gave values above

(>200ng/mg protein) and below (<0.5ng/mg protein) the standards within the kit. Nearly all the non-involved oesophagus samples gave values > 200ng/mg protein hence the small box at the extreme of the chart. High grade dysplasia gave a wide range of values and therefore had a very large box.

5.5 Discussion

As mentioned previously tumour cell invasion involves the attachment of tumour cells to the underlying basement membrane, local proteolysis and migration of tumour cells through this degraded region. Proteases are involved throughout the metastatic process. In order for a cell to metastasise it must be able to move into blood vessels, survive in circulation, arrest, move out of the blood vessels, invade surrounding tissues and grow. All these steps involve interactions between tumour cells, stromal cells, invading lymphocytic cells, endothelial cells and the extracellular matrix. Proteases, which are expressed in these cells (including Cathepsin B and D, Urokinase-type plasminogen activator and receptor, and interstitial collagenase), are believed to participate in many of these steps.

Urokinase-type Plasminogen activator (uPA) is a protein that has a molecular weight of about 54,000 daltons and is composed of 2 disulphide-linked chains, A and B of molecular weights 18,000 and 33,000 respectively. uPA may occur as a single–chain form or as a 2-chain derivative, which is gained by cleavage of the peptide bond between Lys(158) and Ile(159). It has been found the amino acid in position 158 is the main determinate of the functional properties of the single-chain form, but not the 2-chain form. uPA is a prognostic marker in many malignancies including cancers of the lung, breast, stomach and colorectal cancers.

The urokinase-type Plasminogen Activator Receptor (uPAR) is a key molecule in the regulation of cell-surface plasminogen activation and therefore plays an important role in many normal and pathological processes. Plasminogen activator receptor is also known as CD87. uPAR expression *in vivo* is associated predominately with macrophages at inflammatory sites. It is a highly glcosylated protein of about 50kD in monocytes where it is anchored to the plasma membrane by a glycosyl-phosphatidylinositol linkage.

There are two naturally occuring inhibitors for uPA, Plasminogen activator inhibitor type-1 (PAI-1) and Plasminogen activator inhibitor type-2 (PAI-2). PAI-1 is a protein containing 402 amino acids with a predicted non-glycosylated molecular mass of 45kD. It is thought to be a member of the serine protease inhibitor (serpin) superfamily of proteins. PAI-1 comes from endothelial cells whereas PAI-2 comes from placenta, monocytes and macrophages. PAI-2 is a protein containing 450 amino acids with a predicted non-glycosylated molecular weight of 46kD. PAI-2 is also a member of the superfamily of serine proteases in which the target specificity of each is determined by the amino acid residue located at its reactive centre.

Although a large body of information has accumulated on the biology, biochemical and clinical aspects of PAI-2, suggesting it is involved in many physiological processes, its precise role in placenta and inflammatory conditions as well as diagnostic and therapeutic possibilities of PAI-2 remain to be established (Kruithof 1995). PAI-2 is thought to serve as a primary regulator of plasminogen activation in the extravascular compartment. High levels of PAI-2 are found in keratinocytes, monocytes and the human trophoblast, the latter

suggesting a role in placental maintenance or in embryo development. The primarily intracellular distribution of PAI-2 may also indicate a unique regulatory role in a protease-dependent cellular process such as apoptosis (Dickinson 1995).

The progression of Barrett's oesophagus through increasing degrees of dysplasia to adenocarcinoma is characterised by the ability of epithelial cells to invade the basement membrane. Changes in the expression of components of the plasminogen activation system which allows increased production of plasmin are thought to have an important role in malignant cell invasion, but little is known about the expression of these components in the premalignant stages of Barrett's oesophagus. Both uPA and uPAR are implemented in the invasion of oesophageal cells. It has been shown utilising oesophageal cells derived from both squamous cell carcinoma and Barrett's associated adenocarcinoma that when uPA and uPAR are co-expressed the cells display stronger invasiveness. Anti-sense oligonucleotide inhibition of both uPA and uPAR resulted in a marked reduction in invasiveness (Morrissey 1999). Co-expression of uPA and uPAR is therefore required for functional involvement of the plasminogen activator system in the invasion of neoplastic oesophageal cells. Here the components of the plasminogen activation system have been studied during the metaplastic:neoplastic progression of Barrett's Oesophagus using both immunohistochemistry and ELISA techniques.

5.5.1 Tissue distribution of the components of the Plasminogen Activator system

The localisation of the protein and mRNA of uPA, uPAR and the PA inhibitors varies from tumour type to tumour type. It has been shown that in colon carcinoma uPA is expressed in cancer cells, uPAR is found in cancer cells and macrophages surrounding cancer cells and PAI-1 and PAI-2 are found in endothelial cells (Pyke 1994 and Naitoh 1995). In Breast cancer uPA, uPAR and PAI-2 are expressed in cancer cells and the surrounding stromal cells (Pyke 1993 and Umeda 1997). A study by Shiomi *et al* used immunohistochemistry and *in situ* hybridisation to investigate the expression of uPA, uPAR and PAI-2 in oesophageal squamous cell carcinoma found uPA and PAI-2 were expressed in cancer cells as well as in adjacent fibroblasts. In contrast uPAR was only expressed in cancer cells at the periphery of the tumours (Shiomi 2000). These data suggest that the differential expression of uPA, uPAR and PAIs may contribute to the organ and cellular specificity of various malignant characteristics.

Here the IHC results appear to show that staining levels of all the components of the plasminogen system are increased in Barrett's associated adenocarcinoma. The levels appear to stay relatively low in non-dysplastic Barrett's metaplasia, low grade and high grade dysplasia. This implies the expression of these molecules is a late event in the progression of Barrett's associated cancer. Surprisingly levels are lower in lymph node metastasis than adenocarcinoma samples, as the components of the plasminogen activator system are associated with cancer invasiveness and metastasis.

Examples of uPA immunoreactivity during the progression of Barrett's oesophagus to adenocarcinoma are shown in figure 5.2. The immunohistochemical staining of uPA in non-dysplastic Barrett's metaplasia was strongest towards the basement membrane of the cells. There was also strong sub-nuclear staining and staining in the stroma between the metaplastic cells. There was moderate peri-nuclear/nuclear staining for uPA in high grade dysplasia. In adenocarcinoma there were increased levels of staining for uPA through the tissue towards the inner oesophagus. This pattern of staining was also seen in the sections stained for uPAR implying co-localisation of uPA and uPAR. Examples of uPAR immunoreactivity during the progression of Barrett's oesophagus to adenocarcinoma are shown in figure 5.3.

In vitro uPAR-bound to pro-uPA is activated much more quickly than is the fluid based uPAR protein. uPAR is considered to enhance the ability of uPA to mediate invasiveness by localising uPA to the surface of the tumour cell (Shiomi 2000). A resent study by Morrissey *et al* shows that co-expression of uPA and uPAR is required for maximum invasiveness of oesophageal carcinoma cell-lines *in vitro* (Morrissey 1999).

Blood vessel spread stained weak to negative for uPA, but strongly for uPAR. Staining for uPA becomes stronger as the cancer becomes less differentiated. There was no staining for uPA in signet ring cancer cells. These mucin containing cells may express uPA, but not strongly, the high levels of mucin may have diluted it. There is strong staining for uPA in nerve cells, muscle and oesophageal glands. Where there is staining for uPAR in the nucleus of cells

there is also weak staining in the cytoplasm. There was also strong staining for uPAR in mast cells.

uPA is present in circulation in minute quantities. When uPA binds to the receptor or inhibitor it is incorporated into the cellular cytoplasm (Naitoh 2000). Therefore even if uPA antigen is detected it does not mean it was produced there. This could be true for all the other components of the plasminogen system. For this reason identification of the mRNA of the components of the plasminogen activation system is needed. This could be investigated utilising an *in situ* hybridisation method.

Examples of PAI-1 and PAI-2 immunoreactivity during the progression of Barrett's oesophagus to adenocarcinoma are shown in figures 5.4 and 5.5 respectively. Staining for PAI-1 and PAI-2 was strongest in the nucleus of cells. Blood vessel spread was very strongly stained for both the inhibitors. There was very strong staining for PAI-1 in the stroma between cells. Again signet ring cells appeared as negative holes in the staining for PAI-1. The staining for PAI-1 was diffuse in cancer cells. In cardiac and fundic Barrett's metaplasia there was moderate staining towards the basement membrane for PAI-1. Peri-nuclear staining was seen in non-dysplastic metaplasia cells. Diffuse staining for PAI-1 was seen in dysplastic and intestinal metaplasia. The muscle in blood vessel walls and nerve cells also stained positively for PAI-1. There was strong staining for PAI-2 in the stroma between the cells as seen in sections stained for PAI-1. In high grade dysplasia there was focal peri-nuclear staining for PAI-2. Non-involved oesophagus was strongly stained for all components of the plasminogen system.

There are few reports on the levels of PAI-1 in tumour tissues. Reilly *et al* observed PAI-1 expression in cancer cells in breast tumours and suggested that either PAI-1 is necessary in the process of cancer invasion and metastasis mediated by uPA or PAI-1 represents a defence mechanism against cancer invasion and metastasis (Reilly 1992). The incidence of Lymph node metastasis has been reported to become lower with in increasing amount of PAI-2 in the cancer tissues of the stomach and breast (Naitoh 2000), but these results have been contradicted by Gleeson *et al* in endometrial cancer (Gleeson 1993).

A study by Naitoh *et al* looking at uPA, PAI-1 and PAI-2 in colon cancer found that uPA expression in cancer cells was significantly more frequently detected in cases with lymph node metastasis than in cases without metastasis (Naitoh 2000). Here when metastatic Barrett's adenocarcinoma and non-metastatic cases were compared (figure 5.7) staining for uPA was increased in cases with lymph node metastasis. This was also seen in the staining for uPAR, but to a lesser extent. Interestingly, staining levels for the inhibitors PAI-1 and PAI-2 was decreased in cases with lymph node metastasis. This implies all the components of the plasminogen activator system are involved in the metastatic progression of Barrett's associated adenocarcinoma.

The increased levels of uPA and uPAR in metastatic tumours will increase the production of plasmin the protease involved in the degradation of the basement membrane during cellular invasion. The decreased levels of the inhibitors PAI-1 and PAI-2, which inhibit the production of plasmin by binding to uPA, show a decline in the cellular control of invasion in metastatic tumours.

In contrast to other current methods for the detection of the components of the plasminogen activator system in tumour tissues, (e.g., Western blots and ELISA) immunohistochemistry provides the opportunity to directly analyse exactly which cells (e.g., tumour or stroma cells) are positive or negative for protein expression in the tissue sample. Furthermore, immunohistochemistry staining of paraffin-embedded tissues facilitates prognostic studies of a large archival series of one tumour type. The validity of immunohistochemistry has been shown by direct comparison with quantitative methods (ELISA) of uPA measurement (Kobayashi 1994). Here the ELISA and immunohistochemistry results cannot be compared as different cases and samples were assessed in each study. Fresh frozen samples were utilised in the ELISA experiments and paraffin embedded tissues were used for immunohistochemistry.

A possible mechanism for the over expression of uPA in tumour samples could be the modulation of the plasminogen activation by inflammatory mediators. It has been shown that the amount of stromal inflammatory cell reaction has a significant influence on the prognosis of patients with breast and oesophageal cancers (Torzewski 1997). Alternatively, strong cytoplasmic staining for uPA in tumour cells could be caused by increased and rapid internalisation of inactive uPA:PAI-1 and uPA:PAI-2 complexes. Both these mechanisms alone or combined could result in the increased availability of uPA on the cell surface and determine the capacity for the cell to invade and metastasise.

Previous studies have shown that in some tumour types intense staining for uPA is found in the stromal cells surrounding tumour cells as well as the tumour cells themselves (Torzewski 1997). In line with previous studies here intense staining

for uPA has been observed in non-cancerous elements of the tumour tissues. The staining was of equal intensity between the cell types.

Epithelial cadherin (E-cad) is the primary regulator of cell adhesion in epithelial tissues. Cadherin function is regulated in part by interactions with cytoplasmic proteins called catenins. Levels of E-Cad were assessed during this study as a positive control for the immunohistochemistry technique. The levels of staining for E-cad follow the patterns of previous studies. In the past it has been shown there is a reduction in the expression of the E-cadherin/catenin complexes in Barrett's oesophagus biopsy specimens during the progression to adenocarcinoma, (Swami 1995, Washington 1998). A significant reduction in the expression of E-cadherin was also shown by Bailey *et al* to occur during the progression of Barrett's oesophagus to adenocarcinoma (Bailey 1998).

The cellular localisation of E-cadherin would be expected to be on the surface of the cells in the membrane as it is an adhesion molecule. This was seen in non-involved oesophageal sections, but in metaplastic, dysplastic and adenocarcinoma tissues the antigen was cytoplasmic. The reason for this is unclear, although it could result from a change in the mechanism required to insert the protein in the membrane. The result is that E-cadherin loses the ability to perform as an adhesion molecule during disease progression. The reduced levels of E-cadherin during disease progression could be due to mutation or down regulation of the gene. The epitope recognised by the antibody could be altered resulting in loss of antibody specificity.

5.5.2 Analysis of the levels of the components of the Plasminogen Activator System

The ELISA results show levels of uPA increased with the progression from non-involved mucosa through non-dysplastic Barrett's metaplasia, dysplasia, to high levels in adenocarcinoma. There were similar changes in the levels of plasminogen activator receptor. Plasminogen Activator Inhibitor type-1 levels were higher in both non-dysplastic and dysplastic Barrett's than in non-involved oesophagus, but less than in adenocarcinoma of the oesophagus. Plasminogen Activator Inhibitor type-2 levels were very high in high grade dysplasia when compared to non-involved oesophagus, Barrett's metaplasia, low grade dysplasia and adenocarcinoma.

Various studies into the levels of the components of the plasminogen activator system have been carried out using ELSIA techniques by Hewin *et al* on tumours of the gastrointestinal tract (Hewin 1994 a and b). These studies are summarised in table 5.5. The median levels of the components were very different to those found here, but the patterns of expression are similar. Levels of plasminogen activator and its receptor were elevated in the tumours when compared to matched normal and non-involved samples. Levels of plasminogen activator inhibitor type-1 were undetectable in previous studies, but detected here and were increased in tumour samples. A large increase in levels of PAI-1 is seen between gastric and oesophageal mucosa. Levels of inhibitor type 2 were reduced in tumour samples when compared to matched normal and non-involved cases. The differences in median values could be explained by the small number of samples assessed in the previous studies.

In ELISA experiments carried out in this study protein samples were extracted from tissue sections using CHAPS lysis buffer. CHAPS is an ionic detergent. Ionic detergents are strongly amphiphilic and therefore tend to denature proteins. Amphiphilic substances are both hydrophobic and hydrophilic and breakdown these interactions in the tertiary structure of proteins. This could effect the ELISA detection methods for the components of the plasminogen system. The protein samples were originally extracted from fresh frozen tissue to detect active telomerase not the components of the plasminogen activator system. Extraction with CHAPS could denature the proteins and alter the antibody epitopes. A neutral detergent such as Triton X-100 could be used instead to confirm this.

No previous studies have assessed the cellular levels of the components of the plasminogen activator system during the metaplastic:neoplastic progression of Barrett's, but here patterns in the levels of the components of the plasminogen activator system have been shown. Levels of plasminogen activator, its receptor and inhibitor type-1 increase during disease progression, whereas levels of inhibitor type 2 decrease. These changes appear as a late event during the progression of the disease showing the invasive phenotype of Barrett's oesophagus allowing cells to invade the basement membrane does not appear until severe dysplasia or neoplasia is present in the oesophagus.

Table 5.5. Summary of previous studies into the components of the plasminogen activator system in the gastrointestinal tract using ELISA techniques.

Antigen levels in ng/mg protein.

Previous studies		uPA	uPAR	PAI-1	PAI-2
Hewin *et al* 1994a	Oesophageal adenocarcinoma	6.2	Not in study	Not detected	2.4
	Normal mucosa	1.5	Not in study	Not detected	30.8
	Oesophageal carcinoma	20.7	Not in study	Not detected	8.2
	Normal mucosa	0.8	Not in study	Not detected	105.2
Hewin *et al* 1994b	Gastric carcinoma	1.93	1.72	0.04	2.52
	Normal mucosa	0.28	1.03	0.75	2.78
Hewin *et al* 1996	Oesophageal adenocarcinoma	2.16	2.01	Not detected	6.0
	Normal mucosa	0.61	0.49	Not detected	64.77
	Oesophageal carcinoma	4.05	1.95	Not detected	10.68
	Normal mucosa	0.66	0.50	Not detected	46.91
Results of this study	Oesophageal adenocarcinoma	0.93	3.87	6.67	8.09
	Non-involved mucosa	0.11	0.94	1.54	200.00

The ELISA and immunohistochemistry results follow similar patterns of expression for the components of the plasminogen activator system during the progression of Barrett's oesophagus to adenocarcinoma, but both methods have their shortcomings when assessing the validity of molecular markers. Immunohistochemistry allows the analysis of the proteins *in situ* elucidating the cellular location of the molecules within tissue, but gives no information on the levels of expression or activity of the proteins. ELISA techniques allow the quantification of the components of the plasminogen activator system, but as extracted protein samples were utilised localisation and expression of the proteins cannot be assessed. Immunohistochemistry relies upon surgical procedures for the collection of tissues for analysis either biopsy or resection. Smaller amounts of tissue can be assessed using ELISA techniques, either brushed cytological samples from the oesophagus or laser capture micro-dissected samples where a specific cell or cell type can be analysed. For a complete analysis of the plasminogen activator system during the progression of Barrett's associated adenocarcinoma expression of the proteins could be assessed using *in situ* hybridisation techniques to detected mRNA.

The gene map locations of the components of the Plasminogen Activator system are: uPA 10q24 (Tripputi 1985), uPAR 19q13 (Borglum 1991), PAI-1 7q21.3-q22 (Ginsburg 1986), PAI-2 18q21.3 (Antalis 1988). As yet no mutations in these regions of the chromosomes have been discovered during the progression of Barrett's oesophagus to adenocarcinoma. If a mutation in any of these gene locations was found it could explain the changes in the expression of the components of the plasminogen activator system and be utilised as a diagnostic

tool. As yet no investigation into the expression control of the components of the Plasminogen Activator system has been carried out.

5.5.3 The Effect of Telomerase Reactivation on the Levels of the Components of the Plasminogen Activator System

Levels of the plasminogen activator system and telomerase reactivation were assessed here as the same protein samples were utilised during both studies. Levels of plasminogen activator were higher in telomerase negative samples except in cases of low grade dysplasia during disease progression. Levels of plasminogen activator receptor were higher in telomerase positive samples in the early stages of progression, but this relationship was reversed in later stages. Levels of Plasminogen activator inhibitor type-1 were high in telomerase negative samples of non-dysplastic Barrett's metaplasia and in telomerase positive samples of adenocarcinoma. Levels of plasminogen activator inhibitor type-2 exhibited a very different pattern with low levels in all telomerase positive samples. High levels of inhibitor type-2 were seen in telomerase negative samples in the later stages of progression.

The levels of the components of the plasminogen activator system and telomerase reactivation are thought to be crucial steps in the development of the ability of a malignant cell to invade and become immortal. There are no published reports of the relation between these two events and human carcinogenesis. The results show there is a complex relationship between invasion and immortality during the progression of Barrett's oesophagus to adenocarcinoma. It is very probable that the processes work independently. Plasminogen activator and its receptor levels in the later stages of progression were associated with telomerase

negativity. This indicated that cells with an invasive phenotype are not capable of cellular immortality. Interestingly, the highest levels of inhibitor type-2 were also found in these late stage telomerase negative samples, implying that the invasive capacity of non-immortal cells maybe held in check by the activity of the plasminogen activator inhibitors. The expression of the components of the plasminogen activator system may still be controlled in mortal cells.

6 The Molecular Characterisation of Two Oesophageal Cell-Lines

6.1 Introduction

It is currently possible to take immortal human cells from tumours and culture them successfully in the laboratory for many generations. In some cases the cells proliferate indefinitely and become what is known as a cell-line. These cultured immortal cells are useful controls in the assessment of new treatments and diagnostic tools for human cancer. Cultured human cells can also be used as experimental model systems for the development of *in vitro* models of the diseases from which they are derived.

As mentioned previously there are two main types of oesophageal tumours, squamous cell carcinoma and Barrett's associated adenocarcinoma. In order to produce *in vitro* models of these tumours two oesophageal cell-lines were characterised according to molecular changes known within the diseases. These cell-lines were,

- OE21, a human oesophageal squamous cell carcinoma cell-line established in 1993 from a squamous carcinoma of the mid oesophagus of a 74 year-old male patient. Also known as JROECL21.
- OE33, a human oesophageal adenocarcinoma cell-line established from the adenocarcinoma of the lower oesophagus (Barrett's metaplasia) of a 73 year-old female patient. Also known as JROECL33 (Rockett 1997).

This chapter describes the molecular characterisation of these experimental model systems utilising a range of molecular biological techniques previously studied in clinical samples from squamous cell carcinoma and Barrett's associated adenocarcinoma. The molecular characteristics assessed included cellular adhesion, proliferation, the presence of an immortal phenotype, and invasiveness, all of which have been extensively studied in clinical cases.

6.2 Materials and Methods

6.2.1 Cell Culture

Human oesophageal cell-lines OE21 and OE33 (Rockett 1997) were cultured as described in section 3.2, in RPM1-1640 modified medium supplemented with 10% foetal bovine serum, 5ml stabilised penicillin-streptomycin solution and 1ml amphotericin B solution per 500ml (all regents from Sigma cell culture, Poole, Dorset, England).

Once established the growth media was replaced every 2 days. Cells were grown in either T25 culture flasks (for Tri-reagent extraction) or slide flasks (for immunohistochemistry). Once the cells were 75-80% confluent they were detached from the T25 flask surface using 1x trypsin-EDTA solution (Sigma cell culture, Dorset, England) and treated for protein extraction. The growth media was removed and the cells washed once in sterile PBS, then 5ml of trypsin solution was added and the flask left to stand for 1 minute. Gentile tapping on the side of the flask helped to detach the cells. Trypsin was inhibited using 5ml of growth medium and the cells pelleted by centrifugation at 100 x g for 5 minutes at 4°C. The pellet of cells could then be used for splitting into further flasks or protein extraction.

6.2.2 Immunocytochemistry on Slide Flasks

Cell-lines were grown on both glass and polystyrene slide flasks (Merck, Poole, Dorset, England). Once the flasks were 70-80% confluent the cells were washed 3 times in PBS and fixed in ice cold methanol for 10 minutes. The slides were then wrapped in foil and stored at -20°C. Slides were removed from the freezer as required and left on the bench for 30 minutes to reach room temperature. The slide was divided up into a grid using a "Dako" pen (Dako Ltd. Cambridge UK) prior to the blocking of non-specific reactions.

Immunocytochemistry was then carried out as described in section 3.9 with the following modifications. Non-specific reactions were blocked using rabbit serum (1:5) (v/v) in PBS. The blocking serum was removed without washing and the primary antibody (see table 6.1 for details of the antibodies and working concentrations) added diluted with PBS containing 1% serum and left in a humid atmosphere at 4°C overnight.

Once bound the antibodies were detected with the ABComplex and washed. The slides were then incubated with DAB Solution for 5 minutes. The slides were then washed in tap water and counterstained with heamatoxylin. Once the slides had been counterstained in haematoxylin they were mounted using Ultramount aqueous mounting medium (Dako Ltd, Cambridge, UK) and were ready for viewing under the light microscope.

Table 6.1 Antibodies used for immunocytochemistry on slide flasks.

Antibody	Type	Working Concentrations	Company
CD44	Monoclonal mouse anti-human	12μg/ml	Dako Ltd. Cambridge, UK
Cytokeratin 8	Monoclonal mouse anti-human (low molecular weight)	10μg/ml	Dako Ltd. Cambridge, UK
Cytokeratin 18	Monoclonal mouse anti-human	6μg/ml	Dako Ltd. Cambridge, UK
CEA	Monoclonal mouse anti-human	6μg/ml	Dako Ltd. Cambridge, UK
P-Cadherin	Monoclonal mouse anti-human	2.5μg/ml	Affiniti Research Products Ltd. Exeter, UK
β-Catenin	Monoclonal mouse anti-human	2.5μg/ml	Affiniti Research Products Ltd. Exeter, UK
p53	Monoclonal mouse anti-human	500μg/ml	Pharmingen International,
p63	Polyclonal mouse anti-human	No data available - dilution 1/1000	Provided by P. Hall
uPA	Polyclonal goat anti-human	4μg/ml	American Diagnostica Inc. Greenwich USA
uPA	Monoclonal mouse anti-human	4μg/ml	American Diagnostica Inc. Greenwich USA
uPAR	Monoclonal mouse anti-human	4μg/ml	American Diagnostica Inc. Greenwich USA
PAI-1	Monoclonal mouse anti-human	4μg/ml	American Diagnostica Inc. Greenwich USA
PAI-2	Polyclonal goat anti-human	4μg/ml	American Diagnostica Inc. Greenwich USA
CD44 (Hermes)	Monoclonal mouse anti-human	No data available –dilution 1/50	Provided by A. C. Woodman
E-Cadherin	Monoclonal mouse anti-human	2μg/ml	Alexis Biochemicals, Nottingham, UK

6.2.3 Production of cDNA (RT-PCR)

Using total RNA samples produced by Tri-reagent extraction (section 3.5) a reverse transcription reaction (RT-PCR) was made as described in section 3.11 with the following modifications. The Promega RT System (Promega Corporation, Southampton, UK) was again utilised using AMV Reverse Transcriptase to synthesise single-stranded cDNA.

The resulting cDNA was then transferred to a PCR reaction and cycling conditions were one cycle of 94°C for 5 minutes when the DNA polymerase was added. This was followed by 30 cycles of 94°C for 1 minute, 1 minute at the appropriate annealing temperature calculated by the T_M of each primer and a 72°C extension step varying between 30 seconds and 2 minutes. The primers and annealing temperature are summarised in table 6.2.

6.2.4 Gel Electrophoresis

PCR products were visualised on agarose gels the percentage of which varied according to the size of PCR product. A 1.8% agarose gel was used for PCR products approximately 200 – 400bp in size. See section 3.8 for further details.

Table 6.2 Primers and specific conditions for PCR.

Further details of these primers are given in appendix 4.

Target gene	Primer	Conditions	Size of Product
β-Actin	F and R	Annealing at 55°C for 1 minute and extension of 2 minutes	~500bp
E-Cadherin exons 4 and 5	4F and 5R	Annealing at 60°C for 1 minute and extension of 30 seconds	380bp
β-Catenin	1F and 1R	Annealing at 55°C for 1 minute and extension of 1 minute	~250bp
β-Catenin exon 3	3F and 3R	Annealing at 55°C for 1 minute and extension of 30 seconds	228bp

6.2.5 TRAPEZE™ ELISA Telomerase detection kit.

For non-quantitative detection of telomerase activity in cells the TRAPEZE™ ELISA telomerase detection kit (Intergen Company, Oxford, UK) was utilised. The kit contains all the reagents necessary to perform the Telomeric Repeat Amplification Protocol (TRAP) and Enzyme-Linked Immunosorbent Assay (ELISA). The assay was carried out according to the manufacture's protocol and as described in section 4.2.

Cultured cells were detached from the bottom of T25 flasks when approximately 75-80% confluent using trypsin/EDTA as mentioned in the cell culture section 3.2. The pellet of cells was then washed once in PBS and all the PBS removed. The pellet was then resuspended in 200μl (3-[(3-Cholamidopropyl) dimethylammonio]-1-propane-sulfonate) CHAPS Lysis buffer. The sample suspensions were incubated on ice for 30 minutes and then spun at 12,000 x g for

20 minutes at 4°C. The supernatant (160µl) was transferred to a fresh tube and 6µl set aside for determination of protein concentration (see section 3.3.6). The kit recommended diluting the sample extracts with CHAPS lysis buffer prior to use according to the following: cell extracts (0.01µg/µl - 0.75µg/µl) using less than 1.5µg per assay. Titre experiments were also carried out to find the optimum concentration of protein required for the assay. The TRAP assay was set up for each sample as described in section 4.2 with no modifications.

6.2.6 ELISA for the detection of the components of the Plasminogen Activator system

The quantitative analysis of the components of the urokinase Plasminogen Activator system were carried out on the same cell-line protein extracts as were analysed using the TRAPEZE™ ELISA. The kits utilised were the IMUBIND® uPA ELISA kit, the IMUBIND® Total uPAR ELISA kit, the IMUBIND®Tissue PAI-1 ELISA kit and the IMUBIND® PAI-2 ELISA kit (American Diagnostica Inc. Greenwich, USA). Analysis was carried out as described in section 5.3 with no modifications.

6.3 Results

The overall results are shown in table 6.3. To characterise the cell-lines various techniques were used. These were:

- Telomerase Repeat Amplification Protocol (TRAP),
- Immunocytochemistry (ICC),
- Reverse Transcription-Polymerase Chain Reaction (RT-PCR),
- Enzyme-Linked Immunosorbant assay (ELISA).

From the table it can be seen that there were differences in some of the results for each cell-line and some of the experiments resulted in no differences between the cell-lines at all. Differences were seen in some of the components of the plasminogen activator system between the cell-lines. Most of the cadherin and catenin results were the same for both cell-lines, the only difference was in the IHC for β-catenin, OE21 was positive and OE33 was negative. There were also no differences in CD44 expression between the cell-lines. Differences were seen in the expression of the cytokeratins, CEA, p63 and p53.

Table 6.3. Summary of cell-line characterisation results.

Abbreviations: TRAP = Telomerase Repeat Amplification Protocol, ELISA = Enzyme-Linked Immunosorbent Assay, ICC = Immunocytochemistry, and RT-PCR = Reverse Transcription-Polymerase Chain Reaction.

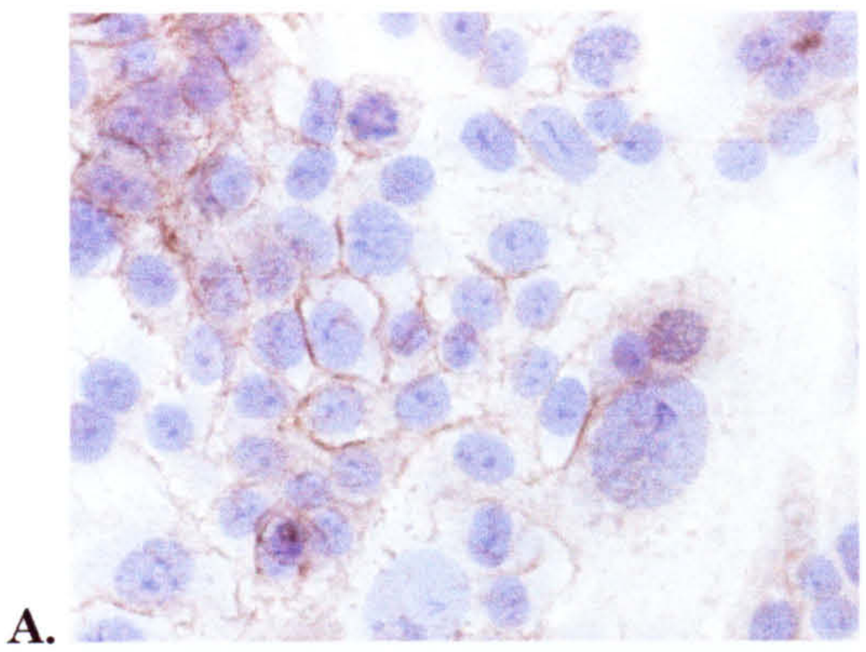
Molecule/Test	OE21 (squamous cell carcinoma)	OE33 (Barrett's adenocarcinoma)	Different?
Telomerase/TRAP	Activity	Activity	NO
p63/ICC	Nuclear staining	Nuclear staining	NO
p53/ICC	Weak nuclear staining in odd cells	Strong nuclear staining in all the cells	YES
β-actin/RT-PCR	Strong band at ~500bp	Strong band at ~500bp	NO
E-Cadherin/ICC	Strong staining in all membranes	Strong staining in all membranes	NO
E-Cadherin/RT-PCR	4F + 5R Clear band at 380bp	4F +5R Clear band at 380bp	NO
P-Cadherin/ICC	Negative	Negative	NO
β-catenin/ICC	Staining in cell membranes	Negative	YES
β-catenin/RT-PCR	1F and 1R strong band at 240bp	1F and 1R strong band at 240bp	NO
β-catenin/RT-PCR	3F and 3R two bands at 200bp and >1000bp	3F and 3R two bands at 200bp and >1000bp	NO
CEA/ICC	Negative	Staining in membranes of half the cells	YES
CD44/ICC	Very strong staining all over the membranes	Very strong staining all over the membranes	NO
Cytokeratin 8/ICC	Negative	Strong staining of the membranes in half of the cells	YES
Cytokeratin 18/ICC	Weak staining of membranes in most cells	Strong staining of the membranes in a few of the cells	YES
Urokinase-type plasminogen Activator/ICC	Strong staining in the cytoplasm around the nucleus of the largest cells	Strong staining in the cytoplasm	NO

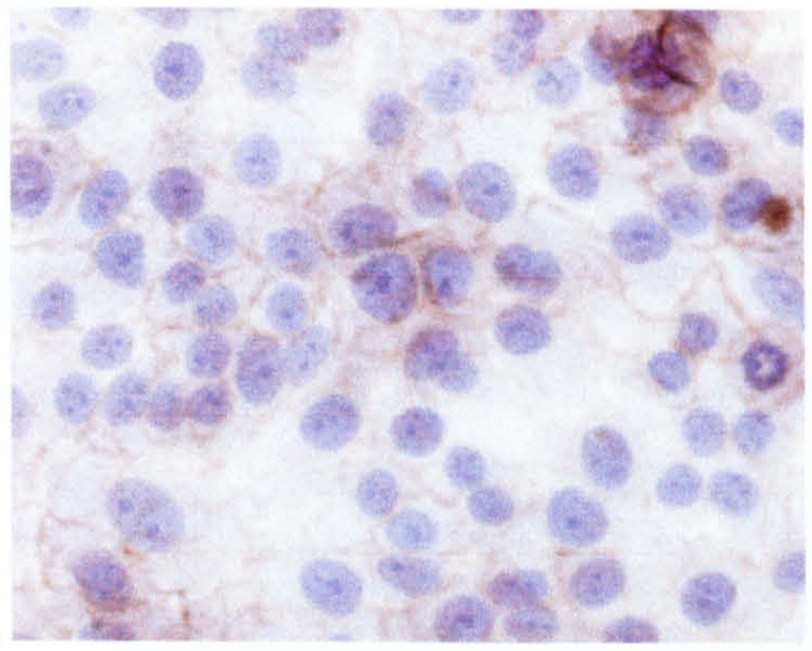
Molecule/Test	OE21 (squamous cell carcinoma)	OE33 (Barrett's adenocarcinoma)	Different?
Urokinase-type plasminogen Activator receptor/ICC	Weak staining in the cytoplasm	Weak staining in the cytoplasm	NO
Plasminogen Activator Inhibitor type-1/ICC	Moderate staining in the cytoplasm	Moderate staining in the cytoplasm	NO
Plasminogen Activator Inhibitor type-2/ICC	Weak staining in the cytoplasm	Strong staining in the cytoplasm	YES
Urokinase-type Plasminogen Activator/ELISA	3.25ng/mg protein	>10ng/mg protein	YES
Urokinase-type Plasminogen Activator Receptor/ELISA	5.417ng/mg protein	3.934ng/mg protein	NO
Plasminogen Inhibitor type-1/ELISA	>200ng/mg protein	0.984ng/mg protein	YES
Plasminogen Inhibitor type-2/ELISA	42.1ng/mg protein	>200ng/mg protein	YES

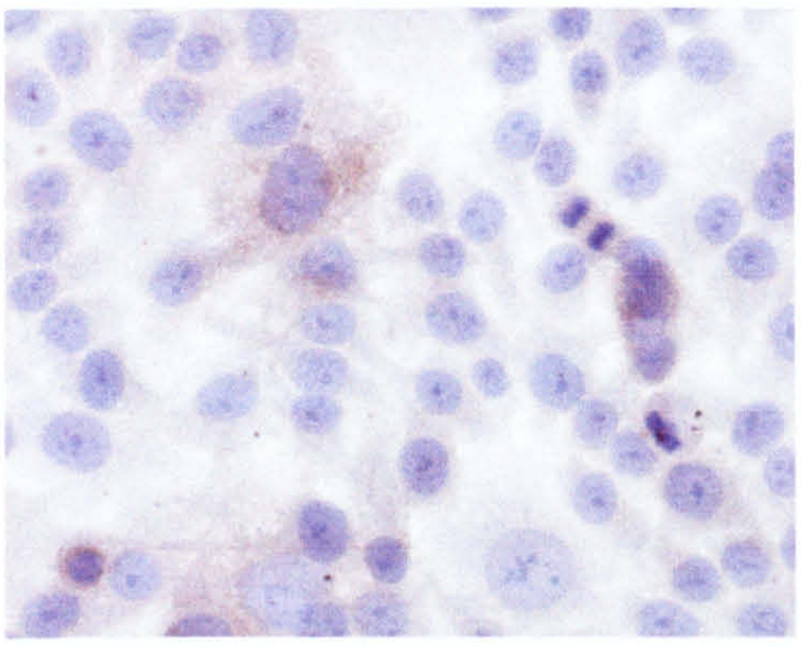
The TRAP assay was initially optimised using cell lysates derived from the oesophageal cell-lines, OE21 and OE33. The optimum protein concentration for the cell-lines was found to be 0.015μg/μl, but the assay was sensitive enough to detect telomerase in dilutions of a hundred times less. All the other cell-line protein extracts were diluted to a similar concentration before use in the assay.

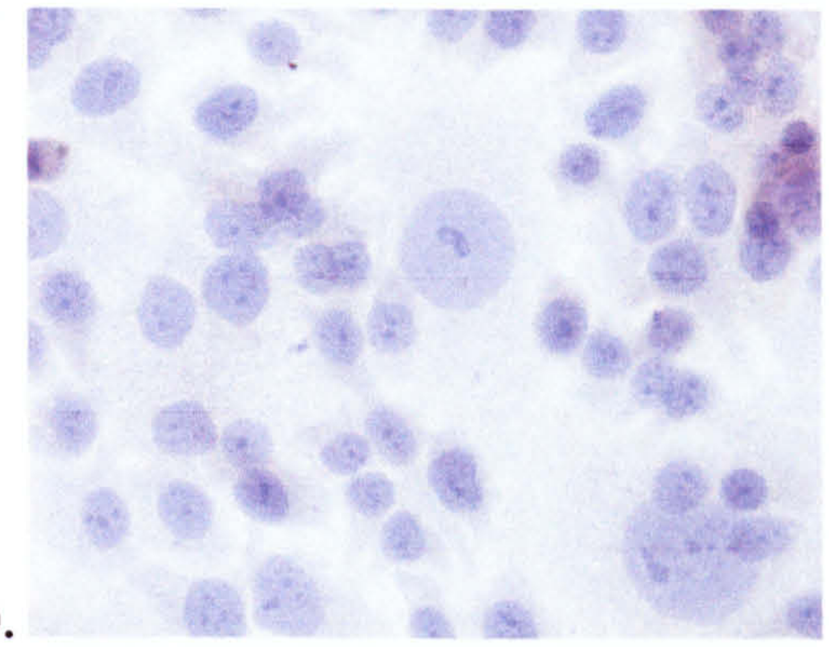
The cell-lines were grown on both polystyrene and glass slide flasks (Merck Ltd. Poole, Dorset, UK) for the immunocytochemical techniques. It was observed that OE33 did not grow very well on the glass slide flasks. When the cells were washed in PBS before fixing with methanol they fell off the slides whereas OE21 grew well on both the glass and plastic slide flasks.

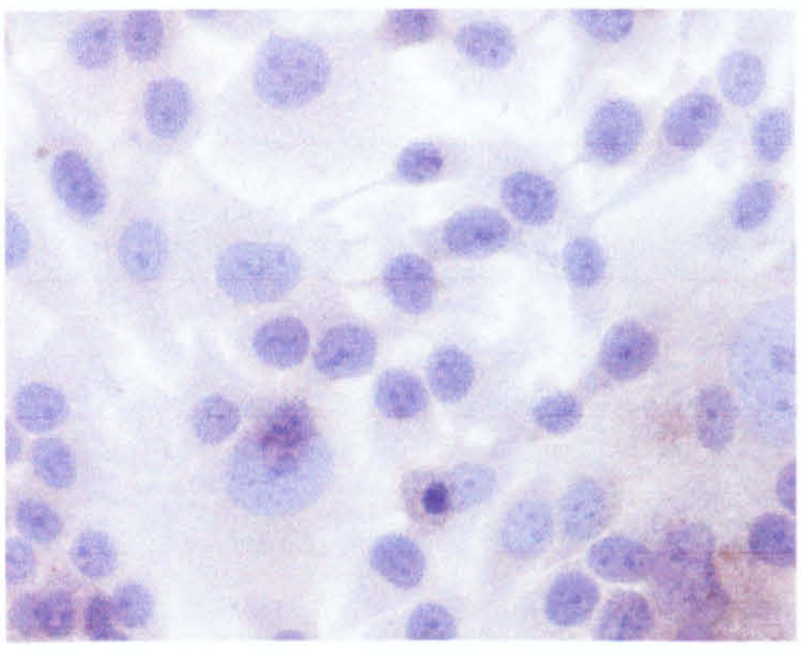
Examples of the immunoreactivity for CD44, E-cadherin, uPA, uPAR, PAI-1 and PAI-2 in OE21 and OE33 are shown in figures 6.1 and 6.2 respectively. Immunocytological staining for CD44, E-cadherin, and cytokeratin 18 was localised to the membranes of both cell-lines, although the staining for cytokeratin 18 was weaker in OE21 compared to OE33. There was membranous staining for CEA and cytokeratin 8 in OE33, but OE21 was negative for these markers. There was membranous staining for β-catenin in OE21 whereas OE33 was negative. Both cell-lines were negative for P-Cadherin. There was nuclear staining for both p53 and p63 in both cell lines, but staining for p53 was weaker in OE21 compared to OE33. There was strong cytoplasmic staining for the components of the plasminogen activator system in both cell-lines. The only difference in staining was for PAI-2, staining was weaker in OE21 compared to OE33.











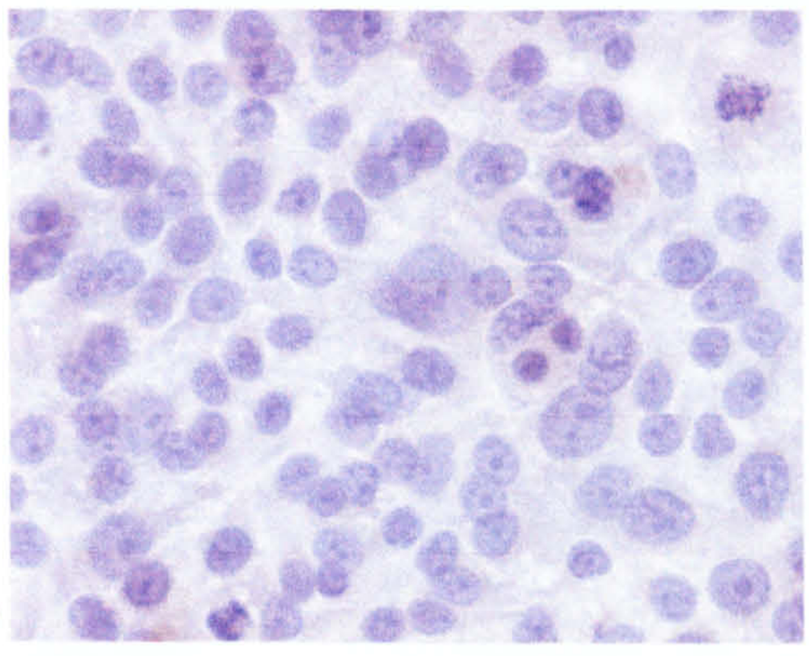
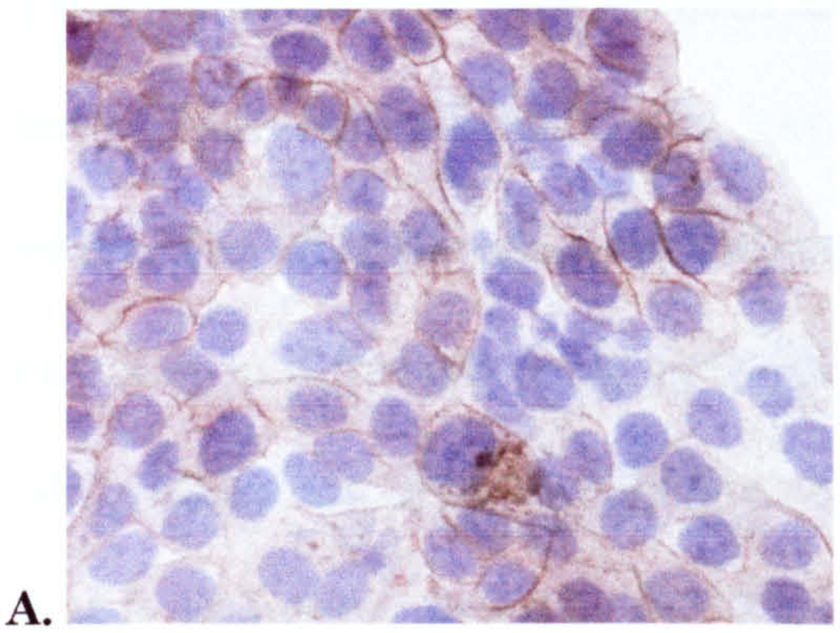
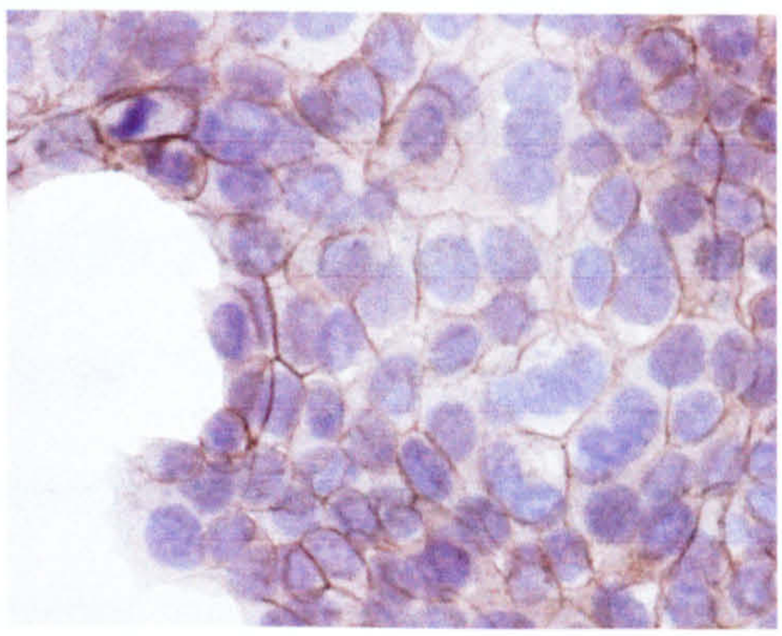


Figure 6.1. Examples of immunocytochemical staining of the squamous cell carcinoma cell-line OE21 with: A. CD44, **B.** E-Cadherin, C. uPA, **D**. uPAR, **E**. PAI-1, and **F**. PAI-2. (Magnification x40).


A.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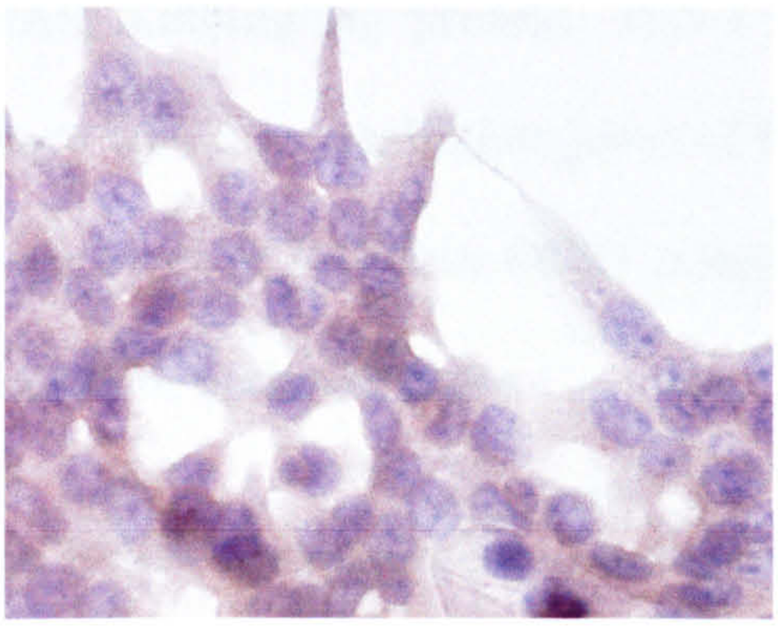
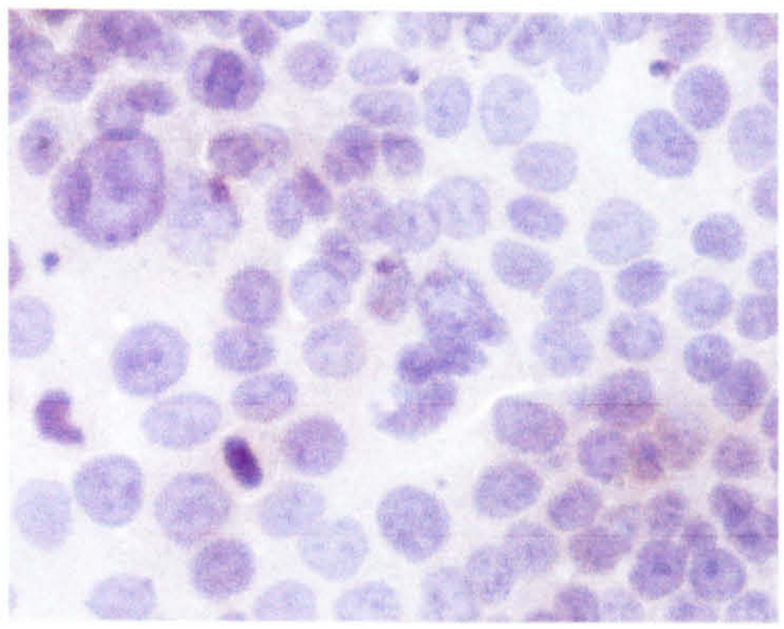
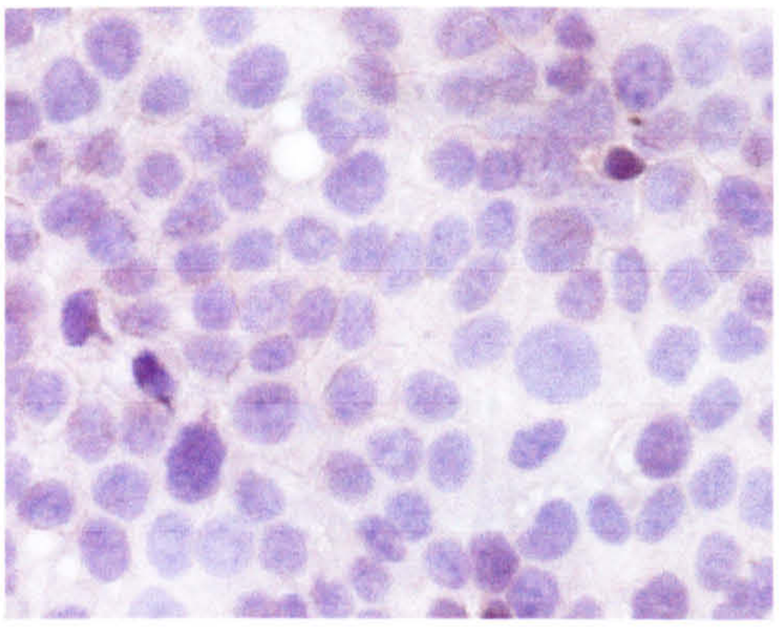

C.


D.


E.

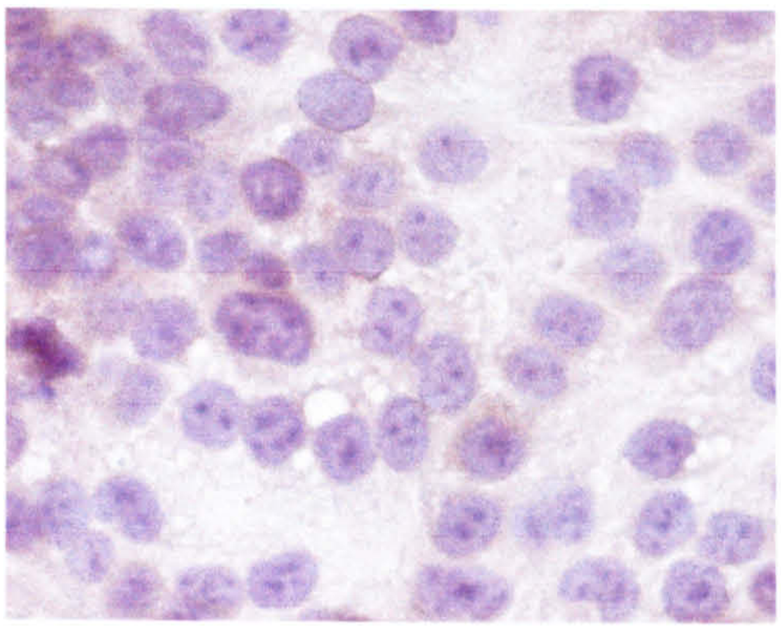

F.

Figure 6.2. Examples of immunocytochemical staining of the Barrett's associated adenocarcinoma cell-line OE33 with: A. CD44, **B**. E-Cadherin, C. uPA, **D.** uPAR, **E**. PAI-1, and **F.** PAI-2. (magnification x40).

Telomerase activity was present in both cell-lines as expected because they are both immortal cell-lines that have been cultured in the laboratory for many years. The ELISA assays for the components of the plasminogen activator system resulted in different levels between the cell-lines. OE21 had the following levels: uPA 3.25ng.mg protein, uPAR 5.417ng/mg protein PAI-1 >200ng/mg protein and PAI-2 42.1ng/mg protein. OE33 had the following levels: uPA >10ng/mg protein, uPAR 3.934ng/mg protein, PAI-1 0.984ng/mg protein and >200ng/mg protein. There was a much higher level of PAI-1 in OE21 compared to OE33 and a much lower level of PAI-2 in OE21 compared to OE33. Levels of uPA and uPAR were similar in both cell-lines.

The RT-PCR experiments showed that mRNA for both E-cadherin and β-catenin were present in both the cell-lines. No differences were seen in the gels, the resulting bands were identical for both cell-lines (results not shown).

6.4 Discussion

The cell-lines, OE21 and OE33, used for this part of the work were developed in 1997. Molecular and cellular analysis was carried out on the lines and clones derived from them. These included chromosome analysis, flow cytometric analysis, transforming growth factor β1 (TGF-β1) sensitivity and production were tested and RT-PCR carried out for the detection of TFG-β1 mRNA. Both the cell lines were found to show epithelial morphology and to express epithelial cytokeratins and are also tumourigenic in nude mice (Rockett 1997). Both the oesophageal cell-lines are held in the European Collection of Cell Cultures.

In order to characterise the cell-lines various methods of analysis were used these included RT-PCR, immunocytochemistry (ICC), the TRAPEZE™ ELISA telomerase detection kit and ELISA kits for the detection of the components of the plasminogen acticator system. In order to characterise the cell-lines to produce *ex vivo* models of oesophageal adenocarcinoma and oesophageal squamous cell carcinoma molecular markers were selected that have previously been analysed in clinical samples.

6.4.1 p53 and p63

The expression of p53 has been widely studied in Barrett's oesophagus. Wild type p53 appears to be a regulator of cell kinetics. When a cell sustains DNA damage p53 may either repair it or induce cell death. Abnormal p53 represents two defects: deletion of one allele in the short arm of chromosome 17 and a functionally inactivating mutation of the other allele. The abnormal p53 protein has a longer half-life than the wild type protein, due to an amino acid

substitution causing over expression allowing the protein to be detected by IHC. The p53 abnormality occurs in less than 50% of Barrett's adenocarcinomas, therefore malignant progression is possible without the involvement of the p53 gene (Kubba 1999).

This study has shown that there was strong nuclear staining in all the cells for p53 in the cell-line OE33, the Barrett's adenocarcinoma, whereas the squamous carcinoma cell-line, OE21, had weak nuclear staining in some of the cells. In the past, p53 abnormalities have been used as a measure of genetic instability in Barrett's metaplasia, but their use is far from well established or accepted (Kubba 1999). From these results it could be concluded that OE33 is more genetically unstable than OE21.

The tumour protein p63 was cloned by Yang *et al* in 1998 and was shown to have strong homology to the tumour suppresser p53 and the p53-related protein p73. p63 has been detected in a variety of human and mouse tissues, but unlike p53 the p63 gene encodes multiple isotypes with the ability to transactivate p53 reporter genes and induce apoptosis (Yang 1998). p63 is also known as KET, p73L and p51. As p63 has strong homology to p53 it is thought that abnormalities in expression will be found in cancer cells, although very little research into this area has been carried out.

A study by Hall *et al* has looked at the expression of p63 in the neoplastic sequence of Barrett's oesophagus and correlated the results with the expression of p53 protein. It was found there was exact concordance between the expression of p53 and p63 in the advanced stages of neoplastic progression in Barrett's oesophagus. In the proliferative compartment of non-neoplastic oesophageal

tissues p63 was detectable whereas p53 was not (Hall 2001). The Barrett's associated adenocarcinoma cell line (OE33) IHC results mimic the results seen in tissue samples. The nuclear staining of p53 and p63 were similar in this cell line. If p63 activates p53 and induces apoptosis it would be expected the protein would be expressed in the nucleus of the cells as seen in this study.

6.4.2 Cadherins and catenins

Epithelial cadherin (E-cad) is the primary regulator of cell adhesion in epithelial tissues. There is reduced expression of E-cad during the progression of Barrett's oesophagus to adenocarcinoma (Bailey 1998). No differences were seen in E-cad protein and mRNA expression in either of the oesophageal cell-lines. There was strong staining in the membranes and cytoplasm of both OE21 and OE33 cells. E-Cad mRNA expression was the same for both the cell-lines as shown by RT-PCR. A PCR product of 380bp was seen for each reaction. As E-cad is a cell adhesion molecule it would be expected that it would be expressed on the surface of the cell as seen in both the IHC results. According to research into Barrett's adenocarcinoma the level of E-cad expression should be lower in cancer when compared to Barrett's metaplasia, but the levels of expression seemed to be high for both cell-lines. Research has also shown reduced levels of cadherin in poorly differenticated invasive oesophageal carcinomas (Sanders 1999).

Placental cadherin (P-cad) has 58% homology to E-cadherin. P-cadherin expression has been found to be absent in Barrett's metaplasia, but was present in some adenocarcinomas (Bailey 1998). Both the cell-lines showed no presence of P-cadherin protein.

Cadherin function is regulated in part by interactions with cytoplasmic proteins called catenins. β-Catenin binds to E-cadherin, but may also bind to the tumour suppresser protein APC. Expression of β-catenin is also found to decrease in cancers. Expression of β-catenin mRNA was the same for each cell-line, a PCR product of 240bp was amplified in each case. Strangely the IHC results for the presence of β-catenin protein were different for each cell-line. OE21, the squamous cell carcinoma, had strong staining in the cytoplasm and cell membrane, but no staining was seen in the Barrett's adenocarcinoma cell-line, OE33.

It was expected that the results for E-cad and β-catenin expression for each cell-line would be the same as E-cad binds to β-catenin. β-Catenin and E-cad mRNA were present in all the cell-lines, but β-catenin protein was not detected by IHC in OE33. When the cell-lines were grown on glass slide flasks instead of polystyrene the cell-line OE33 did not adhere properly to the glass and the monolayer became dislodged during the fixing procedure. This implies that the adhesion properties of the cell-line OE33 differ from OE21 as the results show. The OE33 cells were also observed to grow much slower on glass than they did on polystyrene.

6.4.3 CEA

Carcinoembyonic antigen (CEA) is a complex immunoreactive glycoprotein comprising of 60% carbohydrate. CEA is an adhesion molecule, which is found in adenocarcinomas of endodermally derived digestive system epithelia and in foetal colon. CEA immunoassay is useful in the diagnosis and monitoring of cancer patients for recurrent disease or response to therapy, particularly in colonic

cancer (Oikawa 1987). The presence of CEA in the progression of Barrett's oesophagus to adenocarcinoma has been established by immunohistochemistry by Ferrando *et al.* CEA was found in 100% of oesophageal adenocarcinoma samples tested (Ferrando 1998). In oesophageal carcinoma CEA levels have been found to be significantly increased when compared to normal volunteers and the localisation changed from mainly membranous to cytoplasmic. (Sanders 1994). Staining was seen in the membranes and cytoplasm of approximately half the cells of OE33, whereas no staining was seen at all in the OE21 cells. It is thought that the over expression of CEA disrupts normal adhesive forces allowing more cell movement and adoption of a less organised tissue structure (Benchimol 1989). As OE21 was negative you would expect the cells to still possess full adhesive properties. As mentioned previously OE33 cells appear to be less sticky than the OE21 cells, this could be due to expression of CEA in some of the cells. The CEA results seem to confirm the theory that OE33 has less adhesion properties than OE21.

6.4.4 CD44

CD44 is an integral cell membrane protein, which is thought to have a role in cell adhesion. The CD44 protein is encoded by a gene comprising of 20 exons, which has been mapped to the short arm of chromosome 11. Numerous isoforms are generated by molecular mechanisms of mRNA alternative splicing and post translational modifications. The standard form detected here is encoded by the 10 consecutively expressed exons (exons 1-5 and 16-20) is an 80-90kDa protein that has been implicated mainly in cell-cell and cell-matrix binding.

A study by Lagorce-Pages *et al* into the expression of the standard form of CD44 in Barrett's associated adenocarcinoma tissue showed immunohistochemical staining was localised to the cellular membrane in 70% of adenocarcinoma samples analysed (Lagorce-Pages 1998). As expected the results discussed in this chapter show very strong staining for CD44 which was also localised to the cell membrane in both cell lines. No differences were seen between the staining pattern for either cell-line.

6.4.5 Cytokeratins

Cytokeratins are subunit proteins of epithelial cell intermediate filaments which are genetically determined (Salo 1996). Squamous cell carcinoma of the oesophagus has been found to express CK6, CK14, CK17, CK19 in high amounts and CK8 in weak to moderate amounts, whereas Barrett's adenocarcinoma has been found to express CK8, CK18 and CK19 in high amounts (Moll 1982). It has been suggested that CK18 and CK8 expression are diagnostic markers for Barrett's metaplasia (Salo 1996).

OE21 the carcinoma cell-line was found to be negative for CK8 protein and have weak staining for CK18. OE33 the Barrett's adenocarcinoma cell-line was found to be strongly positive for both CK8 and CK18 as expected. As CK8 and 18 have been used to identify Barrett's adenocarcinoma in the past the IHC results seem to confirm that the cells are in fact derived from a Barrett's adenocarcinoma. The fact that OE21 was negative for CK8 and weakly positive for CK18 may call into question the exact cell type of OE21 as it does not match the cytokeratin distribution found by Moll *et al.*

6.4.6 Telomerase

Telomerase is expressed in 85% of human cancers (Shay 1997b and Mackenzie 1999). It is a sign of immortality and allows the cells to divide without end replication problems. Both Barrett's adenocarcinoma (Morales 1998) and squamous cell carcinoma (Koyanagi 1999) have been found to be telomerase positive from analysis of clinical samples. Both oesophageal cell-lines were found to be telomerase positive. This is as expected, as both are immortal cell-lines that have been growing in laboratories for many years and were derived from oesophageal tumours in 1993.

6.4.7 Components of the Plasminogen System

The localisation of the protein and mRNA of uPA, uPAR and the PA inhibitors varies from tumour type to tumour type. A study by Shiomi *et al* used immunohistochemistry and *in situ* hybridisation to investigate the expression of uPA, uPAR and PAI-2 in oesophageal squamous cell carcinoma found uPA and PAI-2 was expressed in cancer cells as well as in adjacent fibroblasts. In contrast uPAR was only expressed in cancer cells at the periphery of the tumours (Shiomi 2000). The carcinoma cell-line, OE21, has strong staining of uPA, moderate staining of inhibitor type-1 and weak staining of uPAR and inhibitor type-2. From these results there is no way of telling exactly what type of cancer cells the cell-lines are, but to some extent the IHC results of the cell-line mirror those of the tissues, strong staining of the activator and inhibitors and weaker staining of the receptor.

Nekarda *et al* carried out a study in 1998 to assess the level of expression and significance of uPA and PAI-1 in resected adenocarcinoma of the oesophagus

using ELISA techniques. This study found that uPA and PAI-1 levels were significantly elevated when compared with the normal mucosa (Nekarda 1998). The adenocarcinoma cell-line, OE33, showed strong staining for uPA and PAI-1 similar to the results found by Nekarda *et al.*

There is a difference in the amount of staining for PAI-2 in the cell-lines. In oesophageal carcinoma it has been shown levels of PAI-2 decreased compared to normal mucosa (Hewin 1994). PAI-2 levels also decrease in oesophageal adenocarcinoma, but to a lesser extent than squamous cell carcinoma. The level of staining in the cell-lines follows a similar pattern to the findings in tissue samples.

A resent study by Morrissey *et al* shows that co-expression of uPA and uPAR is required for maximum invasiveness of oesophageal carcinoma cell-lines *in vitro* (Morrissey 1999). There is evidence of co-expression of the activator and receptor in both cell-lines when comparing the cells under the microscope, although there is less staining of the receptor than the activator.

In chapter 5 of this report the levels of the components of the PA system in tissue were assessed using the same ELISAs as used here to study the cell lines. The results are summarised in table 6.4 compared to the results of a previous study into the levels of the components of the PA system in the two oesophageal tumours.

There is good concordance in the levels of both uPA and its receptor in the cell-line OE33 and adenocarcinoma tissues. Levels of PAI-1 were not detected in the previous study although high levels were found in the adenocarcinoma cell-line and adenocarcinoma tissues. There is an anomaly in PAI-2 levels. In the cell-line

OE33 the level is significantly higher than in the adenocarcinoma samples and previous studies.

Table 6.4. Comparison of levels of components of the PA system in oesophageal tumours.

Red rows are the results from the cell-line characterisation.

	uPA	**uPAR**	**PAI-1**	**PAI-2**
OE21 Carcinoma cell-line	3.25ng/mg protein	5.417ng/mg protein	>200ng/mg protein	42.1ng/mg protein
Hewin *et al* 1994 Carcinoma	20.7ng/mg protein	Not in study	Not detected	8.2ng/mg protein
OE33 Adenocarcinoma cell-line	>1ng/mg protein	3.93ng/mg protein	0.98ng/mg protein	>200ng/mg protein
Values from chapter 5 Adenocarcinoma	0.93ng/mg protein	3.87ng/mg protein	6.67ng/mg protein	8.09ng/mg protein
Hewin *et al* 1994 Adenocarcinoma	6.2ng/mg protein	Not in study	Not detected	2.4ng/mg protein

The differences in the results from the previous study, the cell-lines and results from chapter 5 could be explained by the small number of samples assessed in a previous study by Hewin *et al*. Only five resected squamous cell carcinoma cases and five adenocarcinoma cases were studied. In chapter 5, thirty adenocarcinoma cases were analysed for all the components of the Plasminogen Activator system and the cell-lines were analysed with every experiment carried out as a control and the median value of these results expressed here.

Overall the cell-line results seem to correspond to the molecular characterisation of the tumours they are primarily derived from. The cell-line OE33 seems to be a good *in vitro* model of Barrett's associated adenocarcinoma. The results are not as conclusive for the squamous cell carcinoma cell-line, OE21. There are some differences in the molecules expressed in the cell-line when compared to those found in the studies of clinical samples. OE33 has more of the expected characteristics of an invasive cancer, whereas OE21 seems to be partway through the neoplastic change.

7 The Effect of Bile Salts on Two Cultured Oesophageal cell-lines

7.1 Introduction

Experimental work using animal models has implicated a role for bile salts in the pathogenesis of Barrett's oesophagus and adenocarcinoma of the oesophagus. Duodenogastric reflux has been shown to occur in normal individuals, especially at night. Whereas duodenogasro-oesophageal reflux probably occurs infrequently in normal individuals, but evidence is strong that it plays a role in Barrett's oesophagus and adenocarcinoma (Marshall 1997). Duodenogastro-oesophageal reflux is suggested as a cause of Barrett's oesophagus. The duodenal contents suspected of causing oesophageal damage include bile acids, lysolecithin secreted in bile and the pancreatic enzyme trypsin. Conjugated bile acids are an important constituent of duodenal refluxate and can cause oesophageal damage. Mucosal damage by bile salts depends on the conjugation state of the bile acids as well as the pH of the solution. Conjugated bile acids cause damage at an acidic pH, whereas unconjugated bile acids as well as the pancreatic enzyme trypsin cause damage at a pH higher than 7 (Buttar 2001).

Previous studies have looked at the effects of bile salts and pH on both *ex vivo* oesophageal tissue samples and cultured cells. A study by Kaur *et al* (2000) looked at the effects of bile salts with and without acid on cell proliferation in biopsies of oesophageal mucosa. This study concluded that brief exposure to bile salts in the absence of acid increased proliferation, whereas exposure to a combination of acid and bile salts together inhibited proliferation (Kaur 2000).

Shirvani *et al* studied the expression of Cyclo-oxygenase 2 (COX-2) in Barrett's oesophagus using biopsies of Barrett's epithelium exposed to acid alone, acid and bile or bile alone. This showed COX-2 expression increased significantly when exposed to acid or bile salts (Shirvani 2000). The effects of bile salts and acid on the apoptotic and proliferative activity of cultured oesophageal cell-lines was studied by Whittles *et al* (1999). It was found that bile salts effected apoptosis during acidic conditions.

In order to assess the effects of conjugated bile salts and acidic conditions on the expression of telomerase, the components of the urokinase-type Plasminogen Activator system and the adhesion molecules CD44 and E-Cadherin, two oesophageal cell-lines were cultured in growth media of varying pH and treated with conjugated bile salts.

7.2 Methods

7.2.1 Cell Culture

As mentioned previously in chapter 6 two oesophageal cell-lines were cultured as an *ex vivo* model of two types of oesophageal cancer, Squamous cell carcinoma and Barrett's associated adenocarcinoma. These cell-lines were, OE21, a human oesophageal squamous cell carcinoma cell-line established in 1993 from a squamous carcinoma of the mid oesophagus of a 74 year-old male patient, and OE33, a human oesophageal adenocarcinoma cell-line established from the adenocarcinoma of the lower oesophagus (Barrett's metaplasia) of a 73 year-old female patient (Rockett 1997).

The cells were cultured as described in section 3.2 with the folowing modifications. Human oesophageal cell-lines OE21 and OE33 were cultured in RPM1-1640 modified medium supplemented with 10% foetal bovine serum, 5ml stabilised penicillin-streptomycin solution and 1ml amphotericin B solution per 500ml (all regents from Sigma cell culture, Poole, Dorset, England). The cells were cultured in both the original pH of the growth media, which was approximately 7.4, and also in media altered to an acidic pH of 5 with HCl. The cells were also cultured in RPMI-1640 supplemented with 0.1mM concentrations of various conjugated bile salts.

The bile salts used were:

- Deoxycholic acid (DCA) $C_{24}H_{39}O_4Na$
- Glycocholic acid (GCA) $C_{26}H_{42}NO_6Na$
- Glycodeoxyxholic acid (GDC) $C_{26}H_{42}NO_5Na$
- Glycochenodeoxycholic acid (GCDA) $C_{26}H_{42}NO_5Na$
- Taurocholic acid (TCA) $C_{26}H_{44}NO{\bullet}SNa$
- Taurodeoxycholic acid (TDC) $C_{26}H_{44}NO_6SNa$
- Taurochenodeoxycholic acid (TCDA) $C_{26}H_{44}NO_6SNa$

Prior to treatment with bile salts or acidified growth media the cells were grown for a day in either slide flasks for immunocytochemistry for the detection of the components of the plasminogen system or T25 flasks for protein extraction utilised for the TRAPEZE ELISA Telomerase detection kit and the Plasminogen ELISAs. The cultured cells were exposed for one hour to either acidified media, media treated with 0.1mM bile salts or 0.1mM bile salts in acidified media. The cells were then either, fed without rinsing and grown for a further 18 hours

(samples B) or prepared for further analysis (samples A). Slide flasks were washed three times in PBS before they were fixed in ice cold methanol for 10 minutes. The slides were then dried and stored at -20°C wrapped in foil. The cells grown in T25 flasks were rinsed in PBS before the cells were scraped off into a 5ml solution of sterile PBS. The cells were detached using a cell scraper instead of trypsin solution as trypsin has been implicated as a causal factor for Barrett's oesophagus and therefore may have effected the cells. The PBS solution containing the cells was placed in a 1ml eppendorf tube and centrifuged at 1000 x g for 5 minutes to produce a pellet of cells. The pellet was then rinsed in 1ml PBS and transferred to a 2ml eppendorf and spun again. All the PBS was removed before the pellet was quick frozen and stored at -80°C for protein extraction.

7.2.2 TRAPEZE ELISA Telomerase detection kit.

The TRAPeze™ ELISA telomerase detection kit as described in section 4.2 was utilised again to assess telomerase activity in the protein samples extracted from oesophageal cell-lines treated with both acid and bile salts.

Cultured cells were detached from the bottom of T25 flasks once treated with bile salts or acid using a cell scraper. The pellet of cells was then washed once in PBS and all the PBS removed. The pellet was then quick frozen and stored at -80°C until sufficient samples were ready for protein extraction. The pellets were removed from the freezer and each resuspended in 200µl CHAPS Lysis buffer without defrosting. The sample suspensions were incubated on ice for 30 minutes and then spun at 12,000 x g for 20 minutes at 4°C. The supernatant (160µl) was transferred to a fresh tube and 6µl set aside for determination of protein concentration (see section 3.6).

The TRAP assay was then carried out as described previously in section 4.2 and 6.2.5 without any modifications.

7.2.3 Immunocytochemistry on Slide Flasks

To assess the effects to treatment with bile and acid on oesophageal cell-lines immunocytochemistry was carried out using methods previously described in sections 3.9 and 6.2.2. Cell-lines were grown on polystyrene slide flasks (Merck, Poole, Dorset, England). Once the cells had been treated with bile or acid they were washed 3 times in PBS and fixed in ice cold methanol for 10 minutes. The slides were then wrapped in foil and stored at -20°C. Slides were removed from the freezer as required and left on the bench for 30 minutes to reach room temperature. The slide was divided up into a grid using a "Dako" pen (Dako Ltd. Cambridge UK) prior to the blocking of non-specific reactions.

The primary antibodies used on slides flasks treated with bile salts and varying pH are described in table 7.1.

Once the immunocytochemistry method had been carried out and the slides counter stained in haematoxylin they were mounted using Ultramount aqueous mounting medium (Dako Ltd, Cambridge, UK) and were ready for viewing under the light microscope.

Table 7.1 Antibodies used for immunocytochemistry on slide flasks.

Antibody	Type	Working Concentrations	Company
uPA	Polyclonal goat anti-human	4µg/ml	American Diagnostica Inc. Greenwich USA
uPA	Monoclonal mouse anti-human	4µg/ml	American Diagnostica Inc. Greenwich USA
uPAR	Monoclonal mouse anti-human	4µg/ml	American Diagnostica Inc. Greenwich USA
PAI-1	Monoclonal mouse anti-human	4µg/ml	American Diagnostica Inc. Greenwich USA
PAI-2	Polyclonal goat anti-human	4µg/ml	American Diagnostica Inc. Greenwich USA
CD44 (Hermes)	Monoclonal mouse anti-human	No data available –dilution 1/50	Provided by A. C. Woodman
E-Cad	Monoclonal mouse anti-human	2µg/ml	Alexis Biochemicals, Nottingham, UK

7.2.4 Detection of the components of the Plasminogen Activator system using ELISA Techniques.

The same protein samples from the cultured cells as used for the TRAPEZE™ ELISA Telomerase detection kit were used to analyse if the cellular concentrations of the components of the plasminogen system (uPA, uPAR, PAI-1 and PAI-2) were altered with exposure to bile salts and acidic conditions. The kits utilised were the IMUBIND® uPA ELISA kit, the IMUBIND® Total uPAR ELISA kit, the IMUBIND®Tissue PAI-1 ELISA kit and the IMUBIND® PAI-2 ELISA kit (American Diagnostica Inc. Greenwich, USA). Analysis was carried out according to the manufacturer's protocol and detailed methods are described in sections 5.3 and 6.2.6.

7.3 Results

The oesophageal cell-lines OE21 and OE33 were exposed to seven different conjugated bile salts in growth media of both acidic and neutral pH. The cells were exposed to bile salts, acidic growth media or bile salts in acidic growth media for 1 hour and either analysed (samples A) or grown for a further 18 hours before analysis (samples B). This resulted in 32 protein samples for each cell-line. These were used in ELISA experiments to assess the components of the Plasminogen Activator system and in the TRAPEZE™ ELISA to detect telomerase activity.

Cells cultured in the different conditions were also analysed using immunocytochemistry to detect the components of the plasminogen system (uPA, uPAR, PAI-1 and PAI-2) and the adhesion molecules CD44 and E-Cadherin. These results are summarised in table 7.2.

7.3.1 Immunocytological Results

The expression of two adhesion molecules, CD44 and E-Cadherin, were analysed using immunocytochemistry on two cell-lines OE21 and OE33 after treatment with various bile salts and acidified growth media. Examples of the immunoreactivity of the cell-lines to these are molecules are shown in figures 7.1 to 7.4. The immunocytological staining for both CD44 and E-Cad was localised to the plasma membrane of the cells. The staining was strongest in the untreated cells and cells treated with bile salts alone. In the presence of acidified media and acidified bile salts the staining for CD44 and E-cad was significantly less.

The expression of the components of the plasminogen activator system, uPA, uPAR, PAI-1 and PAI-2 were analysed using immunocytochemistry in two cell-lines OE21 and OE33 after treatment with various bile salts and acidified growth media. Examples of the immunoreactivity of the cell-lines to these molecules are shown in figures 7.5 to 7.12. The staining for the components of the plasminogen system was localised to the cytoplasm of the cells. In OE21 the staining for uPA was localised around the nucleus of the largest cells. This was not seen in OE33. There was some staining for the inhibitors within the nucleus of the cells. There were no significant differences in the staining for the components of the plasminogen activator system with exposure to bile salts, acidified media or the combination of both in either cell-line.

There were less of both types of cells on the slides exposed to acidified media and bile salt compared to the non-treated controls and cells exposed to bile salts alone. Also of note is the fact that exposure to bile salts alone appeared to increase the number of the cells on the slide a day after treatment compared to the non-treated controls. The exposure of cells to DCA and acidified media, TDC and acidified media, and GCA and acidified media caused the nuclei of both OE21 and OE33 to stain differently in heamatoxylin. The nuclei appeared transparent and pale compared to non-treated controls and other treated cells.

Table 7.2. Results of immunocytochemistry on cells treated with bile salts and grown in acidified media.

Abbreviations: DCA = Deoxycholic acid, GCA = Glycocholic acid, GDC - Glycodeoxyxholic acid, GCDA = Glycochenodeoxycholic acid, TCA = Taurocholic acid, TDC = Taurodeoxycholic acid and TCDA = Taurochenodeoxycholic acid.

A = Samples analysed directedly after exposure. B = Samples analysed 18 hours after exposure.

Grading of staining: - = Negative, +/- = Weak, + = Moderate, ++ = Strong and +++ = Very Strong.

OE21

Treatment	uPA	uPAR	PAI-1	PAI-2	CD44	E-Cad
Control	++	+/-	+	+/-	++	++
ACID A	+/-	+/-	+	+	-	-
ACID B	+/-	-	+	+	-	-
GDC A	++	+/-	+	+++	+++	+++
GDC B	++	+/-	+++	+++	++	+++
GDC+ ACID A	+/-	+/-	+	++	+/-	-
GDC + ACID B	+	+	+	+	+/-	+/-
DCA A	++	+	++	++	++	++
DCA B	++	++	+	++	+++	+++
DCA + ACID A	+/-	+/-	+	+	+/-	+/-
DCA + ACID B	+/-	+/-	+	++	+	+/-
GCA A	++	+/-	+	+	+/-	+/-
GCA B	+	+/-	++	+/-	+	+/-
GCA + ACID A	+/-	+/-	+	+	+/-	-
GCA + ACID B	+/-	+	+	+/-	+/-	-
TCA A	++	+/-	++	+	+	++
TCA B	++	+/-	+	+	+	++
TCA + ACID A	+	+/-	+	+	+/-	-
TCA + ACID B	+/-	+/-	++	+	+/-	-
TDC A	+	+	++	++	++	++
TDC B	+	+	+++	++	+++	+++
TDC + ACID A	+	+	++	+	+	-
TDC + ACID B	+	+	++	+	-	-
GCDA A	+	+/-	+	++	++	++
GCDA B	+	+/-	+	++	++	++
GCDA + ACID A	+	+/-	+	+	+/-	-
GCDA + ACID B	+	+/-	+	+	+/-	+/-
TCDA A	+	+/-	+/-	++	++	++
TCDA B	+	+/-	+	+	++	++
TCDA + ACID A	+/-	+/-	+	+	+/-	-
TCDA + ACID B	+/-	+/-	+	+	+	+/-

OE33

Treatment	uPA	uPAR	PAI-1	PAI-2	CD44	E-Cad
Control	++	+/-	+	++	++	++
ACID A	++	+/-	+	++	+/-	+/-
ACID B	++	+	++	++	+	+/-
GDC A	++	+	++	++	+++	+++
GDC B	++	++	++	++	+++	+++
GDC+ ACID A	+	-	+	+	+	+/-
GDC + ACID B	+/-	+/-	+	+	+/-	+/-
DCA A	++	+	++	++	+++	+++
DCA B	++	+	++	+++	+++	+++
DCA + ACID A	+	+/-	+	+	+	+/-
DCA + ACID B	+/-	+	++	+	+	+/-
GCA A	++	+/-	++	++	+++	+++
GCA B	+	+/-	++	++	++	+++
GCA + ACID A	+/-	+/-	+	+	+	+/-
GCA + ACID B	+	+/-	+	+	+	++
TCA A	+	+/-	+	+	++	+
TCA B	+	+/-	+	+	+++	+++
TCA + ACID A	+/-	+/-	+	+	+/-	+/-
TCA + ACID B	+	+/-	+	++	++	+
TDC A	++	+	+++	++	+++	+++
TDC B	++	+	++	++	++	+++
TDC + ACID A	+	+/-	+	+/-	+	+/-
TDC + ACID B	+	-	+	+	+	-
GCDA A	++	+	++	++	++	+++
GCDA B	+	+	+	++	++	++
GCDA + ACID A	+	+/-	+	++	+	+/-
GCDA + ACID B	+	+/-	+	+	+	+/-
TCDA A	+	+	+	++	++	++
TCDA B	+	+/-	+	+	+	++
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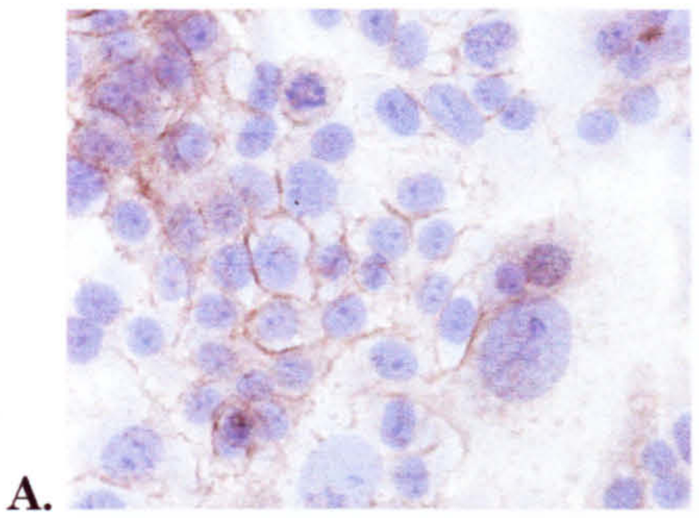
TCDA + ACID B	+/-	+/-	+	+	+	+/-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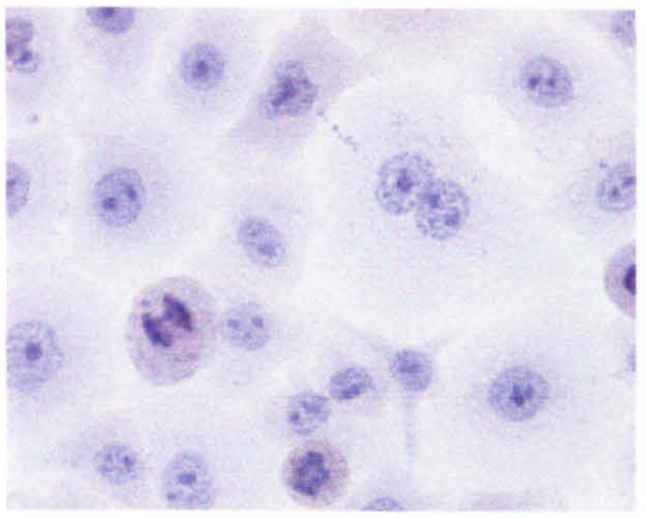
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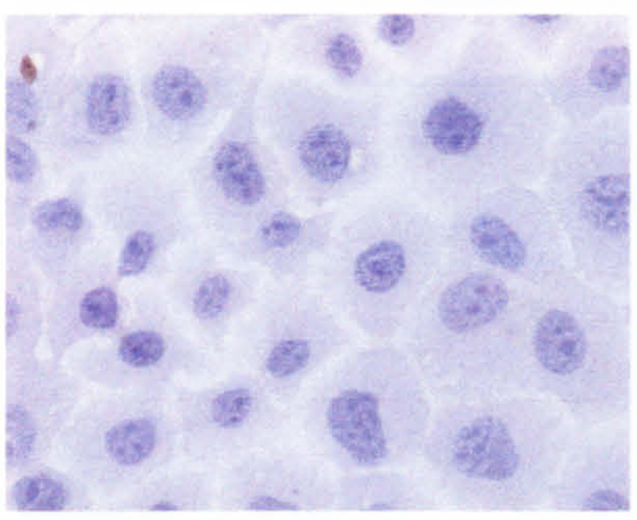
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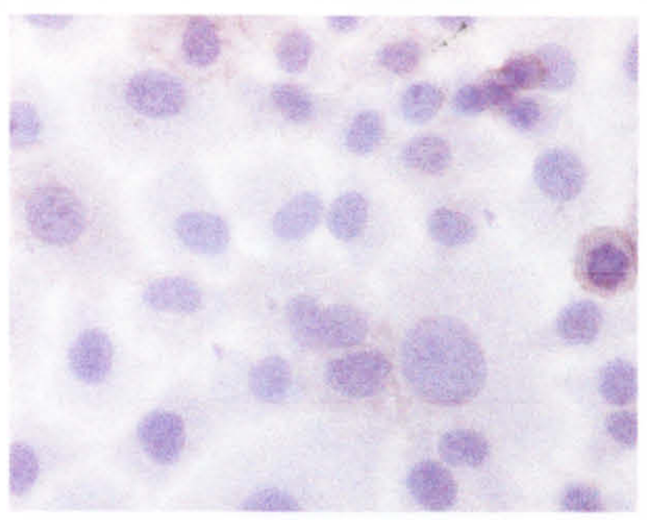
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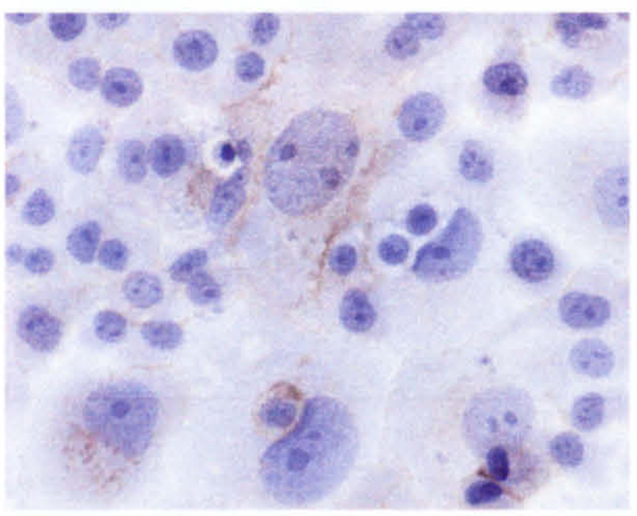
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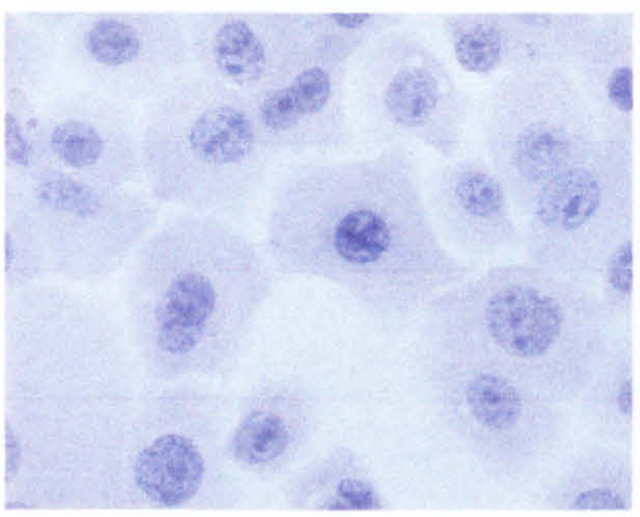
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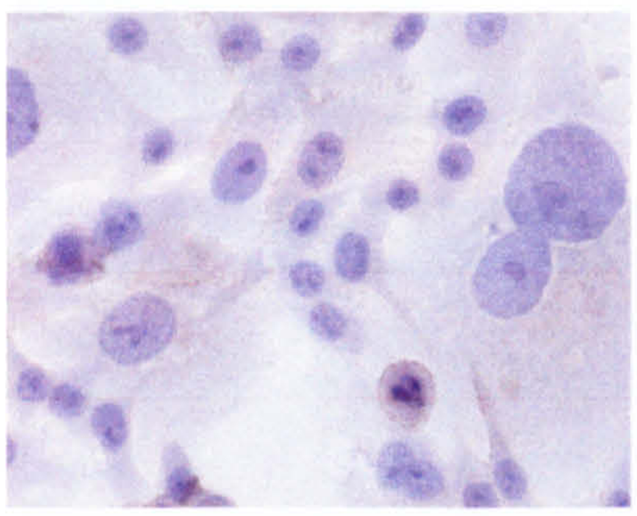
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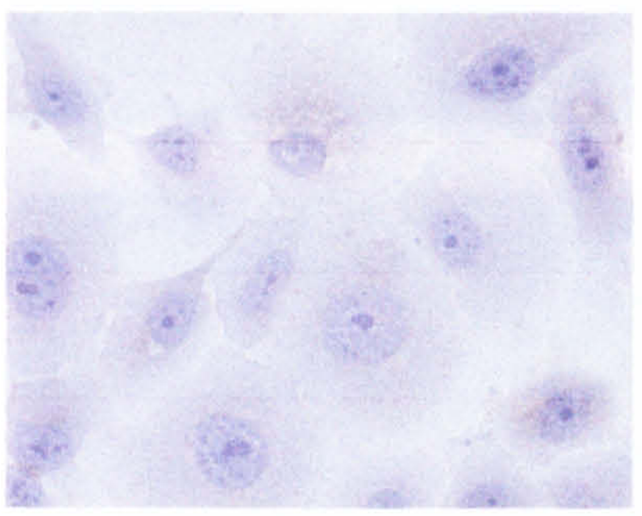
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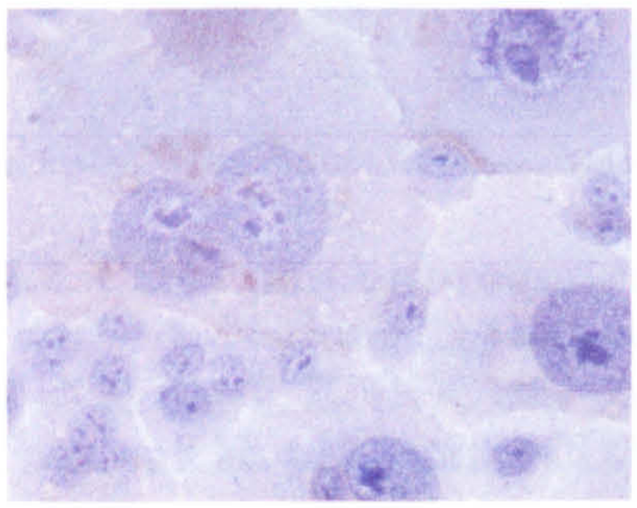
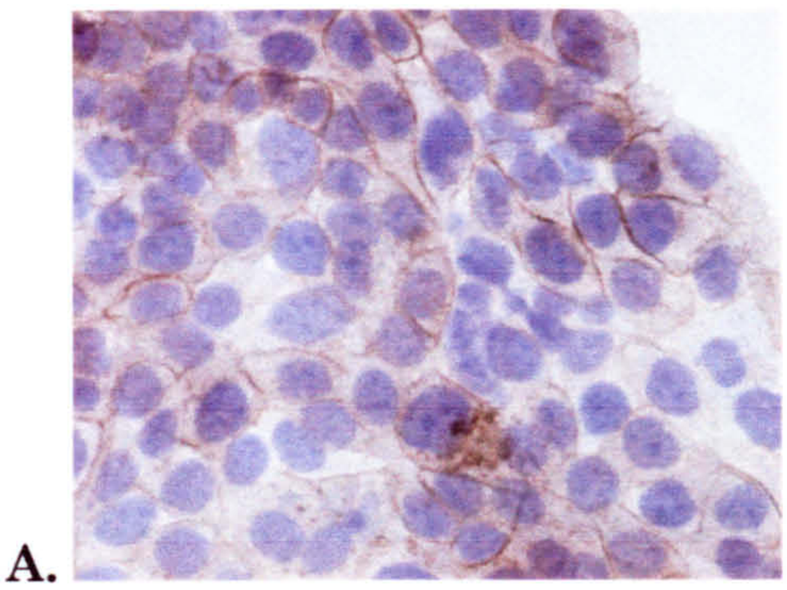
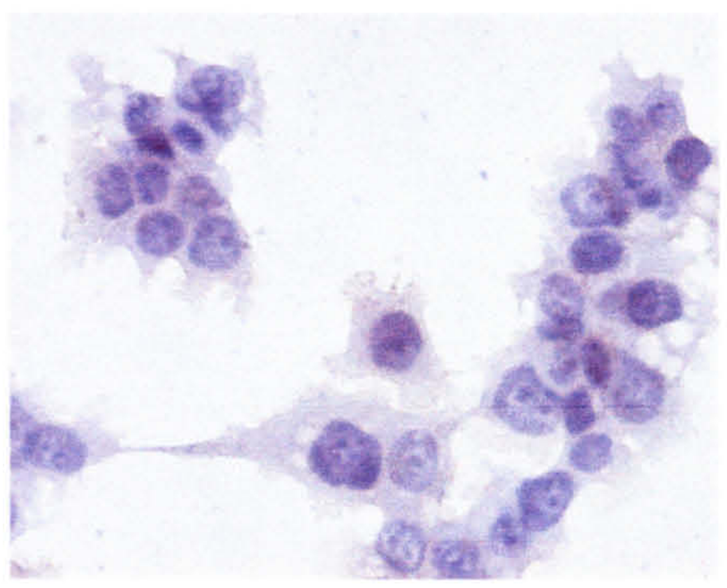
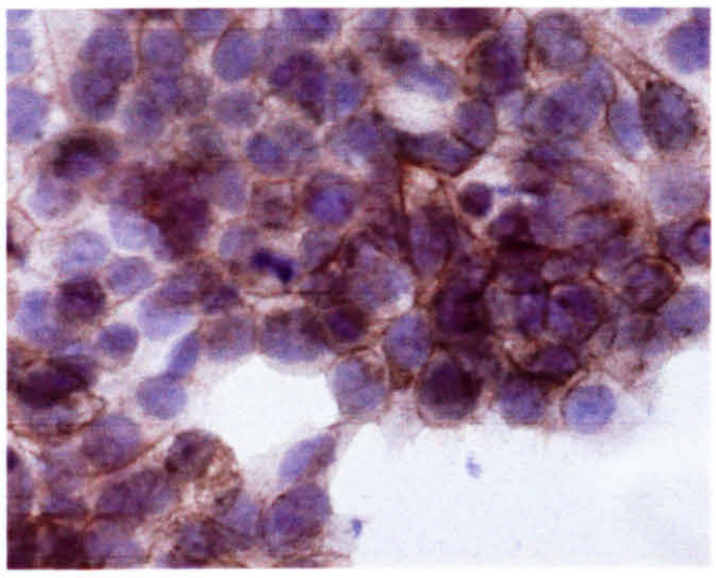
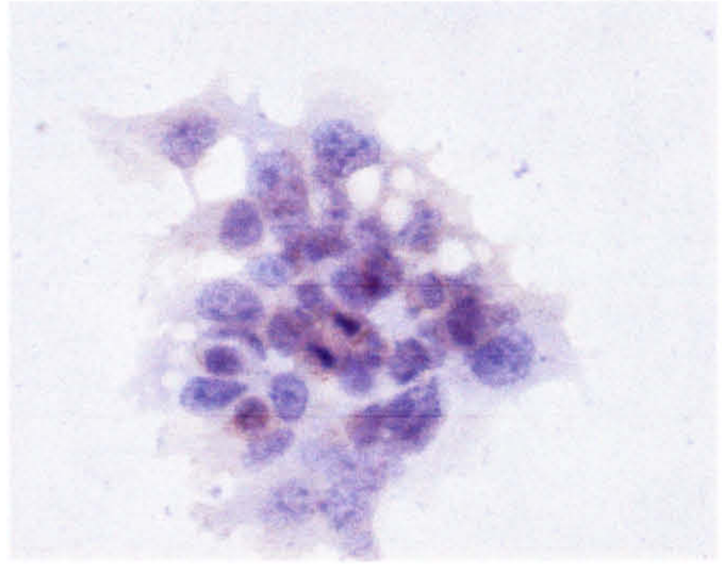

I.

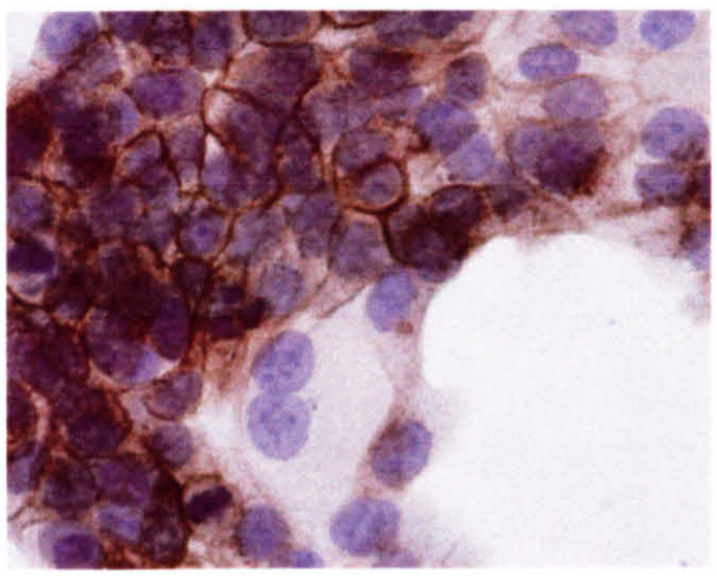
Figure 7.1 CD44 immunoreactivity in the oesophageal cell-line OE21 treated with: A. Nothing day two Control, **B.** pH 5 day one, **C.** pH 5 and GCDA day two, **D.** GCDA day one, **E.** TCA day one, **F.** pH 5 and TCA day 2, **G.** TCDA day two, **H.** pH 5 and TCDA day 2 and **I.** pH 5 and DCA day 2. (Magnification x10)

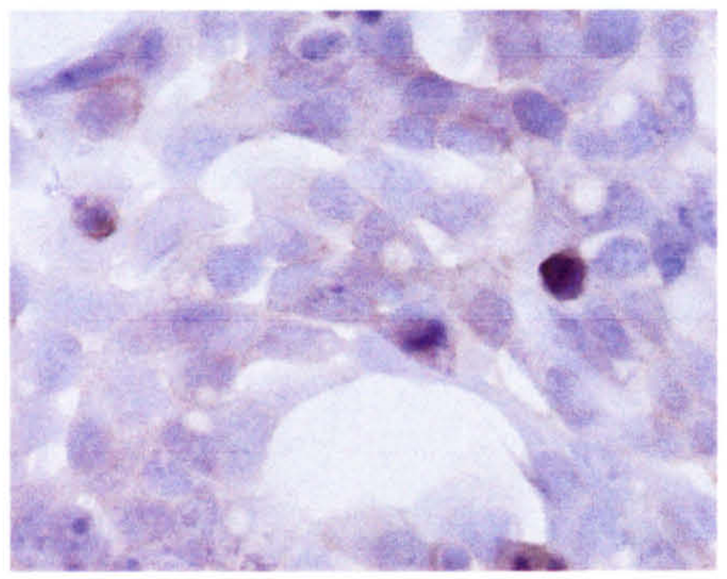


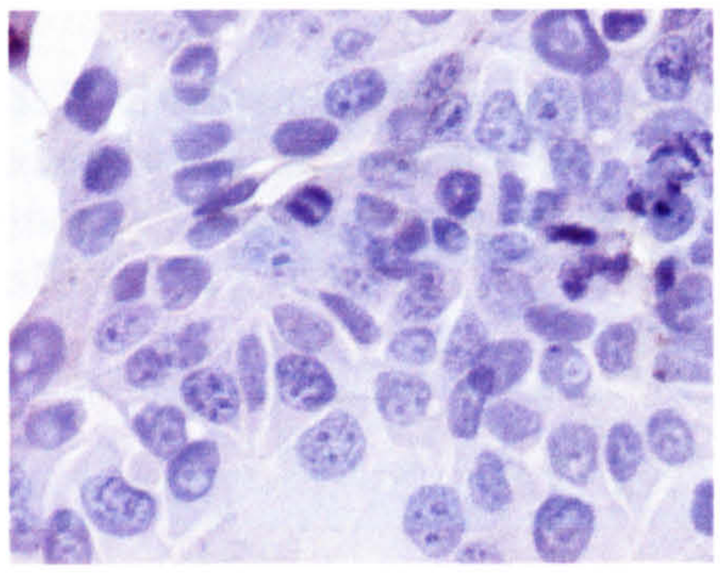












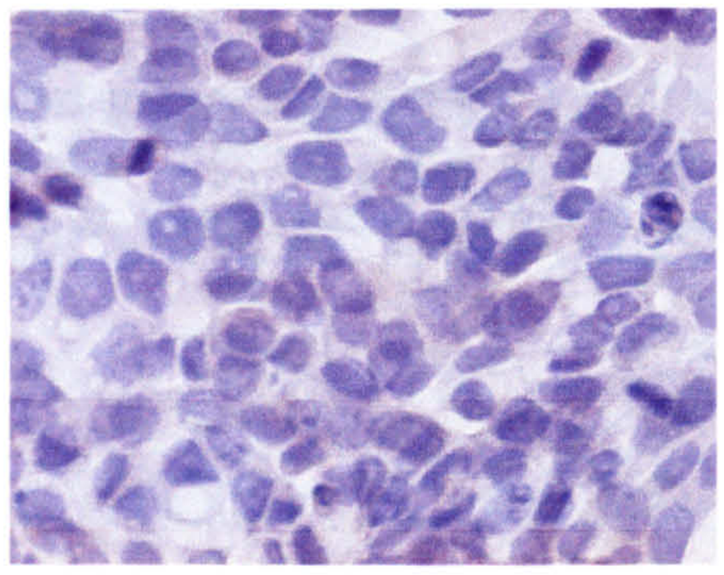


Figure 7.2. CD44 immunoreactivity in the oesophageal cell-line OE33 treated with: A. Nothing day one control, **B.** pH 5 day one, **C.** DCA day one, **D.** pH 5 and DCA day 2, **E.** GDC day one, **F.** pH 5 and GDC day two, **G**. pH 5 and TCA day one and **H.** pH 5 and TCDA day 2. (Magnification x40)

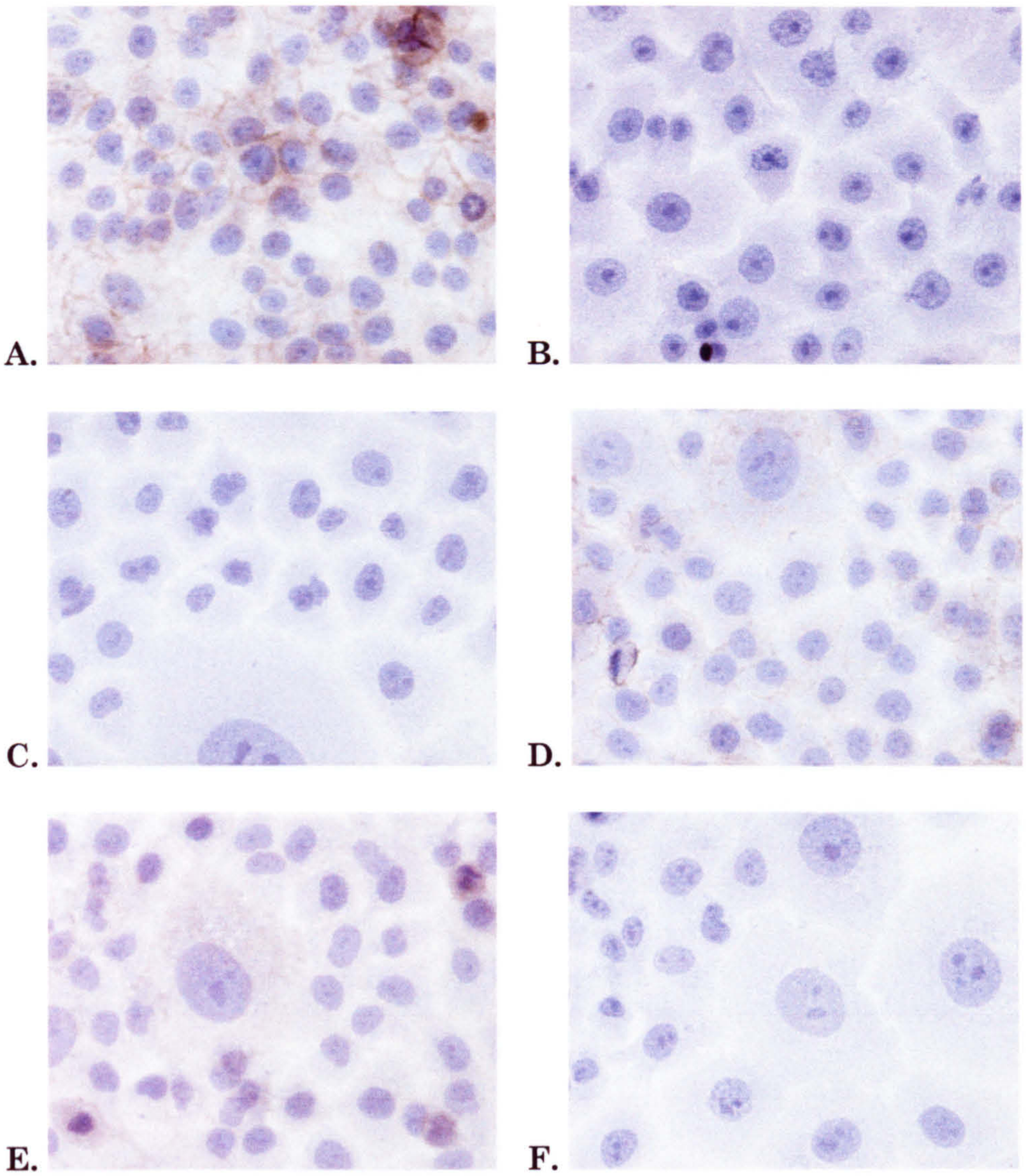


Figure 7.3. E-Cadherin immunoreactivity in the oesophageal cell-line OE21 treated with: A. nothing day two Control, **B**. pH 5 day one, **C.** pH 5 and DCA day one, **D.** DCA day one, **E.** GDCA day one, and **F.** pH 5 and GDC day one. (Magnification x40).

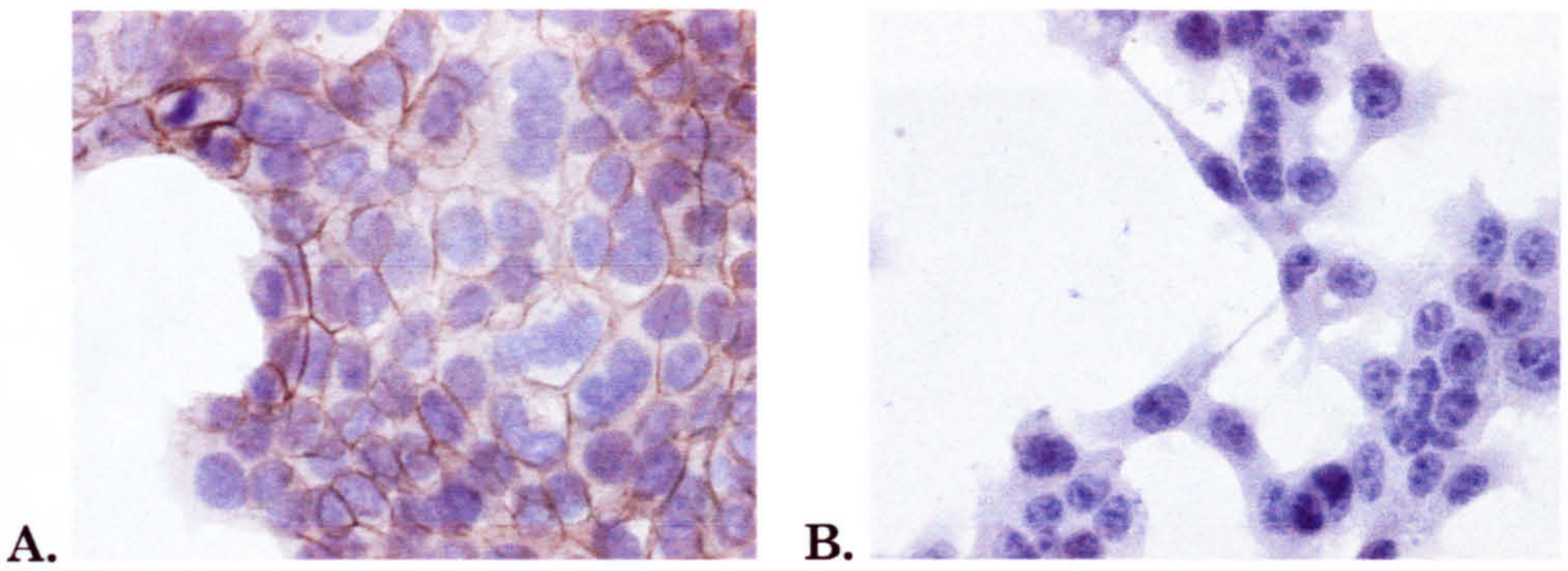
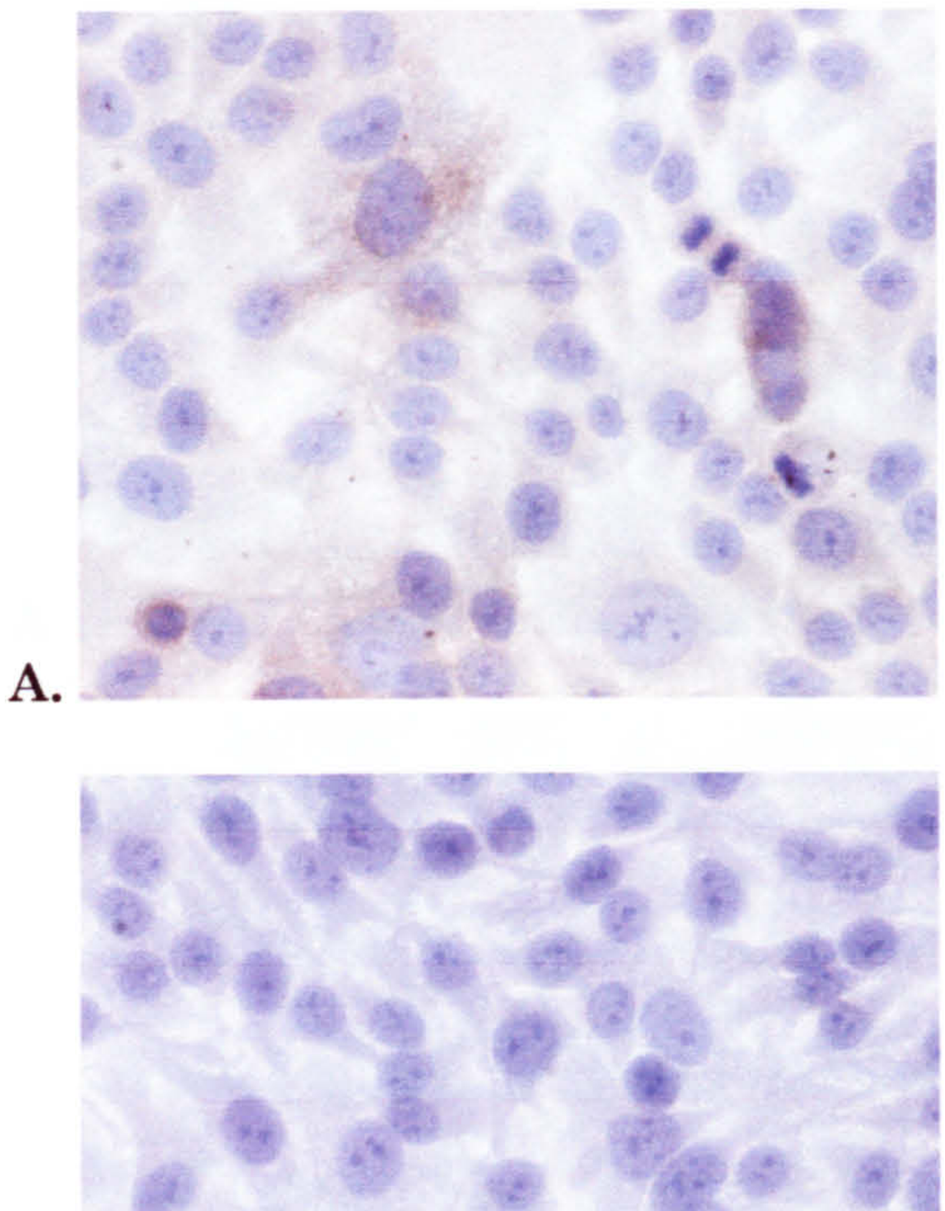
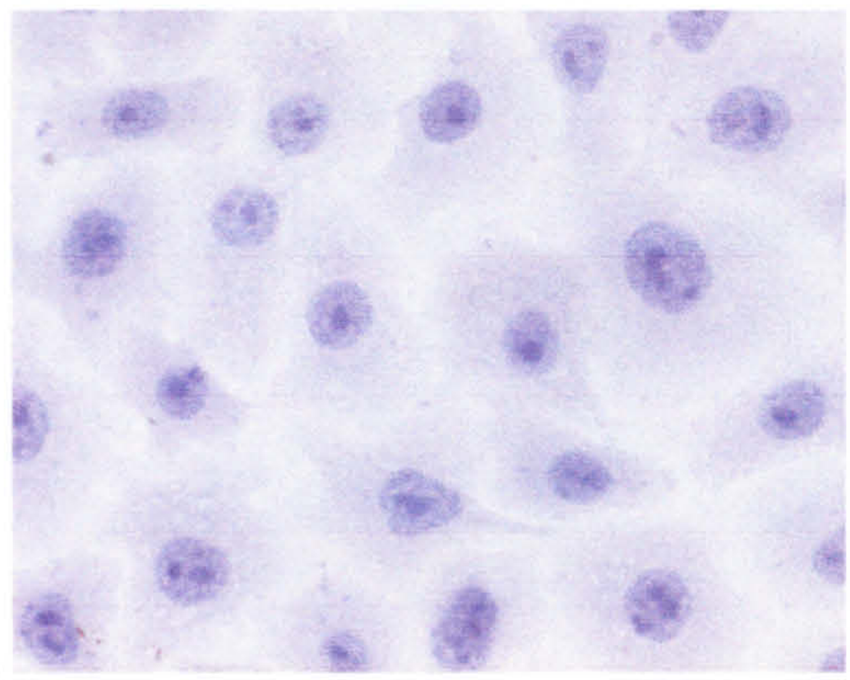
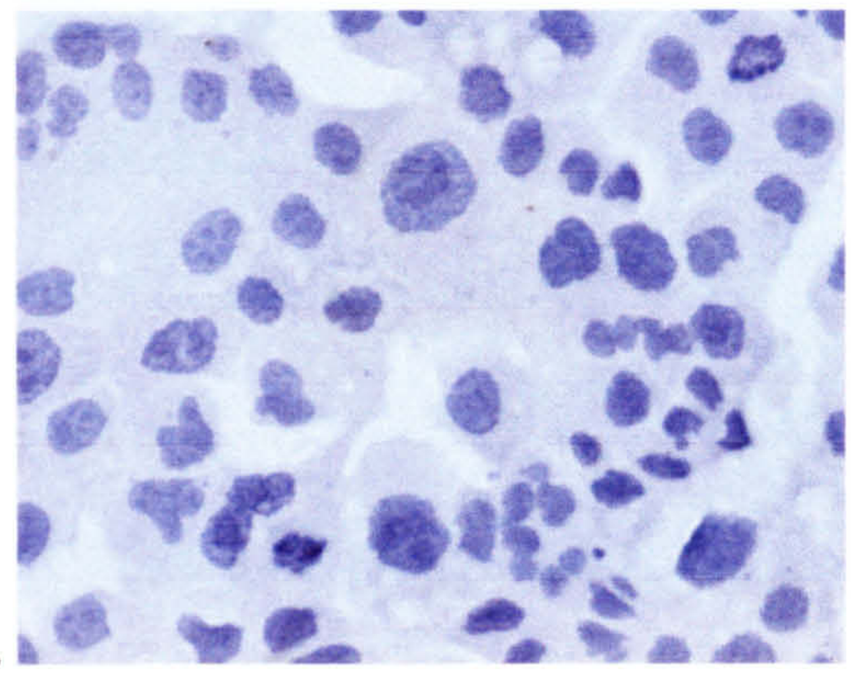


Figure 7.4. E-Cadherin immunoreactivity in the oesophageal cell-line OE33 treated with: A. nothing day one Control, **B.** pH 5 day one, **C.** TDC day one, **D.** pH 5 and TDC day one, **E**. GDC day one, **F.** pH 5 and GDC day one, **G.** TCDA day one, and **H.** pH 5 and TCDA day one. (Magnification x40)







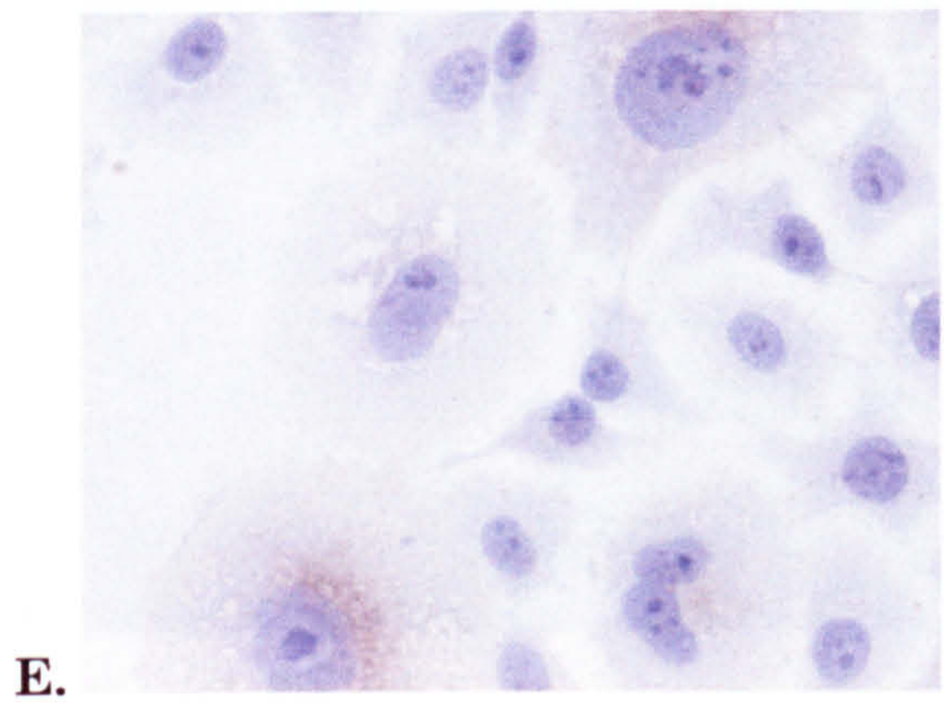
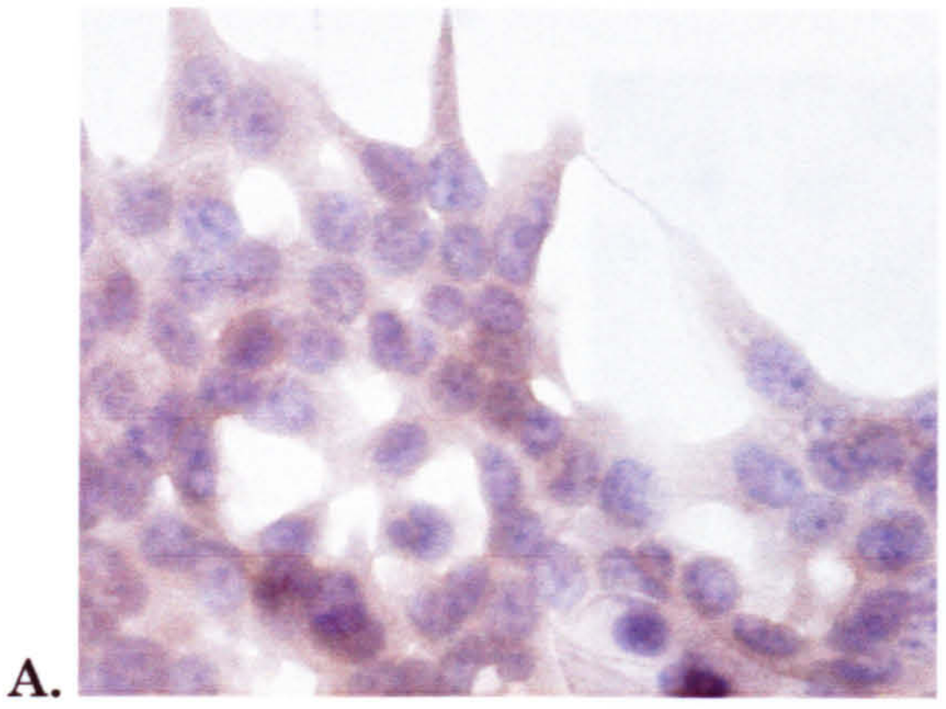
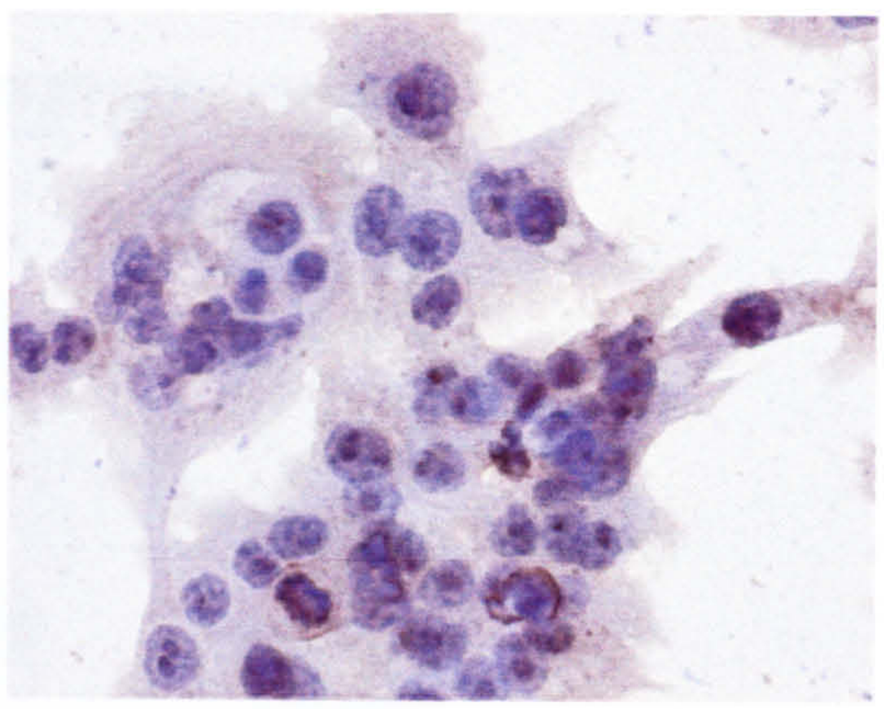
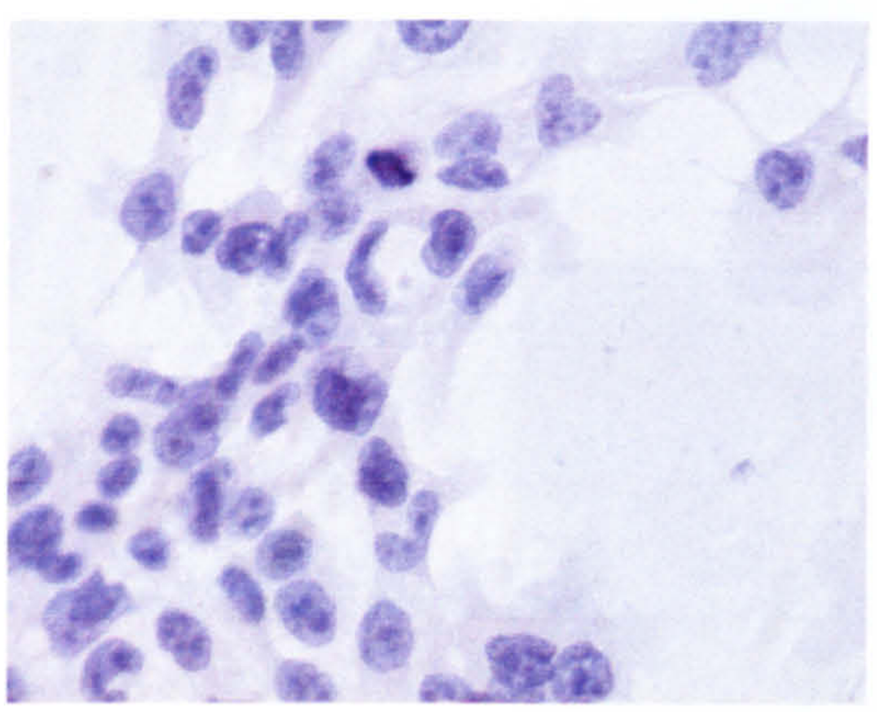
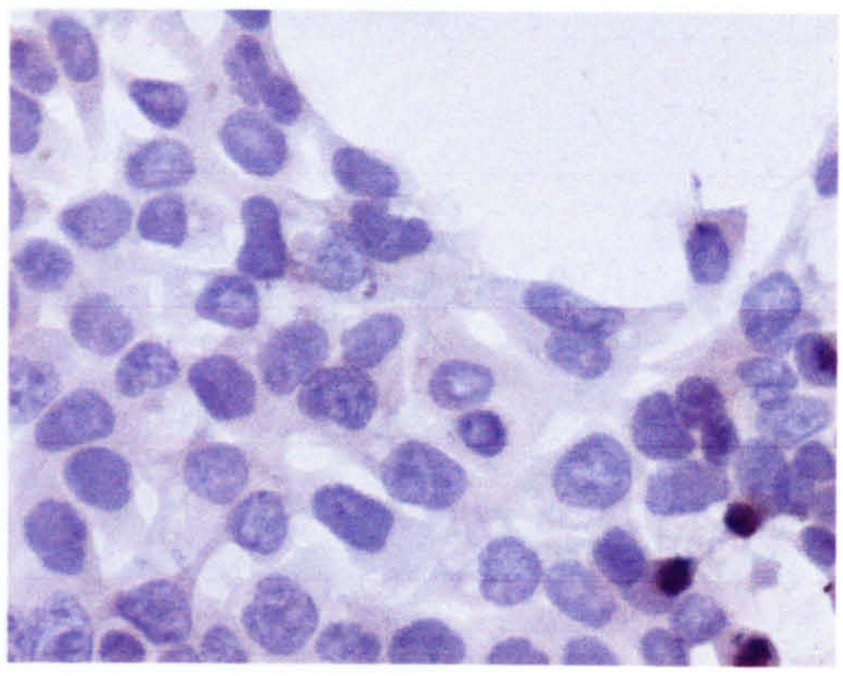


Figure 7.5. uPA immunoreactivity in the oesophageal cell-line OE21 treated with: A. Nothing day one control, **B.** pH 5 day one, **C.** TDC day two, **D.** pH 5 and DCA day two, and **E.** GCA day 2. (Magnification x40).









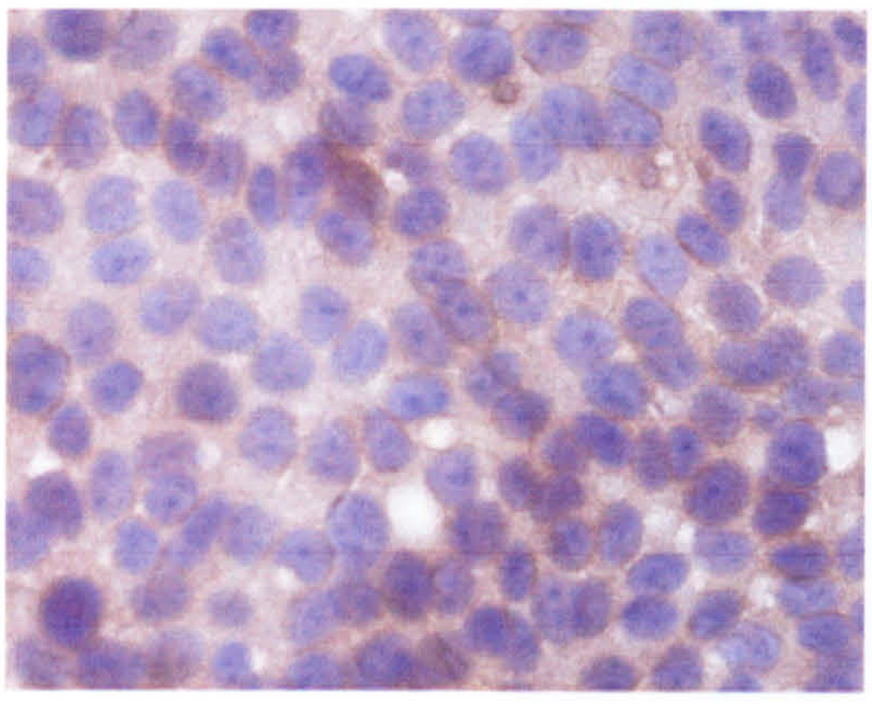
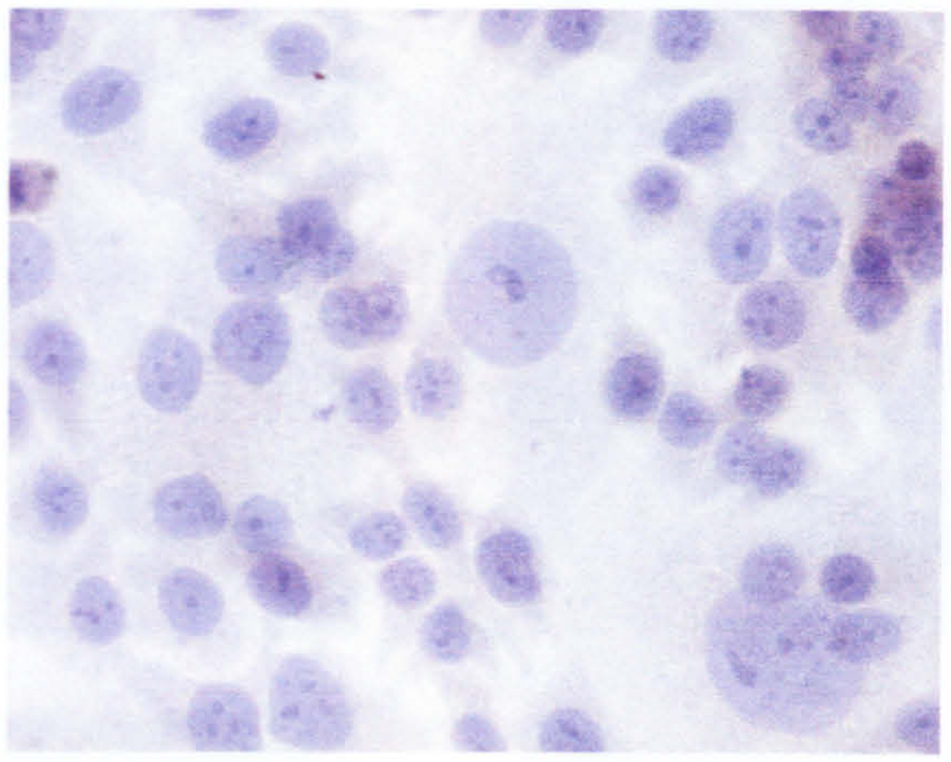
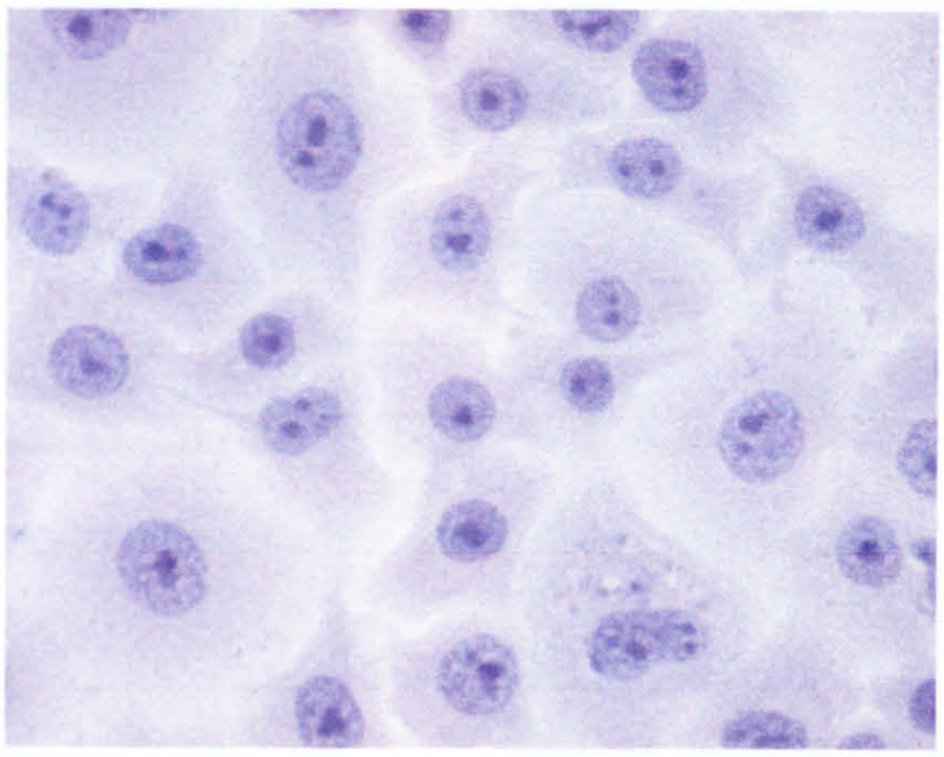


Figure 7.6. uPA immunoreactivity in the oesophageal cell-line OE33 treated with: A. nothing day one control, **B.** pH 5 day one, **C.** pH 5 and TDC day two, **D.** pH 5 and GCDA day two, and **E.** TCA day one. (Magnification x40).



A.



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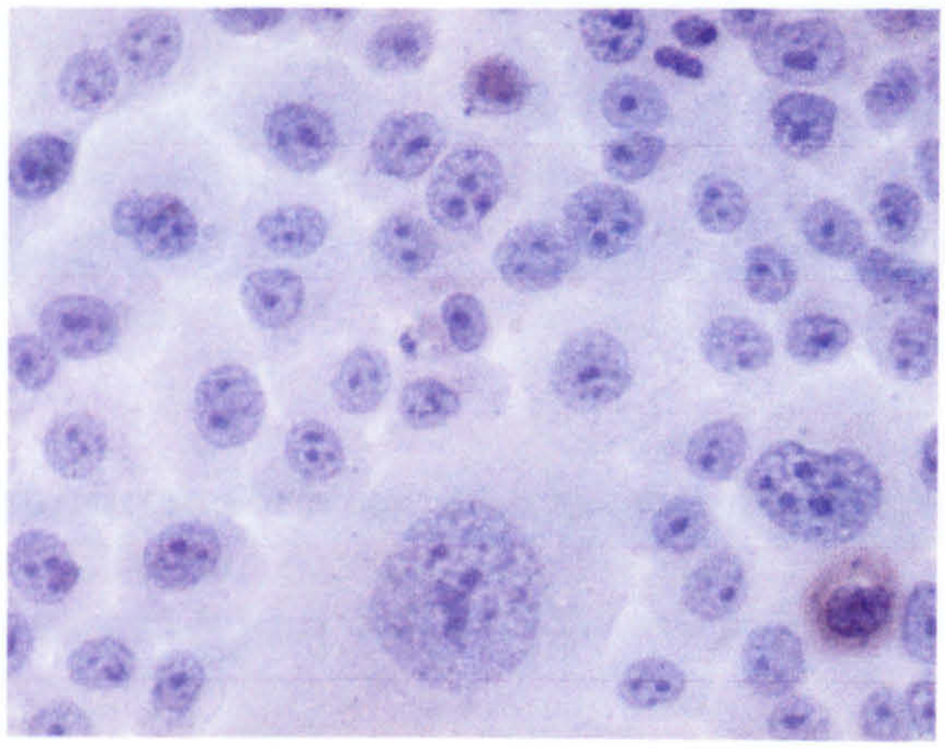


C.

Figure 7.7. uPAR immunoreactivity in the oesophageal cell-line OE21 treated with: A. Nothing day one control, **B.** pH 5 day one, and **C.** pH 5 and TDC day two. (Magnification x40).

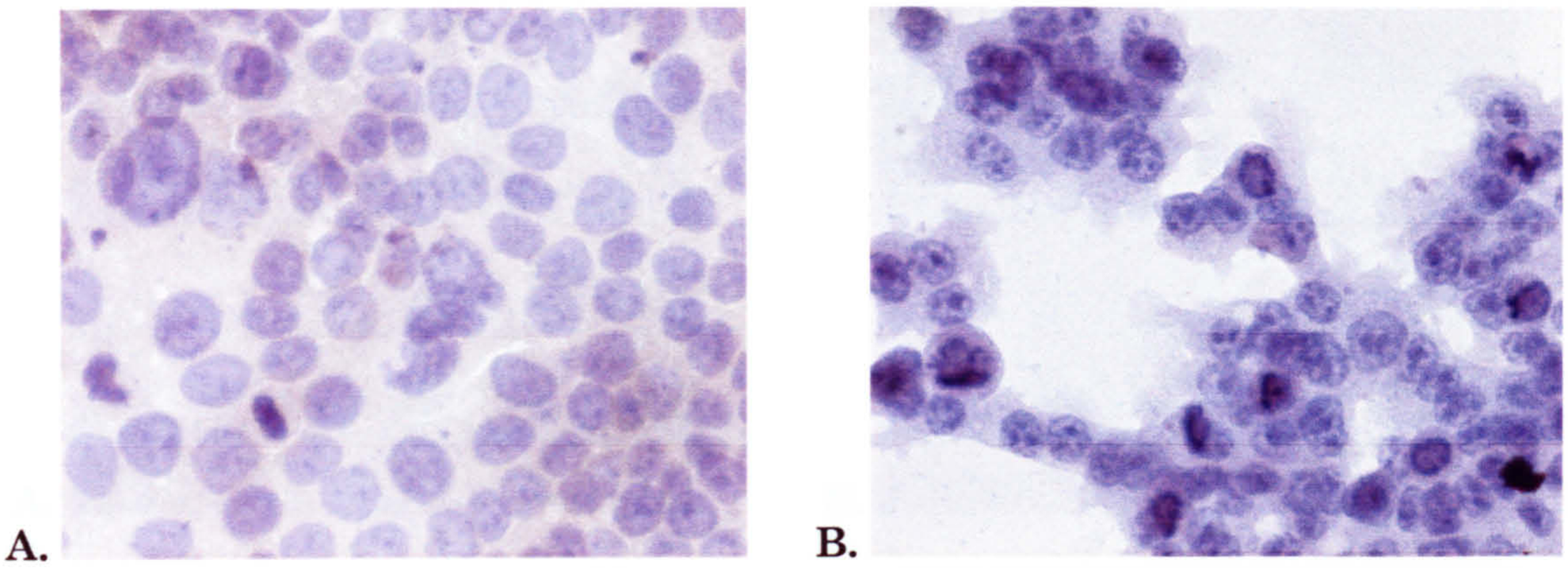


Figure 7.8. uPAR immunoreactivity in the oesophageal cell-line OE33 treated with: A. Nothing day one control, **B.** pH 5 day one, **C.** DCA day one, **D.** pH 5 and DCA day two, **E.** pH 5 and TCDA day two, and **F.** pH 5 and TDC day one. (Magnification x40)

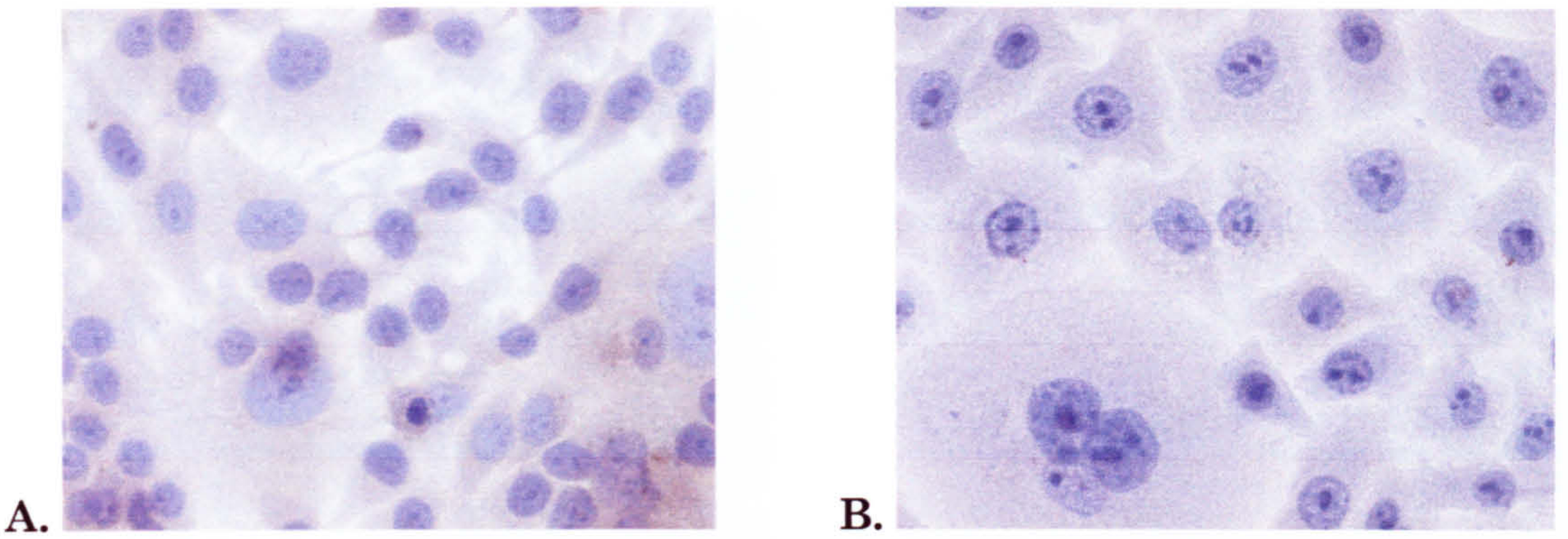


Figure 7.9. PAI-1 immunoreactivity in the oesophageal cell-line OE21 treated with: A. Nothing day one control, **B.** pH 5 day one, **C.** DCA day one, **D.** pH 5 and GCDA day two, **E.** GCA day two, **F.** pH 5 and GCA day one, **G.** pH 5 and TCA day 2, and **H.** pH 5 and TDCA day one. (Magnification x40).

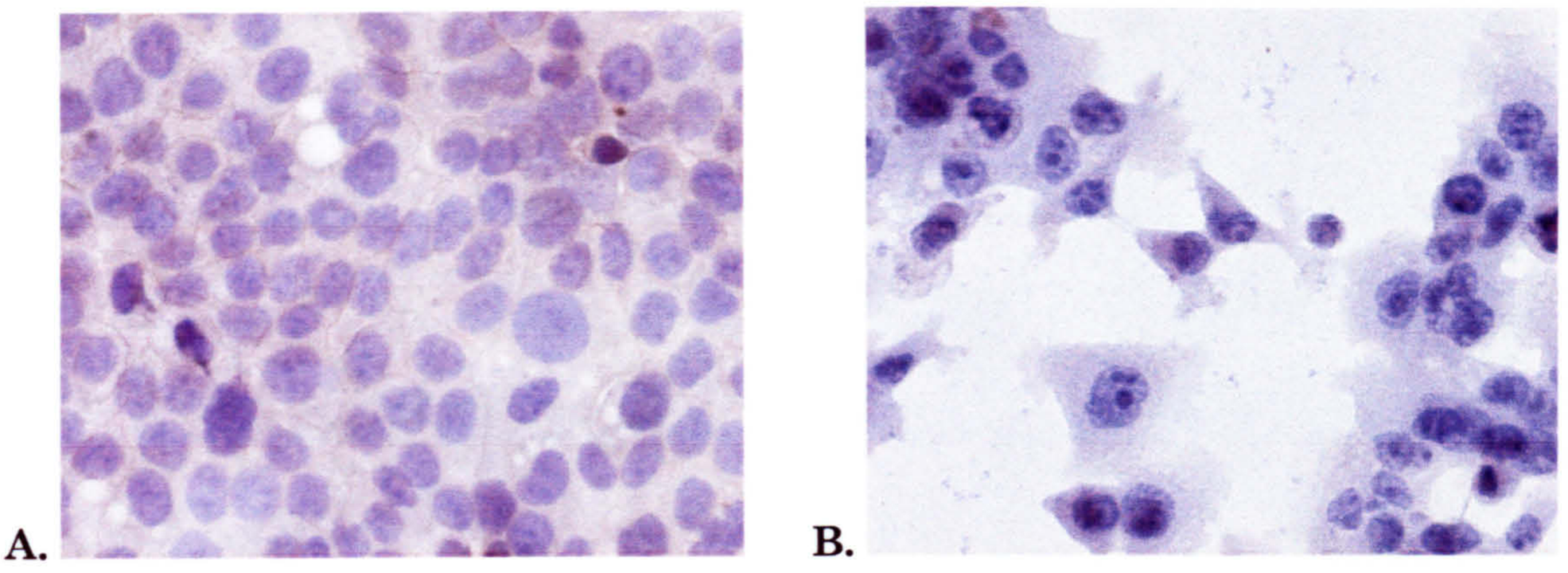
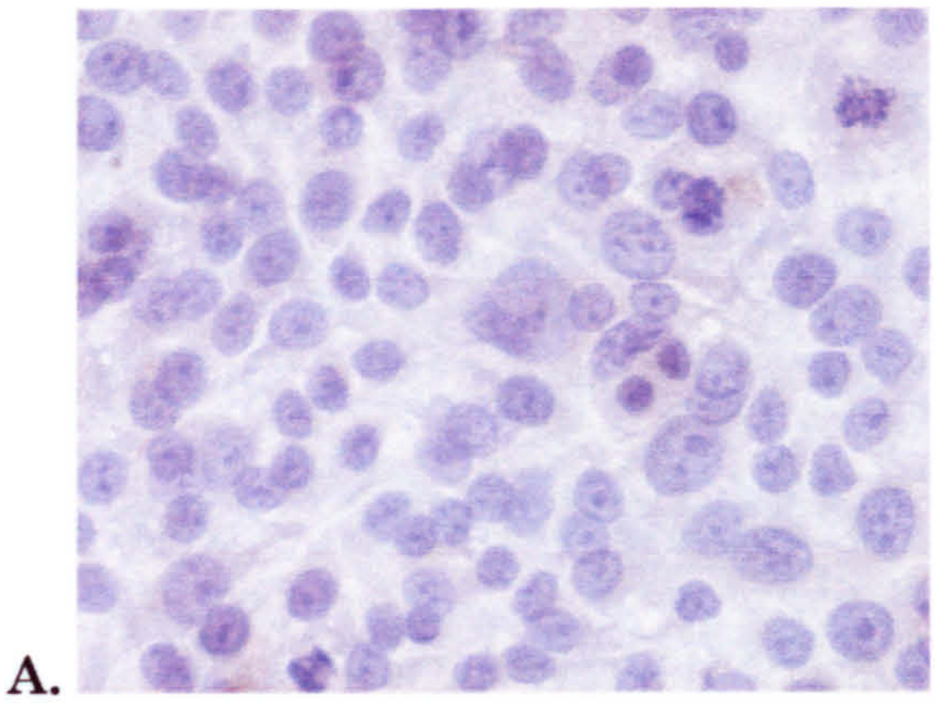
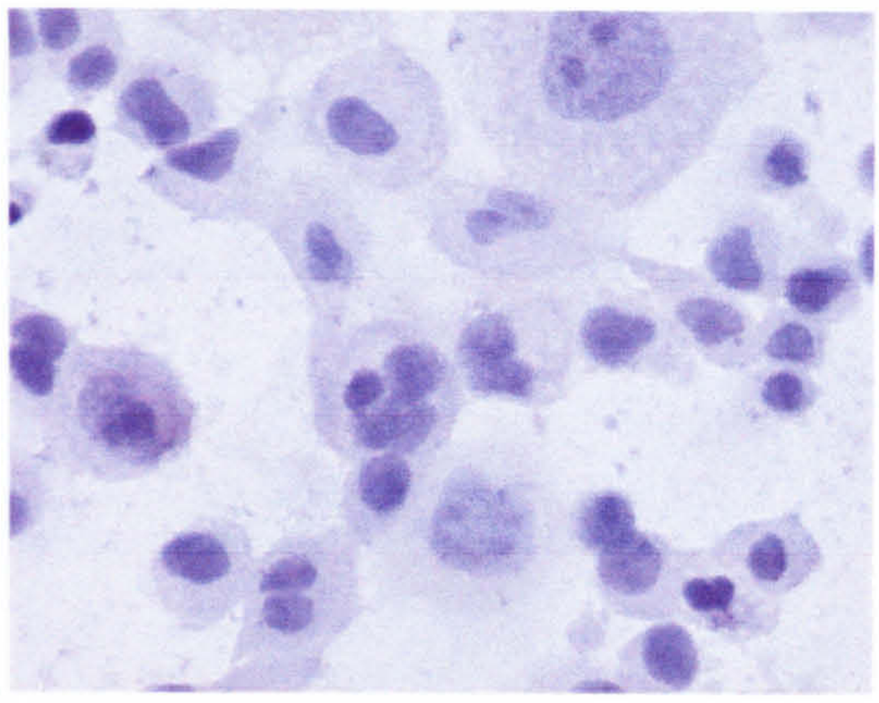
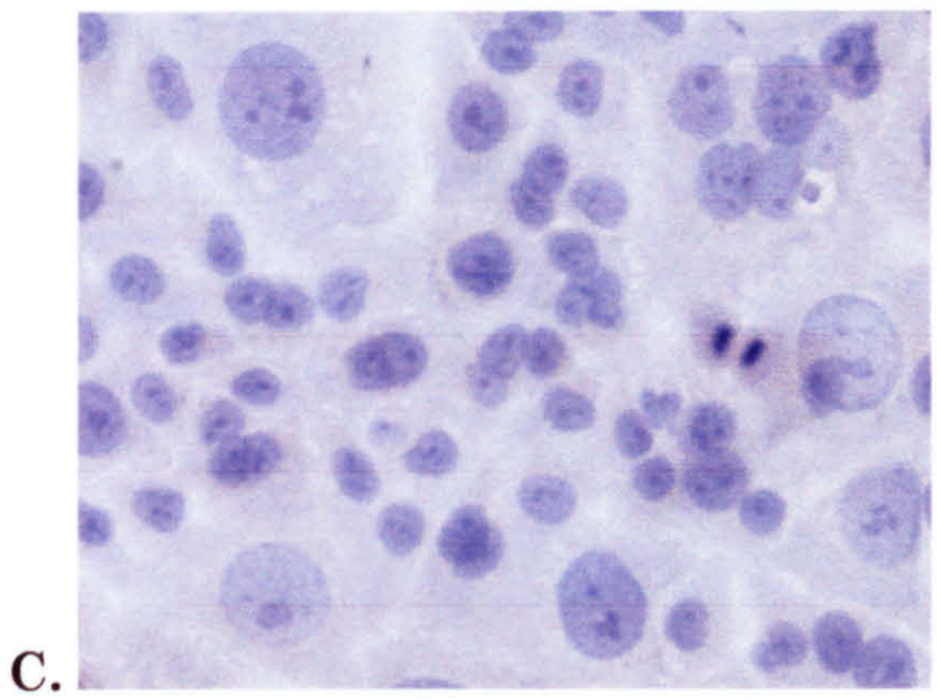
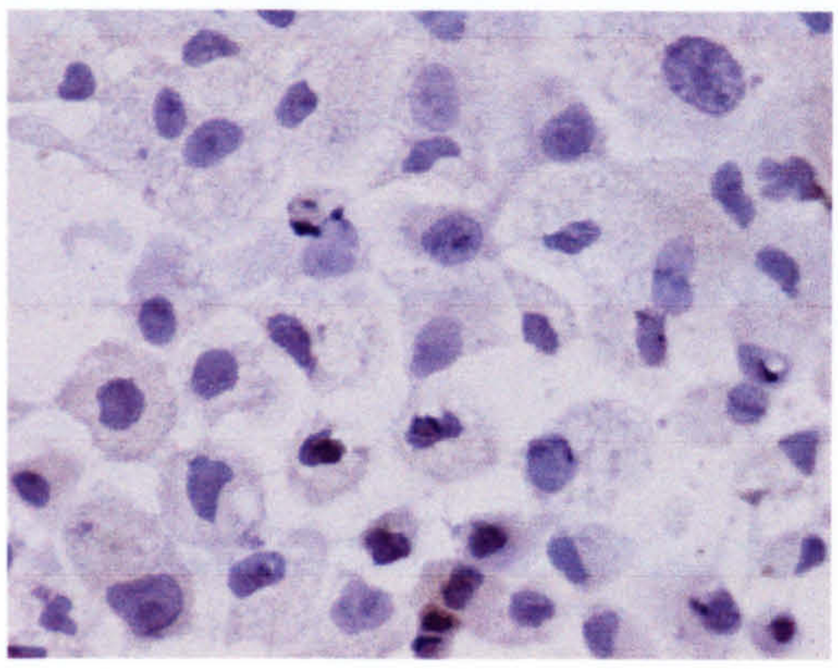


Figure 7.10. PAI-1 immunoreactivity in the oesophageal cell-line OE33 treated with: A. Nothing day two control, **B**. pH 5 day one, **C.** DCA day one, **D.** pH 5 and GCDA day two, **E**. pH 5 and TCDA day one, and **F.** pH 5 and TDC day two. (Magnification x40).









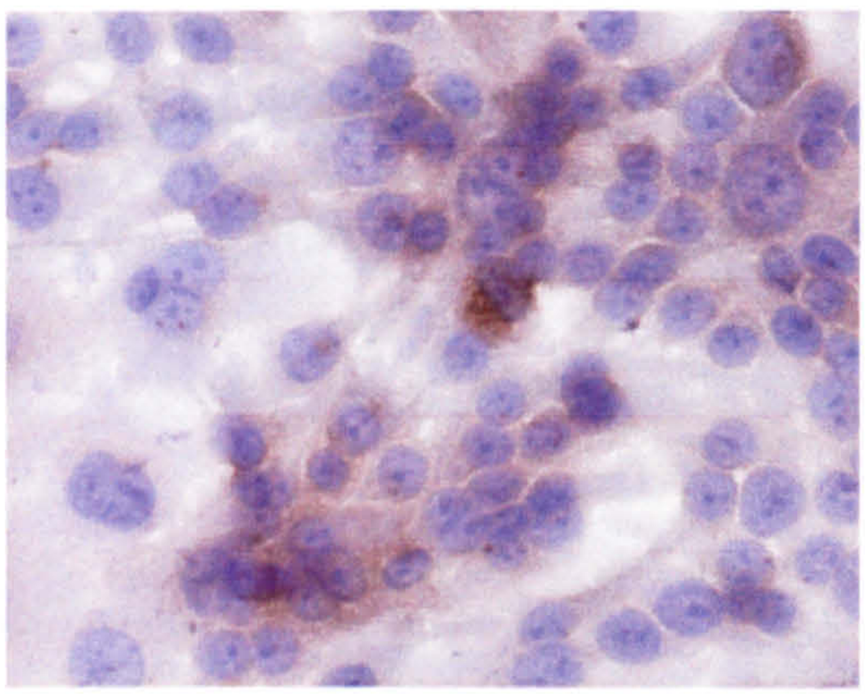


Figure 7.11. PAI-2 immunoreactivity in the oesophageal cell-line OE21 treated with: A. Nothing day 2 control, **B.** pH 5 and GDC day two, **C.** TCA day one, **D.** pH 5 and TCA day one, and **E.** TDC day two. (Magnification x40)

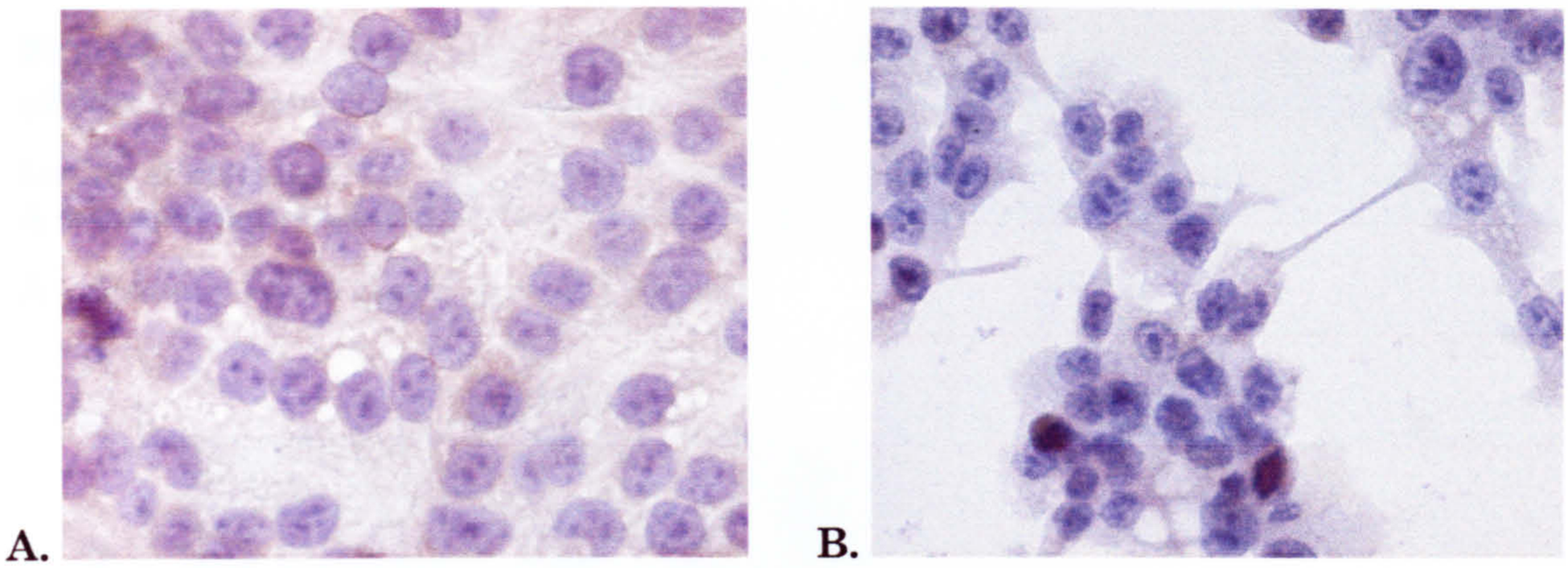
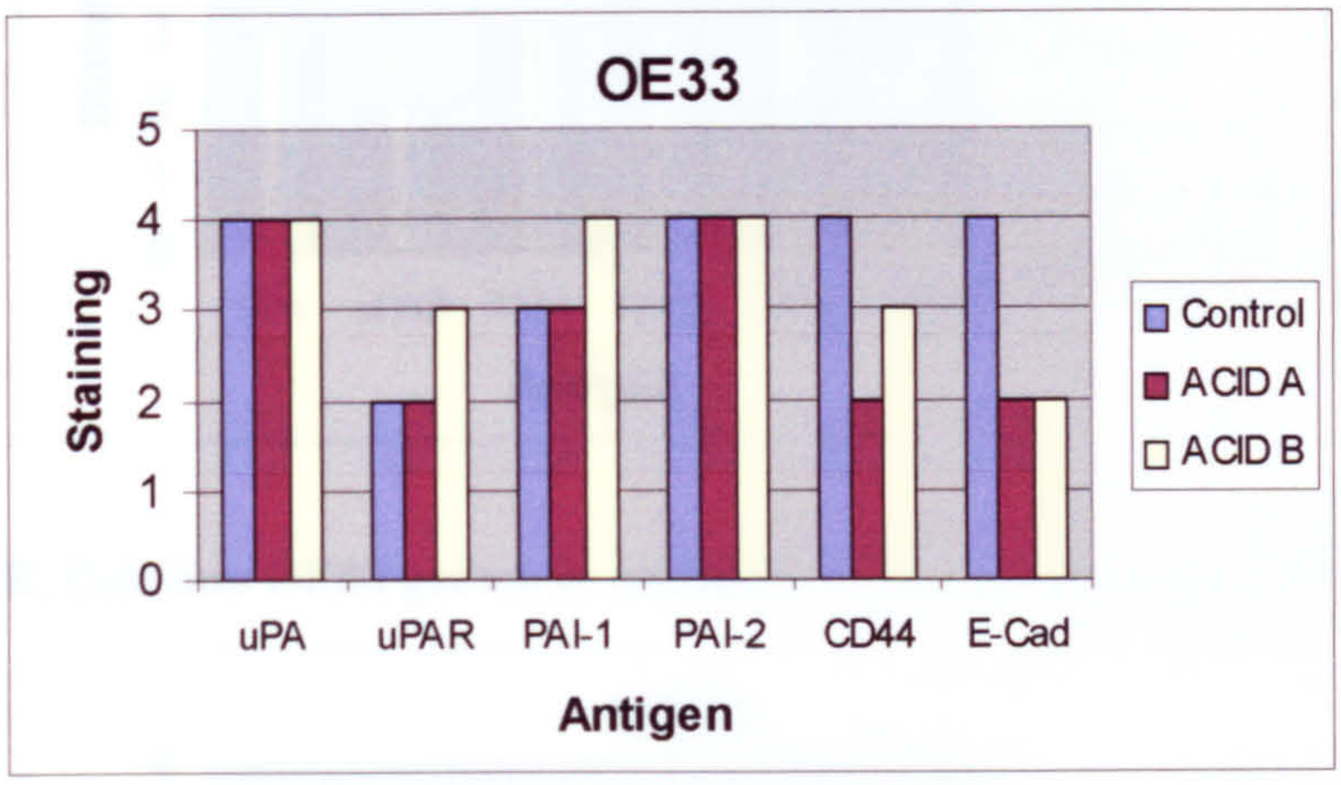


Figure 7.12. PAI-2 immunoreactivity in the oesophageal cell-line OE33 treated with: A. Nothing day one control, **B.** pH 5 day one, **C.** TCA day one, and **D.** pH 5 and DCA day two. (Magnification x40).

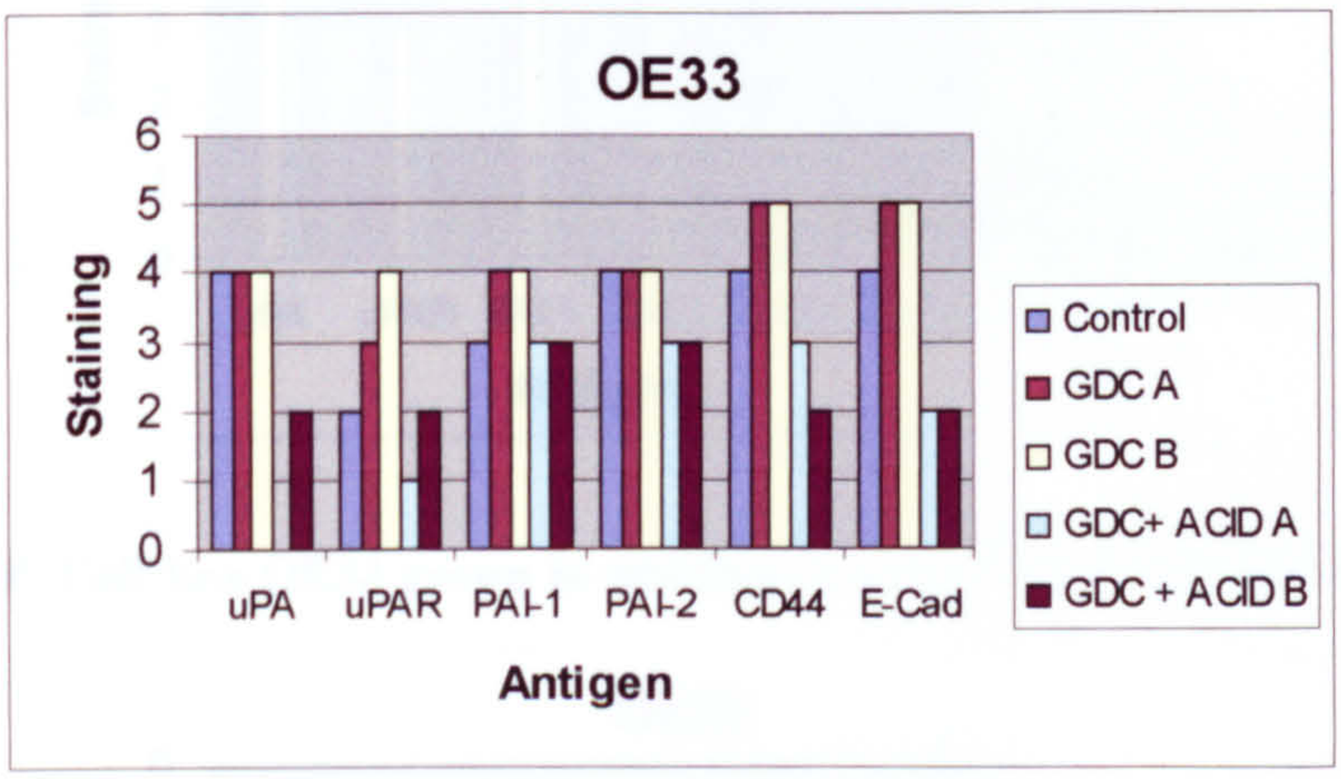
Figure 7.13. Graphical representation of immunohistochemistry results of the cell-line OE33 treated to bile salts and acidic growth media.

Levels of staining: 1 = negative, 2 = weak, 3 = moderate, 4 = strong and 5 = very strong. A = initial exposure and B = one days growth after exposure.

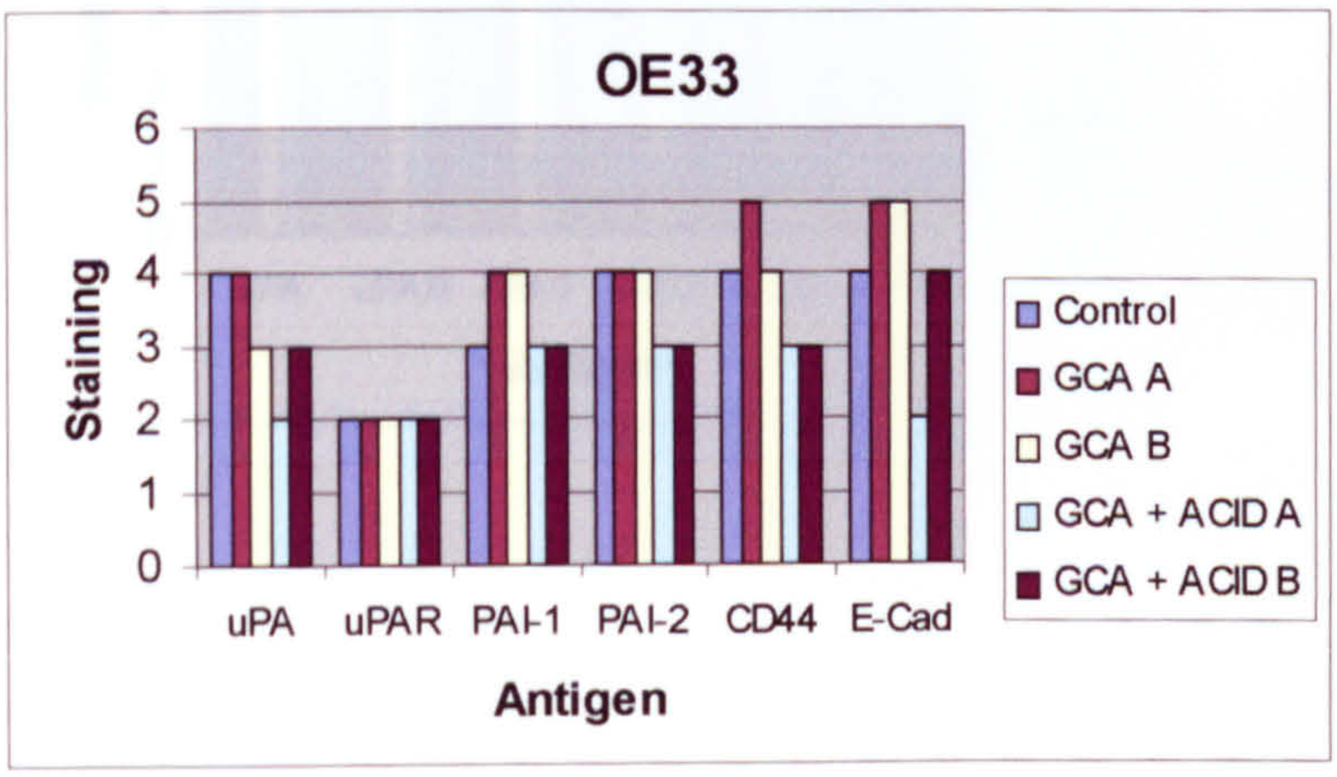
A. Cell-line OE33 grown in acidic media.



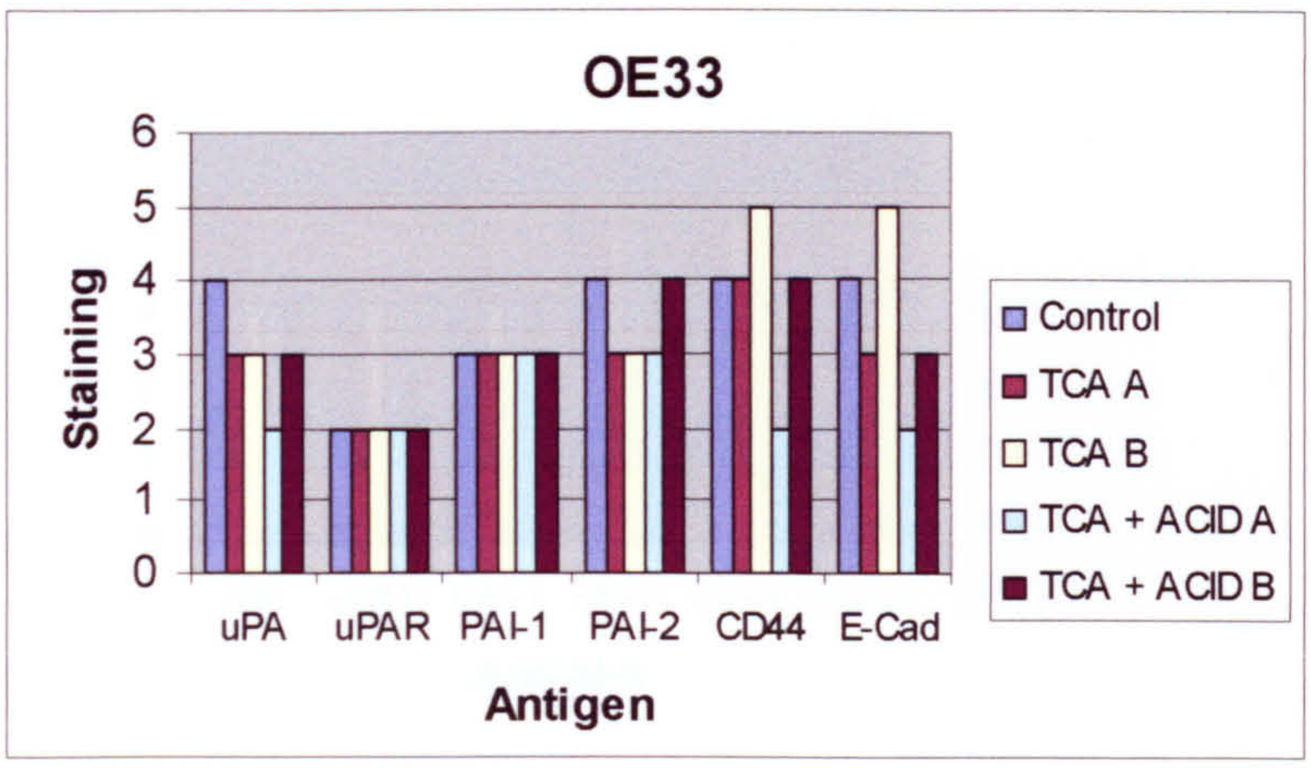
B. Cell-line OE33 grown in acidified media and treated with glycodeoxycholic acid.



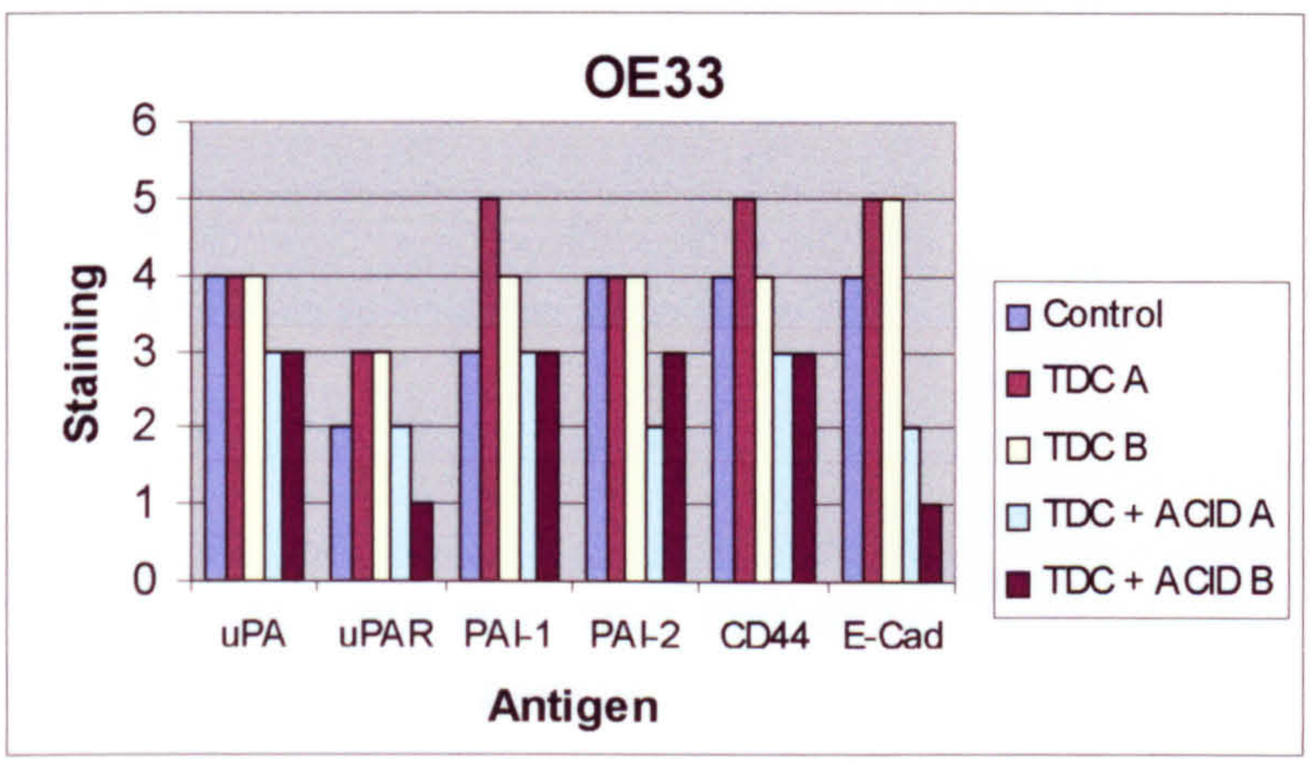
C. Cell-line OE33 grown in acidified media and treated with glycocholic acid.



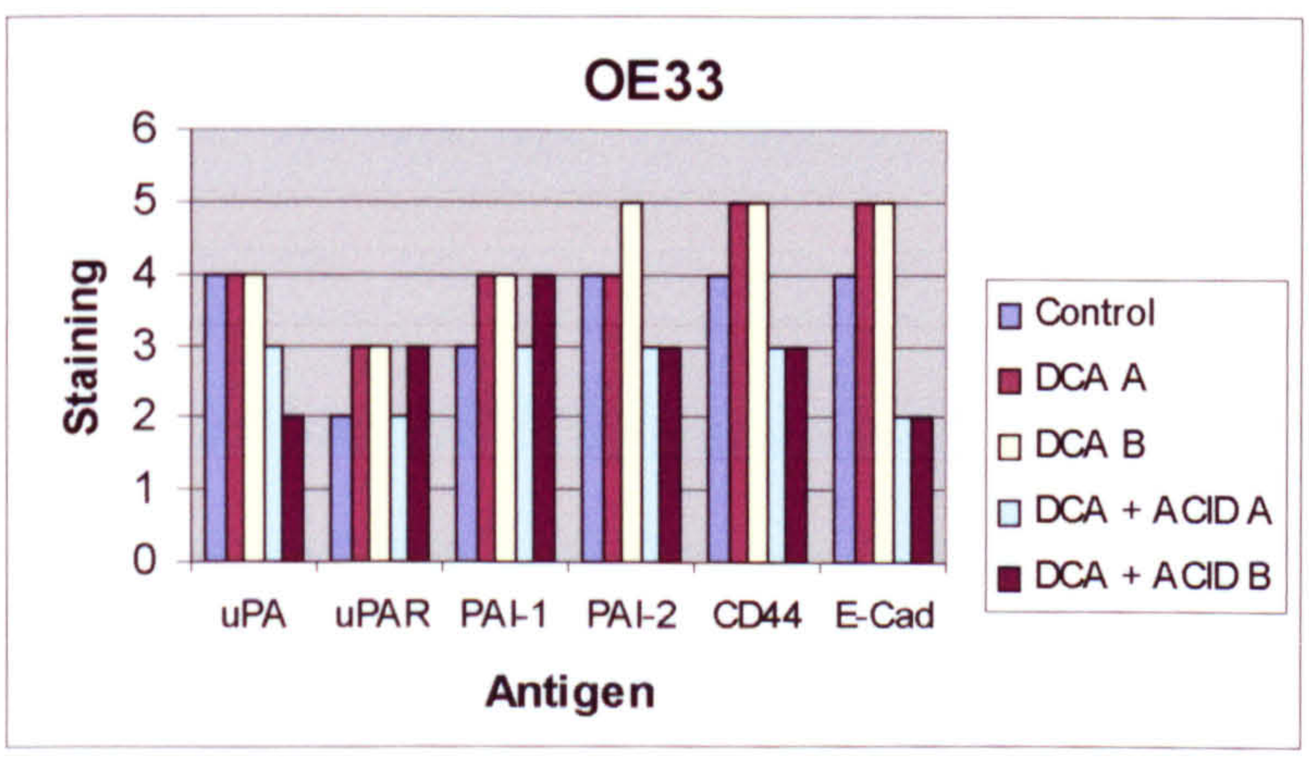
D. Cell-line OE33 grown in acidified media and treated with taurocholic acid.



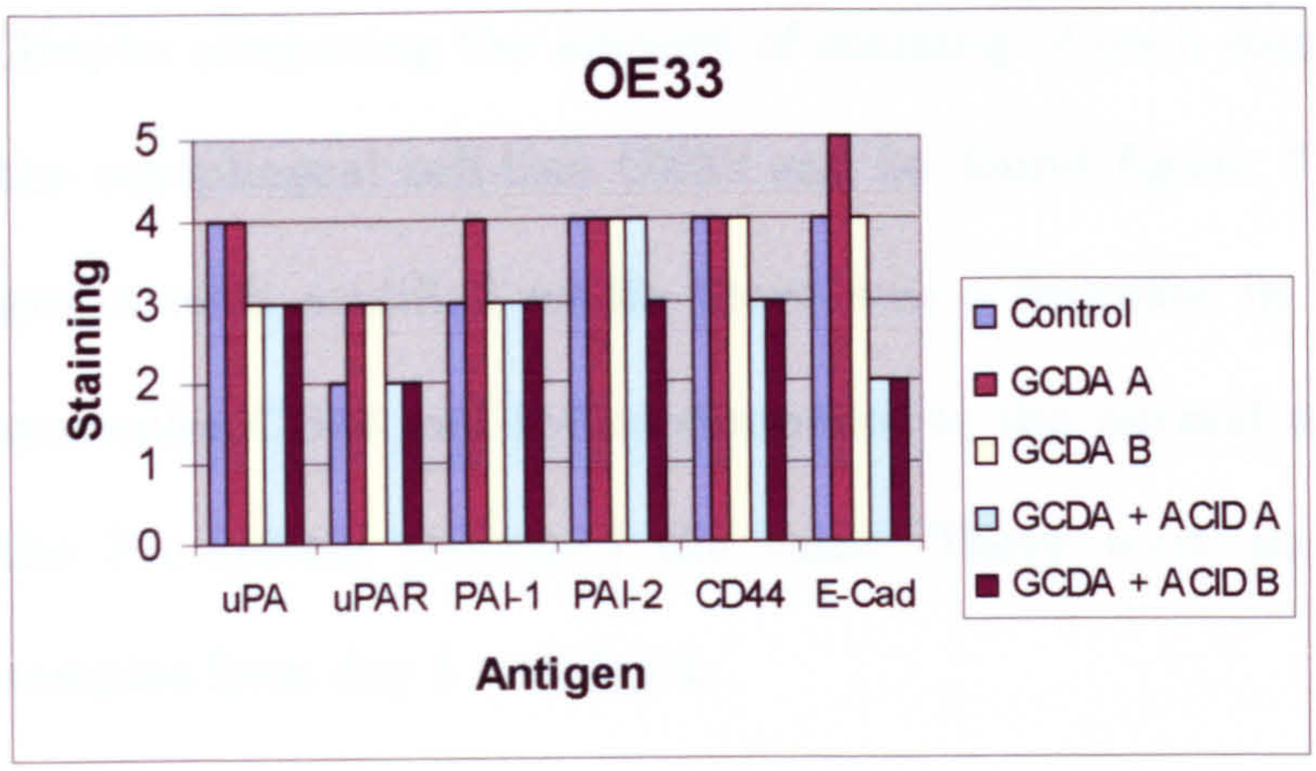
E. Cell-line OE33 grown in acidified media and treated with taurodeoxycholic acid.



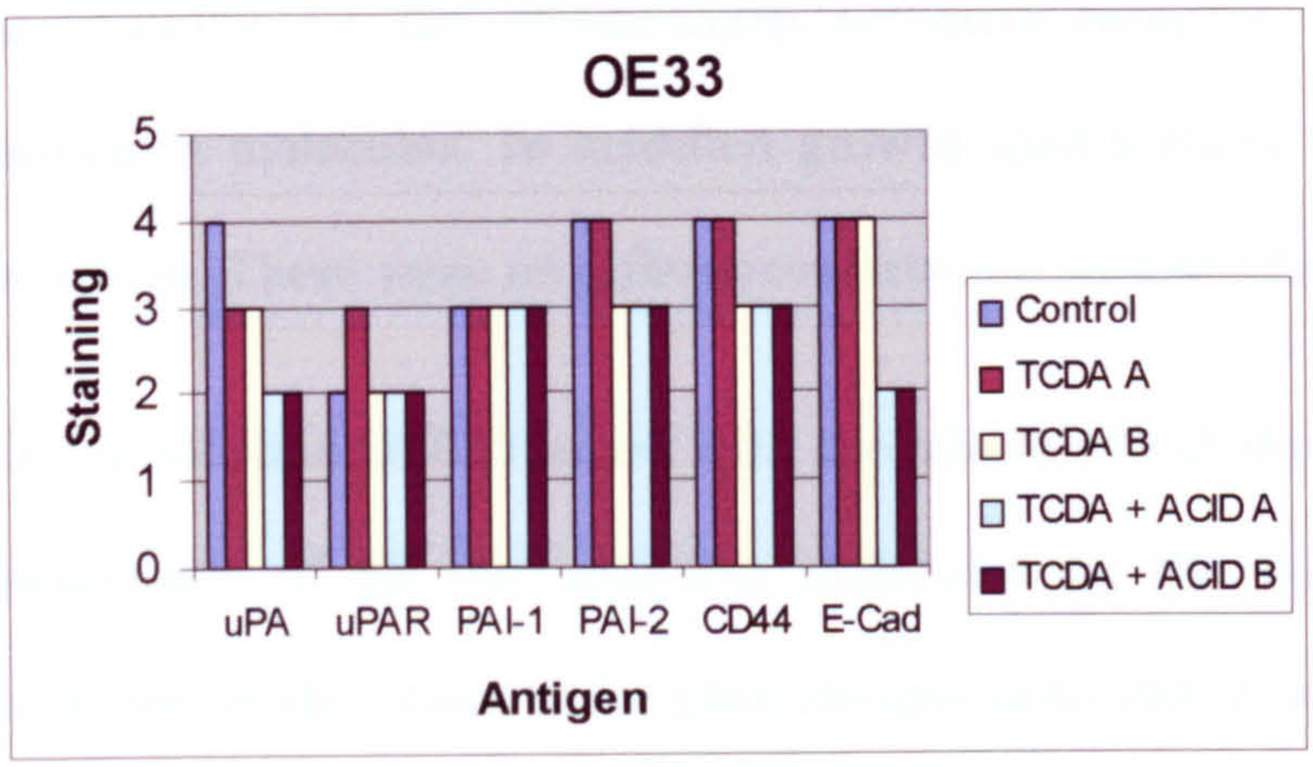
F. Cell-line OE33 grown in acidified media and treated with deoxycholic acid.



G. Cell-line OE33 grown in acidified media and treated with glycochenodeoxycholic acid.



H. Cell-line OE33 grown in acidified media and treated with taurochenodeoxycholic acid.



Graphs comparing the amount of staining of each component with treatment in the oesophageal cell-line OE33 can be found figure 7.13. In the cell-line OE33 grown with acidified media there was a decrease in staining for the adhesion molecules CD44 and E-Cad compared to the normal control. The components of the PA system remained the same. There were no real differences between samples from day 1 and day 2.

In the cell-line OE33 grown treated with the bile salt GDC there was an increase in staining for the plasminogen activator receptor, inhibitor type-1 and the adhesion molecules. In acidified growth media there was a decrease in all the markers. There were no differences between samples from day 1 and day 2.

In the cell-line OE33 treated with the bile salt DCA there was a slight increase in expression of all the adhesion molecules on the second day. There was an increase in the staining for plasminogen activator receptor, inhibitor type-1 and the adhesion molecules on exposure to the bile salt alone. There was a decrease in the expression of all the components in acidic growth media. There were no changes between samples from day 1 and day 2 when treated with acidified growth media.

In the cell-line OE33 treated with the bile salt GCA alone there were no significant changes in any of the molecules. There was a decrease in all the molecules when grown in acidified growth media. There were no changes between samples from day 1 and day 2.

In the cell-line OE33 treated with the bile salt TCA there were no differences in the components of the plasminogen activator system even in acidified growth

media. There was decreased staining for the adhesion molecules CD44 and E-cad in acidified media. When exposed to bile alone there was an increase in staining for CD44 and E-cad in samples from day two.

In the cell-line OE33 treated with the bile salt TDC there was an increase in the expression of the adhesion molecules, plasminogen activator receptor and inhibitor type-1. The levels of staining for all the molecules decreased when grown in acidified growth media. There was a decrease in staining for the plasminogen activator inhibitors and the adhesion molecules on day 2.

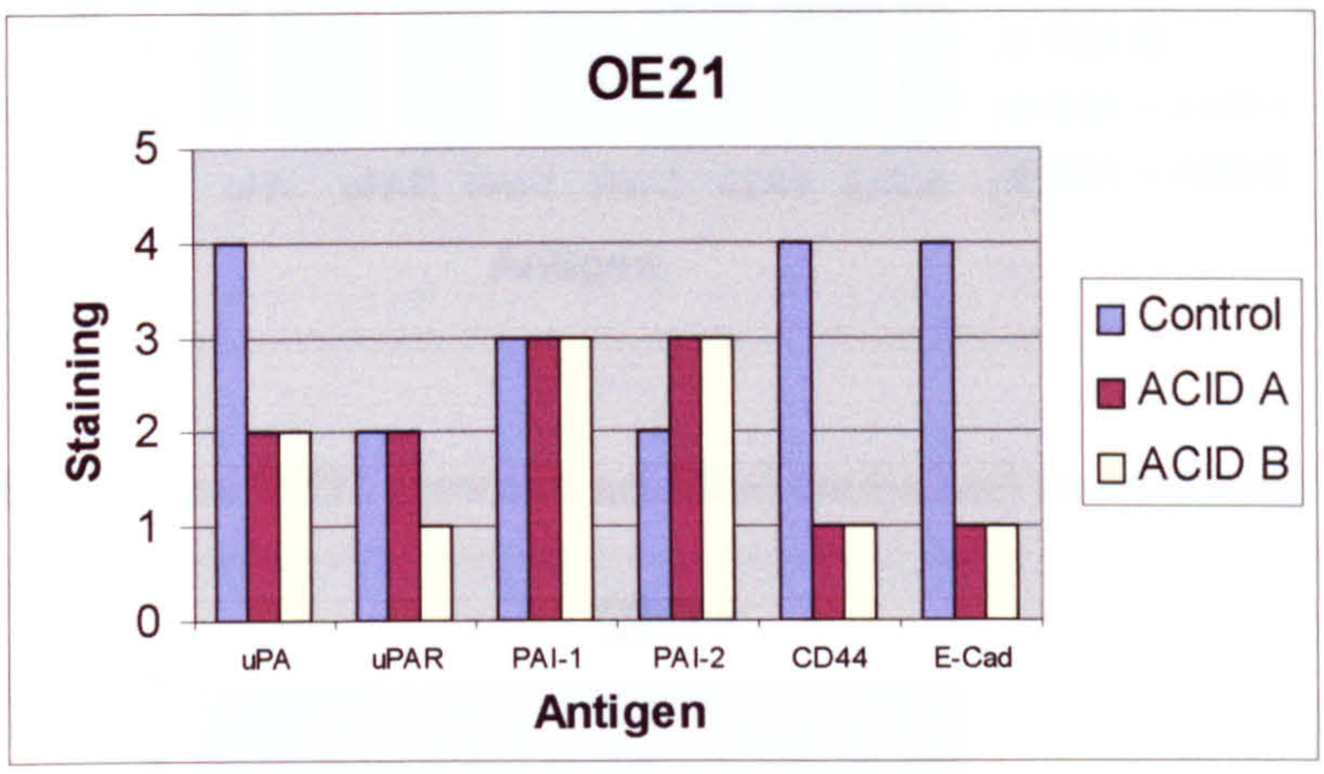
In the cell-line OE33 treated with the bile salt GCDA there was an increase in staining for plasminogen activator receptor, inhibitor type-1 and E-cad. There was a decrease in staining for CD44 and E-cad in acidified growth media. There were no changes between samples from day 1 and day 2.

In the cell-line OE33 treated with the bile salt TCDA there was a decrease in staining in all the samples except plasminogen activator inhibitor type-1 from the second day. There was a decrease in staining for plasminogen activator, CD44 and E-cad in acidified growth media.

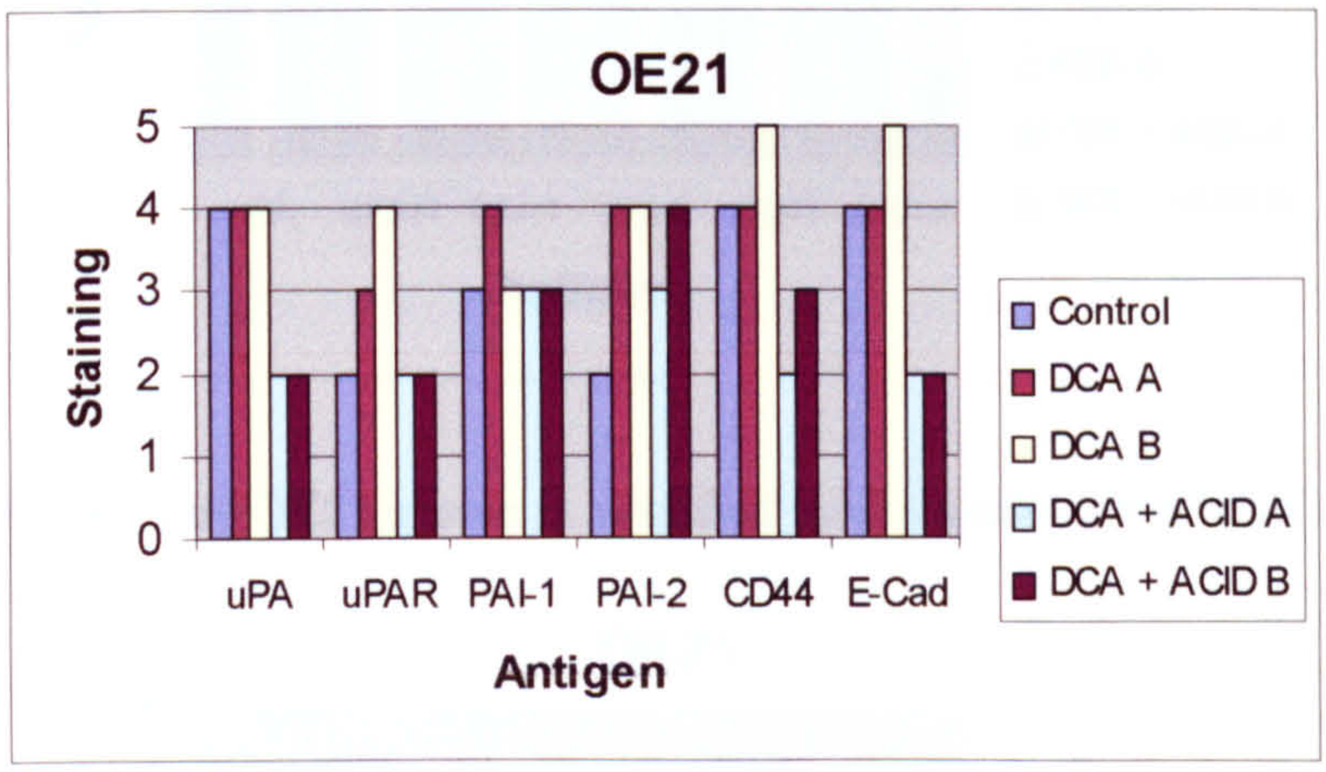
Figure 7.14. Graphical representation of immunohistochemistry results of the cell-line OE21 treated to bile salts and acidic growth media.

Levels of staining: 1 = negative, 2 = weak, 3 = moderate, 4 = strong and 5 = very strong. A = initial exposure and B = one days growth after exposure.

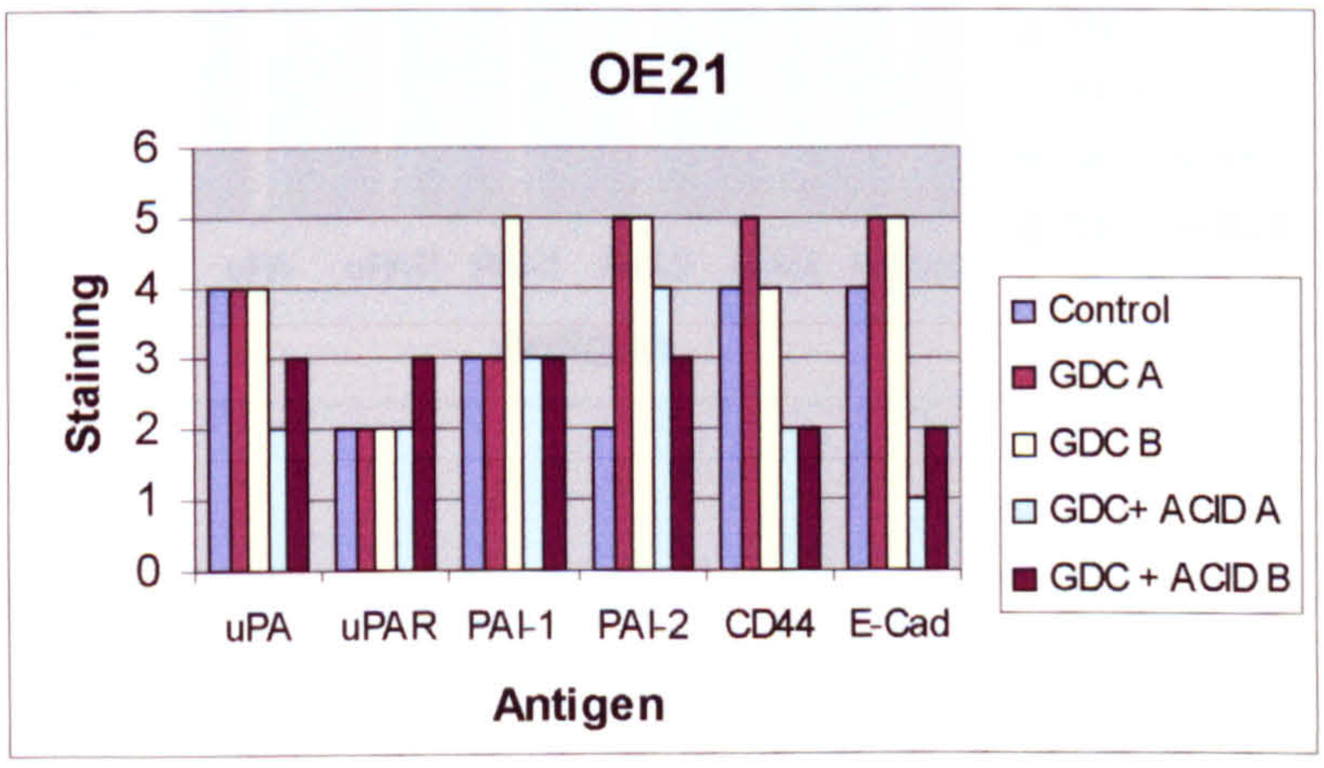
A. Cell-line OE21 grown in acidified media.



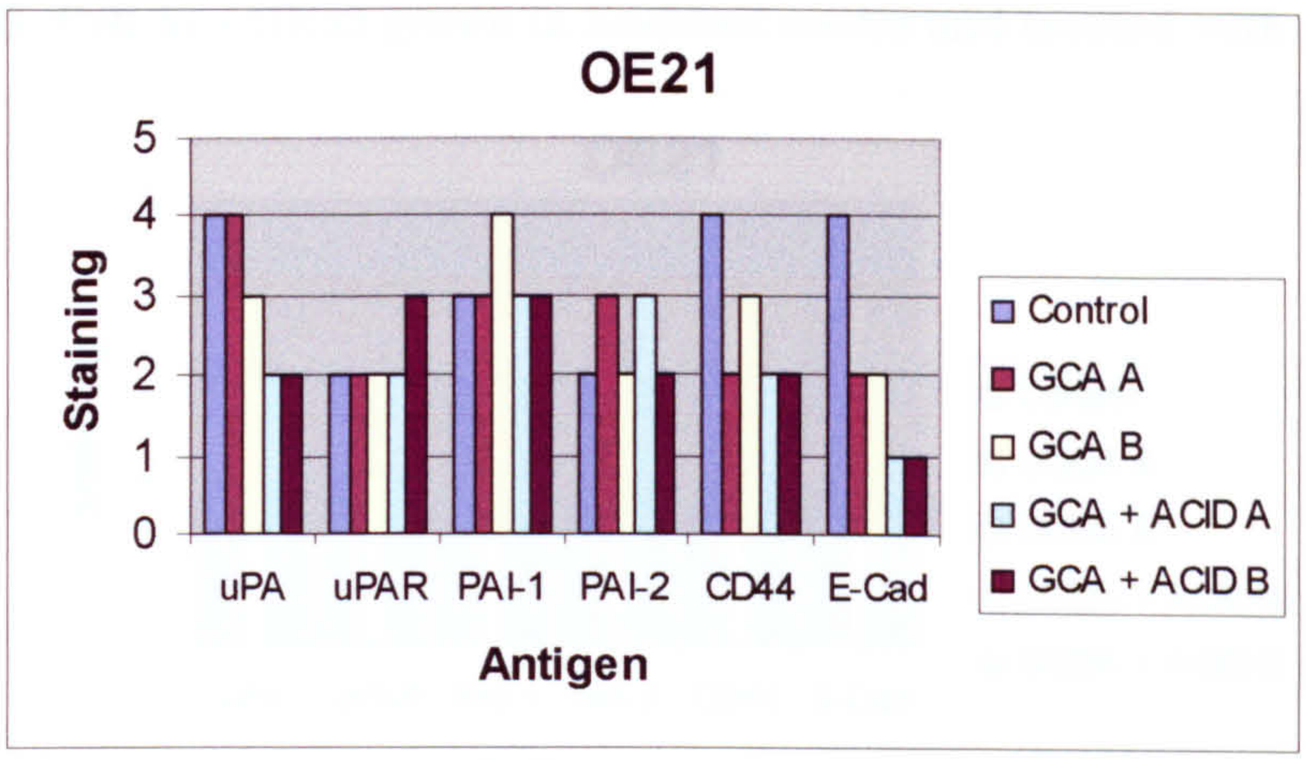
B. Cell-line OE21 grown in acidified media and treated with deoxycholic acid.



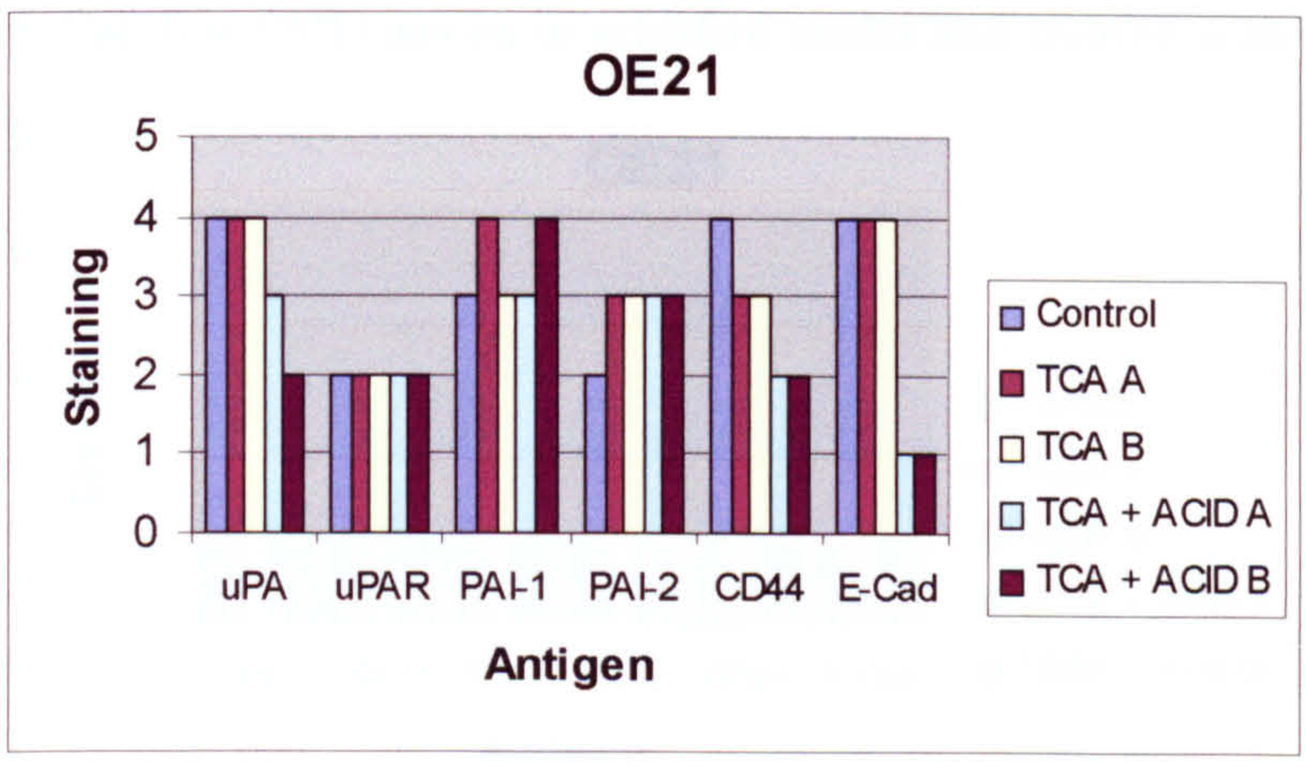
C. Cell-line OE21 grown in acidified media and treated with glycodeoxycholic acid.



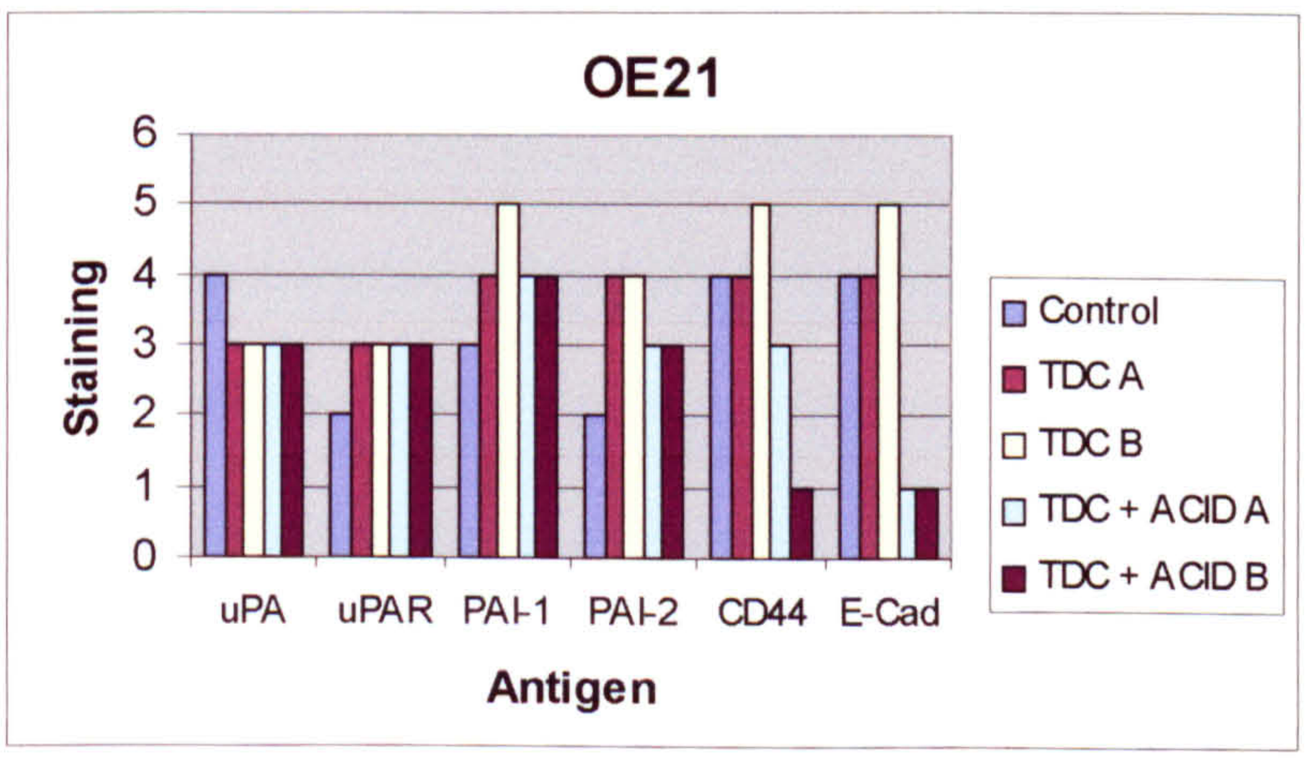
D. Cell-line OE21 grown in acidified media and treated with glycocholic acid.



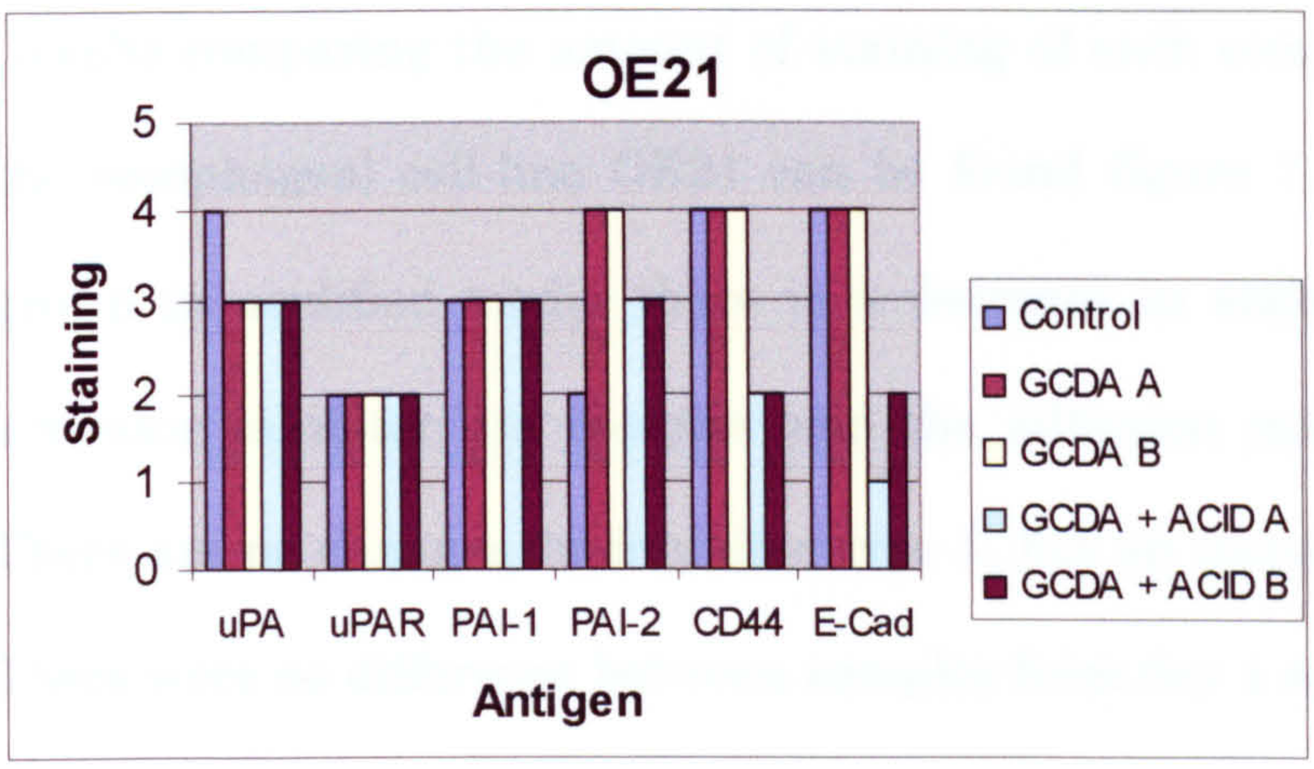
E. Cell-line OE21 grown in acidified media and treated with taurocholic acid.



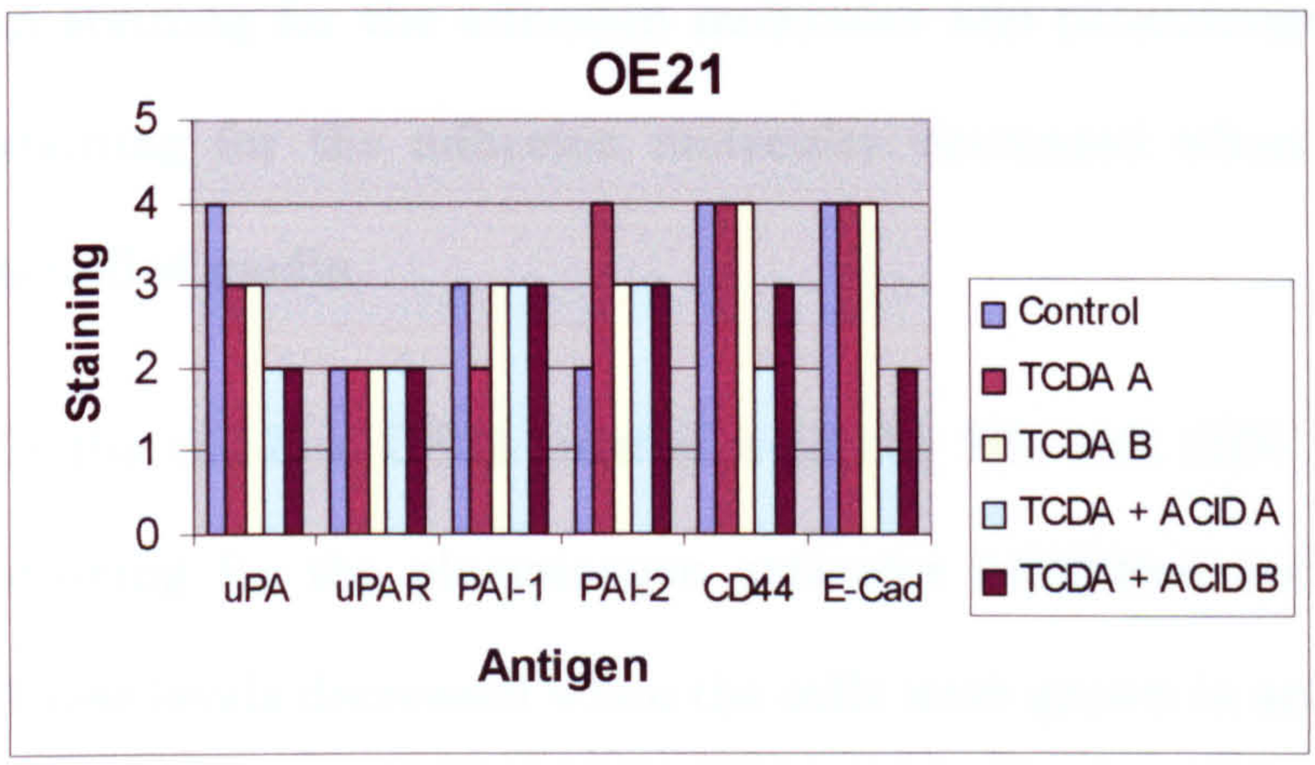
F. Cell-line OE21 grown in acidified media and treated with taurodeoxycholic acid.



G. Cell-line OE33 grown in acidified media and treated with glycochenodeoxycholic acid.



H. Cell-line OE21 grown in acidified media and treated with taurochenodeoxycholic acid.



Graphs comparing the amount of staining of each component with treatment in the oesophageal cell-line OE21 can be found figure 7.14. In the cell-line OE21 grown in acidified media there is a decrease in staining for the plasminogen activator inhibitor, its receptor and the adhesion molecules CD44 and E-cad. There are no changes for inhibitor type-1, but an increase in staining for type-2. There were no difference between samples from day 1 and day 2.

In the cell-line OE21 treated with the bile salt DCA there were was an increase in staining for the adhesion molecules and plasminogen activator receptor. The staining for the adhesion molecules decreased when the cells were grown in acidified media.

In the cell-line OE21 treated with the bile salt GDC there was an increase in staining for the plasminogen activator inhibitors and the adhesion molecules. These levels decreased when the cells were grown in acidified media.

In the cell-line OE21 treated with the bile salt GCA there was a decrease in staining for the adhesion molecules. With exposure to acidified growth media there was a decrease in staining for all the molecules. There were no differences between samples from day 1 and day 2.

In the cell-line OE21 treated with the bile salt TCA there was no change in the staining for any of the molecules. There was decrease staining for plasminogen activator and the adhesion molecules in acidified growth media. There were no significant differences in staining between samples from day 1 and day 2.

In the cell-line OE21 treated with the bile salt TDC there was an increase in staining for the plasminogen inhibitors. There was an increase in staining for the

adhesion molecules on the second day. Levels of staining for the adhesion molecules decreased in acidified growth media.

In the cell-line OE21 treated with GCDA there was no change in the staining for any of the molecules except plasminogen activator inhibitor type-2, which increased. The levels of the adhesion molecules and PAI-2 decreased with exposure to acidified growth media. There were no differences between day 1 and day 2.

In the cell-line OE21 treated with TCDA there was a decrease in the staining for plasminogen activator and an increase in staining for its inhibitor type-2. There is a decrease in staining for the adhesion molecules and plasminogen activator with exposure to acidified growth media.

7.3.2 ELISA and TRAP Results

The cellular concentration of the components of the plasminogen activator system and telomerase activity were also analysed using TRAP and ELISA techniques the results of this are summarised in table 7.3. There were no differences in the activity of telomerase in either cell-line with exposure to acidified media or bile treatment of the cells.

Some differences can be seen in the results from the plasminogen ELISA experiments on exposure to bile and acid treatment, but no real patterns appear in the results. In some cases of the cell-line OE33 there appeared to be an increase in expression of uPA (treatment with GDC, DCA, TDC, and TCDA) and uPAR (treatment with all bile salts) on cells grown in acidified media with bile salts compared to the non-treated control and cells treated with bile salts alone.

No significant difference in the amount of either of the inhibitors can be seen with the various treatments.

A proportion of the treated cells from cell-line OE21 had to be excluded from this study as the extracted protein concentrations were too low for successful completion of the test. Although the cells were washed before the addition of CHAPS lysis buffer the lowered pH seemed to effect the detergent and extraction process.

Table 7.3. Results of ELISA on cells treated with bile salts and grown in acidified media.

Abbreviations: DCA = Deoxycholic acid, GCA = Glycocholic acid, GDC - Glycodeoxyxholic acid, GCDA = Glycochenodeoxycholic acid, TCA = Taurocholic acid, TDC = Taurodeoxycholic acid, TCDA = Taurochenodeoxycholic acid, Y = yes and N = no.

A = Samples analysed directedly after exposure. B = Samples analysed 18 hours after exposure.

*= Samples with a protein concentration that was too low for analysis.

Antigen levels in ng/mg protein.

OE21

Treatment	uPA	uPAR	PAI-1	PAI-2	Telomerase
Control	5	5.417	>200	17.5	Y
ACID A	1	5.833	320	20	Y
ACID B	4.889	7.222	>200	23.333	Y
GDC A	2.727	6.364	338.182	67.273	Y
GDC B	3.487	4.615	297.436	28.718	Y
GDC+ ACID A	*	3.75	215	5	Y
GDC + ACID B	*	6	256	0	N
DCA A	8.533	10	>100	26.667	Y
DCA B	8.533	10	>100	26.667	Y
DCA + ACID A	*	*	146.667	*	Y
DCA + ACID B	*	*	182.857	*	Y
GCA A	3.6	4	242	20	Y
GCA B	17.647	7.059	>200	24.706	Y
GCA + ACID A	*	*	480	*	Y
GCA + ACID B	*	*	293.333	*	Y
TCA A	6.375	4.063	>200	15.625	Y
TCA B	6.537	4.878	>200	24.39	Y
TCA + ACID A	*	28.571	217.143	34.286	Y
TCA + ACID B	*	18.75	162.5	17.5	Y
TDC A	0.842	2.105	>200	58.333	Y
TDC B	2.247	3.146	>200	54.762	Y
TDC + ACID A	*	15	360	0	Y
TDC + ACID B	*	14.444	275.556	0	Y
GCDA A	4.875	8.125	>200	21.359	Y
GCDA B	2	2.241	>200	68.571	Y
GCDA + ACID A	*	22.5	325	5	N
GCDA + ACID B	*	14	296	0	Y
TCDA A	3	0	66.667	0	Y
TCDA B	6.271	2.712	>200	51.186	Y
TCDA + ACID A	0.75	9.375	246.25	0	Y
TCDA + ACID B	1.556	11.111	340	0	Y

OE33

Treatment	uPA	uPAR	PAI-1	PAI-2	Telomerase
Control	>25	3.934	0.984	58.142	Y
ACID A	3.888	4.673	2.243	99.065	Y
ACID B	7.143	18.571	0	57.143	Y
GDC A	7.403	4.328	0.896	57.91	Y
GDC B	7.429	3.429	0	6	Y
GDC+ ACID A	26.429	43.571	0	81.111	Y
GDC + ACID B	29	11.667	10	0	Y
DCA A	5.767	3.023	1.395	30.233	Y
DCA B	5.608	2.941	0.392	45.098	Y
DCA + ACID A	15.333	44.444	0	37.778	Y
DCA + ACID B	10.133	30	0	9.333	Y
GCA A	6.7	4	3.5	107	Y
GCA B	6.36	2.36	2.236	57.143	Y
GCA + ACID A	17.875	35.625	0	52.5	Y
GCA + ACID B	6.476	42.857	0	20.952	N
TCA A	>25	1.553	1.279	23.196	Y
TCA B	>25	2.847	0.694	36.389	Y
TCA + ACID A	18.235	54.118	0	58.824	Y
TCA + ACID B	5.333	23.333	0	14.667	Y
TDC A	4.932	3.609	1.203	11.579	Y
TDC B	6.19	4.167	0.952	10.787	Y
TDC + ACID A	16.111	27.778	0	90	Y
TDC + ACID B	25.538	11.538	6.154	0	Y
GCDA A	>25	3.214	0.714	20	Y
GCDA B	>25	1.748	0	14.655	Y
GCDA + ACID A	24.444	32.771	0	91.111	Y
GCDA + ACID B	35.111	16.667	8.889	0	Y
TCDA A	10.222	0.556	4.444	137.778	Y
TCDA B	11.619	1.429	1.905	101.905	Y
TCDA + ACID A	18.571	38.571	0	0	Y
TCDA + ACID B	23.714	13.571	5.714	0	Y

7.4 Discussion

Bile acid toxicity has been extensively studied, but there is a wide variation in the reporting of the relative toxicity of the individual bile acid fractions. Much information is based on the toxic effects of bile on gastric mucosa, colonic mucosa and hepatocytes, but little information is available on the toxicity of bile on oesophageal mucosa in gastro-oesophageal reflux disease and Barrett's oesophagus. The concentration of bile acids and toxicity is known to change with pH depending on the degree of ionisation of the molecules.

Bile salts have been implicated as having a causal influence in the pathogenesis of Barrett's oesophagus and adenocarcinoma of the oesophagus. Duodenogastro-oesophageal reflux is thought to occur infrequently in normal individuals, but evidence is strong that it plays a role in causing oesophageal damage in Barrett's oesophagus and adenocarcinoma (Marshall 1997). The duodenal contents suspected of causing oesophageal damage include bile acids, lysolecithin secreted in bile, and trypsin. Oesophageal mucosa damage induced by bile salts depends on the conjugation state of the bile salts as well as the pH of the solution. A study by Buttar *et al* found conjugated bile acids cause damage at an acidic pH, whereas unconjugated bile acids as well as the pancreatic enzyme trypsin cause damage at a pH higher than 7 (Buttar 2001).

It has been found that patients with oesophagitis had greater acid reflux than those without, but patients with stricture and Barrett's oesophagus had similar

acid reflux to those with uncomplicated erosive oesophagitis. Pepsin concentrations were highest in patients with stricture and Barrett's oesophagus especially at night (Gotley 1991).

Here the oesophageal cell-lines, OE21 and OE33, which have been characterised as *in vitro* models of oesophageal cancer, have been analysed to see if exposure to conjugated bile salts in both acidic and neutral pH alters the expression of telomerase, the components of the plasminogen system and adhesion molecules. It has been shown that conjugated bile salts cause the most damage in acidic conditions, therefore the bile salts used were all conjugated and included:

- Primary bile acids
    1. Taurocholic acid (TCA)
    2. Glycocholic acid (GCA)
- Secondary bile acids
    1. Deoxycholic acid (DCA)
    2. Taurodeoxycholic acid (TDC)
    3. Glycodeoxycholic acid (GDC)
    4. Taurochenodeoxycholic acid (TCDA)
    5. Glycochenodeoxycholic acid (GCDA)

The main constituents of reflux bile have been found to be taurocholic, glycholic and cholic, therefore the most easily available of these bile acids have been used. Gotley *et al* tried to assess the concentration of bile acids in oesophageal aspirates. He found that chromatograms of oesophageal aspirates contained six

bile acid peaks including, GDA, GCDA, TCDA, TDA, GCA and TCA. It was found that the median concentration of bile acids was <20μmol/l, which was significantly lower than during the night-time period (median 51μmol/l) (Gotley 1988).

7.4.1 Morphology

The overall morphology of the cultured cells treated in the various different ways was assessed during treatment using an inverted phase contrast microscope before harvesting. The cells were also assessed once stained immunocytochemically. Cultured cells treated with conjugated bile salts alone appear no different to the untreated control cells when viewed under the microscope. There does seem to be an increase in cell number in flasks treated with bile and grown on overnight compared to untreated controls. Previous studies have shown that exposure of oesophageal tissues to bile salts increased the proliferation of cells (Kaur 2000), but rates of proliferation have not been assessed here.

Examination of cells under the microscope that were exposed to bile salts in acidic conditions revealed morphological changes characteristic of apoptosis, including loss of adhesion, rounding and membrane blebbing. Cells grown in acidic media appear to shrink in size and gaps appear between cells within the monolayer (see figures 7.1 - 7.12 for examples). This can be seen after the hour treatment and is still evident the following day. Cells treated to conjugated bile salts in acidic conditions also show this shrinkage, and cells become detached from the surface of the flask. This is evident from the small size of the pellets of harvested cells from flasks treated with bile salts in acidic media. In some cases the protein extracted from these pellets was too low for ELISA analysis for the components of the plasminogen activator system. Also significant numbers of loose/nonadherent cells were lost during the fixation and staining steps of the immunocytochemistry. It could also be possible that the acid conditions effected

the detergent based lysis method used here to extract proteins from the cells. All harvested cells grown in acid conditions yielded a low concentration of proteins.

In these experiments protein samples were extracted from the cells using CHAPS lysis buffer. CHAPS is an ionic detergent. Ionic detergents are strongly amphiphilic and therefore tend to denature proteins. Amphiphilic substances are both hydrophobic and hydrophilic and breakdown these interactions in macromolecules. This could effect the ELISA detection methods for the components of the plasminogen system as the samples were also extracted for telomerase detection using CHAPS lysis buffer, which could denature the proteins and alter the antibody epitopes. A neutral detergent Triton X-100 could be used instead to confirm this.

Slide flasks containing OE33 that were treated with bile salts in acidic conditions yielded less cells than corresponding OE21 flasks. In some cases more than 60% of the monolayer of cells became detached from the surface of the flask during the washing and fixing stages of preparation for immunocytochemistry. This could limit the results of the immunocytochemistry as very few cells were left for analysis compared to OE21.

Treatment of both cell-lines with the bile salts DCA, TDC and GCA in acidified media resulted in a change to the appearance of the cell nuclei once counter stained with heamatoxylin. This change was still apparent after a day's growth in normal media. The nuclei appeared to be pale and transparent compared to the non-treated controls and other treated cells.

7.4.2 Effects of bile and acid treatment on molecular markers

Previous studies have looked at the effects of bile salts and pH on both *ex vivo* oesophageal tissue samples and cultured cells. A study by Kaur *et al* (2000) looked at the effects of bile salts with and without acid on cell proliferation in biopsies of oesophageal mucosa. This study concluded that brief exposure to bile salts in the absence of acid increased proliferation, whereas exposure to a combination of acid and bile salts together inhibited proliferation (Kaur 2000).

Shirvani *et al* studied the expression of Cyclo-oxygenase 2 (COX-2) in Barrett's oesophagus using biopsies of Barrett's epithelium exposed to acid alone, acid and bile or bile alone. COX-2 is a membrane bound glycoprotein that functions as a rate limiting enzyme that is induced by various stimuli. Increased COX-2 expression has been demonstrated in human inflammatory diseases. It has been implicated in colorectal and oesophageal carcinogenesis including Barrett's oesophagus and adenocarcinoma. Shirvani showed COX-2 expression increased significantly when exposed to acid or bile salts (Shirvani 2000).

The effects of bile salts and acid on the apoptotic and proliferative activity of cultured oesophageal cell-lines was studied by Whittles *et al* (1999). It was found that bile salts effected apoptosis during acidic conditions. Exposure to the bile salt DCA caused the most apoptosis in the oesophageal cell-lines and also caused the most disruption to intergrins within the cells. It is thought that compared to normal controls, patients with Barrett's oesophagus have high levels of DCA in oesophageal aspirates. Bile salts such as TDC cause extensive damage to the oesophageal mucosa barrier (Whittles 1999).

The expression of the components of the plasminogen activator system, uPA, uPA receptor and the inhibitors PAI-1 and PAI-2 were assessed using immunocytochemistry in oesophageal cell-lines treated with bile salts and acidified growth media. Staining for the components of the plasminogen system was localised to the cytoplasm of the cells. In OE21 the staining for uPA was localised around the nucleus of the largest cells in the treated samples as well as the non-treated controls. Staining for uPA in OE33 was seen universally throughout the cytoplasm of both the treated and non-treated cells. There was some staining for the inhibitors in the nuclei of the cells, but no significant changes were seen with exposure of the cells to either bile salts or acidic conditions.

It has been shown in chapter 5 that changes in the expression of the components of the plasminogen system are a late event during the progression of Barrett's oesophagus to adenocarcinoma. These molecules are expressed when a large amount of oesophageal damage has occurred and dysplasia or *in situ* carcinoma is already present in the mucosa. Gastro-oesophageal and duodenogasro-oesophageal reflux are thought to cause the epithelial changes seen in patients with Barrett's oesophagus. The oesophageal cell-lines are derived from tumour samples from patients who have undergone resection (Rockett 1997). There is increased expression of the components of the plasminogen system in tumours and therefore the levels are high in the cell-lines. Exposure to bile and acid conditions appears to have little effect on these molecular markers.

In cells treated with acidified growth media and bile salts the expression of two adhesion molecules, CD44 and E-Cad, were assessed using

immunocytochemistry. In the non-treated controls the immunocytological staining was localised to the plasma membranes of the cells. Once cells had been treated with either acidified media or bile salts in acidified media the staining for both CD44 and E-cad was significantly less. CD44 and E-cad are adhesion molecules and are involved in cell to cell interactions. As mentioned earlier cells exposed to acidified media became detached from the surface of the flask. This was most obvious in the cell-line OE33. Growth media of pH5 disrupted the adhesion molecules on the surface of the cells causing the cells to shrivel and shrink. As discussed previously (Chapter 6) levels of E-cad are low in OE33 and in some cases cells have lost adhesion properties before treatment with bile and acidic growth media.

Cell membranes are made of a lipid bilayer matrix. Their lipid fractions consist of complex mixtures of lipids and proteins that vary according to the type of cell they are part of. Membrane proteins carry out dynamic processes associated with membranes and therefore vary from cell to cell. Membrane proteins are classified according to how tightly they are associated with the membrane. Intrinsic proteins are tightly bound to the membrane by hydrophobic interactions and can only be separated by treatment with agents that disrupt the membranes such as detergents, (e.g. CHAPS). Extrinsic proteins are dissociated from the membrane by relatively mild procedures that leave the membrane intact (e.g. changes to pH).

In this study changes to the pH of the growth media alters the levels of the membrane bound proteins CD44, E-cad, and uPAR. It is possible that in the normal mucosa of the oesophagus gastro-oesophageal and duodenogasro-

oesophageal reflux causes the membrane bound proteins such as CD44, E-Cad and integrins (Whittles 1999) to become dissociated from the membrane.

Proteins bear numerous functional groups such as carboxyl and amino groups that can undergo acid-base reactions, therefore many properties of these molecules vary with the acidity of the solutions into which they are immersed. The exposure of cells to acidic conditions in the oesophagus and in these experiments could effect the way the proteins tested function in ways that could not be detected with immunocytochemistry and ELISA techniques.

The effects of free bile acids (cholic, deoxycholic and chenodeoxycholic acids) have been studied in the small intestine. These bile acids inhibit the main active transport system for glucose, amino acids and sodium in the small intestine and cause extensive damage with loss of villi (Nehra 1999).

It has been suggested that bile acids damage the mucosa not by a detergent effect on the surface membrane, but by being adsorbed into the epithelial cells, causing disorganisation of the intracellular membranes and interfering with cellular metabolism (Marshall 1997).

The role that the various components of duodenal juice play in the development of oesophageal injury has been the subject of may experimental animal studies. Attempts have been made to define the roles of hydrochloric acid, pepsin, bile salts and trypsin alone and in combination (see table 7.4).

These studies show that more oesophageal damage is induced by bile acids in the presence of acidic conditions or the presence of HCl. Incidence of the damaging effects of duodenal contents on the oesophageal mucosa in humans remains less

clear-cut as there is no reliable technique for detecting duodenogastro-oesophageal reflux. Various direct and indirect methods have been used, but all have their drawbacks. pH monitoring is the most widely used method to detect reflux from the duodenum, but the results are full of contradiction.

Table 7.4. Summary of animal studies investigating the effect of gastric and duodenal contents on oesophageal mucosa.

Abbreviations: HCl = hydrochloric acid, TCA = taurocholic acid, TDCA taurodeoxycholic acid, CA cholic acid, DCA = deoxycholic acid, CDCA = chenodeoxycholic acid.

(Adapted from Marshall *et al* 1997).

Reference	Year	Substances tested	Extent of damage
Henderson *et al*	1972	HCl, TCA, TDCA, GCA	HCl+ bile salt > HCl or bile salt alone
Kivilaakso *et al*	1980	TCA, DCA, CDCA, Pepsin, Lysolecithin	TCA at pH 3.5 and DCA or CDCA at pH7 > pepsin at pH 3.5 > lysolecithin.
Harmon *et al*	1981	TDCA, TCA, CA, DCA, HCl	HCl alone damages at pH1, TDCA and TCA at pH 2, DCA and CA at pH 7.
Salo *et al*	1983	HCl, Pepsin, TCA, DCA	Pepsin or TCA + HCl > pepsin or TCA alone.
Kiroff *et al*	1987	TCA, HCl	TCA + HCl > TCA alone.
Gotley *et al*	1992	"Bile acid mixture", pepsin	Bile acids + pepsin = pepsin > bile acids alone.

A study by Nehra *et al* tried to determine the spectrum of bile acids refluxed in patients with GORD and its relation to oesophageal pH using combined oesophageal aspiration and pH monitoring. It was found that the predominant bile acids detected were cholic, taurocholic and glycocholic, but there was significantly greater proportion of secondary bile acids, deoxycholic and taurodeoxycholic acids in patients with erosive oesophagitis and Barrett's oesophagus/stricture (Nehra 1999). A consistent finding of secondary bile acids in patients with Barrett's oesophagus suggests that these bile salts may contribute to the metaplastic change.

The overall results of this small study into the effects of bile acids and pH on molecular markers for Barrett's oesophagus back up those of previous studies. There were more morphological and molecular changes in cells treated with both bile acids and lowered pH than those treated to bile salts alone. The most changes were seen in cells treated with taurocholic bile acids (TDC, TCA and TCDA). Changes were also seen in cells treated with DCA. These bile salts have been detected in patients with GORD, oesophagitis and Barrett's oesophagus and therefore could be implicated in causing the oesophageal damage associated with these disorders.

8 General Discussion and Future Direction

8.1 Adenocarcinoma of the oesophagus in patients with Barrett's oesophagus

The diagnosis of oesophageal cancer carries a dismal prognosis with an overall five year survival of less than 10%. The incidence of oesophageal squamous cell carcinoma has remained constant over the past 20 years, whereas the incidence of oesophageal adenocarcinoma is increasing rapidly. The reasons for this change from a predominance of one histological type of cancer to another are largely unknown. It has been suggested that all oesophageal adenocarcinomas arise in areas of metaplastic epithelium in which the normal squamous mucosa is replaced by specialised intestinal like columnar epithelium known as Barrett's oesophagus. It is thought that there is a progression in this abnormal epithelium through various degrees of dysplasia to neoplasia. The factors influencing this progression are poorly understood.

Barrett's oesophagus is usually diagnosed in patients undergoing endoscopy for gastro-oesophageal reflux symptoms. The diagnosis can only be carried out in these biopsy samples using histological techniques. As mentioned earlier Barrett's metaplasia can be easily identified by endoscopic surveillance, but the detection of dysplasia alone by endoscopy is difficult, because it has no distinguishing endoscopic characteristics. A better understanding of the molecular characteristics of Barrett's metaplasia, dysplasia and adenocarcinoma could generate more sensitive diagnostic methods for malignant change.

8.2 Molecular Markers for the Progression of Barrett's Associated Cancer.

The current knowledge of the molecular characteristics of Barrett's oesophagus are summarised in section 1.15 and include various molecules such as tumour suppresser genes, markers of cellular proliferation, oncogenes, growth factors, enzymes and adhesion molecules. As yet no single molecular marker or group of markers have been found to reliably diagnose the neoplastic progression in Barrett's oesophagus. Therefore dysplasia in biopsy samples can only be diagnosed using histological techniques. This study has focused upon two molecular markers; the components of the plasminogen activator system as a measure of invasiveness and telomerase as a measure of cellular immortality.

Over the last 20 years extensive research has been carried out to investigate the role of proteolytic enzymes in tumour cell biology. The serine protease system consists of plasminogen, which is converted to plasmin by two activators; urokinase-type plasminogen activator and tissue-type plasminogen activator. The urokinase-type activator is thought to have a greater role in the mechanism of tissue invasion and is active at a cell surface receptor. Two naturally occurring inhibitors, PAI-1 and PAI-2 inhibit the activity of this binding. There are no published reports of the levels of the components of the PA system in Barrett's oesophagus or associated cancers.

Another characteristic feature of many tumours is the absence of programmed cell death and the resultant cellular immortality. The activity of telomerase is critical in the prevention of DNA damage and cell death. In most normal somatic tissues telomerase expression is suppressed, but it is reactivated during tumour

development and is thought to be a potential marker of the development of a malignant phenotype. There are currently no extensive studies of telomerase activity in the progression of Barrett's oesophagus to adenocarcinoma.

This work has investigated several areas of cancer biology relevant to oesophageal adenocarcinoma where there is no previously published work. The levels of the components of the plasminogen system in the progression of Barrett's associated cancer are described for the first time. Also there are no other studies of the relationship between telomerase activity and the plasminogen activator system in any tumour type.

8.3 Telomerase and Barrett's Associated Adenocarcinoma

Here a study has been carried out into the activity of telomerase during the metaplasic-dysplasic-neoplastic progression of Barrett's oesophagus utilising the TRAP method of analysis. Telomerase activity increases as the disease progresses. Telomerase reactivation appears to be an early event during the progression of Barrett's associated adenocarcinoma. Telomerase activity is present in intestinal metaplasia, but not in the other non-dysplastic Barrett's metaplasia. It has been suggested that it is the intestinal metaplasia that progresses to dysplasia and adenocarcinoma and therefore telomerase activity could be a useful molecular marker for finding patients who's disease is starting to progress.

The diagnostic tool utilised here (the TRAPEZE™ ELISA) is a simple test that could in the future detect telomerase in exfoliated cells from the oesophagus. In some institutions an endoscopic brush or balloon is used during surveillance to

collect cells from the surface of the oesophagus of patients with minimal discomfort. Currently two studies have looked at the possibility of diagnosing Barrett's oesophagus using balloon cytology (Fennerty 1995 and Falk 1997). Both studies observed the abrased cells microscopically for abnormalities and the results were disappointing, although the potential cost savings of balloon cytology compared to endoscopic cancer surveillance in Barrett's oesophagus support further studies into this technique. In the future Balloon abrasion for the collection of oesophageal cells and the use of molecular rather than cytological analysis may become a central procedure in the surveillance strategy for Barrett's oesophagus.

The main disadvantage of using the TRAP method for analysing telomerase in clinical tissue samples is there is no way of telling exactly which cells within the tissue are expressing telomerase. Some of the resected tissue samples assessed during this study contained a range of tissue types. In order to get a complete picture of telomerase activity within the progression of Barrett's metaplasia to adenocarcinoma a non-isotopic method of *in situ* hybridisation for the detection of the RNA component of telomerase was developed.

Three methods of *in situ* hybridisation for the detection of the RNA component of telomerase were investigated. The most successful of these was Method 3 loosely based on that described by Chang *et al* 1999 and utilised DIG labelled RNA sense and anti-sense probes detected by the reaction of the enzyme alkaline phosphatase with BCIP/NBT. In the future this method of non-isotopic *in situ* hybridisation could easily be utilised to carry out a full assessment of the localisation of telomerase in the metaplasia:neoplastic progression of Barrett's

oesophagus and its correlation with telomerase activity detected with the TRAP assay.

8.4 The Components of the Plasminogen Activator System and Barrett's Associated Adenocarcinoma

No previous studies have assessed the levels of the components of the plasminogen activator system during the progression of Barrett's oesophagus to adenocarcinoma. Here patterns have been shown in the cellular localisation and levels of the components of the plasminogen activator system. Changes appear as a late event during the progression of the disease showing the invasive phenotype of Barrett's oesophagus allowing cells to invade the basement membrane does not appear until severe dysplasia or neoplasia is present in the oesophagus.

The components of the plasminogen system were detected using an ELISA based technique, so again these molecules could be detected in abrased cells from brush or balloon endoscopy. In the future detection of the components of the plasminogen system could be used as a diagnostic tool to assess patients with a poor prognosis and increased risk of metastasis.

When plasminogen activator binds to its receptor or inhibitors it is incorporated into the cytoplasm of the cell (Naitoh 2000). Therefore even if the plasminogen activator antigen is detected in a certain location within the cell it does not mean it was produced there. This could be true for all the other components of the plasminogen system. For this reason identification of the mRNA of the components of the plasminogen activation system is needed. Messenger RNA can be detected within the cell utilising an *in situ* hybridisation method. In the future

this could be used to further enhance the knowledge of the expression and localisation of the components of the plasminogen activator system during the progression of Barrett's oesophagus to adenocarcinoma.

8.5 Potential Clinical Benefits

This study offers three main groups of potential clinical benefits for patients with or at risk of developing oesophageal cancer. These are diagnostic, prognostic and therapeutic.

8.5.1 Diagnostic

As mentioned there is considerable controversy over the way in which patients with Barrett's oesophagus should be managed within the NHS. Some hospitals use surveillance programs where patients undergo repeated endoscopy and biopsy in the hope of identifying the progression of the metaplasia to dysplasia, to allow early intervention before the development of malignancy. As a vast majority of patients with non-dysplastic Barrett's metaplasia do not go on to develop cancer the yield of these surveillance programs is very low. The analysis of telomerase activity and the components of the plasminogen activator system in biopsy samples may help to identify patients whose disease is starting to progress and maybe used in conjunction with standard pathological examination to provide a more accurate estimate of the risk of progression to cancer.

8.5.2 Prognostic

Many studies have been carried out relating the levels of expression of the components of the plasminogen system to prognosis in a variety of solid tumours. It is thought that tumours possessing the ability to overcome the extracellular

matrix and metastasise will behave more aggressively and give patients a poor prognosis. In the future the analysis of telomerase activity and measurement of the components of the plasminogen system in samples taken by biopsy or after resection may add to the prognostic accuracy and act as a guide to the clinician to recommend treatment for the patient.

8.5.3 Treatment

A novel technique in cancer therapy is proteolysis inhibition. There have been several phase three clinical trials of inhibitors of proteolysis in patients with a variety of cancers. The main target for inhibition with drugs is the matrix metalloproteinases system, which shows varying degrees of success. There are an increasing number of inhibitors of the plasminogen activator system under development some of which it is hoped will provide some therapeutic benefit to patients in limiting or preventing metastasis in tumours. The results presented here will add strength to the idea that Barrett's associated cancer should be included in the list of potential targets for these therapies.

8.6 The Analysis of Immortal Cell-lines as *in vitro* Models of Oesophageal Cancers

In order to produce *in vitro* models of the two main types of oesophageal tumours two oesophageal cell-lines were characterised, these were OE21 (squamous cell carcinoma) and OE33 (Barrett's associated adenocarcinoma). In order to characterise the cell-lines various methods of analysis were used to detect a number of previously studied molecular markers for these diseases.

Overall the molecular characteristics of the cell-lines correspond to those of the tumours from which they were derived. The cell-line OE33 seems a valid *ex vivo* model of Barrett's associated adenocarcinoma. The molecular characteristics of the squamous cell carcinoma cell-line OE21 were not as conclusive. There were differences in the molecules expressed in the cell-line when compared to those found in the studies of clinical cases. OE33 has more of the expected characteristics of an invasive cancer, whereas OE21 seems to express different characteristics to the related tumour.

In the future these cell-lines could be utilised in studies for the treatment and diagnosis of oesophageal tumours, either as controls or for initial analysis of techniques. OE21 and OE33 could also be used as described in chapter 7 as *in vitro* models of oesophageal tumours analysing the effects of environmental changes on the molecular characteristics of oesophageal tumours.

8.7 The Effect of Bile and Acid Exposure in Barrett's Oesophagus

Both animal and human studies have suggested that hydrochloric acid is a major factor in oesophageal mucosa damage, but that bile acids and trypsin in synergy with each other and with hydrochloric acid play a significant role in the pathogenesis of Barrett's oesophagus. Here a small study into the effects of bile acids and pH on molecular markers for the progression of Barrett's oesophagus was carried out in two oesophageal cell-lines OE21 (derived from squamous cell carcinoma) and OE33 (derived from Barrett's associated adenocarcinoma).

The overall results of this small study back up those of previous studies. There were more morphological and molecular changes in cells treated with both bile

acids and lowered pH than those treated to bile salts alone. Most changes were seen in cells treated with taurocholic bile acids (taurodeoxycholic acid, taurocholic acid and taurochenodeoxycholic acid). Changes were also seen in cells treated with deoxycholic acid. These bile salts have been detected in patients with gastro-oesophageal reflux disease and Barrett's oesophagus and therefore could be implicated in causing the oesophageal damage associated with these disorders.

The incidence of the damaging effects of duodenal contents on the oesophageal mucosa in humans remains unclear, as there is currently no reliable technique for detecting duodenogastro-oesophageal reflux. Various direct and indirect methods have been used previously, but all have their shortcomings. In this study human derived oesophageal cell-lines were used as an *ex vivo* model of the human oesophagus to analyse the effects of bile salts and pH on the oesophageal mucosa, but in the future *in vivo* studies will be needed to decipher the exact effects of acid and bile on the human oesophagus.

In this study cells were only exposed to the environmental changes for a short period of time (1 hour) and either analysed then or grown for a further 18 hours. In the future this study could be extended using multiple exposures to the conditions or exposure for longer periods. Also the length of time after exposure could be extended to see if the bile acids or acidified growth media hàve long term effects to the cells. The effects of trypsin, pepsin and lysolecithin could also be assessed in cell-lines exposed to bile acids and lowered pH.

9 Conclusions

The aim of this study was to investigate molecular markers for the progression of Barrett's oesophagus to adenocarcinoma with reference to the components of the urokinase-type Plasminogen Activator system and the reactivation of telomerase. The molecular characterisation of two oesophageal cell-lines was also carried out with the aim of producing *ex vivo* models of oesophageal cancer.

Investigation into both telomerase activity and the plasminogen activator system have provided new information concerning the nature and timing of molecular changes which occur during the Barrett's metaplasia/dysplasia/adenocarcinoma sequence.

The expression of the components of the plasminogen activator system is altered in Barrett's associated adenocarcinoma when compared to non-involved oesophageal and gastric mucosa. The progression of Barrett's oesophagus to adenocarcinoma is characterised by an increase in levels of urokinase-type plasminogen activator and its receptor and a slight decrease in its inhibitors type-1 and -2.

The development of an immortal phenotype during the progression of Barrett's oesophagus to adenocarcinoma has been assessed. Telomerase appears to be reactivated early in disease progression in 50% of the intestinal type Barrett's metaplastic tissue assayed. Telomerase was detected in 76% of adenocarcinoma tissues assayed. A non-isotopic method of *in situ* hybridisation for the detection of the RNA component of telomerase has been successfully developed and utilised

on a small scale to investigate the cellular localisation of telomerase in Barrett's associated adenocarcinoma.

The human oesophageal cell-lines OE21 and OE33 have been shown to have molecular characteristics similar enough to the malignant diseases to be used as *ex vivo* models of squamous cell carcinoma and Barrett's associated adenocarcinoma respectively. The treatment of these cell-lines with bile acids and acidified growth media demonstrated morphological and molecular alterations to their malignant characteristics.

The aim of this thesis was the study of molecular markers for the progression of Barrett's oesophagus to adenocarcinoma, to identify markers that can be used as diagnostic tools. To some extent this aim has been achieved in that telomerase reactivation and the plasminogen system have been assessed as possible molecular markers for the progression of Barrett's oesophagus, but only telomerase detection has the potential to be used as a diagnostic tool for progression. In the future a randomised clinical trial of patients with intestinal metaplasia could be carried out to assess whether this potential is really relevent for diagnosing patients likely to progress to dysaplasia and adenocarcinoma of the oesophagus.

10 Publications Arising from this Study

10.1 Abstracts

Hewin D. F., Cadd V. A., Barr H., Warner P. J., Woodman A. C., Shepherd N. A., and Vipond M. (September 2001). An investigation of the plasminogen activator system in Barrett" oesophagus and adenocarcinoma of the gastro-oesophageal junction. *Association of Upper GI surgeons Meeting*, Edinburgh.

Cadd V. A., Shepherd N. A., Vipond M. N., Barr H., Warner P. J., and Woodman A. C. (May 2001). Telomerase and the plasminogen system during the metaplastic: neoplastic sequence of Barrett's oesophagus. *Clinical Chemistry and Laboratory Medicine* Vol. 39 S165 PO-F031.

Cadd V. A., Licchesi J., Morgan S. L., Warner P. J., and Woodman A. C. (March 2001). Putting molecular diagnosis into practise. *Set for Britain*, presentations by Britain's young scientists, The House of Commons, London.

Cadd V. A., Shepherd N. A.,Hewin D., Warner P. J., and Woodman A. C. (November 2000). Cellular distribution of urokinase-type plasminogen activator, its receptor, plasminogen inhibitor type-1 and –2 in the metaplastic neoplastic sequence of Barrett's oesophagus. *Reporting back the results of R & D, NHS Executive South West.* Abstract #19.

Cadd V. A., Shepherd N. A., Barr H., Warner P. J., and Woodman A. C. (July 2000). Development of an immortal phenotype in the metaplastic neoplastic sequence of Barrett's Oesophagus. *Pathological Society of Great Britain and Ireland 181st Meeting* Abstract #45.

10.2 Papers

None published at present, some papers in progress.

Appendix 1 Ethical Approval

GLOUCESTERSHIRE
HEALTH AUTHORITY

VICTORIA WAREHOUSE
THE DOCKS
GLOUCESTER GL1 2EL

TEL: (01452) 300222
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(01452)
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(01452)

Our Ref: k;r&d/lrecs/1999/wglrec/Outcome99_10W(10_.6)a

Please ask for: Hazel Moynihan

11 June 1999

Professor Hugh Barr
Consultant Surgeon
Ward 9
Gloucestershire Royal Hospital
Great Western Road
GL1 3NN

Dear Professor Barr

99/10W: Investigation of cancerous and pre-cancerous changes in Barrett's Oesophagus using optical and microbiological techniques to advance the early diagnosis of malignancy

Thank you for responding to the points that were made. We are satisfied that these meet the committee's requirements and I am therefore happy to grant you full approval to proceed subject to terms and conditions set out in our previous letter.

Yours sincerely

Jane Maxwell

Jane Maxwell
Vice Chair, West Gloucestershire LREC

cc Catherine Fulljames, Cranfield University, GRH

GLOUCESTERSHIRE
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Please ask for: Hazel Moynihan

10 June 1999

Professor Hugh Barr
Consultant Surgeon
Ward 9
Gloucestershire Royal Hospital
Great Western Road
GL1 3NN

Dear Professor Barr

99/10W: Investigation of cancerous and pre-cancerous changes in Barrett's Oesophagus using optical and microbiological techniques to advance the early diagnosis of malignancy

Thank you for attending the West Gloucestershire LREC yesterday with your colleagues. The committee gives you full approval to proceed subject to your submitting revisions to the Patient Information Sheet, Consent Form and Protocol.

Specifically, the committee asked:-

- That the protocol includes details of what the samples will be used for.
- That the Patient Information Sheet be in a larger type face, and that some of the technical details of what happens to the samples be left out of the Patient Information Sheet.
- That the Patient Information Sheet and Consent Form are on headed note paper.
- The Consent Form should have provision for the subject to answer Yes or No to each of the sentences.

The Committee draws your attention to:

a) The responsibility of the investigator to notify the LREC immediately of any information received by him/her of which he/she becomes aware which would cast doubt upon, or alter, any information contained in the original application, or a later amendment application, submitted to the LREC and/or which would raise questions about the safety and/or continued conduct of the research.

b) The need to comply with the Data Protection Act 1984.

iii

c) The need to comply, throughout the conduct of the study, with good clinical research practice standards.

d) The need to refer proposed amendments to the protocol to the LREC for further review and to obtain LREC approval there to prior to implementation (except only in cases of emergency where the welfare of the subject is paramount).

e) The requirement to furnish the LREC with details of the progress of the research project periodically (usually annually) and **failure to do this could result in approval to continue with the study being withdrawn.** Please also inform us of the conclusion and outcome of the research project and inform the LREC should the research be discontinued or any subject withdrawn altogether

A list of the members of the West Gloucestershire LREC may be supplied if required.

Yours sincerely

Jane Maxwell

Jane Maxwell
Vice Chair, West Gloucestershire LREC

cc Catherine Fulljames, Cranfield University, GRH

THE INSTITUTE OF MEDICAL SCIENCES

Cranfield UNIVERSITY

GLOUCESTERSHIRE ROYAL NHS TRUST

PROTOCOL

DONATION OF SAMPLES TO INVESTIGATE OPTICAL & MOLECULAR BIOLOGY TECHNIQUES TO ADVANCE THE EARLY DIAGNOSIS OF MALIGNANCY

The samples must be processed as soon as is practically possible.

Samples Required
Samples of non-involved, inflammatory, Barrett's, dysplastic and malignant oesophagus.

Equipment
Cryogenic vials, liquid nitrogen, pathology cards (for biopsy samples) and logbook (for resection material) will be provided. A password-protected database will be used to store patient details and assign a unique patient identification number, which is then used in the labelling of the samples.

Collection of biopsy samples
1. Inform the patient about the project and tissue donation.
2. Obtain signed consent to obtain tissue samples for research.
3. Fill in patient details on a pathology card (or use a sticker from their notes), the assigned patient identification number and the date of collection.
4. Take the samples during routine surveillance endoscopy. Place the biopsies on card in the cryovials. These are labelled with the patient identification number, tissue type and sample name.
5. Place the vials in liquid nitrogen at once to snap freeze the samples. Retain the samples in the liquid nitrogen store until required.
6. When the samples are used frozen H&E sections will be cut for histopathological analysis.

Collection of resection samples

1. Inform the patient about the project and tissue donation.
2. Obtain signed consent to obtain tissue samples for research.
3. Fill in patient details in log-book (or use a sticker from their notes), the assigned patient identification number and the date of collection.
4. Take the samples during routine pathological analysis. Place the biopsies on card in the cryovials. These are labelled with the patient identification number, tissue type and sample name.
5. Place the vials in liquid nitrogen at once to snap freeze the samples. Retain the samples in the liquid nitrogen store until required.
6. When the samples are used frozen H&E sections will be cut for histopathological analysis.

The samples collected as detailed above will be used in the following ways:-

Optical Investigation of Samples

Optical analysis will involve the measurement of samples using Raman, Infrared and other spectroscopic techniques. The spectra will be correlated with the pathology as determined from the H&E stained frozen sections. A spectral library will be built and its validity tested.

Molecular Biology Investigation of Samples

Molecular characterisation of oesophageal cell lines and human tissue from each of the stages in the Barrett's adenocarcinoma sequence will be carried out using molecular and cellular techniques. These include immunoassay, PCR, immunohistochemistry, in situ hybridisation. Markers to be examined include oncogenes, adhesion molecules, tumour supressors and telomerase.

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PATIENT INFORMATION

DONATION OF SAMPLES TO INVESTIGATE OPTICAL & MOLECULAR BIOLOGY TECHNIQUES TO ADVANCE THE EARLY DIAGNOSIS OF MALIGNANCY

Background

The staff of Gloucestershire Royal Hospital and scientists at Cranfield University are working together to find new ways of providing advanced clinical support for the local community.

An area where we feel we can make major advances is in improving our ability to detect oesophageal disease, pre-cancer and cancer. Using advanced rapid tests we hope to be able to easily detect this disease without the need to bring patients into hospital or undergo long uncomfortable procedures.

Before these investigations can be carried out on patients, it is necessary to assess its suitability with human tissue in the laboratory.

Please help us.

Your doctor has explained to you that he/she will be removing some oesophagus tissue during the course of your care. Specialist staff within the hospital will examine this tissue and your care guided by what they find. After all these examinations have been undertaken there may be a small amount of tissue remaining. If you are willing to allow us, we can use this tissue to help us develop new diagnostic tests.

Your care, treatment or method of assessment will not be altered in any way by your decision to donate or otherwise. Furthermore no additional procedures will be undertaken as a result of your consent.

We will be collecting samples from many people under surveillance for Barrett's oesophagus. Therefore this request does not mean that your diagnosis is unsure.

Thank you for your help and co-operation. If you have any questions please ask your doctor or contact Professor H. Barr, Ward 9, Gloucestershire Royal Hospital. Tel: (01452) 394679

THE INSTITUTE OF MEDICAL SCIENCES

Cranfield UNIVERSITY

GLOUCESTERSHIRE ROYAL NHS TRUST

CONSENT FORM

DONATION OF SAMPLES TO INVESTIGATE OPTICAL & MOLECULAR BIOLOGY TECHNIQUES TO ADVANCE THE EARLY DIAGNOSIS OF MALIGNANCY

- I have read the attached information leaflet, discussed it with my doctor and fully understand what is required. YES / NO

- I request that any.tissue samples taken during the course of my care at Gloucestershire Royal Hospital, may be made available for research after all procedures required for my clinical management have been completed. YES / NO

- I understand that my care will be unaffected by my consent. YES / NO

....

Name of Patient Hospital Number

....

Signature Date

Your doctor will retain this form.

CONFIDENTIAL

Appendix 2:Suppliers and Equipment

A) Suppliers

Ambion, Austin, Texas, USA

American Diagnostica Inc. Greenwich, CT. USA

Amersham Life Sciences Ltd. Rainham Essex

BDH, Poole, Dorset

BioRad Laboratories Ltd. Hemel Hempstead, Herts

Dako Ltd, High Wycombe, Bucks

Gibco BRL/Life Technologies, Paisley

Intergen Company, Purchase, NY, USA

Invitrogen, Leek, The Netherlands

Perkin Elmer, Warrington, Cheshire

Promega, Madison, WI, USA

Roche Molecular Biochemicals, Mannheim, Germany

Sigma-Aldrich, Poole, Dorset

Transduction Laboratories, Lexington, KY, USA

Vector Laboratories Inc. Burlingame, CA, USA.

B) Equipment

Balances – Sartorius Analytical BP110S and Salter Todd Oertling HC22

Cell culture Incubator – Napco Environment controller

Centrifuges – ALC Multispeed Refidgerated Centrifuge PK 131R

Electroblotter – Hoefer Semiphor

Electrophoresis – EmbiTech OneRun and Mini Protean II

Hybridisation oven – Hybaid minioven MKII

Laminar flow cabinet – Microflow Biological Safety cabinet MS1425/2

Microfuge – MSE Microcentur

Microscopes – Nikon TMS Inverted Microscope and Zeizz Axioscope with Axiocam digital camera

pH Meter – Orion Model 410A

Plate reader – Dynex Technologies MRX

Power packs – Hoefer EPS 2A200

Rocking table – Stuart Scientific Platform Shaker STR6

Stirrers – Bibby Scientific Magnetic Stirrer and Hotplate HB502.

Thermal Cyclers – Hybaid Touchdown and Techne Genius

UV Spec – WPA Lightwave

UV Visualisor – UVP Transluminator and Syngene Gene Genius bio-imaging system.

Waterbaths – Grant

C) Consumables

Consumable	Source/Catalogue number
ECL Western Blotting Detection Reagents	Amersham Pharmacia Biotech RPN 2106
Polystyrene slide flasks	BDH Merck (NUNC) 402/0333/04
Tissue culture flasks T25	BDH Merck 402/0311/04
Tissue culture flasks T75	BDH Merck 402/0311/08
Protein Dye Concentrate	Bio-Rad 500-0006
Glass Slide flasks	BDH Merck 402/0333/02
RPMI 1640 medium (with L-glutamine, sodium bicarbonate)	Sigma R-8758
Amphorericin B 250µg/ml	Sigma A-2942
Foetal Calf Serum	Sigma F-7524
10,000units/ml penicillin/10mg/ml streptomycin	Sigma P-4333
10x solution trypsin/EDTA	Sigma T-4174
Glycocholic acid (GCA)	Sigma G-7132
Deoxycholic Acid (DCA)	Sigma D-6750
Glycochenodeoxycholic acid (GCDA)	Sigma G-0759
Glycodeoxycholic acid (GDC)	Sigma G-3258
Taurodeoxycholic acid (TDC)	Sigma T-0875
Taurochenodeoxycholic acid (TCDA)	Sigma T- 6260
TRAPEZE ELISA Telomerase detection kit	Intergen S7750
IMUBIND uPA ELISA kit	American Diagnostica #894
IMUBIND uPAR ELISA kit	American Diagnostica #893
IMUBIND PAI-1 ELISA kit	American Diagnostica #821
IMUBIND PAI-2 ELISA kit	American Diagnostica #823
Precision molecular mass standard	BioRad 170-8207
Tri reagent	Sigma T-9424
PBS Tablets	GibcoBRL 1 8912-014
RNase Zap	Ambion #9780
RNase Zap Wipes	Ambion #9786
NucAway™ Spin Columns	Ambion #10070
mRNAlocator™-Hyb Kit	Ambion #1800
mRNAlocator™-Biotin	Ambion #1805
MAXIscript™ SP6/T7 Kit	Ambion #1308
Nuclear Fast Red	Vector Laboratories H-3403
VectaMount™	Vector Laboratories H-5000

Alkaline Phosphatase Substrate kit IV BCIP/NBT	Vector Laboratories SK-5400
Ultramount	Dako S1964
Histoclear	Fisher Chemicals H/0468/17
HybriSlips Plastic coverslips	Sigma H-0784
DIG RNA Labeling kit (SP6/T7)	Roche 1 175 025
DIG Wash and Block Buffer Set	Roche 1 585 762
DIG Easy Hyb	Roche 1 603 558
BM Purple AP Substrate	Roche 1 442 074

Appendix 3: Solutions

Solutions for Immunohistochemistry

Tris Buffered Saline (TBS)

0.05M Tris; 0.15M Sodium Chloride pH 7.6

Phosphate Buffered Saline

Made with sterile tablets (GibcoBRL Life Technologies). One tablet per 500ml RO water.

Rabbit Serum Block

1 part rabbit serum to 5 parts PBS

Antibody Diluent

1% Rabbit serum in PBS

Endogenous peroxidase blocker

3% Hydrogen peroxide in methanol.

DAB Solution

50mg DAB plus 100µl Hydrogen Peroxide in 100ml TBS.

Solutions for Western Blotting

Run buffer

25mM Trzma Base; 250mM lycine; 0.1% (w/v) SDS

Sample Buffer

100mM Tris-HCl, pH 6.8; 4% (w/v) SDS; 20% (v/v) Glycerol; 2% (w/v) Bromophenol Blue.

Transfer Buffer

10mM Tris-HCl; 10mM Glycine; 0.1% (w/v) SDS; 20% (v/v) Methanol

Block Buffer/Antibody Diluent

5% (w/v) Marvel /TBS

Wash buffer

0.5% Tween 20/TBS

Solutions for Nucleic acid electrophoresis

TAE Buffer

50X Stock TAE: 605g Trizma Base; 36.5g EDTA (acid free); 125ml glacial Acetic Acid made up to 2.5litres with RO water.

Solutions for *in situ* Hybridisation

20X SSC

3M Sodium Chloride; 0.3M Sodium Citrate pH 7.0

Solutions for bacterial cloning

LB Broth

1% (w/v) tryptone; 0.5% (w/v) Yeast Extract; 1% (w/v) Sodium Chloride, pH to 7.5 with Sodium Hydroxide. Antibiotic (Ampicillin) added 100μg/ml after autoclaving

LB Agar plates

15g/l agar was added before autoclaving and antibiotic (ampicillin) added at 100μg/ml when cooled to approximately 50°C before pouring onto 10cm plates.

Appendix 4: PCR Primers

F = Forward (sense) primer; R = Reverse (anti-sense) primer; all written 5' to 3'

E-Cadherin Primers

4F: AGG CCT CCG TTT GTG GAA TC

5R: CTT CAG CCA TCC TGT TTC TC

β-Catenin Primers

3F: AGC TGC TTT GAT GGA GTT GG

3R: GCT ACT TGT TCT TGA GTG AAG G

1F and 1R unknown gift from T Bailey.

β-Actin Primers

F: TGA CGG GGT CAC CCA CAC TGT

R: CTA GAA GCA TTT GCG GTG GAC

Appendix 5:Plasminogen IHC Raw Data

Abbrieviations: uPA = urokinase-type plasminogen activator, uPAr, urokinase-type plasmingeon activator receptor, PAI-1 plasminogen activator inhibitor type-1, PAI-2 plasminogen activator inhibitor type-2, and E-cad = Epithelial Cadherin.

Grading of staining: - = negative, +/- = weak, + = moderate, ++ = strong and +++ = very strong.

Sample	Diagnosis	PuPA	MuPA	uPAR	PAI-1	PAI-2	CD44	E-Cad
1	Barrett's	-	+	-	+	+/-	++	+
2	Barrett's	+	-	++	+	-	++	-
3	Barrett's	-	+/-	-	+/-	-	+	-
4	Barrett's	-	++	++	+	+	-	-
5	Barrett's	+	+	++	+	+	-	++
6	Barrett's	-	+	-	+	+/-	-	-
7	Barrett's	-	+	+	+	-	-	-
8	Barrett's	-	+	+	+	+	-	+
9	Barrett's	+	+	+/-	+/-	+/-	-	-
10	Barrett's	+/-	-	-	+	+	-	-
11	Barrett's	-	-	-	+/-	-	-	-
12	Barrett's	+	-	+	-	+/-	-	+
13	Barrett's	-	+/-	+	+/-	+/-	-	-
14	Barrett's	+/-	+/-	-	+/-	+	-	+/-
15	Barrett's	-	-	-	+/-	+/-	-	+/-
16	Barrett's	-	-	-	+	-	+/-	+/-
17	Barrett's	+	++	++	+++	+++	-	++
18	Barrett's	+	+	+/-	++	+	-	-
19	Barrett's	+	+	+/-	+	+/-	-	-
20	Cancer well diff	++	+/-	+	++	++	-	++
21	Cancer lymph node	+	+	++	+	-	++	+/-
22	Cancer poorly diff	+	+/-	-	+++	++	++	+/-
23	Cancer well diff	++	++	+++	+++	++	+/-	++
24	Cancer Lymph node	++	++	+	+++	+++	-	+
25	Cancer well diff	-	-	+++	+++	+++	-	-
26	Cancer Lymph node	+	+++	+++	++	+++	-	-
27	Cancer poorly diff	+	++	+	+++	+	-	-
28	Cancer poorly diff	+/-	+++	+	+++	++	-	-
29	Cancer mod diff	++	++	++	++	++	+/-	-
30	Cancer poorly diff	++	++	++	+	++	+	-
31	Cancer Lymph node	++	+	++	+/-	+	+/-	-
32	HGD	-	-	-	+/-	-	-	-
33	HGD	+	-	-	+/-	-	+	-
34	LGD	-	+	-	+/-	-	-	-
35	HGD	+	++	+	++	++	-	+
36	HGD	-	++	++	+	+/-	-	++
37	HGD	-	++	++	+	+/-	-	++
38	Barrett's	+	+	+/-	+/-	+/-	-	+/-
39	HGD	+	+	+/-	+	+/-	-	+/-
40	HGD	+/-	+/-	-	+	+	-	+/-
41	HGD	-	+/-	+/-	+	++	-	+/-
42	LGD	+/-	+/-	-	+	+/-	-	-
43	LGD	+/-	+/-	+/-	+	+/-	-	+/-

44	LGD	+/-	+/-	-	+	+/-	-	+/-
45	HGD	+/-	+	+/-	+	+	-	+
46	LGD	-	+/-	-	-	+/-	-	-
47	HGD/LGD	+	+	+	++	+	-	-
48	LGD	+	+	+/-	+	+	+/-	+
49	LGD	+/-	+/-	+	+	+	-	+
50	HGD	-	-	-	+/-	+/-	-	+/-
51	HGD	+/-	+	+	+	+/-	++	+/-
52	HGD	+	+	+/-	+	+	+/-	-
53	HGD	-	+/-	+/-	+	+/-	+	-
54	HGD/LGD	-	++	-	+++	+++	-	-
55	LGD	+	+	+	+	+	-	+/-
56	HGD	++	++	+	+	++	+/-	+
57	HGD	+	-	++	++	+	-	++
59	Barrett's	+/-	+/-	+/-	+	+	-	+/-
60	Barrett's	+	+	+	++	+	-	+/-
61	Barrett's	+/-	+	+	+	+	-	+
62	Barrett's	+/-	+/-	+	+	+/-	-	+/-
63	Barrett's	+	+	+/-	-	-	+/-	-
64	Barrett's	+	+	+/-	+	-	-	-
65	Barrett's	++	+	+/-	+	+	-	-
66	Barrett's	+	+	+/-	+	+	-	-
67	Barrett's	+	+	+/-	+	+/-	-	+/-
68	Barrett's	+/-	++	-	+/-	+/-	+/-	-
69	Barrett's	+/-	+	+/-	+	+/-	-	+/-
70	Normal Oesophagus	-	++	+	++	+/-	+/-	+/-
71	Barrett's	-	+/-	-	+	+/-	-	-
72	Barrett's	-	++	+	+/-	+	-	+/-
73	Barrett's	-	+	+	+	+/-	-	-
74	Barrett's	+/-	+	+/-	+/-	+/-	-	-
75	Barrett's	+/-	+/-	+	-	-	-	+/-
76	Barrett's	-	+	+	+/-	+/-	-	+/-
77	Barrett's	+	+	-	++	+	-	+/-
78	Barrett's	++	+	+/-	+	+/-	-	+/-
79	Barrett's	+	+	-	+	+/-	-	-
80	Cancer well diff	+	++	++	++	+++	-	-
81	Cancer Lymph node	++	++	+	+++	++	-	-
82	Cancer poorly diff	++	++	++	+++	+++	-	+/-
83	Cancer well diff	+	++	++	+++	++	-	-
84	Cancer Lymph node	+++	+++	+	+++	++	+/-	-
85	Cancer poorly diff	+++	++	++	+++	++	-	+/-
86	Cancer mod diff	+	+/-	+	+/-	+	-	-
87	Cancer poorly diff	++	++	++	++	++	+/-	-
88	Cancer	-	-	-	+	+	+	+
89	Cancer	+	-	-	+++	+++	+	-
90	Cancer Lymph node	-	+	++	++	++	+/-	-
91	Cancer well diff	+	+	++	++	++	+/-	-
92	Cancer Lymph node	+	+	++	+++	+++	-	+/-
93	Cancer poorly diff	+	+	+	+++	+++	-	-
94	Cancer poorly diff	+++	+++	+++	+++	++	+/-	+
95	Cancer poorly diff	++	++	++	+	++	++	+/-
96	Cancer poorly diff	++	+++	++	++	++	-	-
97	Cancer well diff	++	+++	++	+++	+++	-	-
98	Cancer poorly diff	++	++	+++	++	++	+/-	+/-
99	Cancer poorly diff	+++	++	++	++	++	-	-

100	Cancer Lymph node	+	++	++	++	+++	-	+
101	Cancer poorly diff	++	++	++	++	+++	-	-
102	Cancer Lymph node	++	++	++	++	++	-	+
103	Cancer poorly diff	+	+	+	+++	+	-	-
104	Cancer poorly diff	++	+++	+	+++	++	-	-
105	Cancer well diff	+++	++	++	+++	+++	-	-
106	Cancer Lymph node	+	+/-	+/-	++	+	+	-
107	Cancer poorly diff	+	+/-	+/-	++	++	+/-	-
108	Cancer mod diff	+	+	+	+++	+++	+	+/-
109	Cancer well diff	+++	++	+++	+++	+++	-	+/-
110	Cancer	++	+	+	++	+++	-	-
111	Cancer poorly diff	+/-	-	-	++	++	+	-
112	Cancer well diff	++	+++	++	+++	++	-	-
113	Cancer	+/-	++	++	+++	++	-	+/-
114	Cancer mod diff	++	++	+	+++	++	-	-
115	Cancer Lymph node	++	-	-	++	+++	+	-
116	Cancer	+/-	+	+/-	++	++	+/-	-
117	Cancer poorly diff	-	-	+/-	++	++	+/-	-
118	Cancer Lymph node	-	+/-	+	++	++	-	-
119	Cancer well diff	+	+	+/-	++	++	+/-	+
120	HGD	-	+/-	-	+/-	+/-	-	-
121	HGD	++	++	+	+++	+	+/-	++
122	HGD	+/-	+/-	+/-	+	+	-	+/-
123	Cancer poorly diff	++	++	++	+++	++	+	++
124	Cancer lymph node	+	+	+	++	++	+/-	+
125	Cancer well diff	+	++	+	+++	++	+	+
126	Normal Stomach	+	+	+	++	-	-	-

Appendix 6: Plasminogen ELISA Raw Data

Antigen Levels in ng/mg protein

Non-Involved Stomach

	uPA	uPAR	PAI-1	PAI-2
1	0.10	0.36	1.52	0.50
2	0.09	0.61	2.80	0.50
3	0.07	0.12	2.35	0.47
4	0.16	9.00	1.00	34.00
5	0.57	18.10	16.19	22.86
6	0.07	0.03	1.97	22.30
7	0.05	0.37	0.82	0.65
8	0.11	0.03	3.16	0.50
9	0.15	0.03	2.64	3.77
10	0.16	0.16	3.22	0.66
11	0.11	0.06	4.20	0.86
12	0.13	6.88	2.50	2.50
13	0.09	0.58	1.89	0.93
14	0.08	0.62	0.93	2.17
15	0.10	0.40	1.82	0.50
16	0.17	1.38	2.90	10.73
17	0.08	0.65	2.34	0.78
18	0.17	0.72	2.27	0.41
19	0.20	0.98	1.31	200
20	0.13	0.62	2.00	0.50
21	0.19	0.86	1.89	3.78
22	0.08	0.98	3.68	0.79
23	0.18	2.21	2.21	1.48
24	0.15	2.30	0.81	1.84
25	0.32	2.53	1.80	3.37
26	0.25	1.86	2.19	3.58
27	0.13	1.03	0.49	1.22
28	0.14	1.42	2.75	2.28
29	0.21	0.91	1.67	2.42
30	0.15	0.99	2.04	1.79
31	0.30	1.77	1.69	4.90

Non-involved Oesophagus

uPA	uPAR	PAI-1	PAI-2
0.07	0.74	0.74	200
0.05	1.12	0.81	200
0.05	0.27	0.81	200
0.24	1.87	1.33	3.5
0.07	0.03	1.05	39.6
0.10	0.138	1.72	62.1
0.11	0.03	3.33	200
0.05	0.94	1.62	200
0.11	0.98	1.43	200
0.04	0.26	0.71	200
0.32	4.02	5.37	200
0.08	0.34	1.17	200
0.05	0.38	2.28	200
0.12	0.25	1.18	200
0.17	0.64	1.74	200
0.46	1.57	2.29	200
0.46	0.25	2.32	200
0.11	0.75	10.97	200
0.21	1.03	2.89	200
0.31	1.39	1.54	75.1
0.22	1.36	0.81	200
0.28	1.88	1.50	200
0.47	2.33	1.86	4.70

Non-dysplastic Barrett's Metaplasia

	uPA	uPAR	PAI-1	PAI-2
1	0.20	0.03	3.00	4.00
2	0.17	0.03	1.67	0.50
3	0.16	1.35	1.08	1.62
4	0.05	1.19	0.95	0.50
5	0.27	1.71	1.42	1.65
6	0.69	2.41	4.14	50.35
7	1.13	3.75	157.5	95.00
8	0.83	5.00	6.67	1.67
9	0.10	0.34	1.68	0.50
10	0.60	0.03	3.00	2.00
11	0.21	0.03	3.45	1.38
12	0.59	5.19	5.93	0.50
13	0.62	3.08	6.15	35.39
14	0.39	2.40	1.15	2.31

Low Grade Dysplasia

uPA	uPAR	PAI-1	PAI-2
0.17	0.03	2.50	0.50
0.36	0.03	3.03	0.50
0.23	0.03	1.54	0.50
0.17	0.53	1.28	0.85
0.27	0.33	0.67	14.00
0.25	1.67	3.33	5.83
0.30	4.55	3.64	0.5
0.82	0.91	3.64	6.36
0.22	1.80	1.71	0.63
0.63	2.11	4.21	200

	High Grade Dysplasia				Adenocarcinoma			
	uPA	**uPAR**	**PAI-1**	**PAI-2**	**uPA**	**uPAR**	**PAI-1**	**PAI-2**
1	0.46	1.54	3.08	4.62	0.28	0.90	0.30	3.40
2	0.28	0.35	2.76	2.07	1.21	3.23	12.93	5.25
3	0.77	1.70	2.98	5.96	1.19	5.17	4.79	200
4	0.48	0.03	2.40	9.60	1.00	3.75	6.67	10.83
5	0.4	0.03	8.00	112	2.37	5.05	38.45	33.01
6	0.39	0.39	3.08	9.23	1.54	0.03	6.15	7.69
7	0.86	2.57	5.14	200	1.55	4.78	13.22	3.65
8	0.75	0.51	2.29	67.43	0.96	0.01	3.95	2.47
9	0.51	2.18	2.91	200	0.64	2.03	5.8	5.80
10	0.71	2.86	7.14	200	0.57	1.42	3.27	17.14
11					1.39	2.38	6.96	17.39
12					2.20	10.67	12.14	200
13					0.59	5.08	2.30	1.64
14					0.56	2.00	3.60	8.80
15					1.03	5.59	89.39	4.69
16					0.17	1.43	4.00	12.57
17					0.26	1.07	2.42	3.18
18					0.26	0.75	4.37	68.39
19					1.28	3.99	11.28	200
20					0.80	3.20	17.60	13.60
21					1.97	12.28	11.93	14.04
22					2.35	4.94	44.56	3.21
23					0.87	6.51	6.79	2.83
24					0.58	2.84	6.56	6.18
25					0.93	4.51	5.13	10.77
26					1.03	7.35	6.67	8.49
27					0.94	8.51	20.43	1.70
28					0.55	4.85	3.64	6.06
29					0.79	5.56	21.17	4.62
30					0.60	3.00	7.00	130

Appendix 7: Statistical Analysis of Plasminogen ELISA

Standard Deviation — reflects the varience of the data from the mean.

Varience — square of the standard deviation.

Median — middle value.

Urokinase-type Plasminogen Activator

	N Stom	N Oes	Barrett's	LGD	HGD	Cancer
Number	31	23	14	10	10	30
Mean	0.158	0.180	0.429	0.342	0.561	1.015
Median	0.140	0.110	0.330	0.260	0.495	0.935
Lower Quartile	0.090	0.07	0.170	0.220	0.400	0.580
Upper Quartile	0.180	0.28	0.620	0.360	0.750	1.280
Varience	0.010	0.02	0.102	0.046	0.038	0.366
Standard Deviation	0.100	0.141	0.320	0.214	0.196	0.605
Standard Error of the mean	0.018	0.029	0.086	0.068	0.062	0.110

Urokinase-type Plasmingen Activator Receptor

	N Stom	N Oes	Barrett's	LGD	HGD	Cancer
Number	31	23	14	10	10	30
Mean	1.359	1.035	1.896	1.199	1.216	3.569
Median	0.790	0.940	1.530	0.720	1.025	3.490
Lower Quartile	0.400	0.270	0.30	0.03	0.350	1.715
Upper Quartile	1.420	1.390	3.080	1.800	2.180	5.065
Varience	3.753	0.833	2.003	2.003	1.172	4.873
Standard Deviation	1.937	0.913	1.415	1.415	1.082	2.208
Standard Error of the mean	0.354	0.190	0.448	0.448	0.342	0.417

Plasminogen Activator Inhibitor Type-1

	N Stom	N Oes	Barrett's	LGD	HGD	Cancer
Number	31	23	14	10	10	30
Mean	2.842	2.151	3.099	2.555	3.978	10.172
Median	2.190	1.540	3.000	2.765	3.030	6.670
Lower Quartile	1.690	1.050	1.420	1.540	2.760	4.000
Upper Quartile	2.800	2.290	4.140	3.640	5.140	12.140
Varience	8.800	4.792	4.222	1.427	4.233	104.261
Standard Deviation	2.967	2.189	2.005	1.195	2.057	10.211
Standard Error of the mean	0.533	0.456	0.570	0.378	0.651	1.896

Plasminogen Activator Inhibitor Type-2

	N Stom	N Oes	Barrett's	LGD	HGD	Cancer
Number	31	23	14	10	10	30
Mean	4.435	164.591	14.098	3.297	81.091	10.669
Median	1.635	200	0.500	0.630	38.515	6.120
Lower Quartile	0.650	200	0.500	0.500	5.960	3.40
Upper Quartile	3.580	200	4	5.830	200	12.570
Varience	62.597	4914.207	774.343	21.843	7952.423	185.195
Standard Deviation	7.912	70.101	27.827	4.674	89.176	13.609
Standard Error of the mean	1.444	14.617	7.437	1.558	28.2	2.669

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