Biomarkers of Oesophageal Neoplasia

A thesis submitted towards the degree of

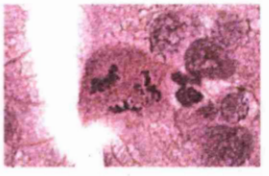
Master of Science in the Faculty of Medicine, University of Glasgow

by

Lisa J. Neilson BSc (Hons)

September 2003

-o-0-o-



Tripolar mitosis in
dysplastic Barrett's mucosa

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Abstract

Background. Symptomatic oesophageal cancer is usually advanced and terminates fatally. There is a need for biomarkers to help pathologists recognise patients at increased risk of squamous and glandular oesophageal cancers (in the case of Barrett's oesophagus). The work described in this thesis looks at four topics: cytokeratin 7 and 20 phenotypes in Barrett's intestinal metaplasia; the distribution of cardiac, cardio-oxyntic and 'specialised' intestinal-type Barrett's mucosa in relation to glandular dysplasia; dysregulated expression of the proliferation marker Ki67 and the DNA replication-licensing proteins Mcm2 and Mcm5 in glandular and squamous dysplasia; and relates different types of complete (type I) and incomplete (types IIA/IIB) intestinal metaplasia to dysplasia/neoplasia in Barrett's oesophagus.

Results. Cytokeratin 7 and 20 phenotypes are complex and variable, so likely clinical utility is not immediately obvious. The ubiquitous presence of intestinal metaplasia (IM) in Barrett's mucosa is confirmed, and a very strong association of IM with dysplasia. A positive association exists between dysplasia and type IIB intestinal metaplasia, and an even stronger negative association with type I intestinal metaplasia. This identifies a possibility that robust markers of (complete) small intestinal metaplasia may be associated with low dysplasia risk in Barrett's oesophagus, whereas a potentially colonic phenotype (type IIB IM) is associated with greater risk.

Over-expression of the cell-proliferation related antigen Ki67 and the DNA replication licensing proteins Mcm2 and Mcm5 in the differentiated surface compartment of squamous oesophageal epithelium and Barrett's mucosa confirm the Mcm proteins are promising markers of oesophageal dysplasia meriting further evaluation, e.g. in the field of non-endoscopic oesophageal cytology.

Contents

List of abbreviations

Ck	Cytokeratin
DAB	Diaminobenzidine
EDTA	Ethylene diamine tetra-acetic acid
GORD	Gastro-oesophageal reflux disease
HGD	High grade dysplasia
IM	intestinal metaplasia
LGD	Low grade dysplasia
LSBE	Long segment Barrett's oesophagus
Mcm	Minichromosome maintenance proteins
OGJ	Oesophago-Gastric Junction
ORC	Origin of replication complex
Cdc	Cell division cycle
PPI	Proton-pump inhibitor
Pre-RC	pre-replication complex (of DNA replication)
SCC	Squamous cell carcinoma
SSBE	Short segment Barrett's oesophagus
TRIS	Tris(hydroxymethyl)aminomethane

List of Tables

List of figures

Acknowledgements

I thank my supervisors, Dr James Going and Mr Robert Stuart in the Departments of Pathology and Surgery at Glasgow University, for giving me the opportunity to do this work and their encouragement and support during the practical work and subsequent writing of this thesis.

Declaration

I performed and am responsible for the work described in this thesis. References to work or data generated by others are specifically identified where they occur.

Lisa J. Neilson

September 2003

Chapter 1: General Introduction

The Clinical Challenge of Oesophageal Cancer

Squamous carcinoma and adenocarcinoma of the oesophagus together rank amongst the top ten cancers in the world, and oesophageal adenocarcinoma was increasing more rapidly than any other cancer in the United States and Europe during the 1990s [1,2].

Two distinct types of dysplasia are associated with oesophageal cancer: glandular dysplasia in Barrett's oesophagus is considered precancerous for adenocarcinoma, and squamous dysplasia for squamous cell carcinoma (SCC). Barrett's adenocarcinoma is frequent in the United States and other western countries, whereas squamous cancer is particularly common in Asia, including China and Japan.

Mortality for oesophageal cancer is close to its incidence, as most patients present late with symptoms of advanced disease, when treatment is relatively ineffective [2]. In Barrett's oesophagus, progression may be detected in patients attending an endoscopic surveillance program, which is a recommended clinical management strategy for this disease. However, despite a better prognosis for surveillance detected cancer [3], there is debate whether the low absolute risk of oesophageal cancer in Barrett's oesophagus justifies the expense of surveillance endoscopy and biopsy. While studies of the incidence of oesophageal cancer suggest a three- to five-fold increase over the last 30-40 years, the overall number of individuals with oesophageal adenocarcinoma remains relatively low [4,5]. It has been calculated that even if there was a treatment that halved the risk of progression to carcinoma in Barrett's oesophagus, 400 patients would have to be treated to prevent one case of cancer per annum [6].

Better understanding of molecular events leading to carcinoma of the oesophagus may, ultimately, allow treatment to be focused towards minimising disease progression in those most at risk. This applies equally to patients in the West, with Barrett's oesophagus and elevated adenocarcinoma risk, and Asian populations susceptible to squamous cancer.

Oesophageal Squamous Cancer

Squamous epithelial dysplasia is thought to be the precancerous lesion in SCC: approximately 70% of patients with squamous dysplasia eventually develop SCC with a 5 year survival rate of 5-35% [7]. Up to the 1990s, when it was overtaken by adenocarcinoma, squamous cell carcinoma was the predominant form of oesophageal cancer in the United States. However SCC of the oesophagus remains one of the most common cancers in China with ~250,000 cases diagnosed every year [8].

Epidemiological studies have identified tobacco smoking [9], alcohol drinking, dietary habits and environmental agents, such as nitrosamines, as major risk factors in squamous cell carcinomas of the oesophagus [8,10].

The incidence of oesophageal SCC varies markedly throughout China but even in high-risk areas only a small proportion of people develop it, suggesting possible host susceptibility factors [8]. Genetic polymorphisms in carcinogen-metabolizing enzymes may modify individual susceptibility to cancer. Tan *et al* [8] demonstrated a significant association between oesophageal SCC and genetic polymorphisms of *CYP2E1*, an enzyme responsible for the metabolic activation of many carcinogens including nitrosamines, which is expressed at significant levels in human oesophagus. Such genetic markers could prove useful in targeting resources at groups most at risk of disease progression.

Oesophageal Adenocarcinoma

In western countries, especially the US and UK, the frequency of adenocarcinoma of the oesophagus has increased substantially over several decades [11]. Adenocarcinomas of the oesophagus mainly develop from Barrett's oesophagus (figure 1), a condition in which a variable length of the non-keratinising stratified squamous epithelium, which lines the normal oesophagus, is replaced by an acid-resistant columnar, glandular mucosa.

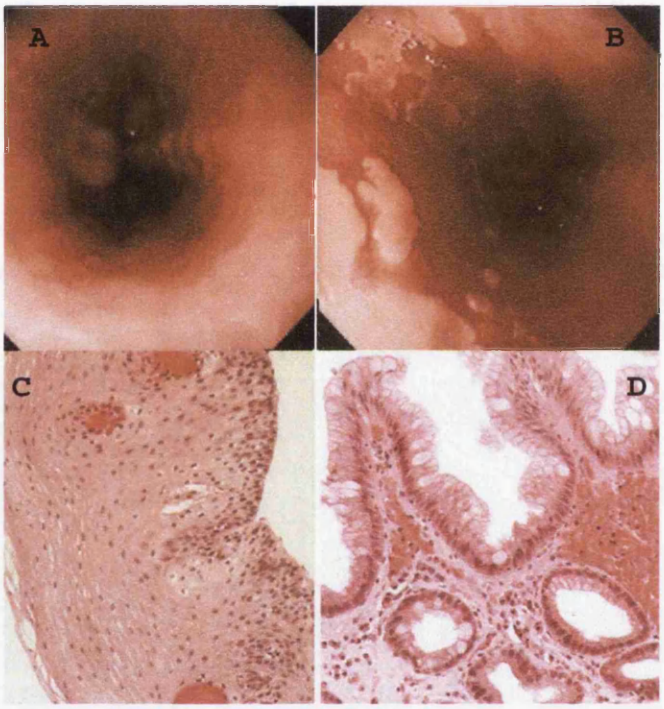


Figure 1/1: endoscopic and histological appearance of normal and Barrett's oesophagus. A: Normal endoscopy looking towards OG junction. B: Red velvety Barrett's mucosa contrasts with pale squamous mucosa. C: Oesophageal stratified squamous epithelium. D: Barrett's mucosa. The goblet cells are characteristic of intestinal metaplasia.

Glandular metaplasia is thought to follow chronic gastro-oesophageal reflux, and patients with Barrett's oesophagus have up to a 100 fold higher risk of developing adenocarcinoma of the oesophagus than

the general population [12]. Unfortunately, most Barrett's cancers are incurable at diagnosis, with overall 5 year survival only 7% [13].

Diagnosis of Barrett's Oesophagus

It is estimated 30% of adults in western countries complain of heartburn at least once a month, a third of whom will have endoscopic evidence of oesophagitis. 40% of patients with oesophagitis will improve spontaneously, but 50% will have persistent oesophagitis and 10% will progress to Barrett's metaplasia [14]. Barrett's oesophagus is much more common among men than women and is present in approximately 10% of all patients undergoing routine GI upper endoscopy with chronic gastroesophageal reflux symptoms, but how well this reflects the prevalence of the condition in the general population is not known [13,14]. The prevalence of Barrett's oesophagus in the population is also uncertain, as many people with Barrett's oesophagus do not have reflux symptoms, perhaps due to the greater resistance of the columnar-lined oesophagus to acid reflux. Lastly, many patients have symptoms which are not investigated, so there is potentially a large number of undiagnosed cases. Based on an autopsy series, Cameron *et al* [15] estimated that for every known patient with Barrett's oesophagus there might be 20 unrecognised cases in the general population.

Three mucosal patterns have been described in the columnar lined oesophagus. These resemble either gastric or intestinal mucosa [16,17];

a) Gastric cardiac (so-called junctional type) mucosa resembles normal cardiac epithelium and has typical mucus secreting cells lining the surface pits and simple mucus-secreting glands.

b) Gastric fundic type mucosa is characterised by mucus cells on the surface with chief and parietal cells in the deeper parts of the glands.

c) Intestinal type mucosa has a villous surface lined by columnar and goblet cells. Deep in the mucosa are clear mucus secreting glands

with enterochromaffin cells and sometimes Paneth cells. This is often called 'specialised' mucosa.

No correlation has been found between the type of mucosa in Barrett's oesophagus and the degree of inflammation, ulceration, stricture formation or length of columnar segment. In most cases there is a mixture of phenotypes with specialised intestinal-type mucosa the most common [16-19]. Classically, a diagnosis of Barrett's oesophagus is established when columnar epithelium extends for several cm above the oesophago-gastric junction (OGJ) [20,21]. An alternative classification does not rely on endoscopic measurements, which may be imprecise, but on the presence of a columnar-lined oesophagus regardless of length so long as it is associated with 'specialised' intestinal metaplasia [20].

Long segment Barrett's oesophagus is defined as the abnormal presence of specialised intestinal metaplasia in a segment of columnar epithelium extending 3cm or more into the oesophagus from the OGJ. Short segment Barrett's oesophagus is defined as the presence of specialised intestinal metaplasia in a segment less than 3cm above the OGJ [21].

The prevalence of short segment Barrett's oesophagus has been reported at 8-32% in routine upper gastrointestinal endoscopy. There is the same white male predominance in both short and long segment Barrett's oesophagus, although the male predominance may be less in short segment Barrett's oesphagus. The length and duration of upright and supine acid exposure are greater in long segment Barrett's oesophagus and there is a greater lower oesophageal sphincter incompetence and impairment of oesophageal peristalsis [21]. Reflux symptoms are reported by 58-82% of short segment Barrett's oesophagus patients and 60% of long segment Barrett's oesophagus patients [22].

Early adaptive responses to increased cell loss in reflux oesophagitis is trophic stimulation by locally produced growth factors leading to an increased thickness of the proliferative zone and increased proliferative zone length with folding of the basal epithelium and

papilla formation. The functional stem cells at the tip of the papillae remain in a relatively superficial position leaving them exposed to reflux.

Intestinalization of the cardiac mucosa occurs when cardiac-like columnar cells develop acid rather than neutral mucins and goblet cells appear, but the specific cellular events that induce the change from cardiac mucosa to specialised intestinal mucosa are unknown.

De Meester and De Meester [23] suggested that patients with >3cm of intestinalized Barrett's mucosa had, on average, a longer duration of reflux symptoms (10 v. 5 years) and a greater exposure to refluxed bilirubin than patients with <3cm of Barrett's oesophagus without intestinal metaplasia. Barrett's oesophagus in children differs from the adult variety in that intestinal mucins and cytokeratins are not present, and typically adults show an inflammatory cell infiltrate which is less common in juvenile Barrett's metaplasia.

Barrett's oesophagus has been described following caustic burns and chemotherapy, but it is accepted that the replacement of oesophageal squamous epithelium by columnar mucosa is usually acquired secondary to gastro-oesophageal reflux [14].

When a patient is found to have Barrett's oesophagus, the length of time it has been present is usually unknown. Cameron *et al* [24] estimated the mean age of development of Barrett's to be 40 years with a mean age of diagnosis 63 years.

Pathogenesis of Barrett's oesophagus

Intraoesophageal pH recordings have established that patients with Barrett's oesophagus have more acid reflux events and longer acid clearance times compared to oesophagitis patients without columnar mucosa [25]. Patients with Barrett's oesophagus frequently have impaired oesophageal motility with low contraction amplitudes and an increased frequency of abnormal waveforms in the distal oesophagus compared with

normal subjects [23]. Bremner [26] argued that the columnar-lined oesophagus was a protective response to acid reflux and thought it unlikely that further acid reflux would be involved in the pathogenesis of Barrett's complications. Barrett's oesophagus has developed after total gastrectomy, so acid reflux is not the sole factor responsible for this disease [27]. Elevated intragastric bile acid concentrations in patients with complications of Barrett's oesophagus such as strictures, ulcers, or adenocarcinomas may indicate that these patients have a diffuse motor abnormality of the upper gastrointestinal tract involving the lower oesophageal sphincter and the pylorus [25]. Lagergren et al [28] found that lower oesophageal sphincter relaxing medications were associated with increased risk for oesophageal adenocarcinoma, probably through promotion of reflux.

Structurally defective lower oesophageal sphincters have been demonstrated in more than 90% of those with Barrett's oesophagus. Patients who reflux both gastric and duodenal juice have been shown to have a higher prevalence of oesophagitis than those who only reflux gastric juice. Additionally, among patients with Barrett's oesophagus, significantly greater oesophageal bilirubin exposure (a marker of duodenal reflux) has been demonstrated in those in whom dysplasia develops [23].

Evidence is accumulating that bile salts are a toxic component in refluxed duodenal juice and their ability to cause cellular injury is pH dependent. For bile salts to enter mucosal cells and cause injury they must be soluble and un-ionized. At pH 7 > 90% of bile salts are in solution and completely ionized. Acidification of bile to a pH of < 2 produces irreversible precipitation so, under normal physiologic gastric conditions, bile acids precipitate and are of minimal significance. However, in a less acid gastric environment, as can occur with pharmacological acid suppression, bile salts are partially dissociated at pH 3-5 and a mixture of ionized salts and un-ionized salts are present. Un-ionized bile salts can rapidly cross mucosal cell membranes. Once

inside the alkaline environment of the cell they are converted back to their ionized form, so become trapped within the cell, accumulate and become toxic to the mitochondria.

For bile acids to remain completely ionized and innocuous in a patient with reflux disease, a pH of 7 would have to be maintained 24 hours a day. One study demonstrated that normal volunteers taking 20mg of Omeprazole twice a day spend more than 30% of a 24 hour period with a gastric pH of less than 4, therefore insufficient doses of medication may allow oesophageal mucosal cell injury to occur while the patient remains relatively asymptomatic [29].

The hydrogen-ion ('proton') pump inhibitor (PPI) Omeprazole is largely metabolised by cytochrome P450 isozyme CYP2C19. This isozyme is absent in about 3% of caucasians and 12-20% of orientals. Poor metabolizers, heterozygous extensive metabolizers and homozygous extensive metabolizers represent 3%, 30% and 67% of caucasians. Poor metabolizers are exposed to Omeprazole to a larger extent than extensive metabolizers and as Omeprazole is widely used to inhibit gastric acid secretion in the treatment of gastro-oesophageal reflux disease, the individual's capacity to metabolize the drug will influence its efficacy [30]. As many patients with Barrett's oesophagus have few or no reflux symptoms, probably as a consequence of an altered sensitivity of the metaplastic epithelium to the refluxed acid, their symptoms are an unreliable guide to treatment and eradication of heartburn does not ensure elimination of acid reflux. Increasing the dose of Omperazole until symptoms were alleviated was shown to be an unreliable measure of effective therapy by Katza et al as 80% of these patients were found to have abnormal oesophageal acid exposure on 24-hour pH monitoring [31].

The main aim of therapy for Barrett's oesophagus is to control symptoms. Patients must continue medication indefinitely, usually by proton pump inhibition, but even high dose therapy may not prevent nocturnal acid regurgitation and the advent of increasingly potent inhibitors of acid production over the last 20 years has not prevented

the rising incidence of oesophageal adenocarcinoma [28,32]. There is no medical treatment as yet for alkaline reflux of bile or pancreatic juice and standard medical antireflux therapy does not reduce the length of the Barrett's segment [17]. Antireflux surgery with fundoplication can be performed to control acid exposure in the short term, but adenocarcinoma progression after fundoplication has been documented [17,33]. There is speculation that medical therapy of prolonged and inadequate acid supression may actually promote the development of Barrett's oesophagus. One study reported that the risk of oesophageal adenocarcinoma was three times higher among patients who used medication for symptoms of reflux compared to those who did not [34].

The presence of a gastric type mucosa within the oesophagus is a prerequisite for Helicobacter pylori (*H. pylori*) colonisation and *H. pylori* may contribute to the severity of inflammation in Barrett's epithelium. There is wide variation in the reported prevalence of *H.pylori* in Barrett's oesophagus. Henihan et al [35] found *H. pylori* in gastric mucosa only and more commonly associated with chronic moderate to severe inflammation. In the presence of *H. pylori* 89.5% of Barrett's patients had moderate to severe chronic inflammatory changes in contrast with 36.5% of those without.

H. pylori produces urease, vacuolase cytotoxin (vac A), cytotoxin-associated gene A (cag A) and phospholipidases which may directly injure the mucosa. *H. pylori* oesophageal infection is invariably associated with gastric colonisation. In some patients with *H. pylori* gastritis, serum gastrin is elevated with diminished somatostatin expression, which leads to elevated gastric acid secretion and may worsen oesophageal acid injury.

In Henihan's study [35] none of those with evidence of dysplasia or adenocarcinoma associated with Barrett's oesophagus had evidence of *H. pylori* colonisation. Henihan et al postulated that altered properties of

the intercellular junction (the usual site for *H. pylori* attachment) were preventing *H. pylori* adherence.

Oxygen-derived free radicals, the superoxide anion especially are involved in the pathogenesis of reflux oesophagitis. Neutrophils, macrophages and monocytes are the main sources of this radical and anti-inflammatory drugs may reduce acid and pepsin induced injury. A mixed inflammatory infiltrate is common in peptic oesophagitis, especially in the stem-cell rich areas of the basal mucosal compartment and papilla. This infiltrate is initially composed of acute inflammatory cells, subsequently T lymphocytes become more numerous, especially in tissues in which metaplastic foci develop. Even if acid reflux is suppressed, Barrett's metaplasia maintains a mild chronic inflammatory infiltrate [36].

Genetic factors may play a role as individuals with Barrett's oesophagus, oesophageal adenocarcinoma or oesophagogastric junctional adenocarcinoma are more likely to have a positive family history of these diseases than individuals without Barrett's oesophagus, oesophageal adenocarcinoma or oesophagogastric junctional adenocarcinoma. Barrett's oesophagus has a familial association and may occur in twins. Many patients with gastro-oesophageal reflux disease (GORD) have relatives with reflux symptoms and several families have multiple members having Barrett's oesophagus and sometimes oesophageal adenocarcinoma, often involving more than one generation [37]. Some family members had oesophagitis without Barrett's oesophagus, suggesting that the inherited factor might be a propensity to reflux. Patients with a first degree relative with GORD are twice as likely to have GORD themselves whereas those reporting a spouse with GORD are not [38]. So a positive family history could be of consideration when making decisions about screening patients with gastro-oesohageal symptoms.

Stratifying Cancer Risk in Barrett's Oesophagus

2-5% of patients with Barrett's oesophagus will eventually develop oesophageal adenocarcinoma, which appears usually to develop through a metaplasia-dysplasia-adenocarcinoma sequence [2,14]. Although different studies quote varying levels of risk, there is an estimated 30-40 times greater incidence of adenocarcinoma of the oesophagus in patients with Barrett's oesophagus than in the general population [39].

In order to target resources towards surveillance and treatment of those most at risk of developing oesophageal adenocarcinoma, it is necessary to utilise known risk factors associated with progression of the disease.

It is recognised that specialised intestinal-type Barrett's metaplasia measuring any length is associated with all grades of dysplastic change and carries a higher risk of malignant change [40,41]. Skinner [42] suggested and Reid [13] and then Haggitt [17] confirmed that only the intestinal type of columnar mucosa is definitely premalignant, although cases of adenocarcinoma associated with the pure fundic or junctional types of columnar metaplasia have also been reported [43]. Specialized intestinal metaplasia of the oesophagus is retrospectively diagnosed in 79-100% of oesophageal adenocarcinomas and in 42-73% of adenocarcinomas at the OGJ [3].

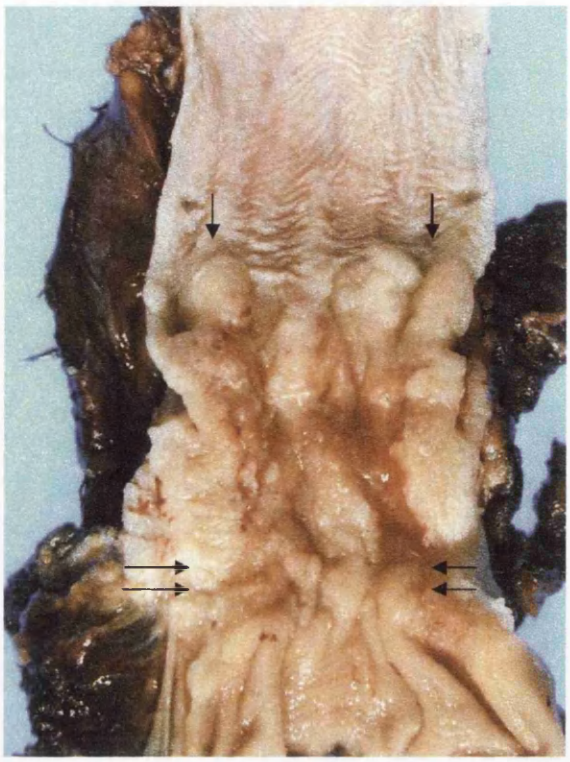


Figure 1/2: Adenocarcinoma arising in Barrett's oesophagus. This advanced lesion presented with dyspagia. The prognosis is poor. Top - squamous lined oesophagus. Bottom - gastric mucosa. Single arrows - proximal limit of Barrett's mucosa. Double arrows - oesophagogastric junction.

Progression to cancer in patients with Barrett's oesophagus is associated with loss of cell cycle regulation, accumulation of multiple genetic abnormalities and the appearance of multiple aneuploid cell populations [10,44-47]. Overexpression of p53 and increased proportions of cells with a G2 DNA content are observed in Barrett's oesophageal metaplastic epithelium, probably as a result of chronic cell and DNA damage induced by gastric reflux [10,45-47]. Subsequent p53 mutations and 17p allelic losses result in the inactivation or alteration of the p53 gene which disrupts the G1/S transition control and aneuploid cell populations develop [10].

Neshat et al [2] found aneuploid cell populations in 86% of high grade and/or adenocarcinoma in Barrett's oesophagus, with different aneuploid populations occupying defined but sometimes overlapping spatial distributions. Apoptosis may also be inhibited late in a proportion of dysplastic cells, and over-expression of various oncogenes and growth factors have been reported [14]. Barrett's oesophagus patients with increased G2 fraction or aneuploid cell populations could be targeted for more frequent endoscopic surveillance. P53 mutation is not essential for malignant transformation in Barrett's oesophagus so p53 abnormality alone may not reliably predict malignant progression [44]. Neither of these possible strategies has been validated in practice.

Recognising Dysplasia

The reported prevalence of adenocarcinoma in Barrett's oesophagus averages approximately 10% at the time the initial diagnosis of Barrett's oesophagus is made [2,17,41]. Adenocarcinoma patients usually present with advanced disease [33,41] and have a 5-year survival of only 5-10%, compared to 50% for early lymph-node negative disease and 80% for high grade dysplasia/carcinoma *in situ* or stage 1 cancer following oesophagectomy [33]. The prognosis of oesophageal cancer is related to the depth of invasion and spread to adjacent lymph nodes [48]. Unfortunately Barrett's adenocarcinomas are rarely discovered in time for cure and 93% of patients who develop adenocarcinoma will die of the disease [2], hence the need for surveillance to detect dysplasia at its earliest stage.

A sequence from mild or low grade dysplasia to severe or high grade dysplasia with progression to carcinoma is thought to occur, based on the frequent finding of high grade dysplasia in the mucosa surrounding adenocarcinoma, and the progression of high grade dysplasia to adenocarcinoma in prospective series [49]. Dysplasia is confined to the basement membrane but does not arise from the basement membrane. In low grade dysplasia the nuclei are enlarged, crowded and hyperchromatic,

but confined to the lower half of the cells. Goblet and columnar cell mucus are diminished. In high grade dysplasia there is distortion of the crypt architecture with 'back to back' glands. The abnormalities seen in low grade dypsplasia are more pronounced in high grade dysplasia and nuclear abnormalities reach the luminal surface of the cells. Epithelium that is atypical but not definitely dysplastic can be classified as 'indefinite for dysplasia'.

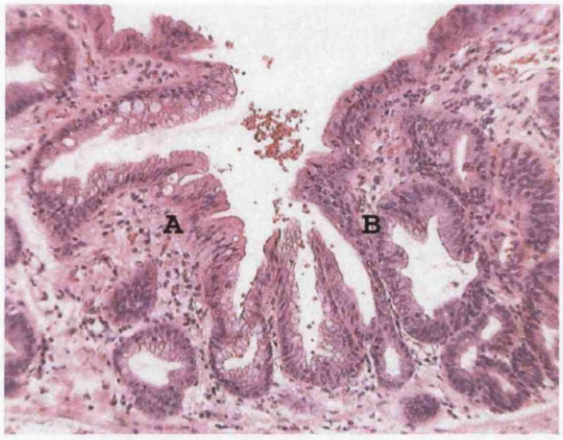


Figure 1/3. On the left of this photomicrograph there is typical 'intestinal type' Barrett's mucosa (A). In the middle there is an abrupt transition to moderate (low grade) glandular dysplasia (B). Characteristically dysplasia involves the mucosal surface.

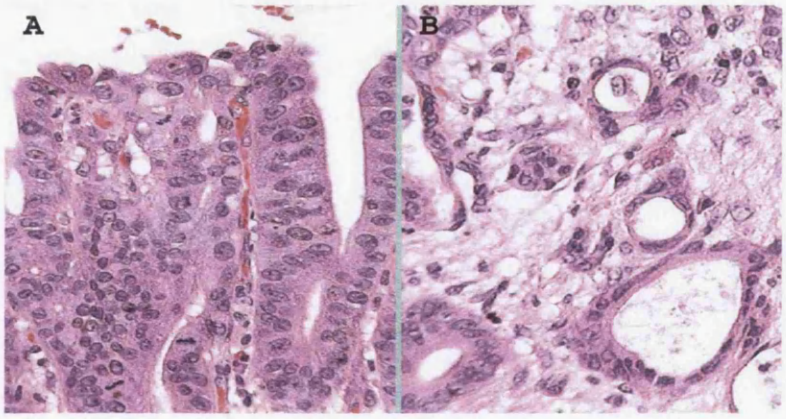


Figure 1/4. A: High grade Barrett's dysplasia (severe dysplasia /adenocarcinoma in situ). ß : intramucosal Barrett's adenocarcinoma.

The ability to detect curable neoplastic disease in patients with Barrett's oesophagus is a prerequisite for prospective endoscopic surveillance. Once invasive adenocarcinoma develops it may intially be difficult to detect as is reliable endoscopic differentiation between HGD alone and HGD with early foci of adenocarcinoma. Dysplasia may occur in the absence of any endoscopic lesion and may be hidden underneath regenerated squamous epithelium [32,50].

Four quadrant biopsies at 2cm intervals or less throughout the length of the Barrett's oesophageal segment plus biopsies of any lesions are usually recommended to minimise sampling error [17]. The intensity of surveillance varies from a 3 yearly interval for those in whom dysplasia has never been seen to 6-12 monthly in low grade dysplastic cases [32]. The treatment of those with high grade dysplasia is controversial, some advocate oesophagectomy [50], others close endoscopic surveillance every 3-6 months [13,51].

The ability to distinguish high grade dysplasia and early adenocarcinoma from lesser histological abnormalities is acceptable but imperfect (average interobserver agreement of 85-87%)[17]. The ability to differentiate intramucosal adenocarcinoma from high grade dysplasia may require examination of numerous tissue sections to detect early invasion. Also, the diagnosis of invasive adenocarcinoma arising from the bases of dysplastic glands may be compromised because endoscopic samples may be inadequate. Significant interobserver and intraobserver variation exists in grading dysplasia at the indefinite/low grade interface [17].

A confirmed endoscopic biopsy diagnosis of early Barrett's adenocarcinoma often leads to surgery, but the diagnosis of high grade dysplasia poses the question of whether surgery should follow.

The risk of progression from high grade dysplasia to cancer is high [17,32,33], with adenocarcinoma that was unidentified preoperatively in approximately 40% of oesophagectomy cases [32,33,50]. These patients are

likely to have resectable disease and have an improved five years survival compared with patients with symptomatic cancer detected outside a surveillance program [32]. On the other hand, some high grade dysplastic cases have regressed, 27% in one study to lesser grades of dysplasia and even to non-dysplastic mucosa [32]. Operative mortality of 3-6% and significant morbidity as high as 40% [32,52] are an obvious consideration especially since high grade dysplasia can sometimes be eradicated by endoscopic treatments including photodynamic therapy [52], which involves light-induced activation of an administered photosensitiser in tissue to produce local necrosis. However, only tumours at an early stage allow a good response to photodynamic therapy, so such treatment appears to have its greatest potential benefit in treating patients with HGD and early Barrett's adenocarcinoma, especially those who are high risk candidates for operation. Another ablation therapy is multipolar electrocautery using a probe that transmits electrical energy to the epithelium to cause a superficial injury, although in some cases ablation therapy has led to re-epithelialization over intestinal metaplasia which may still harbour a neoplastic risk [33,52]. Endoscopic mucosal resection is another minimally invasive therapeutic strategy which also does not carry the risk of major surgery.

Young fit patients with HGD in Barrett's oesophagus should still be offered operation for fear of missing early cancer. In patients at risk, surgery may be reserved until endoscopic surveillance detects evidence of invasion. So there is ongoing debate about the clinical management of patients in whom endoscopic biopsy samples show only high grade dysplasia and any methods that could assist identification of cancer development and reduce diagnostic error in patients with Barrett's oesophagus would prove very useful.

The need for improved surveillance

The cost of endoscopic surveillance is considerable and its value has been questioned [53], which has led to studies evaluating non-endoscopic surveillance techniques for Barrett's patients using balloon cytology [34] and nasogastric brush cytology [54].

Mass screening with balloon cytology is well described in China for the detection of oesophageal squamous cell carcinoma and dysplasia [55]. Falk et al [34] found balloon cytology detected 80% of patients with high grade Barrett's dysplasia and/or adenocarcinoma when sampling was adequate. The potential cost savings of these techniques supports further studies into their potential in the surveillance and follow up of Barrett's oesophageal patients. Theoretically, sampling errors could be reduced by assessing brushings taken from the whole of the Barrett's epithelium, as foci of dysplasia or cancer can seem macroscopically normal so could be missed by endoscopy and biopsy.

Some patients whose biopsies show histologically abnormal tissue will progress to cancer whereas others will remain stable or even regress. A better understanding of the early events in neoplastic progression in Barrett's oesophagus might indicate those patients a risk of subsequent progression to adenocarcinoma. More than three million people in the United States have Barrett's oesophagus. There is a clear need to stratify the risk for progression to adenocarcinoma in these patients. To date the most predictive factor is degree of dyplasia. New methods of detecting dysplasia and more specific markers for neoplasia should improve the reliability of surveillance.

The work described in this thesis addresses four areas of interest in Barrett's oesophagus. These are: a, the possible existence of characteristic cytokeratin 7 and 20 phenotypes in Barrett's metaplasia (which might be useful in discriminating short and ultra-short Barrett's oesophagus from intestinal metaplasia related to gastric pathology in the proximal stomach); b, the distribution of different mucosal phenotypes

(intestinal, cardiac, cardio-oxyntic) in long-segment Barrett's oesophagus, and their relationship with glandular dysplasia; c, the potential of the cell proliferation marker Ki67 and the DNA replication licensing proteins as markers of glandular and squamous oesophageal dysplasia; and finally, d, whether any relationship can be detected between different subtypes of intestinal metaplasia (type I v. type IIA v. type IIB) and dysplasia in Barrett's oesophagus.

Chapter 2: Recognising Barrett's Oesophagus

Cytokeratin Phenotypes in Barrett's oesophagus

The metaplastic glandular cells of Barrett's mucosa have ultrastructural features of squamous and glandular cells, and also express cytokeratins (CK) of both cell origins [56]. Two distinct Ck 7/20 expression patterns have been described in intestinal metaplasia of Barrett's oesophagus and intestinal metaplasia of the gastric cardia,(table 2/1), and this has been used to distinguish between intestinal metaplasia at the OGJ and SSBE [57].

Dysplasias and cancers tend to retain patterns of Ck expression that characterise the cells and tissues from which they originate, so cytokeratin phenotypes could help to demonstrate whether or not adenocarcinoma of the cardia and the oesophagus originate from distinct cell types.

The cardia, the anatomical region of transition between the oesophagus and stomach, cannot be identified macroscopically. It is visible microscopically as a thin glandular mucosa without acid-secreting oxyntic cells [58]. Intestinal metaplasia occurs at the cardia particularly with chronic inflammation but there is no clear evidence that this metaplasia predisposes to the development of adenocarcinoma of the cardia.

Biopsy specimens obtained from the OGJ may have been obtained from either the distal oesophagus or the gastric cardia and there is debate as to whether adenocarcinomas of the cardia and lower oesophagus share a common pathogenesis. Short segment Barrett's oesophagus is believed to be caused by GORD whereas intestinal metaplasia of the cardia is often associated with *H. pylori* infection, and the dysplasia risk is greater in short segment Barrett's oesophagus compared to cardia intestinal metaplasia [59].

Intestinal metaplasia is detectable in mucosa adjacent to 89% of adenocarcinomas of the oesophagus, 58% of adenocarcinomas of the cardia and 33% of adenocarcinomas of the subcardia [60].

Cytokeratins 7 and 20 are cytoplasmic structural proteins, constituents of the intermediate filaments of epithelial cells, and their expression patterns help to assign likely origins to many epithelial tumours including those of the gastrointestinal tract. A Ck 7 +/20- immunophenotype is present in 90% of Barrett's adenocarcinomas but only 21% of gastric adenocarcinomas[61].

Intestinal metaplasia involving the proximal stomach is histologically indistinguishable from intestinal metaplasia arising in Barrett's oesophagus but it has been claimed that Cytokeratin 20 and 7 expression patterns can reliably discriminate between intestinal metaplasia in these two contexts[62]. Jovanovic *et al* [63] agreed that it was possible to distinguish intestinal metaplasia of the OGJ from Ck 7 and 20 expression patterns. They demonstrated that the CK7/20 immunophenotype of intestinal metaplasia at the gastric cardia resembled that of intestinal metaplasia in the gastric antrum and corpus, and differed from that seen in intestinal metaplasia of long segment Barrett's oesophagus.

Applying Ck7 and 20 antibodies to IM at the cardia discloses keratin phenotypes characteristic of both oesophageal and gastric intestinal metaplasia, and the oesophageal IM pattern is associated with GORD (i.e. SSBE). These antibodies could therefore prove helpful in evaluating the dysplasia risk to patients with intestinal metaplasia in the vicinity of the OGJ, by discriminating between SSBE and gastric intestinal metaplasia, which appear to be associated with different cancer risks.

Tissue Type	Site of graded cells	Ck7 staining	Ck20 staining
Squamous	Superficial	-ve	-ve
	Deep	-ve	-ve
Squamous(adjacent to Barrett's mucosa)	Superficial	Variable	-ve
	Deep	-ve	-ve
Barrett's oesophagus(complete and incomplete IM)	Superficial	+ve	+ve
	Deep	+ve	-ve
Normal Gastric (Cardiac, Fundic & Antral)	Superficial	-ve	+ve
	Deep	-ve	-ve
Gastric Complete IM(Cardiac, Fundic & Antral)	Superficial	-ve	+ve
	Deep	-ve	+ve
Gastric Incomplete IM(Cardiac, Fundic & Antral)	Superficial	Variable	Variable
	Deep	Variable	Variable

Table 2/1. Cytokeratin 7 and 20 staining patterns as described by Ormsby *et al* [57].

The objective of this part of the study was to confirm the distinctive Ck7/20 phenotype described as characteristic of Barrett's mucosa [57]. For this purpose we chose to examine only established LSBE, in order to avoid confounding our data with cases in which the differential diagnosis of SSBE v. gastric type IM at the OGJ was unclear.

Cytokeratin 7 and 20 patterns were examined throughout the length and different histological phenotypes of long segments of Barrett's mucosa and the corresponding gastric tissue for both non-dysplastic and dysplastic cases.

Materials and Methods

Cytokeratin 7 and 20 immunostaining was performed on formalin-fixed paraffin embedded oesophageal and gastric biopsies from 50 patients diagnosed by endoscopy and biopsy with long segment Barrett's oesophagus.

1] Four micron sections dewaxed in xylene were rehydrated through graded alcohols to water.

2] Slides were washed in 3% aqueous hydrogen peroxide for ten minutes to eliminate endogenous peroxidase in the tissue, thus avoiding any confusion with the artificially attached peroxidase label, followed by a water wash.

3] Three antigen retrieval methods were compared: trypsin enzymatic digestion and microwave heating in either citrate or ethylenediamine tetraacetic acid (EDTA). Heating in EDTA (pH8) gave best results. EDTA in tris[hydroxymethyl]aminomethane (TRIS base) was brought to the boil in a microwaveable pressure cooker for 13 minutes prior to adding the sections which were then microwaved for a further 8 minutes (5 minutes of which was at full pressure) then left to cool in the antigen retrieval fluid for 20 minutes. This was followed by a water wash and 3 minute TRIS saline wash.

4] Horse serum (Vector) diluted 1:5 in TRIS saline was applied to the sections and left at room temperature for 15 minutes in order to block any non-specific background staining.

5] After blotting off the serum block, primary antibody diluted in antibody diluent (Dako) was applied to the sections and left at room temperature for 30 minutes and followed by a 3 minute TRIS saline wash. (Optimum primary antibody concentrations were determined by titration as follows; Ck 7 1 in 1000, CK 20 1 in 500)

6] Horse anti mouse biotin secondary antibody (Vector) was then applied for 30 minutes at room temperature.

7] Avidin/ peroxidase labelled biotin (prepared half an hour in advance to allow complex formation) was applied for 30 minutes at room temperature followed by a 3 minute TRIS saline wash. The proportion of avidin to biotin is such that some binding sites on the avidin are left free to attach to the horse anti-mouse biotin.

8] Sections were then left in 3,3'-diaminobenzidine tetra chloride (DAB)/ H_2O_2/ TRIS saline solution for 10 minutes which enables the formation of visible brown reaction product at the antigenic site.

9] Sections were then left in Copper (II) sulphate 5-hydrate (BDH)/ saline solution for 10 minutes, the copper ions turning the DAB a deeper shade of brown.

10] After rinsing in water, the sections were counterstained with haematoxylin (Sigma), dehydrated through graded alcohols, cleared in xylene and mounted in a resinous mountant.

The pattern of Ck7 and 20 positive staining was scored throughout the length of each oesophagus and at the OGJ. Each mucosal type at each biopsy site was given a score for superficial and deep cell compartments rating staining intensity from zero to three; zero when there was no staining at all, one for weak staining, two for moderate and three for strong staining. This aimed to reflect the range of patterns of immunostaining seen in the oesophagus with these antibodies. The patterns of positive Ck staining were compared to those described by Ormsby et al [57]. Specific intestinal metaplasia phenotype was ascertained from staining profiles with High Iron Diamine/ Alcian Blue (chapter 5).

Cytokeratin immunostaining was patchy and to deal with this issue the scoring protocol described above was modified by recording the range of staining intensity observed in the relevant tissue compartment. These ranges are given in the tables presenting the results below. Thus a score of "1-2" would imply weak to moderate staining; 2-3, moderate to strong; and so on. In the tables the weaker staining is on the horizontal axis and the stronger staining on the vertical axis. Entries

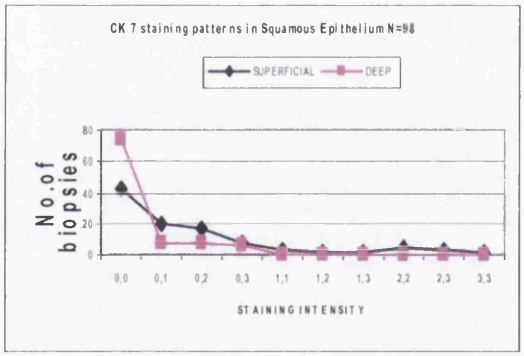
on the diagonal (0-0,1-1,2-2,3-3) imply homogeneous staining of the given intensity.

In order to make the data as comprehensible as possible, the background in each cell in the tables has been shaded grey in proportion to the percentage of the scored biopsies in that group falling into that cell. This device was employed rather than giving the percentage figures in parentheses as it was felt that this would merely create a less graspable mass of data than the approach adopted.

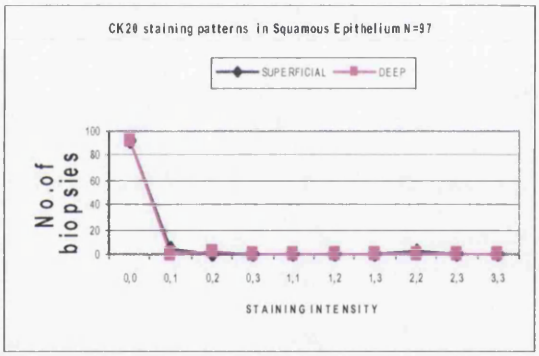
Results

The results are presented in the graphs and tables which follow.

A



B



A

Squamous epithelium, superficial				
3	7	1	3	1
2	16	1	4	
1	19	3		
0	43			
N 98	0	1	2	3

Squamous epithelium, deep				
3	6	0	0	0
2	8	0	0	
1	7	0		
0	74			
N 95	0	1	2	3

B

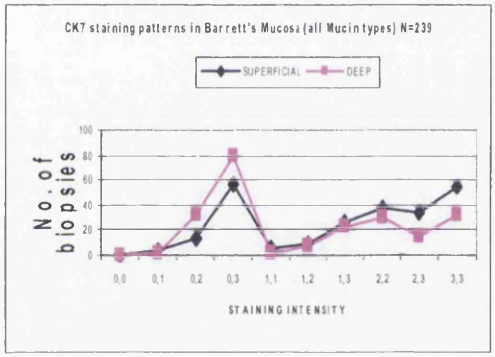
Squamous epithelium, superficial				
3	0	0	0	0
2	0	0	1	
1	3	0		
0	93			
N 97	0	1	2	3

Squamous epithelium, deep				
3	0	0	0	0
2	1	0	0	
1	0	0		
0	93			
N 94	0	1	2	3

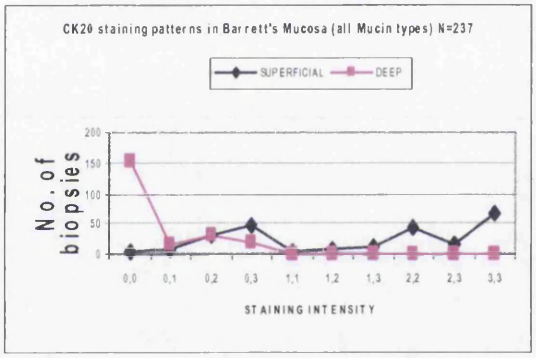
Graphs 2/1, Table 2/1, A and B. Cytokeratin 7 (A) and cytokeratin 20 (B) expression in oesophageal squamous epithelium.

Ck 20 expression is almost entirely negative in oesophageal squamous epithelium. Ck7 expression is variable, often negative, but may be positive, particularly in the differentiated superficial (luminal) compartment). This pattern differed somewhat from the Ck7-/Ck20- staining mentioned by Ormsby et al [62,63] in relation to native oesophageal squamous epithelium, but can be related to the Ck7 ±/Ck20- phenotype they mention in relation to squamous 'islands' often observed 'floating' on Barrett's mucosa, and this intermediate phenotype may reflect a less determinate differentiation (as has been described in electron micrographs also).

A



B



A

Barrett's mucosa, superficial				
3	57	26	34	54
2	13	9	38	
1	3	5		
0	0			
N 239	0	1	2	3

Barrett's mucosa, deep				
3	79	22	16	33
2	33	7	31	
1	1	1		
0	0			
N 223	0	1	2	3

B

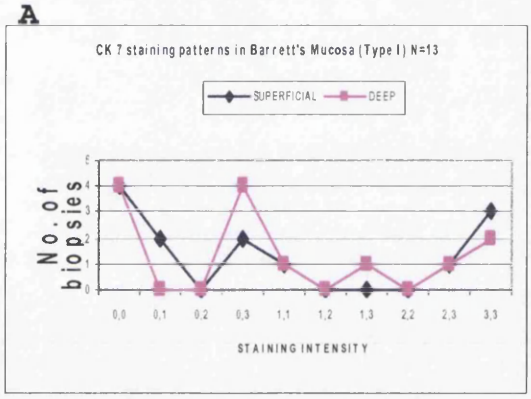
Barrett's mucosa, superficial				
3	46	12	15	66
2	30	9	42	
1	9	5		
0	3			
N 237	0	1	2	3

Barrett's mucosa, deep				
3	19	0	0	1
2	33	0	0	
1	16	1		
0	151			
N 221	0	1	2	3

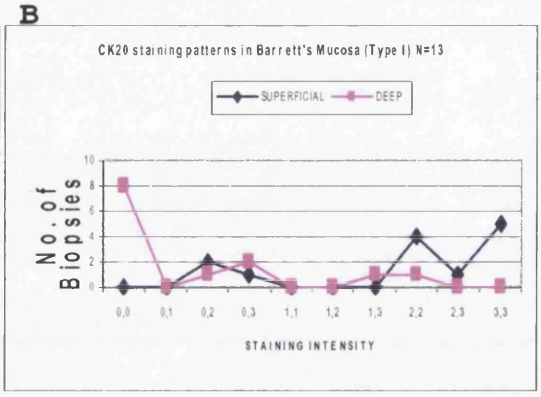
Graphs 2/2, Table 2/2, A and B. Cytokeratin 7 (A) and cytokeratin 20 (B) in intestinal-type Barrett's mucosa (all mucin phenotypes)

Intestinal Barrett's mucosa as a whole shows rather similar patterns for both Ck7 and Ck20 in the superficial compartment, with marked heterogeneity of staining between cases. Heterogeneity of staining is also a marked feature for Ck7 in the deep compartment, with Ck20 more usually negative. While broadly in agreement with Ormsby's description of Ck7 positivity superficial and deep, and Ck20 positivity superficial only, heterogeneity is pronounced.

A



B



A

Type I IM, all loci, superficial				
3	2	0	1	3
2	0	0	0	
1	2	1		
0	4			
N 13	0	1	2	3

B

Type I IM, all loci, superficial				
3	1	0	1	5
2	2	0	4	
1	0	0		
0	0			
N 13	0	1	2	3

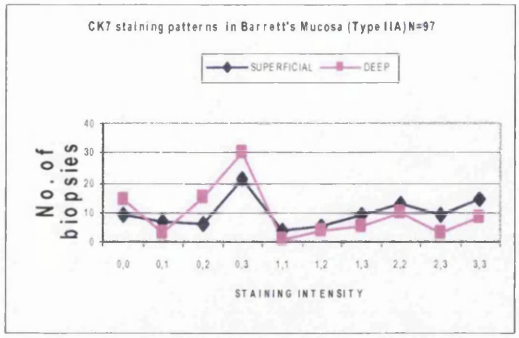
Type I IM, all loci, deep				
3	4	1	1	2
2	0	0	0	
1	0	1		
0	4			
N 13	0	1	2	3

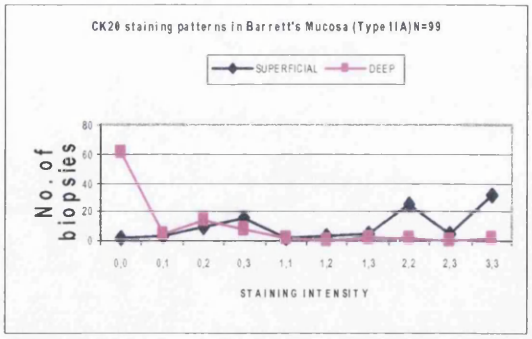
Type I IM, all loci, deep				
3	2	1	0	0
2	1	0	1	
1	0	0		
0	8			
N 13	0	1	2	3

Graphs 2/3, Table 2/3, A and B. Cytokeratin 7 (A) and cytokeratin 20 (B) in Type I intestinal-type Barrett's mucosa.

The smaller number of cases with type I intestinal mucosa in Barrett's oesophagus make definitive evaluation difficult, but there is a suggestion that Ck7 is more likely to be negative in the deep compartment.

A **B**





A

Type IIA IM, all loci, superficial				
3	21	9	9	14
2	6	5	13	
1	7	4		
0	9			
N 97	0	1	2	3

Type IIA IM, all loci, deep				
3	30	5	3	8
2	15	4	10	
1	3	1		
0	14			
N 93	0	1	2	3

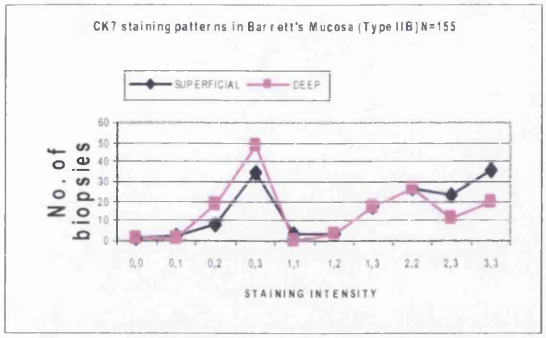
B

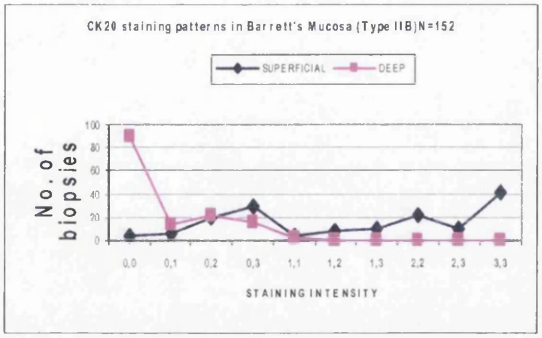
Type IIA IM, all loci, superficial				
3	15	4	5	32
2	9	3	25	
1	3	2		
0	1			
N 99	0	1	2	3

Type IIA IM, all loci, deep				
3	8	1	0	2
2	14	0	1	
1	4	1		
0	61			
N 92	0	1	2	3

Graphs 2/4, Table 2/4, A and B. Cytokeratin 7 (A) and cytokeratin 20 (B) in Type IIA intestinal-type Barrett's mucosa.

A **B**





A

Type IIB IM, all loci, superficial				
3	35	17	23	36
2	8	4	26	
1	2	3		
0	1			
N 155	0	1	2	3

Type IIB IM, all loci, deep				
3	49	17	12	20
2	19	3	26	
1	1	0		
0	1			
N 148	0	1	2	3

B

Type IIB IM, all loci, superficial				
3	30	9	10	42
2	20	7	22	
1	6	3		
0	3			
N 152	0	1	2	3

Type IIB IM, all loci, deep				
3	16	0	0	0
2	21	0	0	
1	13	1		
0	91			
N 152	0	1	2	3

Graphs 2/5, Table 2/5, A and B. Cytokeratin 7 (A) and cytokeratin 20 (B) in Type IIB intestinal-type Barrett's mucosa.

These compare Ck7 and 20 phenotypes in type IIA and type IIB intestinal metaplasia. It will be seen that the staining distribution is virtually identical in the two cases, and that no possible distinction could be made in individual cases.

This particular group of cases did not include enough examples of gastric intestinal mucosa, *H. pylori* positivity or dysplasia for definitive analysis so this is not attempted.

Chapter 3: Mucosal Phenotypes, Zoning and Oesophageal Dysplasia

Patients with Barrett's oesophagus usually present with a variable, sometimes asymmetrical length of columnar mucosa showing intestinal, cardiac and fundic phenotypes in which occurs islands of squamous epithelium [64]. However it is the specialised intestinal type mucosa which is thought to have the greatest risk of dysplastic development [40,41]. Some studies have commented on the appearance of zonation of the mucosal phenotypes in long segment Barrett's oesophagus with some mucosal phenotypes regularly seen more proximally to others but often a mixture of phenotypes is seen at a given level [64]

Some endoscopic studies have suggested that the upper limit of columnar epithelium may extend proximally at successive examinations in the presence of continuing reflux, which suggests a process of creeping substitution of squamous by columnar mucosa [65], and with increasing severity of gastro-oesophageal reflux, the squamocolumnar junction shifts proximally, resulting in an increase in the length of cardiac-type columnar mucosa within the oesophagus [66]. In addition, studies using 24 hour pH monitoring have shown that as acid exposure increases, the length of columar mucosa within the oesophagus significantly increases as well. However, Barrett's oesophagus may present as islands of columnar epithelium remote from the gastric cardia [25]. The tissue of origin for Barrett's metaplasia is not clear and three theories exist.

1] De novo metaplasia theory: stem cells of inflamed squamous mucosa in the exposed papillae are damaged, the resulting metaplasia in these cells produces Barrett's stem cells.

2] Transitional zone metaplasia theory: cells at the oesophago-gastric junction colonise the gastric cardia or distal oesophagus in response to noxious luminal agents.

3] Duct cell metaplasia theory: stem cells located in the glandular neck region of oesophageal ducts might selectively colonise the oesophagus when mucosal damage occurs.

Chandrasoma [64] conducted a review of the oesophago-gastric junction in a large number of autopsies without known history of GORD. He determined that in most children and adults younger than 20 years of age the squamous epithelium of the oesophagus gave way directly to oxyntic mucosa of the gastric fundus with no interposed segment of cardiac type mucosa. Recognisable cardiac mucosa appeared in specimens from those older than 20 years and at a length of less than 1cm.

Cardiac mucosa is the simplest form of columnar epithelium with no specialised cells and it could be that cardiac mucosa in the oesophagus develops from reflux-induced injury to squamous epithelium. Both clinical and experimental animal evidence support this theory. Follow-up studies in patients who have undergone partial oesophagogastrectomy with an intrathoracic anastamosis of the oesophagus to the fundus of the stomach, which results in near constant bathing of the remaining oesophagus in refluxed gastric juice, have demonstrated over a period of months to years (mean time 8.2 years) the development of cardiac mucosa proximal to the anastamosis in what was squamous oesophagus. Experimental evidence comes from a 1970 study by Bremner et al [67] where dogs underwent stripping of the distal oesophageal squamous mucosa with or without cardioplasty to destroy the function of the lower oesophageal sphincter. They noted extensive squamous cell re-epithelialization in the animals without gastro-oesophageal reflux whereas squamous regeneration was absent or minimal in animals with cardioplasty induced reflux in which the oesophagus was replaced by a columnar epithelium that lacked submucosal glands and parietal cells equivalent to human cardiac mucosa.

The murine antibody DAS-1, which stains 'specialized' (intestinal-type) columnar mucosa, also reacts positively with cardiac mucosa and on repeat biopsies histological evidence of intestinalization later developed in 6 of 7 patients [1]. This could suggest that cardiac mucosa

is the precursor of intestinalized columnar epithelium as postulated by De Meester et al [23].

Analysing patterns of mucosal development in the columnar lined oesophagus could improve our understanding of the early stages of Barrett's oesophagus, the precursors in the metaplastic, dysplastic sequence towards adenocarcinoma in this disease.

Materials and methods

Thirty-two consecutive patients with biopsy-proven long segment Barrett's oesophagus attending Glasgow Royal Infirmary were recruited as part of a study of telomerase activity in Barrett's mucosa, not part of this study. Apart from five newly diagnosed patients, all were already undergoing annual endoscopy and biopsy with three biopsies for every 2cm of columnar-lined oesophagus (every 1cm in patients with a previous diagnosis of dysplasia). Patients with previously diagnosed invasive adenocarcinoma were excluded. In all, the 32 patients had experienced 152 endoscopies with biopsy, including 77 before the 32 study endoscopies, and 43 after. All patients had more than 3 cm of columnar mucosa in the distal tubular oesophagus. Median length of Barrett's oesophagus was 8 cm (range 3 - 16 cm). Mean and median age of patients at the time of the study endoscopy was 64 and 70 years (range 37 to 84). There were 24 males and 8 females. Dysplasia was identified in at least one biopsy in ten of these patients, and indefinite changes not amounting to confirmed dysplasia in a further seven patients. The study was approved by Glasgow Royal Infirmary ethics committee and patients gave informed written agreement to participate.

Biopsy protocol

At each endoscopy, three biopsies were taken for histology each from the following locations: original squamous mucosa proximal to the Barrett's segment, columnar side of the squamo-columnar junction (Z line), Barrett's segment every 2cm, anatomical oesophago-gastric

junction(defined by the most proximal gastric folds), gastric corpus and antrum. All biopsies were fixed overnight in 4% neutral buffered formaldehyde, embedded in paraffin wax, and four micron sections cut at three histological levels. Dewaxed sections were stained with haematoxylin-eosin and three biopsies each from a total of 268 sites (n=794) were examined histologically. In addition, histological review was performed of all oesophageal and gastric sites previously (n=488) and subsequently (n=401) biopsied, representing in all 1057 sites and 3171 individual mucosal biopsies.

Biopsy review

One pathologist with an interest in Barrett's oesophagus (JJG) was responsible for initial reporting of all biopsies from all endoscopies. Subsequently, all biopsies were reviewed by the same pathologist 'blind' to his original report. Presence or absence of the following mucosal types was recorded for each biopsy. 1. Full-thickness squamous epithelium. 2. Immature squamous 'islands' overlying glandular mucosa. 3. 'Specialised' Barrett's mucosa, defined by goblet cells. 4. Mucosa resembling gastric cardia, without goblet cells or oxyntic cells. 5. Cardio-oxyntic mucosa with oxyntic cells, or fully-developed fundic mucosa. 6. Antral mucosa (in gastric biopsies). Intestinal metaplasia in biopsies from gastric sites was separately recorded.

Glandular dysplasia was evaluated, also by JJG, using published criteria [17,68] in conformity with the Vienna classification [69,70] in the following groups: 1. No dysplasia. 2. Mild changes, possibly reactive: indefinite for dysplasia. 3. Definite dysplasia of mild or moderate severity: low grade dysplasia. 4. Severe dysplasia / adenocarcinoma in situ: high grade dysplasia. While category 4 may also include biopsies in which invasion is suspected, our series did not include such biopsies. Phenotype of the dysplastic mucosa and biopsy level were also recorded. At any biopsy site the most severe dysplasia category was recorded and analysed.

Published studies show good inter- and intra-observer agreement in recognising high grade glandular dysplasia in Barrett's oesophagus [71,72], but less robust discrimination between no dysplasia, indefinite and low grade dysplasia. To evaluate the observer's consistency of dysplasia grading in this study, all biopsies were re-scored 'blind' to the original reading, after a minimum delay of six months. The review dysplasia coding was compared with the grading originally assigned. If these were in agreement, that grading was accepted. If discrepant, a third 'blind' review was undertaken, and final allocation of dysplasia grade was based on the majority reading. Reproducibility of dysplasia grading was evaluated by comparison of the first and second overall readings for each biopsy group from a specific site. While application of diagnostic criteria has been reviewed with histopathologist colleagues in the same and other institutions, inter-observer agreement was not evaluated formally.

Derived calculations and statistical analysis

The spatial distribution of mucosal phenotypes in Barrett's oesophagus was examined by graphing the percentage of biopsy groups in which each mucosal phenotype was present as a function of the distance from the Z line to the anatomical oesophago-gastric junction. The distance along each Barrett's oesophagus was also expressed as a percentage, to compensate for the variable length of Barrett's oesophagus in different patients. This was done by mapping the percentage of biopsies in which a particular mucosal type was present in each centimetre of a particular Barrett's oesophagus to the corresponding section of the graph, and averaged for all 32 patients. The mean percentage was calculated for every length centile by summation (Σ) over all 32 patients as $\Sigma(100n_1/L)/\ \Sigma(100n_2/L)$, where n_1 is the number of biopsy groups containing the feature in question, n_2 the total number of biopsy groups, and L is the length of each Barrett's oesophagus in centimetres. This strategy gives equal weight to all biopsies. Zonal distribution of dysplasia was examined identically, taking n_1 as the

number of biopsy sites found to be dysplastic, and n_2 as the number of sites in which 'specialised' Barrett's mucosa was confirmed (because dysplasia was almost exclusively associated with the intestinal phenotype).

The kappa statistic [73] was calculated as a measure of agreement using Analyse-It version 1.48 (Analyse-It Co, Leeds, UK) in Microsoft Excel 97. Group comparisons were made using Fisher's Exact Test.

Results

Repeatability of dysplasia reading

To demonstrate reproducibility of dysplasia grading in this biopsy series, repeat scores are presented for individual biopsy sites (N=612) in table 3/1. The overall kappa score (0.62) represents good agreement.

There is most discrepancy between no dysplasia and indefinite for dysplasia, and least between low and high grade dysplasia.

Second reading	First reading			
	No dysplasia	Indefinite	Low grade dysplasia	High grade Dysplasia
No dysplasia	469	34	8	0
Indefinite dysplasia	20	15	5	0
Low grade dysplasia	9	6	46	0
High grade dysplasia	0	0	6	21

Table 3/1. Repeatability of dysplasia grading by Dr J.J. Going in 612 individual Barrett's biopsy sites. Overall kappa score = 0.62 (good agreement).

Merging 'no dysplasia' with 'indefinite for dysplasia' creates 3 categories (no definite dysplasia, low grade dysplasia, high grade dysplasia) with a kappa score representing excellent repeatability (0.79). Although changes suspicious of invasion are often associated

with high-grade dysplasia, such changes were not identified in these patients, probably because only five of our patients were recently diagnosed. The others had been biopsied previously, and patients with evidence of invasion excluded.

Zonal distribution of mucosal types in Barrett's oesophagus

In order to investigate the zonal distribution of mucosal types in Barrett's oesophagus, biopsies were taken for histology from the following locations: original squamous mucosa proximal to the Barrett's segment, columnar side of the squamo-columnar junction (Z line), Barrett's segment every 2cm, anatomical oesophago-gastric junction (defined by the most proximal gastric folds), gastric corpus and antrum. Presence or absence of the following mucosal types was recorded for each biopsy. 1. Full-thickness squamous epithelium. 2. Immature squamous 'islands' overlying glandular mucosa. 3. 'Specialised' Barrett's mucosa, defined by goblet cells. 4. Mucosa resembling gastric cardia, without goblet cells or oxyntic cells. 5. Cardio-oxyntic mucosa with oxyntic cells, or fully-developed fundic mucosa. 6. Antral mucosa (in gastric biopsies). Intestinal metaplasia in biopsies from gastric sites was separately recorded.

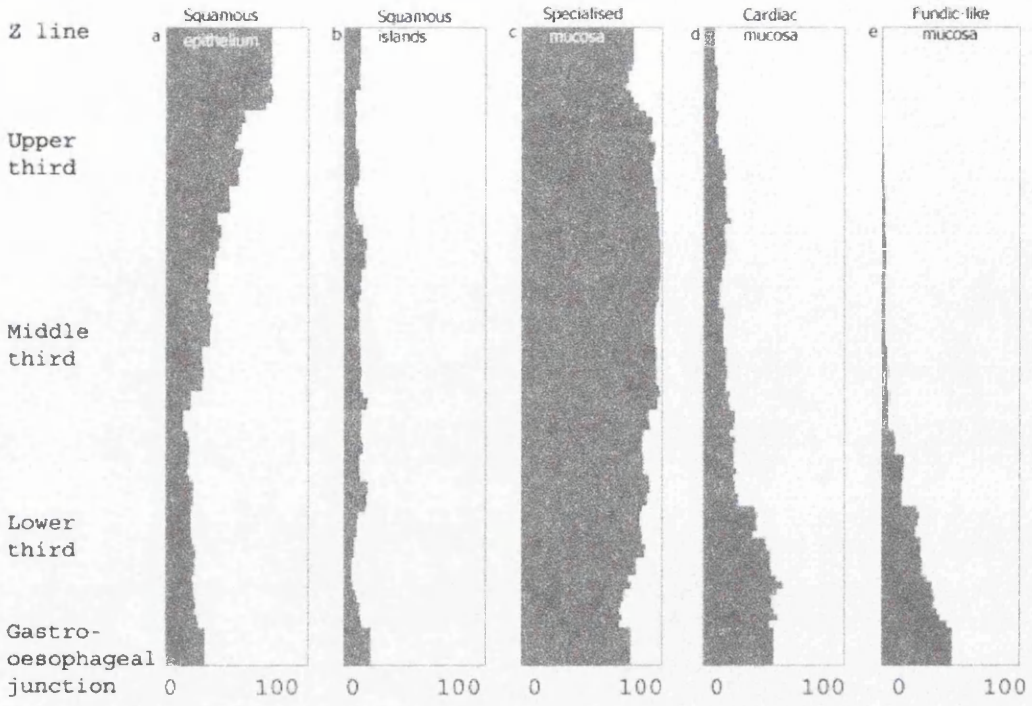


Figure 3/1. Data for 32 patients with long segment Barrett's oesophagus. These graphs indicate the mean probability (expressed as a percentage) of different mucosal types being present in Barrett's oesophagus as a function of normalised anatomical level, on the vertical axis, from the Z line proximally (top), down to, but not including, the anatomical oesophago-gastric junction distally (bottom). The grey shaded area in each box shows how often that particular component is found at that level. The five boxes represent a, full thickness squamous epithelium; b, squamous islands over glandular mucosa; c, 'specialised', intestinal-type Barrett's mucosa; d, cardiac type mucosa; and e, cardio-oxyntic or fundic mucosa.

This analysis showed a pronounced proximal-to-distal zonation of mucosal phenotype in long segment Barrett's oesophagus which was reflected in the probability of detecting different mucosal types at different levels in the Barrett's segment. Figure 3/1 is a more detailed graph of the mean probability for all 32 patients of different mucosal types being present in Barrett's oesophagus as a function of anatomical level from the Z line to the most distal Barrett's oesophagus, excluding

the oesophago-gastric junction. Table 3/2 is an overview of the data looking at Barrett's oesophagus by thirds.

Location	Squamous Mucosa	Squamous Islands	Specialised Barrett's Mucosa	Cardiac Mucosa	Fundic-like Mucosa
Z Line	32 100%	10 31%	31 97%	7 22%	0 0%
Z Line and upper third	32 100%	14 44%	32 100%	10 31%	2 6%
Middle third	19 59%	18 56%	32 100%	17 53%	5 16%
Lower third	16 50%	15 47%	32 100%	27 84%	23 72%
OG junction	7 22%	8 25%	18 56%	23 72%	30 94%

Table 3/2. Zonation of mucosal type in 32 cases of Barrett's oesophagus. Columns show the number and percentage of cases in which different mucosal types were present at various levels including the Z line alone and the upper, middle and lower third of the Barrett's segment.

Full thickness squamous epithelium was often detectable in the upper third of the Barrett's segment, diminishing in frequency distally. Superficial squamous islands overlying glandular mucosa were present less often but at all levels without much variation in frequency. Cardiac-like mucosa occurred at all levels in Barrett's oesophagus, but more often distally. Oxyntic differentiation hardly occurred above the lower third. In this location some biopsies may actually derive from native gastric mucosa, but there is no doubt that oxyntic differentiation in an otherwise cardiac-like mucosa can be found in the true oesophagus, confirmed by the presence of oesophageal submucosal glands or their ducts. In contrast, 'specialised' intestinal-type Barrett's mucosa was likely to be found at all levels in every case, confirming its ubiquitous status in Barrett's oesophagus. Equally characteristic in individual cases was the occurrence of several different mucosal phenotypes at a single anatomical level: i.e. mucosal zonation in Barrett's oesophagus is

present but does not create horizontal bands of uniform mucosal type, but a patchwork of mucosal types varying in proportion with anatomical level.

Dysplasia and mucosal phenotype

Our data confirm that dysplasia in Barrett's oesophagus is especially likely to occur in 'specialised' intestinal type-mucosa, and not in other mucosal types [74,75,76]. Definite dysplasia, low or high grade, was present at 87 sites biopsied; in 85 of these the dysplasia was in continuity with histologically confirmed 'specialised' intestinal type, found at 616 sites (14%) and only two dysplastic sites were in continuity with histologically confirmed cardiac-type mucosa, found at 156 oesophageal sites (1.3%)($P<0.00001$).

Zonal distribution of dysplasia

This was examined in the same way as the zonal distribution of mucosal phenotype. Figure 3/2 shows the average probability of dysplasia for ten cases in which there was definite dysplasia, and a further seven cases indefinite for dysplasia. Although some fluctuation in the probability of dysplasia being present was observed, there was no evidence of any major proximal to distal trend.

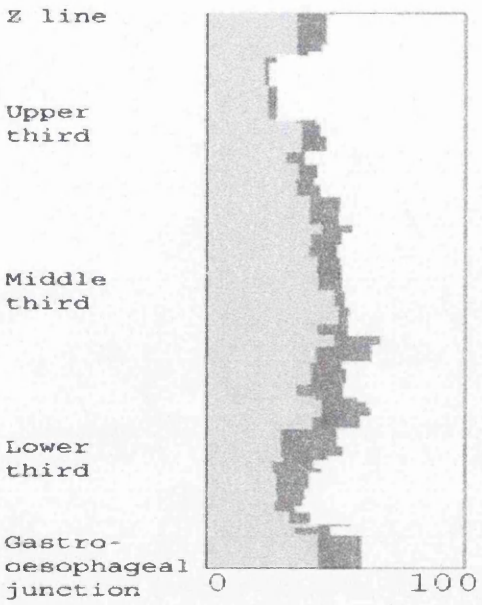


Figure 3/2. This graph plots the average probability (as a percentage on a scale of 0 to 100) of dysplasia being present in 'specialised' intestinal Barrett's mucosa as a function of normalised anatomical level. Data are averaged for ten patients with long-segment Barrett's oesophagus and definite biopsy-proven dysplasia, and seven patients indefinite for dysplasia. There is no evidence of any significant proximal-to-distal gradient in dysplasia frequency. Light grey area represents definite dysplasia, darker grey biopsies indefinite for dysplasia.

Chapter 4: DNA Replication Licensing Proteins

In normal oesophageal squamous epithelium, stem cells divide slowly in the basal layer giving rise to daughter cells which supply a population of mature cells at the luminal surface [77]. In Barrett's oesophagus the proliferative compartments are similar to those in gastric mucosa, with maximum proliferation in a zone beneath the mucosal surface [78].

Organization of proliferation and differentiation zones appears to break down in dysplastic epithelia. This failure could potentially identify tissue at a preneoplastic stage with proliferating and atypical cells identifiable on the luminal surface, (as seen characteristically in dysplastic mucosae), if a suitable marker of proliferative capacity was available. The Mini-chromosome maintenance (Mcm) proteins are candidates for this role.

Initiation of DNA replication in eukaryotic cells is a highly regulated process that requires the ordered assembly of many proteins at multiple origins of DNA replication to form a competent prereplicative chromosomal state [79-82]. The eukaryotic initiator proteins form the origin of replication complex (ORC) where proteins interact to form a pre-replicative complex (pre-RC). This can only happen at certain stages of the cell cycle [79]. Mini-chromosome maintenance proteins form an essential part of this complex with Cdc6 being required for their loading onto the ORC. [79-82]. The assembly of the pre-RC containing: ORC, cell division cycle protein 6(Cdc6) and Mcm proteins, occurs at a point defined by cyclin CDK activity [83]. After initiation a post-RC is formed and cyclin CDK activity blocks the assembly of the pre-RC. Competence to initiate DNA replication in vitro arises suddenly following release from quiescence coinciding with maximum Cdc6 accumulation and binding of the Mcm protein complex to chromatin. Mcm proteins become bound to chromatin during late mitosis and remain there until they are gradually removed as S phase progresses [82], so newly replicated sections of chromatin are

free of Mcm proteins and therefore cannot replicate during the same cell cycle. It follows that Mcm expression is required for cell proliferation potential to ensure DNA replication occurs only once per cell cycle [84].

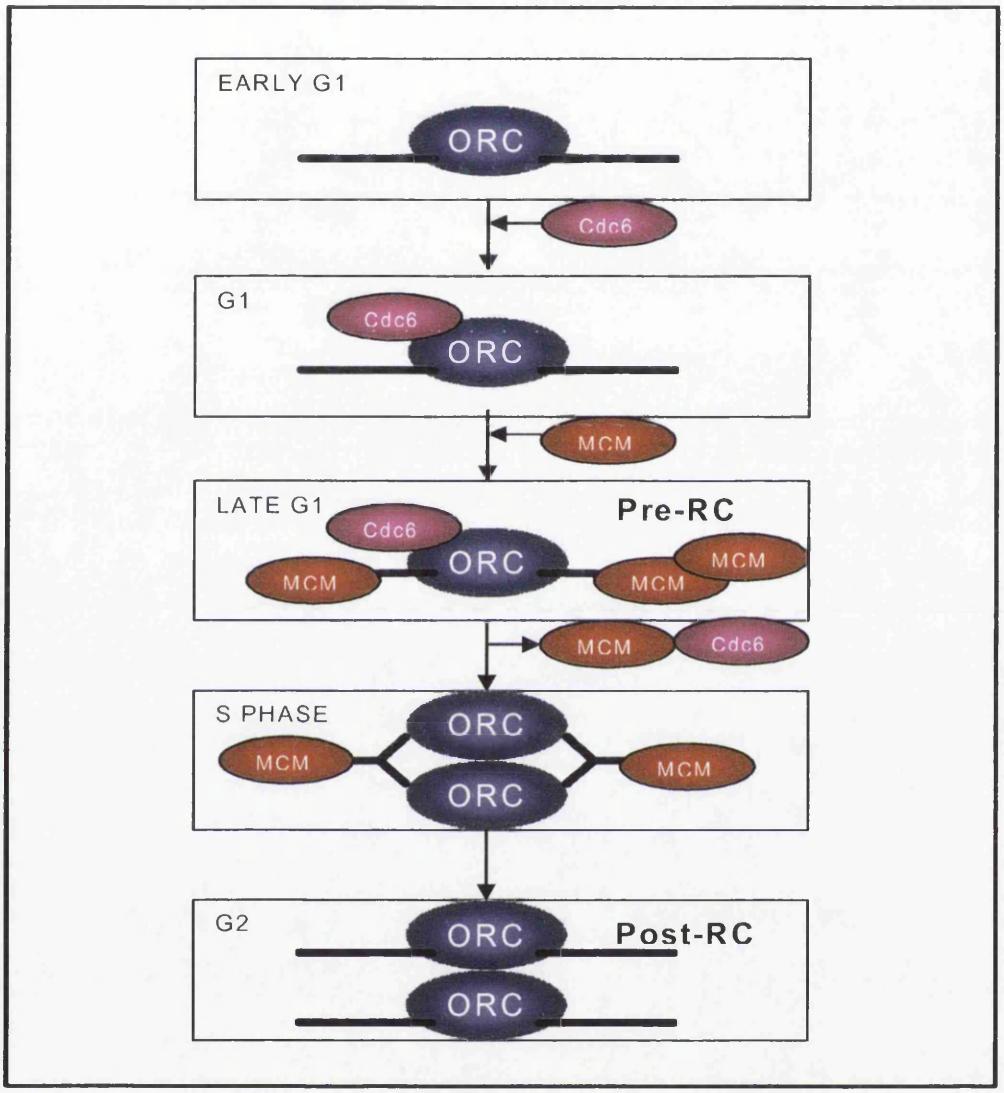


Figure 4/1. A model for the role of Mcm proteins in the control of DNA replication. It shows the ordered assembly of the Pre-RC containing: ORC, Cdc6p and the Mcm proteins. After initiation of replication a Post-RC is formed. The cycle of assembly and disassembly of the initiation proteins has a role in blocking the re-firing of origins in the same cell cycle.

The Mcm proteins were first identified in a screen for mutations that caused defects in the maintenance of plasmids in yeast [80,85]. There are six Mcm proteins (labelled 2-7) and they have a central core of homology [85]. Released nucleoplasmic Mcm proteins are found as large multiprotein complexes which have the tendency to disintegrate into more stable subcomplexes one of which is a dimer of Mcm 3 and 5, another a trimer of Mcm 4, 6 and 7, with Mcm 2 usually found loosely associated with these subcomplexes. Mcm 5 is quite easily separated from other Mcm proteins so is described as a peripheral protein, Mcm 4 and 6 associate very strongly in what is thought to be a core complex, Mcm 4 being essential for Mcm 2 to bind to this core complex [86]. During late mitosis there is a brief window when the Mcm proteins are able to bind to chromatin, during this period they associate to a form that has chromatin binding potential, a step that may be initiated by dephosphorylation of the Mcm complex [87].

Immunodepletion of Mcm proteins from Xenopus extract was found to block DNA synthesis [88]. Reduced dosage of the Mcm proteins allowed replication but prevented completion of S phase, resulting in DNA damage and genomic instability. It could be that when Mcm protein levels are reduced, only a few active Mcm complexes are assembled, sufficient to fire a subset of origins but not enough to complete genome replication.

The Mcm proteins are bound to chromatin at levels that far exceed the number of active replication origins [89], so they probably have functions in addition to replication licensing. They may aid replication movement as they have a conserved DNA dependent ATPase domain shared with helicases or they could be involved in the assembly or disassembly of replisomes at initiation sites or in the assembly of replication factories composed of large numbers of replisomes [83,85,87,89].

By isolating the soluble cellular proteins in urine, an immunoassay assay of Mcm5 proteins has been used sucessfully to detect cancers of the urinary tract [90]. Additionally, anti-Mcm5 antibodies can improve the Papanicolaou smear test for cervical dysplasia and neoplasia [91],

therefore it seemed appropriate to examine their pattern of expression in the non-dysplastic and dysplastic oesophagus.

Chapter 4: Materials and Methods

We examined cell proliferation in non-dysplastic and dysplastic squamous epithelium and Barrett's mucosa in biopsies by immunostaining with antibodies directed against Ki67, and replication licencing with antibodies against minichromosome maintenance proteins 2 and 5, donated by Professor Gareth Williams (Cambridge).

Immunohistochemical staining was carried out as described (chapter 2). The Ki67 antibody was clone MIB1 from DAKO (a mouse monoclonal). The anti-Mcm antibodies were rabbit polyclonals. After titration, the optimum primary antibody concentrations were as follows: Ki 67 1/100, Mcm 2 1/4000, Mcm 5 1/ 4000. Biopsies from a total of 77 patients were studied: formalin fixed, paraffin embedded tissue blocks were cut at 4μm and fixed onto silane coated slides. Patients were from cohorts undergoing diagnostic endoscopy enrolled in a programme of yearly surveillance of Barrett's oesophagus, or had surgical resection of Barrett's associated oesophageal carcinoma.

Dysplastic changes were assessed on haematoxylin and eosin stained sections of paraffin embedded endoscopic biopsies and tissue blocks from resection specimens. These were shown to represent a range of morphologies from normal oesophageal squamous epithelium through low and high grade squamous dysplasia, to invasive squamous carcinoma (tables 4/1-2). Similarly, examples of Barrett's mucosa without dysplasia, low grade dysplasia and high grade dysplasia in Barrett's mucosa and invasive Barrett's adenocarcinoma were selected.

WITHOUT BARRETT'S OESOPHAGUS (25)	**WITH BARRETT'S OESOPHAGUS (45)**
NO SQUAMOUS DYSPLASIA (13)	NO DYSPLASIA (25)
LOW GRADE SQUAMOUS DYSPLASIA (3)	LOW GRADE DYSPLASIA (10)
HIGH GRADE SQUAMOUS DYSPLASIA (6)	HIGH GRADE DYSPLASIA (8)
LOW & HIGH GRADE SQUAMOUS DYSPLASIA (3)	LOW AND HIGH GRADE DYSPLASIA (2)
4 ALSO HAD INVASIVE SQUAMOUS CARCINOMA	*4 ALSO HAD INVASIVE ADENOCARCINOMA*

Table 4/1. The number of biopsies immunostained from different tissue categories.

Category	Ki67	Mcm2	Mcm5
Squamous, non-dysplastic	53	54	51
Low grade squamous dysplasia	5	5	3
High grade squamous dysplasia	11	11	7
Invasive squamous carcinoma	6	6	5
Barrett's mucosa	34	35	33
Low grade Barrett's dysplasia	19	19	18
High grade Barrett's dysplasia	10	7	9
Invasive Barrett's adenocarcinoma	4	4	4

Table 4/2. Number of biopsies stained for Ki67, Mcm2 and Mcm5 in different categories.

Immunostaining of cell nuclei was scored positive or negative using a semi-quantitative scoring scheme. Intensity of staining did not vary much and was not scored. Four compartments were recognised in the squamous epithelium; (1)the most basal single layer of cells and the thickness of the epithelium above that divided into (2)parabasal, (3)middle and (4)luminal thirds. In Barrett's mucosa four strata were also defined; (4)the surface epithelium between crypts, the underlying crypts divided into (3)upper and (2)lower halves and(1)the deepest layer, a differentiated glandular zone (fig 4/2).

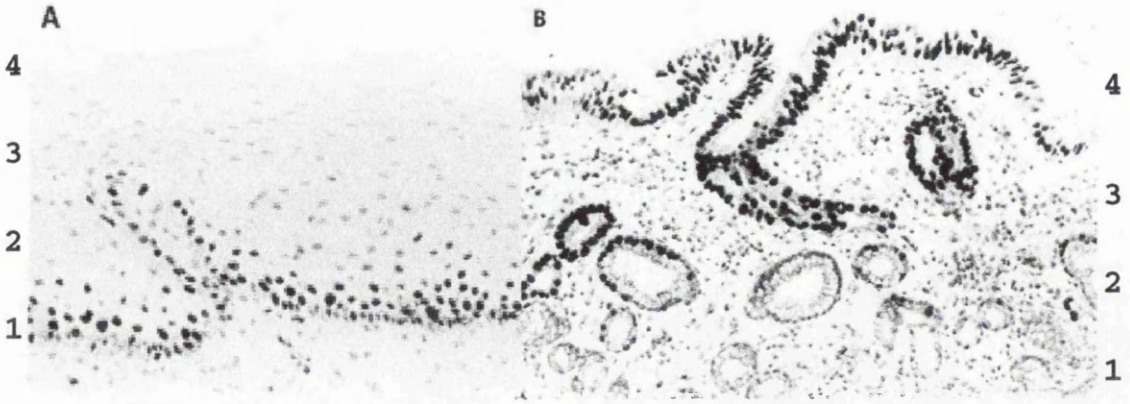


Figure 4/2. Mcm 2 expression in squamous epithelium (A) and Barrett's mucosa (B), showing the four scoring compartments.

These compartments corresponded to known areas of cell proliferation, as defined by Lauwers *et al* (90), and within each compartment the estimated percentage of positive cells was allocated to scoring bands as follows: 0: no staining 1: <10% +ve stained cells. 2: 10-30% +ve stained cells. 3: 30-70% +ve stained cells. 4: 70-90% +ve stained cells. 5: >90% +ve stained cells. 6: 100% +ve stained cells. Actual cell counts were not attempted.

Statistical significance of the differences between staining scores for the same tissue compartments in different biopsies or within the same biopsy was established using a Mann-Whitney test (table 4/3).

Results

Both the Mcm 2 and the Mcm5 antibodies yielded clear nuclear staining although the Mcm5 staining was less clean, with some staining of cytoplasm and cell membranes in the glandular mucosae. Ki67 with monoclonal antibody MIB-1 yielded clean nuclear staining as expected.

In the non-dysplastic mucosae (both squamous and glandular) both showed maximal proliferation in the expected compartments, suprabasal in the case of squamous epithelium and in the lower crypt in Barrett's mucosa. Expression of both the cell cycle marker Ki67 and the replication licensing proteins were downregulated in the mature

compartments as expected, i.e. towards the surface of the squamous epithelium and on the surface and in the deep glands in the glandular mucosae. In the presence of dysplasia, however, this spatial organization of the mucosae broke down, with persistence of proliferation and especially persistence of replication licensing into compartments which would not normally be so licensed (figures 4/1, 4/2). Figure 4/3 has been constructed to summarise a large body of data for squamous and Barrett's mucosae with no dysplasia, low grade dysplasia, or high grade dysplasia. Most striking feature in both squamous and Barrett's mucosa is the progressively increasing expression of Ki67 and especially of the Mcm proteins on the mucosal surface with increasing dysplasia.

		Ki67	Mcm5	Mcm2
Squamous epithelium	Normal v LGD	$P < 0.0001$	$P < 0.0005$	$P < 0.0029$
	Normal v HGD	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Barrett's Mucosa	Normal v LGD	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
	Normal v HGD	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

Table 4/3. Univariate analysis of significance of differences in Ki67 and Mcm protein expression between normal and dysplastic epithelium on the surface of oesophageal squamous and Barrett's mucosa.

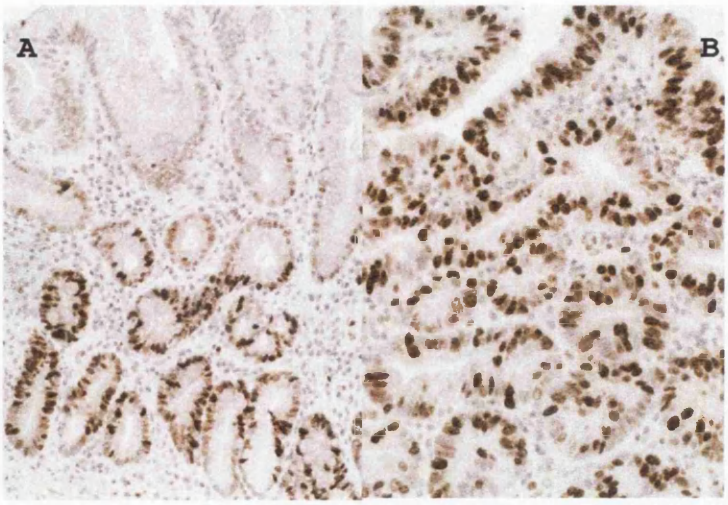


Figure 4/3. Distribution of proliferating cells in Barrett's mucosa without dysplasia (A) and with high grade dysplasia (B). Immunohistochemistry for Ki67 (monoclonal antibody MIB-1). Distinct proliferation and differentiation compartments in the non-dysplastic mucosa are lost in dysplasia.

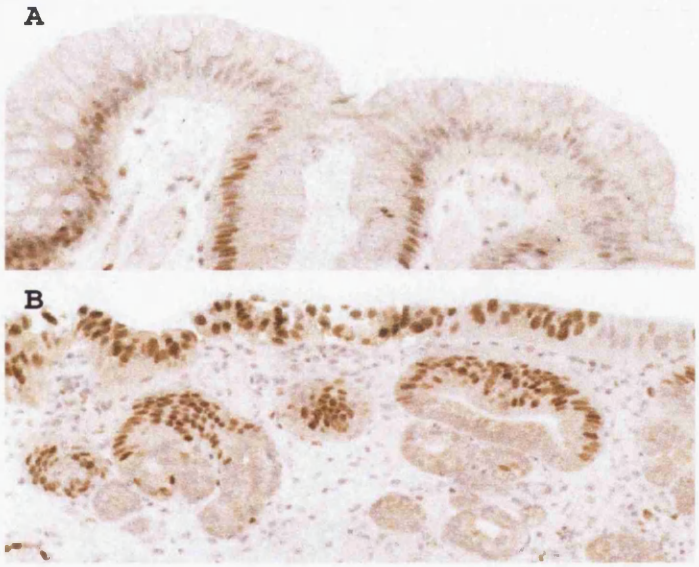


Figure 4/4. Distribution of DNA replication licensed cells in non-dysplastic Barrett's mucosa (A) and low-grade Barrett's mucosa (B). In the absence of dysplasia cells lose their replication as they migrate on to the mucosal surface, but retain that capability in the dysplastic mucosa. Notice the abrupt transition between licensed and unlicensed cell populations on the right side of the lower figure.

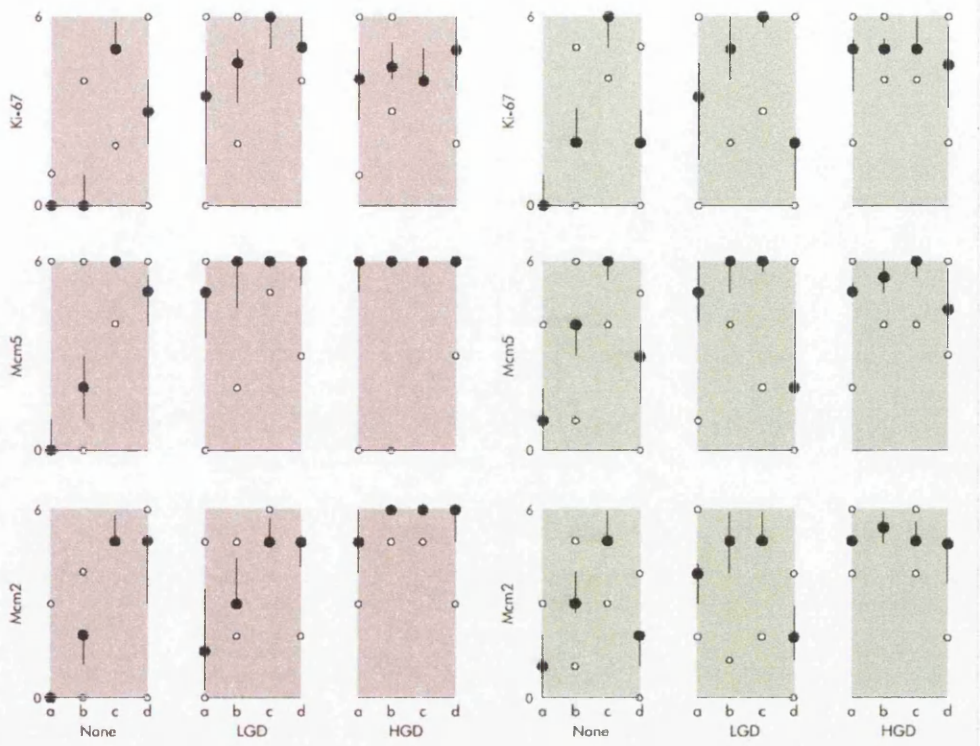


Figure 4/5. Expression of minichromosome maintenance proteins 2 and 5 and Ki67 in squamous epithelium (left, pinkish background) and Barrett's mucosa (right, greenish background). Each of the 18 small plots indicates the distribution of one marker in four layers from most superficial on the left (a) to deepest (d) on the right, as described in the methods section. All the plots in one row are for the same marker protein and the dysplasia status of the mucosa is indicated at the foot of each column. Large blobs represent medians; bars the interquartile range; and small circles the range in each case.

Although persistent expression of Mcm proteins is a very characteristic feature of dysplastic squamous and Barrett's epithelium, this is clearly not an absolute feature of the neoplastic state, as we did observe cases in which viable but Mcm-negative tumour cells were present in both invasive adenocarcinoma and invasive squamous carcinoma of the oesophagus.

Chapter 5: Mucin Phenotypes

Based on the anticipated risk of malignancy, the spectrum of Barrett's mucosa can be divided into the following two types [92]: a) Columnar epithelium without specialised intestinal metaplasia composed of columnar mucosa, but lacking goblet cells. b) Columnar epithelium with specialised intestinal metaplasia including goblet cells and columnar non-goblet cells. Specialised intestinal metaplastic epithelium in the oesophagus can further be subdivided by the mucin profile and appearance of the columnar cells [19,20]:

a) Complete intestinal metaplasia (type 1), in which the columnar cells resemble normal enterocytes without mucus secretion and with well-developed brush borders. This type of intestinal metaplasia is relatively uncommon in Barrett's mucosa.

b) Incomplete intestinal metaplasia (also known as type 2A), in which columnar cells secrete neutral mucins or sialomucins, but not sulphomucins and there is an incomplete or absent brush border. The goblet cells may contain sulphomucins.

c) Incomplete intestinal metaplasia (type 2B), in which columnar cells secrete sulphomucins.

Adenocarcinoma of the stomach is often associated with type 2B intestinal metaplasia of the adjacent mucosa and histological identification of goblet cells is regarded as the hallmark of Barrett's oesophagus patients at higher risk of adenocarcinoma and therefore candidates for endoscopic surveillance [18-20,92]. Sulphomucins in columnar cells have also been considered a marker of dysplasia in Barrett's oesophagus [18]. The role of the columnar non-goblet cells in the progression towards malignancy is not yet clarified but they have been shown to express the same intestinal enzymes as neoplastic cells of associated adenocarcinomas [92].

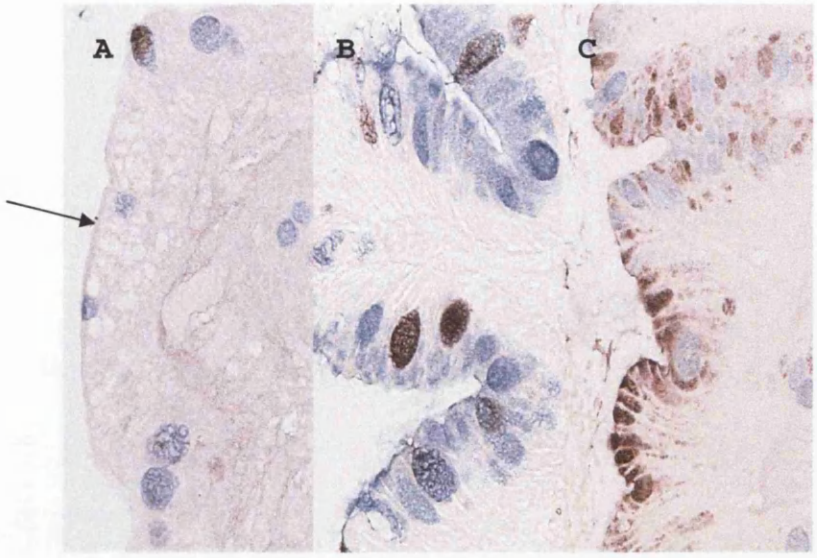


Figure 5/1. Different IM types.(A):Type 1. Note brush border (arrow).(B):Type IIa. High iron diamine staining (sulphomucins) confined to goblet cells.(C):Type IIb. Sulphomucin-positive columnar cells are numerous.

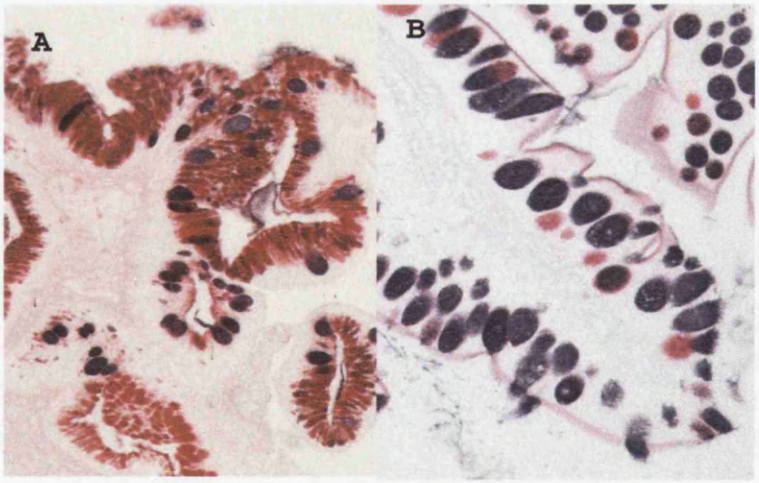


Figure 5/2. Incomplete (A) and complete (B) intestinal metaplasia. Strongly alcianophilic goblet cells and weakly alcianophilic columnar cells are present in incomplete intestinal metaplasia. This could be type IIA or type IIB. The brush border of the enterocytes and their lack of cytoplasmic mucin are characteristic of complete intestinal metaplasia.

There is a range of routine stains available to identify mucin profiles of the columnar lined oesophagus. Periodic acid - Schiff stains neutral mucins of both gastric fundic and cardiac types of Barrett's mucosa [20]. Alcian blue at pH 2.5 stains acidic mucins (sialomucins and sulphomucins) and can be combined with high iron diamine staining for sulphomucins.

We established sulphomucin profiles of the columnar cells in intestinal metaplasia of oesophageal and gastric biopsies by the high iron diamine/Alcian blue staining technique on non-dysplastic and dysplastic tissue to investigate whether an association could be detected between the type 2B incomplete intestinal metaplasia phenotype and early events in the progression to oesophageal adenocarcinoma.

Chapter 5: Materials and Methods

Sections of 1240 formalin-fixed paraffin-embedded gastric and OGJ biopsies taken from patients identified as showing intestinal metaplasia on routine pathology reports were stained using a High Iron Diamine/Alcian Blue technique to establish their mucin phenotype and this was subsequently compared to the presence and degree of dysplasia present in these sections.

High Iron Diamine/Alcian Blue staining protocol

1] Sections were dewaxed through xylene, rehydrated through graded alcohols and washed in water.

2] Sections left for 24 hours in a solution of freshly prepared N,N-dimethyl-m-phenylene diamine dihydrochloride (Sigma) and N,N-dimethyl-p-phenylene diamine hydrochloride (Sigma) dissolved in distilled water and 40% ferric chloride (BDH).

3] Sections were rinsed rapidly in water and left in Alcian Blue (Sigma) for 1 hour.

4] Sections were then dehyrated rapidly through graded alcohols, cleared in xylene and mounted in a resinous mountant.

Scoring

Type 1 complete intestinal metaplasia was indicated by surface columnar cells which resemble normal enterocytes with well developed brush borders.

Type IIA intestinal metaplasia was defined by blue or blue-black staining goblet cells containing sialomucins or sulphomucins scattered among columnar cells containing either neutral mucins or blue staining sialomucins, with no evidence of a brush border or high iron diamine staining.

Type IIB intestinal metaplasia was defined black/blue staining of columnar cells, indicating the presence of sulphomucins, with goblet cells containing either sialomucins, sulphomucins, or both.

Reproducibility of scoring was tested by independent allocation of a random sample of 43 biopsies to the types indicated above (I/IIA/IIB) by LN and also by the supervisor (JJG). The Kappa statistic was calculated from the resulting 3x3 contingency table as a measure of agreement.

Results

Reproducibility of mucin phenotyping.

Forty-three biopsies were scored independently by LN and JJG. The 3x3 contingency table (5/1) represents the results of this exercise:

		Classification of mucin phenotype by JJG		
		I	IIA	IIB
Classification of mucin phenotype by LN	I	3	3	0
	IIA	1	17	5
	IIB	0	0	14

Table 5/1. Comparison of classification of mucin phenotype by LN and JJG

The kappa score for these values is 0.56, which indicates reasonable if not outstanding agreement. While the agreement could have been better, agreement was good enough for the data to be analysed, therfore no attempt was made to improve it.

Mucin phenotype, dysplasia and neoplasia (all sites)

Initially the distribution of mucin phenotypes was looked at as a function of the presence and degree of dysplasia at all sites. The following categories were extracted from the pathology reports: 0, no dysplasia or atypia. 1, reactive atypia. 2, indefinite for dysplasia. 3, low grade dysplasia, 4, high grade dysplasia. 5, invasive carcinoma. The following table shows the raw data analysed for all biopsies with the worst atypia/neoplasia score of the biopsy taken from the original pathology report:

	Worst atypia/neoplasia score					
Mucin	**0**	**1**	**2**	**3**	**4**	**5**
I	136	13	0	0	0	5
II (NOS)	1	0	0	0	0	0
I/IIA	83	21	1	2	0	5
I/IIB	27	12	3	1	0	4
IIA	341	89	26	24	6	11
IIB	332	169	59	58	19	13

Table 5/2. Mucin phenotypes for all biopsies stratified by atypia.

This table can be simplified by combining groups in order to show how many biopsies contain the relevant types of metaplastic mucosa:

	Worst atypia/neoplasia score					
Mucin	**0**	**1**	**2**	**3**	**4**	**5**
I	246	46	4	3	0	14
IIA	424	110	27	26	6	16
IIB	359	181	62	59	19	17

Table 5/3. Mucin phenotypes for all biopsies stratified by atypia. Some biopsies are represented in row 1 and row 2 because both types of IM were present. Type IIB IM appears only in row 3.

or

	Worst atypia/neoplasia score					
Mucin	**0**	**1**	**2**	**3**	**4**	**5**
I	246	46	<	7	>	14
IIA	424	110	<	59	>	16
IIB	359	181	<	140	>	17

Table 5/4. As 5/2, but combining dysplasia groups.

or

	Worst atypia/neoplasia score					
Mucin	**0**	**1**	**2**	**3**	**4**	**5**
I	292(264)		<	7(40)	>	14(9)
IIA	534(513)		<	59(77)	>	16(18)
IIB	540(588)		<	140(89)	>	17(20)

Table 5/5. As 5/3, but combining the groups with no atypia or reactive atypia and the dysplasia groups. Expected values are given in brackets.

Inspection of the pooled data (table 5/4) shows that the distribution of IM phenotypes does not differ significantly from expected in this large biopsy series for dysplasia groups 0 and 1 (no dysplasia, reactive atypia) or for the cancer group (5), but there is a strikingly non-random distribution within the dysplastic biopsies (dysplasia groups 2-4). $\chi^2 = 72.165$ (d.f. = 2, $P < 0.0005$). This very large χ^2 value is almost entirely due to the deficiency of type I IM and the excess of type IIB IM in this group.

Without getting involved in excessive subgroup analysis, it seemed appropriate to repeat this analysis separating the 812 true oesophageal biopsies from gastric sites and the OG junction (N = 649).

Mucin phenotype, dysplasia and neoplasia (oesophagus)

	Worst atypia/neoplasia score					
Mucin	**0**	**1**	**2**	**3**	**4**	**5**
I	27	7	0	0	0	0
I/IIA	14	3	0	0	0	0
I/IIB	8	5	2	0	0	1
IIA	148	57	23	22	3	2
IIB	224	139	54	51	16	5

Table 5/6. Mucin phenotypes for oesophageal biopsies by atypia.

	Worst atypia/neoplasia score					
Mucin	**0**	**1**	**2**	**3**	**4**	**5**
I	49	25	2	0	0	1
IIA	162	60	23	22	3	2
IIB	232	144	56	51	16	6

Table 5/7. Mucin phenotypes for oesophageal biopsies stratified by atypia. Some biopsies are represented in row 1 and row 2 because both types of IM were present. Type IIB IM appears only in row 3.

	Worst atypia/neoplasia score					
Mucin	**0**	**1**	**2**	**3**	**4**	**5**
I	49	25	<	2	>	1
IIA	162	60	<	48	>	2
IIB	232	144	<	123	>	6

Table 5/8. As 5/7, but combining dysplasia groups.

	Worst atypia/neoplasia score					
Mucin	**0**	**1**	**2**	**3**	**4**	**5**
I	74(60)		<	2(16)	>	1
IIA	222(214)		<	48(55)	>	2
IIB	376(397)		<	123(102)	>	6

Table 5/9. As 5/8, but combining the groups with no atypia or reactive atypia and the dysplasia groups. Expected values are given in brackets except where the expected numbers are <5.

Using the figures in table 5/9 but omitting column 5 (because the expected values are all <5) gives $\chi^2 = 21.40$, and again this large χ^2 value (d.f. = 1, $P < 0.0005$) is almost entirely due to the deficiency of type I IM and the excess of type IIB IM in the dysplasia group.

Finally the gastric biopsies were examined (including the oesophago-gastric junction and cardia, in more or less equal numbers).

Mucin phenotype, dysplasia and neoplasia (gastric)

	Worst atypia/neoplasia score					
Mucin	**0**	**1**	**2**	**3**	**4**	**5**
I	109	6	0	0	0	5
I/IIA	69	18	1	2	0	5
I/IIB	19	7	1	1	0	3
IIA	193	32	3	2	2	9
IIB	109	30	5	7	3	8

Table 5/10. Mucin phenotypes for gastric biopsies by atypia.

	Worst atypia/neoplasia score					
Mucin	**0**	**1**	**2**	**3**	**4**	**5**
I	197	31	2	3	0	13
IIA	266	50	4	4	2	14
IIB	128	37	6	8	3	11

Table 5/11. Mucin phenotypes for gastric biopsies stratified by atypia. Some biopsies are represented in row 1 and row 2 because both types of IM were present. Type IIB IM appears only in row 3.

	Worst atypia/neoplasia score					
Mucin	**0**	**1**	**2**	**3**	**4**	**5**
I	197	31	<	5	>	13
IIA	266	50	<	10	>	14
IIB	128	37	<	17	>	11

Table 5/12. As 5/7, but combining dysplasia groups.

	Worst atypia/neoplasia score					
Mucin	**0**	**1**	**2**	**3**	**4**	**5**
I	228(224)		<	5(10)	>	13(12)
IIA	316(309)		<	10(14)	>	14(17)
IIB	165(176)		<	17(8)	>	11(9)

Table 5/13. As 5/8, but combining the groups with no atypia or reactive atypia and the dysplasia groups. Expected values are given in brackets except where the expected numbers are <5.

Using the figures in table 5/13 χ^2 = 15.7, (d.f. = 2, P = 0.0005), and this χ^2 value is less entirely due to the deficiency of type I IM than the excess of type IIB IM in the dysplasia group.

Chapter 6: Discussion

Oesophageal cancer remains a substantial challenge. In the West Barrett's cancer is increasingly recognised and in recent years it has overtaken squamous cancer, but remains less common in the Far East where squamous cancer of the oesophagus remains a major killer. Screening for cancer precursors or early cancers is an attractive strategy for reducing the impact for neoplastic diseases, and screening programmes have been implemented for cancer of the cervix and cancer of the breast and there are ongoing programmes which seek to develop screening for colon, prostate, lung and other common cancers. In point of fact, there are rather stringent requirements to be met before the costs of a screening programme can be justified, and in the case of oesophageal cancer these criteria are nowhere near met.

At the very least, a screening programme must have available a test or tests which is sensitive and specific for the disease entity (e.g. cervical intraepithelial neoplasia, early breast cancer, etc) which are to be detected, these values must translate, taking into consideration the actual incidence of the disease in the population being screened, into positive and negative predictive values sufficiently robust to avoid unnecessary anxiety caused by false positives without missing cases (false negatives), and there must be effective and acceptable treatments available, appropriate to the known risks associated with the disease.

In the case of the oesophagus, there are difficulties. In the West, most oesophageal adenocarcinoma presents in the context of Barrett's oesophagus, but occurs in patients not previously known to have had Barrett's oesophagus, so in order to have a substantial impact on oesophageal adenocarcinoma, it would almost inevitably be necessary to screen for Barrett's oesophagus; but there is major controversy about the exact size of the risk which is actually associated with Barrett's oesophagus, the size of that risk may have been substantially exaggerated as a consequence of publication bias [93].

Nevertheless, the possibility of effective screening and intervention remains important. While a case for screening for Barrett's oesophagus would be hard to justify at the present time [96], the fact remains that in the course of investigation for upper GI symptoms, many patients are identified with Barrett's oesophagus, and the question then arises what should be done about them in terms of follow up. Most of these people will never develop oesophageal adenocarcinoma, but some of them will, can those at risk be identified? This remains the key question, and the studies described in this thesis all had this question in the background.

Differentiating primary gastric cancer from oesophageal cancer can be difficult, especially when tumours involve both the stomach and oesophagus. Adenocarcinomas of both the stomach and oesophagus typically arise in a background of intestinal metaplasia. Carcinomas from the gastric cardia differ from those of the rest of the stomach. They share common epidemiological characteristics with oesophageal adenocarcinoma. Goldblum et al (58) found reflux systems to be more frequent in patients with SSBO than in those with cardiac IM. Additionally, patients with SSBO had a high male: female ratio, which is similar to LSBO, whereas patients with cardiac IM were found to be mostly female and to have a lower frequency of GORD systems.

Although dysplastic risk in IM of the cardia remains unclear, Sharma et al (59) prospectively followed patients with SSBO and IM of the cardia and found the risk of dysplasia to be significantly greater in SSBO than in IM of the cardia, indicating two potentially different clinical processes. Regular endoscopic surveillance is required in patients with LSBE and is frequently performed in SSBE, but the need for surveillance in IM of the cardia is unknown. IM in SSBO and below the Z line can be histologically indistinguishable by haematoxylin and eosin sections. Additionally, endoscopically, an irregular Z-Line can be a normal finding and determination of where the oesophagus and the cardia begin can be unreliable.

Ormsby et al (62) first described a Barrett's Ck7/20 immunophenotype as superficial Ck20 staining and strong Ck7 staining of both superficial and deep glands in 94% of oesophageal resection specimens and in 100% of LSBO biopsy specimen or gastric resection specimens in patients with histological evidence of IM. They found the Barrett's Ck7/20 pattern was highly sensitive and specific compared to cases with gastric IM and therefore could reliably identify the location of IM in the oesophagus and stomach. They later described a Ck 7 positive, Ck 20 negative immunophenotype in 90% of patients with Barrett's oesophageal adenocarcinoma and only in 21% of gastric adenocarcinoma cases, so concluded this pattern to be potentially useful in accurate tumour classification.

Jovanovic et al (63) confirmed the immunophenotype described by Ormsby in 94% of LSBO cases, a pattern which was not seen in any of their 36 cases with IM of the stomach. In cases with IM of the cardia, 93% expressed the Ck7/20 immunophenotype seen in the gastric mucosa. On the other hand, studies by some other researchers have not been able to support Ormby's findings. Mohammed et al (95) found that although the Barretts Ck7/20 was observed in many cases of Barrett's oesophagus, the sensitivity and specificity were only moderate (65% and 56% respectively) and that the cardiac IM pattern was variable. Additionally, where Ormsby (57) had described the Barrett's Ck7/20 pattern in 82% of patients with SSBE, not seen in any of their patients with gastric IM, Kurtkaya-Yapicier et al (96) found only 10% of patients with SSBE showed the anticipated Ck7/20 pattern. They found the two patterns of Ck7/20 to have low sensitivity and high false negativity values.

For this study the main purpose of this part of the work was to determine whether different IM phenotypes, in particular types IIA and IIB, might be associated with distinctive cytokeratin phenotypes which, in view of the association (described in chapter 5) between dysplasia risk and mucin phenotype might have been of some practical value. It does not appear that the prospects here are very encouraging. We find no

difference in the Ck 7/ Ck 20 phenotypes for types IIA and IIB intestinal metaplasia and the numbers of type I cases are too small to be definitive. Ormsby et al [58,62,63] describe differences between oesophageal and gastric intestinal metaplasia patterns of Ck 7/ Ck 20 expression which have generated a certain amount of interest as appearing to offer the prospect of being able to discriminate between short and ultra-short Barrett's oesophagus and gastric metaplasia at the gastric cardia, which appear to differ in the associated risk of neoplastic disease. Although this work did not address the question of gastric Ck 7/Ck 20 patterns in many cases, the description of the phenotype recognised as characteristic by Ormsby et al (Gastric incomplete intestinal metaplasia: Ck7 weak superficial and deep, Ck20 patchy superficial and deep) clearly overlap with the patterns we are observing in classical Barrett's intestinal mucosa, and are unlikely therefore to be of great discriminatory power in that context.

Whether the reasons for the differences experienced in looking at the usefulness of Ck7/20 patterns in the oesophagus and stomach are due to inaccurate visualisation of SSBO or interobserver variability or possibly differences in tissue processing and staining techniques, it remains that if the results between groups are not comparable, Ck7/20 immunophenotype cannot be reliably used to differentiate between IM taken from above or below the Z line.

This study supports the observation that Barrett's mucosa is phenotypically complex. It confirms the findings of other groups which describe a mixture of cardiac, fundic and specialised Barrett's in the lower oesophageal sphincter region and a mixture of cardiac, specialised, but rarely fundic mucosal types above this region, with several different mucosal phenotypes visible at a single level. As Barrett's epithelium may show as islands of columnar epithelium remote from the cardia [25] it is unlikely to evolve from a process of creeping substitution from the cardia but may develop from cells intrinsic to the region. It has been postulated that stem cells in the oesophagus possess multipotentiality

for cell differentiation and this could account for the variety of cell types seen in Barrett's epithelium. It could be that the luminal reflux contents may influence the morphology of the epithelium.

Specialised intestinal- type Barrett's mucosa is invariably present in LSBO and the previously reported association of dysplasia with intestinal Barrett's mucosa is very striking in our series [5,17,74].

The absence of obvious zonation of dysplasia in this study supports the previous observation, from relatively small patient numbers, that dysplasia is evenly distributed along the length of Barrett's oesophagus. McArdle et al [75] histologically evaluated the entire mucosal surface of 7 oesophagectomy specimens with high grade dysplasia or early carcinoma. They showed an equal likelihood of high grade dysplasia or early invasive carcinoma occurring throughout the length of Barrett's epithelium with the amount of dysplastic epithelium related to the surface area of Barrett's epithelium but without an association between the extent of dysplasia and the likelihood of carcinoma. As mapping studies show dysplasia involving a variable amount of oesophageal mucosa [17, 75] the current recommendation for uniformly distributed endoscopic biopsies of the entire Barrett's segment is therefore supported.

The impressive association between dysplasia and intestinal metaplasia in our series confirms that endoscopic or molecular markers of intestinal phenotype might allow useful biopsy targeting [76], but only if a practical endoscopic technique of visualisation could be devised.

The data presented in chapter in chapter 4 confirm the presence of disrupted spacial organisation of cellular proliferation and maturation in both squamous oesophageal dysplasia and Barrett's dysplasia. Identifying these changes could be of practical significance in relation to the challenge of screening for squamous dysplasia in Oriental populations and Barrett's dysplasia in the West. The fact that both these conditions are characteristically associated with marked dysregulation of

a family of proteins which are expressed at high levels suggests that these may have the potential to act as useful markers in their own right.

Patients with Barrett's oesophagus may be subjected to frequent endoscopy and biopsy. A sensitive and specific test for dysplasia might allow Barrett's patients to be screened for dysplasia and divided into a cohort without dysplasia, at a low risk of oesophageal adenocarcinoma, for whom less intensive follow up would be safe and a higher risk group with dysplasia, for whom more frequent endoscopic and biopsy surveillance could be appropriate.

Endoscopic surveillance is costly and unpleasant and biopsy sampling may miss focal areas of dysplasia. The superficial expression of Mcm2, Mcm 5 anfd Ki 67 proteins by dysplastic Barrett's mucosa, suggests that exfoliative brush cytology could be used to increase sample areas. Non-endoscopic screening cytology has been attempted in Chinese populations [56] and the changes we have described suggest that this approach supplemented either by immunohistochemistry or biochemical determination of Mcm proteins, could have a useful screening role.

Analogous to their application in a variety of other contexts such as screening the urinary tract for urothelial neoplasia, or screening smears for the lesions which lead to invasive carcinoma of the cervix, [91], this study supports the concept that Mcm protein expression in dysplastic epithelia is associated with preneoplastic cells locked in the cell cycle (confirmed by persistance of positive Ki 67 expression).

In the stomach an incompletely differentiated variant of intestinal metaplasia secreting sulphomucins has been shown in several studies to be associated with the presence of gastric carcinoma of intestinal type. Jass [96] reported a similar association between this variant of IM and well differentiated adenocarcinoma arising in the columnar lined oesophagus and he suggested that the presence of this type of IM in oesophageal biopsies may serve as an important marker for

identification of a sub group of patients at particular risk of developing oesophageal adenocarcinoma.

Haggitt et al [97] investigated the relationship of sulphated mucins, flow cytometry and histologic diagnosis in 152 biopsies from 42 patients with histological diagnosis of dysplasia or carcinoma. They found sulphated mucins in non goblet columnar cells of Barrett's metaplastic epithelium (as detected by the high iron diamine-alcian blue stain) present in 73% of patient with histological diagnosis of dysplasia or carcinoma, 78% in patients whose biopsies were indefinite for dysplasia and in 55% of patients whose biopsies were negative for dysplasia . Abnormal flow cytometry was found in all patients with histological diagnosis of dysplasia or carcinoma, in 33% indefinite for dysplasia and in 5% negative for dysplasia. They concluded that the presence of sulphated mucin did not have sufficiently high sensitivity or specificity for dysplasia or carcinoma to be of value in managing patients whereas abnormal flow cytometry correlated extremely well with histological diagnosis of dysplasia and carcinoma and detected a subset of patients who were histologically indefinite or negative for dysplasia but who had flow cytometric abnormalities similar to those otherwise seen only in dysplasia and carcinoma.

The purpose of this part of the study was to see whether the presence of type IIB intestinal metaplasia, characterised by sulphomucins in columnar epithelium (non-goblet cells) might be strongly enough associated with dysplasia to be a useful biomarker to be exploited in biopsy screening for dysplasia in Barrett's oesophagus. There is indeed a positive association of type IIB intestinal metaplasia with dysplasia both at gastric and intestinal loci, but there is an even stronger association between type I intestinal metaplasia and the absence of dysplasia. Type I (complete) intestinal metaplasia implies a definite small intestinal phenotype and it is striking that this phenotype is negatively associated with dysplasia, given the rarity of dysplasia and primary adenocarcinoma in the small intestine. If incomplete types of

intestinal metaplasia (types IIA and particularly IIB) are manifestations of a colonic mucosal phenotype, this may suggest a link with the much greater risk of neoplasia which exists in large bowel mucosa. Markers of differentiation in different parts of the bowel are increasingly well characterised [98,99] and such markers may allow easier phenotyping and ultimately risk stratification of the metaplastic mucosa in Barrett's. The less than perfect kappa values associated with allocation of intestinal metaplasia to types I, IIA and IIB suggest that these characteristics themselves may not be sufficiently robust for routine clinical use. Such relatively modest agreement is very common in studies of pathological classification and diagnosis [100,101] and presumably reflects the subjective nature of these evaluations.

Conclusions.

It does not appear likely that Ck7/Ck20 phenotypes will be very useful in identifying an 'at risk' type of oesophageal intestinal metaplasia, as the expression patterns seem too variable to be highly distinctive. Nor is the size of the difference in the probability of dysplasia being associated with type IIA vs. type IIB intestinal metaplasia in Barrett's oesophagus likely to form the basis of a useful risk stratification. The decreased probability of dysplasia being found in the presence of Type I intestinal metaplasia seems more striking and interesting, and understanding the molecular underpinnings of these patterns of differentiation may give useful further information. It has sometimes been suggested that patients without evidence of intestinal metaplasia in Barrett's oesophagus are not at increased risk of cancer and need not be subjected to any ongoing surveillance, but an important finding is that such intestinal metaplasia is almost invariably present in Barrett's oesophagus if the density of biopsy taking is sufficient to detect it. It is not likely that many people will escape from surveillance in this manner. A much more plausible scenario is that patients in whom thorough biopsy has detected no evidence of dysplasia at

any site are likely not to require further surveillance for at least several years and some centres are adopting a policy of less intensive surveillance in such patients. A corollary of this approach, of course, is that the detection of dysplasia by the reporting pathologist must be very precise, and we know that there are problems in this area. In this situation the rather robust upregulation of the Mcm proteins in the dysplastic Barrett's mucosa is of interest and deserves evaluation as a potential marker of patients in whom expansion of neoplastic clones and subsequent cancer risk are greatest. This will ultimately require a relatively large scale prospective evaluation in order to be tested fully.

Finally, the distribution of the Mcm proteins to the mucosal surface in dysplastic Barrett's mucosa offers the hope that non-endoscopic methods may eventually be able to sample the mucosal surface widely and that detection of aberrant Mcm protein expression could identify patients requiring more intensive investigation by endoscopy and biopsy.

References

1. Griffel, L.H., Amenta, P.S. and Das, K.M. (2000) Use of a novel monoclonal antibody in diagnosis of Barrett's esophagus. *Dig Dis Sci*, 45, 40-8.

2. Spechler, S.J. and Goyal, R.K. (1996) The columnar-lined esophagus, intestinal metaplasia, and Norman Barrett. *Gastroenterology,* 111, 565-66.

3. Wright, T.A., Gray, M.R., Morris, A.I., Gilmore, I.T., Ellis, A., Smart, H.L., Myskow, M., Nash, J., Donnelly, R.J. and Kingsnorth, A.N. (1996) Cost effectiveness of detecting Barrett's cancer. *Gut*, 39, 574-9.

4. Shaheen, N. and Ransohoff, D.F. (2002) Gastroesophageal reflux, Barrett esophagus, and esophageal cancer: clinical applications. *JAMA*, 287, 1982-6.

5. Nilsson, J., Skobe, V., Johansson, J., Willen, R. and Johnsson, F. (2000) Screening for oesophageal adenocarcinoma: an evaluation of a surveillance program for columnar metaplasia of the oesophagus. *Scand J Gastroenterol*, 35, 10-6.

6. Spechler, S.J. (2003) Managing Barrett's oesophagus. *BMJ*, 326, 892-4.

7. Shamma, A., Yamamoto, H., Doki, Y., Okami, J., Kondo, M., Fujiwara, Y., Yano, M., Inoue, M., Matsuura, N., Shiozaki, H. and Monden, M. (2000) Up-regulation of cyclooxygenase-2 in squamous carcinogenesis of the esophagus. *Clin Cancer Res*, 6, 1229-38.

8. Tan, W., Song, N., Wang, G.Q., Liu, Q., Tang, H.J., Kadlubar, F.F. and Lin, D.X. (2000) Impact of genetic polymorphisms in cytochrome P450 2E1 and glutathione S-transferases M1, T1, and P1 on susceptibility to esophageal cancer among high-risk individuals in China. *Cancer Epidemiol Biomarkers Prev*, 9, 551-6.

9. Mizobuchi, S., Furihata, M., Sonobe, H., Ohtsuki, Y., Ishikawa, T., Murakami, H., Kurabayashi, A., Ogoshi, S. and Sasaguri, S. (2000) Association between p53 immunostaining and cigarette smoking in squamous cell carcinoma of the esophagus. *Jpn J Clin Oncol*, 30, 423-8.

10. Montesano, R., Hollstein, M. and Hainaut, P. (1996) Genetic alterations in esophageal cancer and their relevance to etiology and pathogenesis: a review. *Int J Cancer*, 69, 225-35.

11. Jankowski, J.A., Provenzale, D. and Moayyedi, P. (2002) Esophageal adenocarcinoma arising from Barrett's metaplasia has regional variations in the west. *Gastroenterology*, 122, 588-90.

12. Weston, A.P., Badr, A.S. and Hassanein, R.S. (1999) Prospective multivariate analysis of factors predictive of complete regression of Barrett's esophagus. *Am J Gastroenterol*, 94, 3420-6.

13. Reid, B.J., Blount, P.L., Rubin, C.E., Levine, D.S., Haggitt, R.C. and Rabinovitch, P.S. (1992) Flow-cytometric and histological progression to malignancy in Barrett's esophagus: prospective endoscopic surveillance of a cohort. *Gastroenterology*, 102, 1212-9.

14. Jankowski, J.A., Wright, N.A., Meltzer, S.J., Triadafilopoulos, G., Geboes, K., Casson, A.G., Kerr, D. and Young, L.S. (1999) Molecular evolution of the metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. *Am J Pathol*, 154, 965-73.

15. Cameron, A.J., Zinsmeister, A.R., Ballard, D.J. and Carney, J.A. (1990) Prevalence of columnar-lined (Barrett's) esophagus. Comparison of population-based clinical and autopsy findings. *Gastroenterology*, 99, 918-22.

16. Lapertosa, G., Baracchini, P. and Fulcheri, E. (1992) Mucin histochemical analysis in the interpretation of Barrett's esophagus. Results of a multicenter study. The Operative Group for the Study of Esophageal Precancer. *Am J Clin Pathol*, 98, 61-6.

17. Haggitt, R.C. (1994) Barrett's esophagus, dysplasia, and adenocarcinoma. *Hum Pathol*, 25, 982-93.

18. Rothery, G.A., Patterson, J.E., Stoddard, C.J. and Day, D.W. (1986) Histological and histochemical changes in the columnar lined (Barrett's) oesophagus. *Gut*, 27, 1062-8.

19. Peuchmaur, M., Potet, F. and Goldfain, D. (1984) Mucin histochemistry of the columnar epithelium of the oesophagus (Barrett's oesophagus): a prospective biopsy study. *J Clin Pathol*, 37, 607-10.

20. Johnston, M.H., Hammond, A.S., Laskin, W. and Jones, D.M. (1996) The prevalence and clinical characteristics of short segments of specialized intestinal metaplasia in the distal esophagus on routine endoscopy. *Am J Gastroenterol*, 91, 1507-11.

21. Loughney, T., Maydonovitch, C.L. and Wong, R.K. (1998) Esophageal manometry and ambulatory 24-hour pH monitoring in patients with short and long segment Barrett's esophagus. *Am J Gastroenterol*, 93, 916-9.

22. Donahue, P.E. (1997) Basic considerations in gastroesophageal reflux disease. *Surg Clin North Am*, 77, 1017-40.

23. DeMeester, S.R. and DeMeester, T.R. (2000) Columnar mucosa and intestinal metaplasia of the esophagus: fifty years of controversy. *Ann Surg*, 231, 303-21.

24. Cameron, A.J. and Lomboy, C.T. (1992) Barrett's esophagus: age, prevalence, and extent of columnar epithelium. *Gastroenterology*, 103, 1241-5.

25. Gillen, P., Keeling, P., Byrne, P.J., West, A.B. and Hennessy, T.P. (1988) Experimental columnar metaplasia in the canine oesophagus. *Br J Surg*, 75, 113-5.

26. Bremner, C.G. (1977) The columnar-lined (Barrett's) esophagus. *Surg Annu*, 9, 103-23.

27. Meyer, J.H. (1979) Reflections on reflux gastritis. *Gastroenterology*, 77, 1143-5.

28. Lagergren, J., Bergstrom, R. and Nyren, O. (1999) Association between body mass and adenocarcinoma of the esophagus and gastric cardia. *Ann Intern Med*, 130, 883-90.

29. Kuo, B. and Castell, D.O. (1996) Optimal dosing of omeprazole 40 mg daily: effects on gastric and esophageal pH and serum gastrin in healthy controls. *Am J Gastroenterol*, 91, 1532-8.

30. Sagar, M., Janczewska, I., Ljungdahl, A., Bertilsson, L. and Seensalu, R. (1999) Effect of CYP2C19 polymorphism on serum levels of vitamin B12 in patients on long-term omeprazole treatment. *Aliment Pharmacol Ther*, 13, 453-8.

31. Katza, D.A., Castell D.O. (1994) Successful elimination of reflux symptoms does not insure adequate control of acid reflux in Patients with Barrett's esophagus. *Am J Gastroenterol, 89, 989-991.*

32. Morales, T.G., Bhattacharyya, A., Johnson, C. and Sampliner, R.E. (1997) Is Barrett's esophagus associated with intestinal metaplasia of the gastric cardia? *Am J Gastroenterol*, 92, 1818-22.

33. Wright, T.A. (1997) High-grade dysplasia in Barrett's oesophagus. *Br J Surg*, 84, 760-6.

34. Lagergren, J., Bergstrom, R., Lindgren, A., Nyren, O. (1999) Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. *N Engl J Med,* 340, 825-831.

35. Henihan, R.D., Stuart, R.C., Nolan, N., Gorey, T.F., Hennessy, T.P. and O'Morain, C.A. (1998) Barrett's esophagus and the presence of Helicobacter pylori. *Am J Gastroenterol*, 93, 542-6.

36. Jankowski, J.A., Harrison, R.F., Perry, I., Balkwill, F. and Tselepis, C. (2000) Barrett's metaplasia. *Lancet*, 356, 2079-85.

37. Romero, Y., Cameron, A.J., Locke, G.R., 3rd, Schaid, D.J., Slezak, J.M., Branch, C.D. and Melton, L.J., 3rd (1997) Familial aggregation of gastroesophageal reflux in patients with Barrett's esophagus and esophageal adenocarcinoma. *Gastroenterology*, 113, 1449-56.

38. Locke, G.R., III, Talley, N.J., Carpenter, H.A., Harmsen, W.S., Zinsmeister, A.R. and Melton, L.J., 3rd (1995) Changes in the site- and histology-specific incidence of gastric cancer during a 50-year period. *Gastroenterology*, 109, 1750-6.

39. Gottfried, M.R., McClave, S.A. and Boyce, H.W. (1989) Incomplete intestinal metaplasia in the diagnosis of columnar lined esophagus (Barrett's esophagus). *Am J Clin Pathol*, 92, 741-6.

40. Clark, G.W., Smyrk, T.C., Burdiles, P., Hoeft, S.F., Peters, J.H., Kiyabu, M., Hinder, R.A., Bremner, C.G. and DeMeester, T.R. (1994) Is Barrett's metaplasia the source of adenocarcinomas of the cardia? *Arch Surg*, 129, 609-14.

41. Hamilton, S.R., Smith, R.R. and Cameron, J.L. (1988) Prevalence and characteristics of Barrett esophagus in patients with adenocarcinoma of the esophagus or esophagogastric junction. *Hum Pathol*, 19, 942-8.

42. Skinner, D.B., Walther, B.C., Riddell, R.H., Schmidt, H., Iascone, C. and DeMeester, T.R. (1983) Barrett's esophagus. Comparison of benign and malignant cases. *Ann Surg*, 198, 554-65.

43. Johansson, J., Johnsson, F., Walther, B., Willen, R., Stael von Holstein, C. and Zilling, T. (1996) Adenocarcinoma in the distal esophagus with and without Barrett's esophagus. Differences in symptoms and survival rates. *Arch Surg*, 131, 708-13.

44. Kubba, A.K., Poole, N.A. and Watson, A. (1999) Role of p53 assessment in management of Barrett's esophagus. *Dig Dis Sci*, 44, 659-67.

45. Lagorce-Pages, C., Paraf, F., Dubois, S., Belghiti, J. and Flejou, J.F. (1998) Expression of CD44 in premalignant and malignant Barrett's oesophagus. *Histopathology*, 32, 7-14.

46. Barrett, M.T., Galipeau, P.C., Sanchez, C.A., Emond, M.J. and Reid, B.J. (1996) Determination of the frequency of loss of heterozygosity in esophageal adenocarcinoma by cell sorting, whole genome amplification and microsatellite polymorphisms. *Oncogene*, 12, 1873-8.

47. Wu, T.T., Watanabe, T., Heitmiller, R., Zahurak, M., Forastiere, A.A. and Hamilton, S.R. (1998) Genetic alterations in Barrett esophagus and adenocarcinomas of the esophagus and esophagogastric junction region. *Am J Pathol*, 153, 287-94.

48. Hameeteman, W., Tytgat, G.N., Houthoff, H.J. and van den Tweel, J.G. (1989) Barrett's esophagus: development of dysplasia and adenocarcinoma. *Gastroenterology*, 96, 1249-56.

49. Morales, T.G. and Sampliner, R.E. (1999) Barrett's esophagus: update on screening, surveillance, and treatment. *Arch Intern Med*, 159, 1411-6.

50. Heitmiller, R.F., Redmond, M. and Hamilton, S.R. (1996) Barrett's esophagus with high-grade dysplasia. An indication for prophylactic esophagectomy. *Ann Surg*, 224, 66-71.

51. Levine, D.S., Haggitt, R.C., Blount, P.L., Rabinovitch, P.S., Rusch, V.W. and Reid, B.J. (1993) An endoscopic biopsy protocol can differentiate high-grade dysplasia from early adenocarcinoma in Barrett's esophagus. *Gastroenterology*, 105, 40-50.

52. Barr, H., Shepherd, N.A., Dix, A., Roberts, D.J., Tan, W.C. and Krasner, N. (1996) Eradication of high-grade dysplasia in columnar-lined (Barrett's) oesophagus by photodynamic therapy with endogenously generated protoporphyrin IX. *Lancet*, 348, 584-5.

53. Levi, F., Ollyo, J.B., La Vecchia, C., Boyle, P., Monnier, P. and Savary, M. (1990) The consumption of tobacco, alcohol and the risk of adenocarcinoma in Barrett's oesophagus. *Int J Cancer*, 45, 852-4.

54. Dowlatshahi, K., Skinner, D.B., DeMeester, T.R., Zachary, L., Bibbo, M. and Wied, G.L. (1985) Evaluation of brush cytology as an independent technique for detection of esophageal carcinoma. *J Thorac Cardiovasc Surg*, 89, 848-51.

55. Dawsey, S.M., Yu, Y., Taylor, P.R., Li, J.Y., Shen, Q., Shu, Y.J., Liu, S.F., Zhao, H.Z., Cao, S.G., Wang, G.Q. and et al. (1994) Esophageal cytology and subsequent risk of esophageal cancer. A prospective follow-up study from Linxian, China. *Acta Cytol*, 38, 183-92.

56. Taniere, P., Martel-Planche, G., Maurici, D., Lombard-Bohas, C., Scoazec, J.Y., Montesano, R., Berger, F. and Hainaut, P. (2001) Molecular and clinical differences between adenocarcinomas of the esophagus and of the gastric cardia. *Am J Pathol*, 158, 33-40.

57. Ormsby, A.H., Vaezi, M.F., Richter, J.E., Goldblum, J.R., Rice, T.W., Falk, G.W. and Gramlich, T.L. (2000) Cytokeratin immunoreactivity patterns in the diagnosis of short-segment Barrett's esophagus. *Gastroenterology*, 119, 683-90.

58. Goldblum, J.R. (2000) The esophagogastric junction. *Am J Surg Pathol*, 24, 1171-4.

59. Sharma, P., Weston, A.P., Morales, T., Topalovski, M., Mayo, M.S. and Sampliner, R.E. (2000) Relative risk of dysplasia for patients with intestinal metaplasia in the distal oesophagus and in the gastric cardia. *Gut*, 46, 9-13.

60. Siewert, J.R. and Stein, H.J. (1998) Classification of adenocarcinoma of the oesophagogastric junction. *Br J Surg*, 85, 1457-9.

61. Ormsby, A.H., Goldblum, J.R., Rice, T.W., Richter, J.E. and Gramlich, T.L. (2001) The utility of cytokeratin subsets in distinguishing Barrett's-related oesophageal adenocarcinoma from gastric adenocarcinoma. *Histopathology*, 38, 307-11.

62. Ormsby, A.H., Goldblum, J.R., Rice, T.W., Richter, J.E., Falk, G.W., Vaezi, M.F. and Gramlich, T.L. (1999) Cytokeratin subsets can reliably distinguish Barrett's esophagus from intestinal metaplasia of the stomach. *Hum Pathol*, 30, 288-94.

63. Jovanovic, I., Tzardi, M., Mouzas, I.A., Micev, M., Pesko, P., Milosavljevic, T., Zois, M., Sganzos, M., Delides, G. and Kanavaros, P. (2002) Changing pattern of cytokeratin 7 and 20 expression from normal epithelium to intestinal metaplasia of the gastric mucosa and gastroesophageal junction. *Histol Histopathol*, 17, 445-54.

64. Chandrasoma, P.T., Der, R., Dalton, P., Kobayashi, G., Ma, Y., Peters, J. and Demeester, T. (2001) Distribution and significance of epithelial types in columnar-lined esophagus. *Am J Surg Pathol*, 25, 1188-93.

65. Mossberg, S.M. (1966) The columnar-lined esophagus (Barrett syndrome)--an acquired condition? *Gastroenterology*, 50, 671-6.

66. Csendes, A., Maluenda, F., Braghetto, I., Csendes, P., Henriquez, A. and Quesada, M.S. (1993) Location of the lower oesophageal sphincter and the squamous columnar mucosal junction in 109 healthy controls and 778 patients with different degrees of endoscopic oesophagitis. *Gut*, 34, 21-7.

67. Bremner, C.G., Lynch, V.P. and Ellis, F.H. (1970) Barrett's esophagus: congenital or aquired? An experimental study of esophageal mucosal regeneration in the dog. *Surgery,* 68: 209-16.

68. Riddell, R.H. (1996) Premalignant and early malignant lesions in the gastrointestinal tract: definitions, terminology, and problems. *Am J Gastroenterol*, 91, 864-72.

69. Schlemper, R.J., Kato, Y. and Stolte, M. (2000) Diagnostic criteria for gastrointestinal carcinomas in Japan and Western countries:

proposal for a new classification system of gastrointestinal epithelial neoplasia. *J Gastroenterol Hepatol*, 15 Suppl, G49-57.

70. Schlemper, R.J., Riddell, R.H., Kato, Y., Borchard, F., Cooper, H.S., Dawsey, S.M., Dixon, M.F., Fenoglio-Preiser, C.M. and et al. (2000) The Vienna classification of gastrointestinal epithelial neoplasia. *Gut*, 47, 251-55.

71. Reid, B.J., Haggitt, R.C., Rubin, C.E., Roth, G., Surawicz, C.M., Van Belle, G., Lewin, K., Weinstein, W.M., Antonioli, D.A., Goldman, H. and et al. (1988) Observer variation in the diagnosis of dysplasia in Barrett's esophagus. *Hum Pathol*, 19, 166-78.

72. Montgomery, E., Bronner, M.P., Goldblum, J.R., Greenson, J.K., Haber, M.M., Hart, J., Lamps, L.W., Lauwers, G.Y., Lazenby, A.J., Lewin, D.N., Robert, M.E., Toledano, A.Y., Shyr, Y. and Washington, K. (2001) Reproducibility of the diagnosis of dysplasia in Barrett esophagus: a reaffirmation. *Hum Pathol*, 32, 368-78.

73. Silcocks, P.B. (1983) Measuring repeatability and validity of histological diagnosis--a brief review with some practical examples. *J Clin Pathol*, 36, 1269-75.

74. Schmidt, H.G., Riddell, R.H., Walther, B., Skinner, D.B. and Riemann, J.F. (1985) Dysplasia in Barrett's esophagus. *J Cancer Res Clin Oncol*, 110, 145-52.

75. McArdle, J.E., Lewin, K.J., Randall, G. and Weinstein, W. (1992) Distribution of dysplasias and early invasive carcinoma in Barrett's esophagus. *Hum Pathol*, 23, 479-82.

76. Going, J.J., Stuart, R.C., Downie, M., Fletcher-Monaghan, A.J. and Keith, W.N. (2002) 'Senescence-associated' beta-galactosidase activity in the upper gastrointestinal tract. *J Pathol*, 196, 394-400.

77. Karam, S.M. (1999) Lineage commitment and maturation of epithelial cells in the gut. *Front Biosci*, 4, D286-98.

78. Hong, M.K., Laskin, W.B., Herman, B.E., Johnston, M.H., Vargo, J.J., Steinberg, S.M., Allegra, C.J. and Johnston, P.G. (1995) Expansion of the Ki-67 proliferative compartment correlates with degree of dysplasia in Barrett's esophagus. *Cancer*, 75, 423-9.

79. Weinreich, M., Liang, C. and Stillman, B. (1999) The Cdc6p nucleotide-binding motif is required for loading mcm proteins onto chromatin. *Proc Natl Acad Sci U S A*, 96, 441-6.

80. Donovan, S., Harwood, J., Drury, L.S. and Diffley, J.F. (1997) Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proc Natl Acad Sci U S A*, 94, 5611-6.

81. Liang, D.T., Hodson, J.A. and Forsburg, S.L. (1999) Reduced dosage of a single fission yeast MCM protein causes genetic instability and S phase delay. *J Cell Sci*, 112 (Pt 4), 559-67.

82. Stillman, B. (1996) Cell cycle control of DNA replication. *Science*, 274, 1659-64.

83. Fujita, M., Yamada, C., Goto, H., Yokoyama, N., Kuzushima, K., Inagaki, M. and Tsurumi, T. (1999) Cell cycle regulation of human CDC6 protein. Intracellular localization, interaction with the human mcm complex, and CDC2 kinase-mediated hyperphosphorylation. *J Biol Chem*, 274, 25927-32.

84. Ohtani, K., Iwanaga, R., Nakamura, M., Ikeda, M., Yabuta, N., Tsuruga, H. and Nojima, H. (1999) Cell growth-regulated expression of mammalian MCM5 and MCM6 genes mediated by the transcription factor E2F. *Oncogene*, 18, 2299-309.

85. Sherman, D.A., Pasion, S.G. and Forsburg, S.L. (1998) Multiple domains of fission yeast Cdc19p (MCM2) are required for its association with the core MCM complex. *Mol Biol Cell*, 9, 1833-45.

86. Ritzi, M., Baack, M., Musahl, C., Romanowski, P., Laskey, R.A. and Knippers, R. (1998) Human minichromosome maintenance proteins and human origin recognition complex 2 protein on chromatin. *J Biol Chem*, 273, 24543-9.

87. Kearsey, S.E., Maiorano, D., Holmes, E.C. and Todorov, I.T. (1996) The role of MCM proteins in the cell cycle control of genome duplication. *Bioessays*, 18, 183-90.

88. Stoeber, K., Mills, A.D., Kubota, Y., Krude, T., Romanowski, P., Marheineke, K., Laskey, R.A. and Williams, G.H. (1998) Cdc6 protein causes premature entry into S phase in a mammalian cell-free system. *Embo J*, 17, 7219-29.

89. Ishimi, Y., Komamura, Y., You, Z. and Kimura, H. (1998) Biochemical function of mouse minichromosome maintenance 2 protein. *J Biol Chem*, 273, 8369-75.

90. Stoeber, K., Halsall, I., Freeman, A., Swinn, R., Doble, A., Morris, L., Coleman, N., Bullock, N., Laskey, R.A., Hales, C.N. and Williams, G.H. (1999) Immunoassay for urothelial cancers that detects DNA replication protein Mcm5 in urine. *Lancet*, 354, 1524-5.

91. Williams, G.H., Romanowski, P., Morris, L., Madine, M., Mills, A.D., Stoeber, K., Marr, J., Laskey, R.A. and Coleman, N. (1998) Improved cervical smear assessment using antibodies against proteins that regulate DNA replication. *Proc Natl Acad Sci U S A*, 95, 14932-7.

92. Chaves, P., Cardoso, P., de Almeida, J.C., Pereira, A.D., Leitao, C.N. and Soares, J. (1999) Non-goblet cell population of Barrett's esophagus: an immunohistochemical demonstration of intestinal differentiation. *Hum Pathol*, 30, 1291-5.

93. Shaheen, N.J., Crosby, M.A., Bozymski, E.M. and Sandler, R.S. (2000) Is there publication bias in the reporting of cancer risk in Barrett's esophagus? *Gastroenterology*, 119, 333-8.

94. Ofman, J.J. (2002) Decision making in gastroesophageal reflux disease. What are the critical issues? *Gastroenterol Clin North Am*, 31, S67-76.

95. Mohammed, I.A., Streutker, C.J. and Riddell, R.H. (2002) Utilization of cytokeratins 7 and 20 does not differentiate between Barrett's esophagus and gastric cardiac intestinal metaplasia. *Mod Pathol, 15 (6), 611-6.*

96. Jass J.R. (1981) Mucin histochemistry of the columnar epithelium of the oesophagus: a retrospective study. *J Clin Pathol, 34, 866-7.*

97. Haggitt, R.C., Reid, B.J., Rabinovitch, P.S. and Rubin, C.E. (1988) Barrett's esophagus. Correlation between mucin histochemistry, flow cytometry, and histologic diagnosis for predicting increased cancer risk. *Am J Pathol, 131 (1), 53-61.*

98. Houde, M., Laprise, P., Jean, D., Blais, M., Asselin, C. and Rivard, N. (2001) Intestinal epithelial cell differentiation involves activation of p38 mitogen-activated protein kinase that regulates the homeobox transcription factor CDX2. *J Biol Chem*, 276, 21885-94.

99. Silberg, D.G., Swain, G.P., Suh, E.R. and Traber, P.G. (2000) Cdx1 and Cdx2 expression during intestinal development. *Gastroenterology*, 119, 961-71.

100. McLaren, K.M., Burnett, R.A., Goodlad, J.R, Howatson, S.R., Lang, S., Lee, F.D., Lessells, A.M, Ogston, S., Robertson, A.J., Simpson, J.G., Smith, G.D., Tavadia, H.B. and Walker, F. (2000) Consistency of histopathological reporting of laryngeal dysplasia. *Histopathology,* 37, 460-63.

101. McLaren, K.M., Burnett, R.A., Goodlad, J.R, Howatson, S.R., Lang, S., Lee, F.D., Lessells, A.M, Ogston, S., Robertson, A.J., Simpson, J.G., Smith, G.D., Tavadia, H.B. and Walker, F. (2003) Observer variability in the Goseki grouping of gastric adenocarcinoma in resection and biopsy specimens. *Histopathology,* 42, 472-75.