The Columnar Lined Esophagus: aspects on the assessment of dysplasia and on the relationship with the esophageal submucosal glands

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In **study I**, we devised and tested two clinically applicable methods for immunohistochemical assessment of p53 and Ki67 as surrogate dysplasia markers. Using these methods, the inter-observer agreement improved substantially from mean κ value 0.24 for H&E evaluation to 0.71 and 0.52 for p53 and Ki67 evaluations, respectively. There was a correlation between severity of dysplasia, p53 over-expression and shift of the proliferation zone towards the mucosal surface. We conclude that our methods are reproducible and associated with less inter-observer variation than morphologic dysplasia grading, and that p53 and Ki67 are useful supplementary prognostic markers.

The origin of columnar metaplasia in esophagus is debated. The submucosal glands have been proposed as a stem-cell source, but studies of the human esophageal glands are rare.

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Key words: Columnar lined esophagus, Barrett´s esophagus, submucosal gland, multilayered epithelium, p53, Ki67, CK17, skin appendage

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Contents

List of original studies

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals (I-IV):

I. **Ki67 and p53 immunohistochemistry reduces interobserver variation in assessment of Barrett´s oesophagus.**
Lörinc E, Jakobsson B, Landberg G, Veress B.
Histopathology. 2005;46(6):642-8.

II. **Submucosal glands in the columnar-lined oesophagus: evidence of an association with metaplasia and neosquamous epithelium.**
Lörinc E, Öberg S.
Histopathology. 2012;61(1):53-8.

III. **Hyperplasia of the Submucosal Glands of the Columnar Lined Oesophagus.**
Lörinc E. Öberg S.
Histopathology. 2014. Nov 8. In press.

IV. **Immunohistochemical characterization of the Submucosal Glands of the Columnar Lined Oesophagus**
Lörinc E, Mellblom L, Öberg S.
In manuscript.

List of abbreviations

AMACR	α-Methylacyl-CoA racemase
CM	Columnar metaplasia
EAC	Esophageal adenocarcinoma
GEJ	Gastroesophageal junction
GERD	Gastroesophageal reflux disease
GI	Gastrointestinal
H&E	Hematoxylin and eosin
HGD	High-grade dysplasia
HPF	High-power field
IFD	Indefinite for dysplasia
IHC	Immunohistochemistry
IMC	Intramucosal carcinoma
LGD	Low-grade dysplasia
ME	Multilayered epithelium
MS	**M**etaplastic segment
NFD	Negative for dysplasia
NSCJ	Neosquamocolumnar junction
PAM	Pancreatic acinar metaplasia
SI	Squamous island
SMG	Esophageal submucosal mucinous gland
SS	Squamous segment
TZ	Transformation-zone

Abstract

Columnar metaplasia, where columnar epithelium replaces the normal squamous epithelium in esophagus, is considered to be a precancerous condition in which the development of adenocarcinoma can be followed through various grades of dysplasia. The interpretation of these histological changes is subjective and suffers from considerable inter-observer variation among pathologists.

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Introduction

The normal anatomy, histology and physiology of esophagus

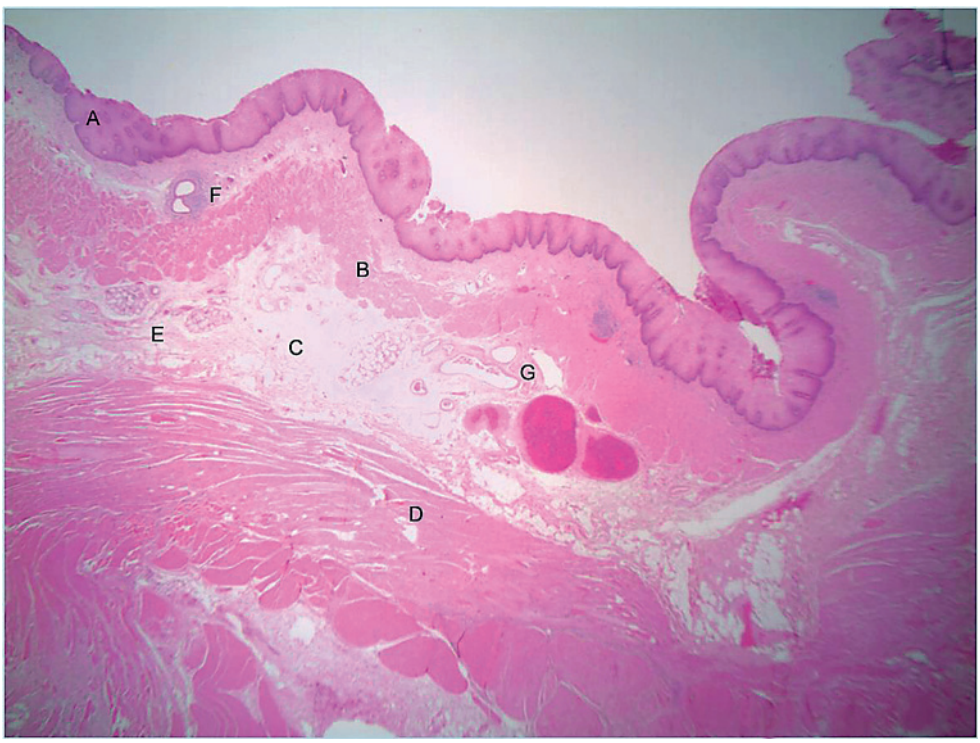


Figure 1. The normal histology of esophagus: A) squamous epithelium B) muscularis mucosae C) submucosa D) muscularis propria E) esophageal submucosal glands F) excretory ducts of submucosal glands surrounded by a lymphoid plaque G) vessels.

Esophagus is a tube-shaped organ connecting the oral cavity and pharynx with the stomach. It has a principal anatomic design analogous with the rest of the gastrointestinal (GI) tract constituted by an outlining mucosa, a vessel and nerve containing

submucosa, a muscularis propria responsible for peristalsis and a peripheral connective tissue adventitia. In addition, as in the oral cavity, the submucosa harbors submucosal mucinous glands and the mucosa is lined by a non-keratinizing stratified squamous epithelium that withstand thermal and mechanical challenges better than the simple columnar epithelium of the rest of the GI channel.

The esophageal submucosal mucinous gland (SMG) is a lobular structure composed of tubuloacinar glands, which ends in intra-glandular ducts that drain into a common excretory duct, and which tortuously traverses the submucosa and muscularis mucosae. The excretory duct, initially lined by a cuboidal simple epithelium, merges with the esophageal squamous epithelium forming a luminal ostium through which the lubricating and buffering mucous can spread over the esophageal luminal surface.[1] (Figure 1)

The anti-reflux barrier that prevents reflux into the esophagus is located in the gastroesophageal junction (GEJ). This barrier includes two sphincters, namely, the lower esophageal sphincter, composed of the intermingling smooth muscles of the muscularis propria of the distal esophagus and the proximal stomach, and the diaphragmatic sphincter composed of striated muscles. A dysfunctional reflux barrier can lead to gastroesophageal reflux disease.[2]

In addition to the anti-reflux barrier, the esophagus also has intrinsic defense mechanisms including luminal acid clearing mechanisms such as gravity, peristalsis and the buffering components of swallowed saliva and secretions of the submucosal glands, as well as the resistance of the esophageal epithelial tissue. The latter comprises pre-epithelial, epithelial and post-epithelial defense. The pre-epithelial defense is considered as weaker than the corresponding mucous buffer-zone on the surface of gastroduodenal epithelium because the salivary and submucosal gland mucins are of the soluble type, capable of lubrication but incapable of forming a fixed viscoelastic protective mucous layer.[3]

The concepts of transformation-zones and of metaplasia

Transformation-zones represent the junction between two types of epithelia and have been proposed to constitute a niche for multipotent stem cells. In humans transformation-zones occur naturally between the esophagus and the stomach, the stomach and the duodenum, the ileum and the colon, the rectum and the anal canal, the ectocervix and the endocervix and between the cornea and the conjunctiva. The epithelium of the squamocolumnar transition-zones expresses proteins associated with wound healing and other hyper-poliferative states. They also constitute well-known predilection foci for tumor development.[4]

Metaplasia is defined as the change in the type of adult cells in a tissue to a form which is not normal for that tissue.[5] Metaplasia generally occur between tissues that are neighbours at the time of formation in the embryo and is thought to occur when a change to the microenvironment or a somatic mutation alter the expression of a tissue specific gene in a stem cell, so that its progenitor cells follow another cellular developmental path. This process is strongly associated with situations of tissue regeneration, because tissue damage means that stem cell niches needs to be repopulated. Furthermore some metaplasias are regarded as precursors to cancer.[6, 7]

Thus transition-zones and metaplasia have many features in common and metaplasia might be regarded as constituting an abnormal transformation-zone. In esophagus, transformation-zones are exemplified by the gastroesophageal junction and metaplasias by pancreatic acinar metaplasia, multilayered epithelium and by columnar metaplasia.

The gastroesophageal junction

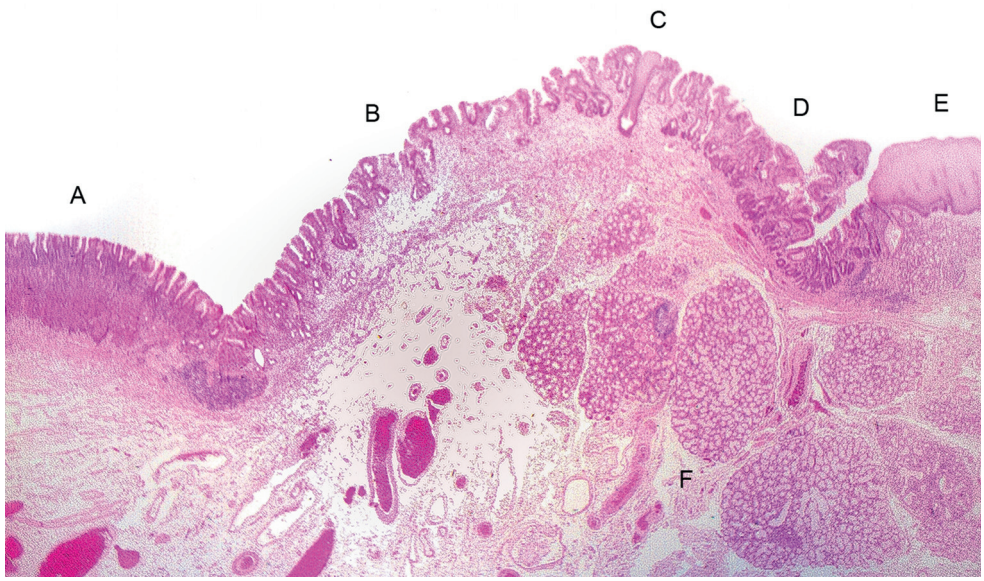


Figure 2. The gastro-esophageal junction: A) gastric oxyntic mucosa B) cardiac mucosa C) squamous epithelial island in connection with the excretory duct of a submucosal gland D) columnar metaplasia with goblet cells E) squamous epithelium F) an accumulation of both hyperplastic and atrophic esophageal submucosal glands.

The gastroesophageal junction is defined as the point at which the esophagus ends and the stomach begins, but from the endoscopist´s point of view, it is located at the

most proximal extent of the gastric folds. Classic anatomic teaching states that the body of the stomach is lined by an oxyntic mucosa containing parietal cells and chief cells and that the most proximal part of the stomach, called the cardia, is lined by a columnar epithelium with tubular mucinous glands. However this has been disputed, and it remains unclear whether cardiac mucosa represents metaplasia or not, and whether it lines the esophagus, the proximal stomach or both.[8, 9] (Figure 2)

In 2010 adenocarcinoma of the esophagogastric junction was introduced as a separate entity in the WHO classification of tumors of the digestive system. This entity shares epidemiologic characteristics, risk factors, a precursor stage of metaplasia, and similarities at the molecular level, mainly with adenocarcinoma of the distal esophagus, but includes also adenocarcinoma of the proximal stomach.[8] Recently The Barrett´s and Esophageal Adenocarcinoma Consortium concluded that there is a strong association between symptoms of gastroesophageal reflux and adenocarcinoma of esophagus that increases with duration and/or frequency. This association is somewhat weaker for adenocarcinoma of the GEJ, possibly because the latter entity represents a mixed cancer-population.[10]

Pancreatic acinar metaplasia in esophagus

Pancreatic acinar metaplasia (PAM) is readily identified histologically as cells with basophilic cytoplasm in the basal part, centrally placed nuclei and acidophilic granular cytoplasm in the luminal parts. The cells can be arranged in a single acinus or multiple acini forming small lobuli and they express pancreatic secretory proteins.[11] Pancreatic acinar metaplasia is a common finding in the stomach, the gastroesophageal junction and the esophagus. The overall prevalence of PAM among patients selected for endoscopy is reported to be 19%, but the prevalence of PAM exclusively above the GEJ is only 6%.[12] It has been suggested that PAM is rather a congenital than a metaplastic phenomenon at the GEJ, however when exclusively located above the GEJ it most probably represents metaplasia, since there it is associated with age, H.pylori, female gender and gastroesophageal reflux.[12, 13] The biological significance of PAM is unclear and there are no reports of association with malignancy.[13] (Figure 3A)

Multilayered epithelium in esophagus

In 1993 Shields et al, in their scanning electron microscopy investigation of the transformation-zone between esophageal squamous epithelium and columnar metaplasia, found a distinct cell type similar to those found in the squamocolumnar trans-

formation-zone of the uterine cervix. These cells displayed both microvilli, which is a feature of glandular epithelium, and intercellular bridges, which is a feature of squamous epithelium. For this reason, the authors postulated that they might represent an intermediate step in the development or healing of columnar metaplasia.[14] Multilayered epithelium (ME) have since then been observed in several studies and defined as a type of epithelium that has morphological and immunohistochemical characteristics of both squamous and columnar epithelium. ME is strongly associated with gastroesophageal reflux disease (GERD). In biopsy material ME is seen mainly in conjunction with columnar metaplasia in the vicinity of the ductal orifices of the submucosal esophageal glands, and is proposed to stem from these gland ducts. ME (Figure 3B) is regarded as a precursor of columnar metaplasia.[15, 16,17,18,19]

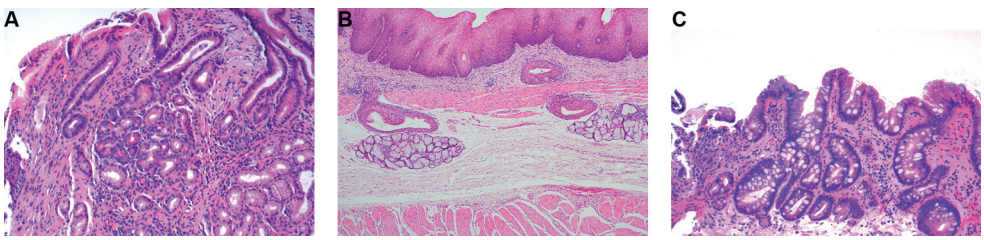


Figure 3. Metaplasias in esophagus: A) pancreatic acinar metaplasia B) multilayered epithelium in the excretory ducts of submucosal glands C) columnar metaplasia with goblet cells, no dysplasia.

A squamous metaplasia-like change has been described in the GEJ-zone and proposed to be an intermediate form in the transition from squamous to columnar epithelium, or from columnar to squamous epithelium. However this metaplasia-like change has a similar ciliated appearance and a similar immunohistochemical profile as respiratory bronchial epithelium, suggesting that it is an entity that differs from ME or columnar metaplasia.[20]

Columnar metaplasia in esophagus

History of name and definition

Columnar metaplasia (CM), where the normal squamous esophageal epithelium is replaced by metaplastic columnar epithelium, was first described in 1906 by Wilder Tileston. For decades this condition carried the eponym "Barrett´s esophagus" after Norman R Barrett who in 1950 interpreted it as a thoracic stomach, which appeared tubular due to congenital shortening of the esophagus, although PR Allison in 1946 more correctly described it as located in the esophagus.[21]

In 1965 it was proposed that the metaplastic epithelium could be of the intestinal type, and in 1976 Paull A introduced a classification into three subtypes, atrophic

gastric-fundic-type with parietal and chief cells, junctional-type with cardiac mucous glands, and distinctive specialized columnar epithelium with a villiform surface, mucous glands and intestinal-type goblet cells.[22, 23]

In the 1980s reports suggested that the intestinal goblet cell containing type of metaplasia is predisposed to malignancy, and population based studies seemed to confirm this.[24, 25] This interpretation was adopted by both researchers and clinicians, so that the term "Barrett´s esophagus" became synonymous with the new definition that required histologically verified CM with goblet cells. The great majority of studies on the topic of CM, from the 80s until recently, are based on this definition.[26] (Figure 3C)

In 1997 Chandrasoma pointed out that the pathophysiology of CM implied that intestinal metaplasia was part of a continuous metaplastic reflux-induced process, and advocated that the eponym be discarded.[27] It has since then been shown that CM without goblet cells expresses intestinal peptides, and harbors DNA content abnormalities to the same extent as CM with goblet cells.[28, 29] Minute adenocarcinomas develops mainly in CM without goblet cells.[30] The risk of developing dysplasia and adenocarcinoma is similar in both groups. Furthermore the chance of finding goblet cells is strongly dependent on the number of biopsies taken and the length of the CM segment.[31] The goblet cell density is increased in the proximal CM segment and correlates with an increase in esophageal luminal pH gradient.[32]

Against this background it is clear that the definition of CM is controversial and has led to discrepancies in terminology between the endoscopy and pathology societies and between different countries.[33] Currently the Swedish Society for Gastroenterology demands endoscopic detection of columnar epithelium proximal to GEJ and histologic confirmation of "specialized intestinal metaplasia" for the definition of Barrett´s esophagus, while the Swedish Society for Pathology advocates the term "columnar lined esophagus" and demands a specific statement of Paull´s subtype for each biopsy.[34, 35] Henceforth in this text, CM will be used for all Paull´s three subtypes unless otherwise stated.

Epidemiology

In children and adolescents selected for endoscopy the prevalence of CM is reported to be 1.4%.[36] Studies addressing CM with intestinal metaplasia in the general population have indicated a prevalence of approximately 0,5-1,5%.[37] There are limited data on corresponding prevalence for CM without intestinal metaplasia, however Ronkainen et al reported 8,7%.[38] A register study from Northern Ireland found an increase of the incidence of CM with intestinal metaplasia of 93% between 1993 and 2005, in spite of the fact that potential confounding was avoided by counting the number of positive cases per 100 endoscopies.[39] There are no data on the incidence of CM without intestinal metaplasia in the general population.

Etiology and risk factors

The only known cause of CM is gastroesophageal reflux disease. Numerous studies have confirmed the association between GERD and CM and also proved that it´s extension is related to dysfunction of the anti-reflux barrier and the degree of esophageal acid exposure.[40, 41] Patients with previous metaplasia are more prone to develop CM in response to acid injury in the cervical esophagus after esophagectomy, indicating an individual predisposition.[42] Familial aggregation of CM has been reported, as have susceptibility loci and germline mutations, proving that genetic factors must also be involved in at least a subset of individuals with CM.[43, 44, 45, 46]

The Montreal definition and classification of GERD states that it is a condition which develops when the reflux of stomach contents causes troublesome symptoms and/or complications.[47] The cardinal symptoms of GERD are heartburn and regurgitation. Factors such as obesity, age, genetics, pregnancy and trauma may all contribute to impairment of the anti-reflux barrier evoking pathological reflux.[48] Based on endoscopic findings, patients with GERD are classified into erosive and non-erosive phenotypes. Most patients with GERD belong to the non-erosive group, in which biopsy material shows discrete histopathological features consisting of dilated intercellular spaces within the esophageal squamous epithelium.[49] The erosive group includes a spectrum ranging from reflux esophagitis to peptic ulcers, strictures and CM. A finding of a basal zone height of >15% and a papillary length of > 66% of the total epithelial thickness is considered positive for reflux esophagitis, confirming squamous epithelial hyperplasia, according to Ismail-Beigi´s criteria for histopathological assessment of reflux esophagitis.[50] However there is at least one study that failed to confirm the value of the Ismail-Beigi criteria as histological markers of acid reflux.[51] Furthermore squamous epithelial hyperplasia is a normal finding in the 2 to 3 cm of lower esophagus.[52] The histologic features of GERD are not specific but can include balloon cells, vascular changes, epithelial hyperplasia, and presence of inflammatory cells such as eosinophils, neutrophils and lymphocytes.[53] Occurrence of ME at the GEJ has also been identified as a marker of GERD.[18, 19]

Historically only epithelial damage caused by hydrochloric acid has been considered in GERD, but the prevalence of CM in patients with GERD is reported to vary between 3% and 15%. This variation can depend on which criteria are used for patient selection.[54] Clearly other factors than hydrochloric acid must also be involved in the process of initiating CM, and bile acids have emerged as one such. In vivo studies have shown that bile acid concentrations are higher in esophageal aspirates of patients with GERD than in controls, and that bile acid triggers GERD symptoms. In vitro studies have demonstrated that bile acids cause oxidative stress, DNA damage and apoptosis in both squamous esophageal cells and CM cells. Furthermore bile acids can induce changes in gene expression patterns and cause an increase in expression of intestinal type genes in CM cells. The human bile acids are conjugated by the liver and their

conjugation status is pH dependent. At a pH of 4 – 7, unconjugated bile acids become predominant in the refluxed juice. Unconjugated bile acids may be especially damaging in this neutral environment, while conjugated bile acids are more dangerous at acidic pH. Thus the bile acid content in the refluxed gastric juice may contribute to GERD and the development of CM.[55]

Other factors identified as conferring an increased risk for CM are older age and male gender. Obesity has been pointed out as a risk factor and recently it has been shown that waist-to-hip ratio, but not fat mass or body mass index, is associated with increased risk for CM, suggesting that abdominal adiposity seems to play a key role.[56] H. pylori infection is associated with a decreased risk for CM. However this association is restricted to patients with corpus atrophy or to those who regularly take antisecretory medications, implying that suppressed gastric acid secretion is probably the true protective factor.[57]

Theories on the origin of columnar metaplasia

The current major theories on the origin of CM are not incompatible since they do not mutually exclude each other. Depending on which biologic level the explanatory model is applied on they may interfere or occur simultaneously. They all share the basic concept of hydrochloric and bile acid induced epithelial injury being the pathophysiological initiating step.

The stem cell level

In the intestinal epithelium and stratified epithelia there is a development from undifferentiated towards mature differentiated cells that are sloughed off during the constant turnover of cells. The classic hierarchic stem cell model implies that epithelia are replenished by stem cells that can divide indefinitely providing both more stem cells and progenitor cells that are destined to differentiate.[6] It is assumed that these stem cells are tissue-specific, however the classic hierarchic stem cell model has been challenged.[58] In vitro experiments have shown that induced pluripotential stem cells can be produced by insertion of a subset of genes in normal fibroblasts.[59] Another source of stem cells might be the circulating bone- marrow derived cells that have been shown to contribute to regeneration of diverse tissues including rat esophagus.[60] A population of residual embryonic cells has been shown to persist at the squamo-columnar junction in adult mice and humans, and in mice these cells can migrate into the esophageal epithelium upon damage to the esophagus, thus constituting yet another possible stem cell source.[61]

More challenges to the classic stem cell model can be found. Already in 1964, Leblond C et al suggested that all cells in the basal layer of squamous epithelium are equal.[62]

A finding further augmented by Barbera M et al who, by using 2D and 3D ex vivo and in vitro models of human esophagus, showed that squamous cells at diverse stages of differentiation have equal capacity for self-renewal and ability to reconstitute epithelial architecture.[63] Recently lineage tracing in human esophageal surgical specimens have shown that in normal esophageal squamous epithelium clonal patches expand laterally and that CM, submucosal glands and their ducts are maintained by multiple stem cells.[64] On a crypt-to-crypt basis, CM is proven to be genetically heterogenous, containing many independent clones.[65] Clearly the theories of stem cell niches for esophageal epithelium and CM are evolving but not yet defined.

The molecular level

From developmental biology it is known that a small number of genes called "master control genes" encode transcription factors whose activity determines choices between the developmental programs of cells, i.e. their differentiation.[66] Switching between developmental programs, as occurs in metaplasia, must arise from the change of expression levels of the key developmental genes, but it is not known whether somatic mutations, epigenetic switching or environmental stimuli initiate these changes.[6] Signaling pathways involved in the development of CM are not yet elucidated. However several candidates have been proposed, such as the Notch pathway and the Sonic Hedgehog pathway.[67, 68]

The tissue level

It is generally accepted that a prerequisite for the cell of origin for esophageal columnar metaplasia is the capability to produce descendants representing all Paull´s subtypes. The exact location of this cell is debated, but three major theories can be considered: 1) the gastroesophageal junction, 2) the squamous esophageal epithelium and 3) the esophageal submucosal gland with its duct.

1) As early as 1953 Allison and Johnstone suggested that CM might originate from upward growth of cardiac mucosa in the process of healing after peptic ulcer in the gastroesophageal junction.[69] In 1977 Hamilton and Yardley showed that CM could develop in the distal esophagus in patients who had undergone partial esophagogastrectomy, and interpreted this finding as evidence for cardiac and/or fundic mucosa as the cellular origin of CM.[70] Arguments against this were raised by Gillen P et al. In 1988 they demonstrated that in canine esophagus, columnar re-epithelialization could occur even when squamous barriers to proximal migration of cardiac columnar epithelium were constructed, thereby proving that the cellular origin of CM must be intrinsic in esophagus.[71] The theory of an origin in the gastroesophageal junction has since then had a renaissance, based mainly on experimental animal models where putative stem cells for CM have been identified in the GEJ.[61, 67, 72] Questions about how these stem cells might migrate into esophagus, and whether the animal models are representative for humans remain to be answered. Finally the GEJ is in fact a transformation zone where stability

in the two different types of structural-proliferative units must be maintained. Tissue damage might destroy some of these units and allow new ones to form, resulting in overgrowth of one of the tissue types analogously with the undulating border between the endo- and exocervix.[73]

2) Since Gillen's experiment, efforts have been made to prove the possibility of a direct conversion from esophageal squamous epithelium to CM.

Some ideas may be found in embryonal development, where the esophagus is initially lined by a thin layer of stratified columnar epithelium followed by the appearance of ciliated cells, and where stratified squamous epithelium emerges only after five months' of gestation.[1] The ciliated squamous metaplasia in adults described by Tabuko et al might well be a remnant of the embryonal epithelium or a reversed differentiation.[74] Based on morphologic and immunophenotypic similarities, De Hertog et al have suggested that fetal simple columnar epithelium may be a precursor of CM.[75] In an in vitro model of esophageal explants from mouse embryos, Yu WY et al could show that during development, direct conversion of columnar cells to basal layer cells of the squamous epithelium takes place, without cell proliferation or apoptosis.[76]

It has also been suggested that gastroesophageal reflux-induced damage to the superficial layers of esophageal squamous epithelium might influence putative stem cells and induce abnormal differentiation.[77, 78] The proposed mechanism behind this influence would be increased permeability to noxious agents and acidification of the squamous epithelium through dilated intercellular spaces.[79, 80, 81] This would in turn ultimately affect master control genes, and lead to a reversed differentiation process where fetal columnar epithelium re-emerges. It is also proposed that when ulceration occurs, bone-marrow derived pluripotent stem cells might be recruited to form new tissue, and that mesenchymal-epithelial transformation might take place.[60, 82]

3) The submucosal esophageal gland and especially its excretory duct have been gaining increasing interest lately as a source for development of CM. This is partly due to the easily recognized shift from simple cuboidal to stratified squamous epithelium in the excretory duct, and partly due to the finding that multilayered epithelium is most frequently observed in connection with these ducts. Both of these findings imply an inherent capability to transform. The excretory duct´s openings into the esophageal lumen can provide an easily accessible way for harmful agents to reach putative stem cells in the ductal or acinar epithelium.

Wright NA was the first to propose a theory of ductal origin. In 1996 he suggested that an acid-induced, ulcer-associated columnar cell lineage emanated from ducts and were responsible for duodenal gastric metaplasia, gastroduodenal pseudopyloral metaplasia as well as for CM. Nevertheless, for the histogenesis of CM he referred to the esophageal cardiac glands that do not display squamous ductal epithelium.[83] In 2001 Glickman et al provided evidence that multilayered epithelium represents a stage in

the development of CM. On the basis of morphological and immunohistochemical similarities they proposed SMG duct epithelium as the locale for progenitor cells.[16, 84]

As a part of healing, newly formed islands of neosquamous epithelium can arise in CM after medical or surgical therapy. These islands can be seen close to the openings of the submucosal gland excretory ducts, and they generally do not share genetic alterations with the surrounding CM. For this reason it has been suggested that the gland ducts may provide a reservoir also for re-growth of esophageal squamous epithelium.[65, 85, 86, 87] However, in one case in each of two studies, neo-squamous epithelium harbored the same mutation as the surrounding CM, indicating that they may share a common progenitor.[64, 65]

The first study on human esophageal material to assess three dimensional tissue relationships by serial sectioning was conducted by Coad RA et al in 2005. They found continuity between CM and 21 of 46 submucosal esophageal gland ducts. They also found that 15 of 15 ducts were continuous with presumed neosquamous islands, and concluded by suggesting an interrelationship.[87] Further strong morphologic and genetic evidence of the common origin of both neosquamous epithelium and CM in the esophageal submucosal gland duct has been provided by Leedham SJ et al In their analysis of laser-capture, micro-dissected human esophageal material they found that multiple independent clones competes to colonize the CM segment. Identical point mutations were revealed in CM and gland ducts that were seen in continuity with each other. Interestingly the same mutation was found in the acini of the associated submucosal gland.[65] Recently evidence of the existence of both mutant and non-mutant cells within either acini of the same submucosal gland or the same duct have been found, and interpreted as indicating that there are at least two stem cell populations in each of these structures.[64] Retinoic-acid has been shown to induce a glandular phenotype, including multilayered epithelium, in the lamina propria of adult human mucosa ex vivo, via a proliferation-independent process. The possibility that this induced glandular epithelium is derived from stem cells located in the submucosal esophageal glands and ducts cannot be ruled out.[82]

The columnar metaplasia to dysplasia to adenocarcinoma sequence in esophagus

In 1952 Morson BC & Belcher JR described a case of esophageal adenocarcinoma (EAC) arising from what they interpreted as atrophic, ectopic gastric mucosa.[88] This report was to be followed by a large number of case reports until in 1972 Hawe A et al in their retrospective archive survey of EAC established CM as a premalignant condition.[89] It has also been established that esophageal adenocarcinoma evolves from CM

through a series of morphologically identifiable intra-epithelial, neoplastic changes often referred to as "the metaplasia - dysplasia - adenocarcinoma sequence".[90, 91, 92]

Adenocarcinoma of the esophagus

Adenocarcinoma of the esophagus is defined as a malignant epithelial tumor of the esophagus with glandular differentiation.[8] In the majority of cases cancer develops in the distal part of a CM segment.[93] Since tumors originating from esophagus may infiltrate the GEJ as can tumors originating from the proximal stomach, these entities are difficult to distinguish. Currently, according to WHO 2010, all adenocarcinomas crossing the GEJ should be classified as adenocarcinomas of the gastro-esophageal junction, while the American Joint Committee on Cancer states that cancers whose epicenter is in the lower thoracic esophagus, GEJ, or within the proximal 5 cm of the stomach, that extend into the GEJ or esophagus are to be grouped with the adenocarcinomas of the esophagus.[94]

EAC used to be a rare disease but the incidence rate has risen during the last 20 years, and in Sweden now exceeds that of esophageal squamous carcinoma. In 1990 we had 1.06 new cases per year per 100 000 inhabitants, while by 2013 the incidence had risen to 5.46 new cases per year per 100 000 inhabitants.[95] Studies from the 1980s and -90s have reported a risk of developing adenocarcinoma from CM varying from 0% to almost 3% per patient-year. This variation is probably related to publication-bias, since there was a strong inverse relationship between the sizes of the studies and the reported cancer risk.[96] In 2003 Murray L et al found that it is only among men over the age of 70 and with intestinal metaplasia that the incidence was greater than 1% per year.[97] In 2008 a systematic meta-analysis of papers published between 1950 and 2006 reported that, when only high quality studies were included, the incidence of adenocarcinoma was only 0.39%, and the combined incidence of high grade dysplasia 0.77% per year among patients with CM.[98] In 2011a Danish nationwide population- based study found that the relative risk of developing adenocarcinoma was 11 times as high among patients with CM as in the general population. This is more than 5 times lower than previously reported.[99] The Swedish Society for Gastroenterology describes the risk of developing esophageal adenocarcinoma from CM as 0.10% per year and the corresponding risk from low-grade dysplasia to 0.5-1.5% per year. They do not recommend screening for CM but if low-grade dysplasia is found, patients are recommended to join a surveillance program with control after one year and then after every second year.[34]

The prognosis for patients with EAC is poor. In the US, at the time of presentation, more than 50% of patients have metastatic disease, nearly 30% have a locally advanced stage, and less than 20% have a localized stage that can be cured. The overall 5-year relative survival for 2002-2008 was 16.9%, but the survival rate was seen to be improving

during the period.[100] There are several treatment modalities. Surgical esophagectomy is the standard but there is a global agreement that neo-adjuvant chemo-radiotherapy is strongly recommended for patients with locally advanced EAC.[101] For early stage adenocarcinoma and high-grade dysplasia, ablative therapies, such as endoscopic mucosal dissection, endoscopic submucosal dissection and radiofrequency ablation have been introduced. However it has been shown that protumorigenic mutations can be found in post-ablation squamous mucosa, as well as mutant submucosal esophageal glands.[102] When ablative therapies are used, it is therefore essential that the whole area of CM is incorporated to avoid relapse and progression. Even when conversion to squamous mucosa is achieved, patients may still need to undergo surveillance.[103]

Histologic assessment of dysplasia and adenocarcinoma

Histopathological diagnosis and grading of CM on biopsy material is essential in the follow-up and treatment of patients. The terminology used for neoplastic changes varies depending on which organ or anatomic site they occur in. For CM the term dysplasia is preferred, and defined as "histologically unequivocal neoplastic epithelium without evidence of tissue invasion".[8] There are two quite similar classification systems: Riddell et al initially proposed for dysplasia in Inflammatory Bowel Disease, and The Vienna Classification system that sought to develop a terminology that could resolve differences between Western and Japanese classifications.[104, 105] Further clarity has been provided by JR Goldblum in 2010 in an attempt at semantic description of the entities. It is worth particular attention and is briefly described here.[106] (Figure 4A-C)

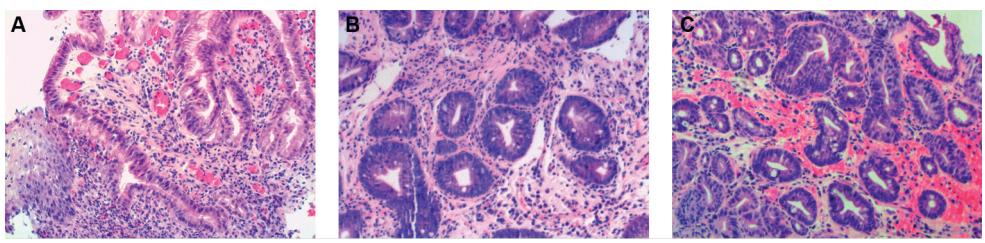


Figure 4. Categories in assessment of dysplasia in columnar metaplasia: A) indefinite for dysplasia B) low-grade dysplasia C) high-grade dysplasia.

CM negative for dysplasia (NFD)

In esophageal columnar metaplasia there is a "baseline atypia" that is always present, and in a sense, must be overlooked to make a diagnosis of dysplasia. This baseline atypia is most pronounced in the glands at the base of the mucosa and does not involve the surface epithelium.

CM indefinite for dysplasia (IFD)

Since biopsies from CM are often inflamed, with both acute and chronic inflammatory cells, the differentiation of regenerative changes from true dysplasia is at times difficult, if not impossible. However the cytological atypia associated with repair is more uniform than is seen in dysplasia. Although regenerative cells may have nuclear size similar to those seen in dysplasia, there tends to be a commensurate increase in the amount of cytoplasm, such that the nuclear-to-cytoplasmic ratio is normal or only mildly increased. In addition, regenerative cells tend to have round and regular nuclear contours.

CM with low-grade dysplasia (LGD)

In low-grade dysplasia, crypt architecture tends to be preserved with only minimal distortion, and cytologically atypical nuclei are limited to the basal half of the crypts. There is evidence to suggest that CM-related dysplasia begins in the crypt bases, and progresses to involve the full length of the crypts and surface epithelium.[107, 108] The nuclei tend to show variable hyperchromasia, overlapping cell borders with nuclear crowding, and irregular nuclear contours. Dystrophic goblet cells may be seen, although typically goblet cell numbers are markedly reduced.

CM with high-grade dysplasia (HGD)

High-grade dysplasia shows more severe cytological and architectural changes than are present in low-grade dysplasia. Architecturally, there tends to be more crypt distortion in HGD, sometimes with a villiform configuration of the mucosal surface and/or branched or cribriform crypts. Cytologically, the cells show more nuclear pleomorphism and hyperchromatism than is seen in low-grade dysplasia, and there is often nuclear stratification to the crypt luminal surface.

The morphological changes ranging from "baseline atypia" to high-grade dysplasia are continuous, without sharp borders between the entities. The assessment of dysplasia is therefore subjective and suffers from considerable inter-observer variation, even among experienced pathologists with a special interest in gastrointestinal pathology. The greatest disagreement is found in distinguishing between NFD, LGD and IFD, while HGD seem to be more readily recognizable. Montgomery et al reported kappa-values as low as 0.15 and 0.32 for inter-observer agreement for IFD and LGD respectively. Scacel et al showed that the association with disease progression increases with the number of pathologists that agree on a diagnosis of LGD.[109, 110, 111, 112] Overdiagnosis of LGD can result in more frequent monitoring of patients, which can have both individual and socio-economic consequences. The Swedish Society for Pathology recommends that at least one GI pathologist is consulted before a definite diagnosis of LGD.[35]

Although the diagnosis of HGD seems to be straightforward, the delineation between HGD and intramucosal adenocarcinoma (IMC) is difficult and accompanied by lack of agreement between experienced GI pathologists.[113, 114] Intramucosal carcinoma is defined as a neoplasm that has invaded into the surrounding lamina propria or muscularis mucosae but not into the submucosa.[106] The evolving endoscopic therapeutic options have put pressure on pathologists to distinguish more accurately between HGD and IMC. A few histologic features in preoperative biopsies have been found to be significantly associated with the presence of concurrent adenocarcinoma in esophageal resections. These include: presence of "never-ending" glands, sheet-like growth, angulated glands, >3 dilated glands with intraluminal debris, and >1 focus of single-cell infiltration into the lamina propria. When strict diagnostic definitions are applied and include consensus between GI pathologists, diagnosis on preoperative biopsies have a good predictive value.[115, 116] Lymphatic channels are present within the esophageal mucosa, giving a small but definite risk of lymph node metastasis, estimated to affect 0.6% to 1.93% of the patients with IMC, but with considerably higher risk for patients with submucosal adenocarcinoma.[117, 118, 119] Diagnosing submucosal cancer invasion is hardly ever possible in mucosal biopsies since the submucosa is rarely present in these tiny tissue pieces. However, the new technique of endoscopic submucosal dissection provides excellent material for evaluation of the submucosa. Diagnostic caution is warranted due to the peculiar and unexplored fact that patients with CM often develop an additional muscularis mucosae above the original layer.[120] (Figure 5) Entrapment of both benign and neoplastic glands occurs between these layers, and since desmoplasia seems not to be a diagnostic feature in early esophageal adenocarcinoma, the diagnostic difficulties are obvious.[121] In one study, however, it has been shown that tumor invasion to the space between these layers carries the same risk for lymph node metastasis and recurrence as for intramucosal adenocarcinoma. This means that the outer muscularis mucosae represent the true border to submucosa but lymphovascular invasion remains an independent predictor of lymph node status and of recurrence-free survival.[122]

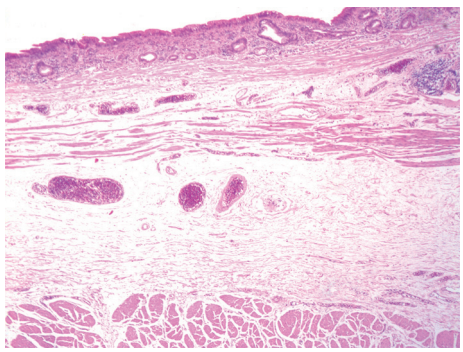


Figure 5. Esophagus with columnar metaplasia and double muscularis mucosae.

Biomarkers for dysplasia and adenocarcinoma in the esophagus

Histopathological assessment is the gold standard for diagnosis of dysplasia and adenocarcinoma, but has limitations due to inter-observer variability and sampling error, which has led to a vigorous search for more precise methods. Biomarkers, defined as biological variables that correlate with biological outcome, should be better than the current diagnostic and prognostic tools and also easy to implement into clinical practice.[123, 124] The process of development of a biomarker for early cancer detection is divided into five phases.[125]

- Phase 1 – Preclinical explanatory studies aimed to identify a biomarker.
- Phase 2 – Clinical assay development aiming to estimate the sensitivity and specificity for the biomarker.
- Phase 3 – Retrospective longitudinal studies to evaluate the capacity of the biomarker to detect preclinical disease.
- Phase 4 – Prospective screening studies.
- Phase 5 – Cancer control studies addressing whether screening reduces the burden of cancer on the population.

A systematic literature search on the topic "Barrett´s esophagus and biomarkers from 1980 to 2011" has been reported to yield 1069 citations. Most biomarker studies in CM includes only Phases 1 – 3 evaluations, a few include Phase 4, and no Phase 5 studies have been conducted.[126] The most well-known of the proposed biomarkers for CM related dysplasia and adenocarcinoma will be described here.

DNA content abnormalities

Abnormalities of DNA contents include aneuploidy (a cell containing an abnormal number of chromosomes) and tetraploidy (4N, a cell having four complete sets of chromosomes instead of two) and have been shown to be associated with increased risk of progression from CM to adenocarcinoma. Detected at the baseline endoscopy, using flow cytometry, the presence of both a 4N fraction of >6% and aneuploid DNA content of >2,7N is a predictor of cancer.[127, 128] Clinical implementation of ploidy measurement has been hindered by the technical need for fresh biopsy material. New techniques allowing flow cytometric analysis on paraffine embedded tissue have been introduced recently. However, some studies applying these new techniques have failed to confirm DNA content abnormalities as a valid biomarker.[129, 130] Furthermore, increased 4N fractions in CM have been shown to be a result of biallellic inactivation of TP53.[131]

Cell proliferation

Increased cellular proliferation has long been hypothesized to be associated with progression to cancer. Ki67 is a nuclear antigen expressed in proliferating cells, but not in resting cells. A linear increase in immunohistochemically detected Ki67 expression in the development from CM towards HGD/EAC has been described, as well as the finding that strong Ki67 over-expression in CM is associated with increased risk of neoplastic progression.[129, 130] In a long-term prospective study, however, they found no association between overall Ki67 expression, as measured by flow cytometric analyses in CM and later development of EAC.[131] Discrepancies in methods, cut-off values and material selection might explain these diverging results. Earlier studies that have also considered the localization of Ki67 expression in the mucosa, have unanimously reported a shift of the proliferation-zone from the normal basal location to the superficial parts of the mucosa, along the CM-to-dysplasia-to-carcinoma progression.[132, 133, 134, 135] Since increased cell proliferation in the lower halves of the glands can represent adaptive changes to reflux, Ki67 over-expression is not a valuable biomarker for differentiating between CM/IFD/LGD, but there is strong evidence of its value in confirming HGD.[135] Unfortunately, reliable assessment of Ki67 expression patterns in CM is limited by the demand for well-orientated tissue sections. (Figure 6 A-B)

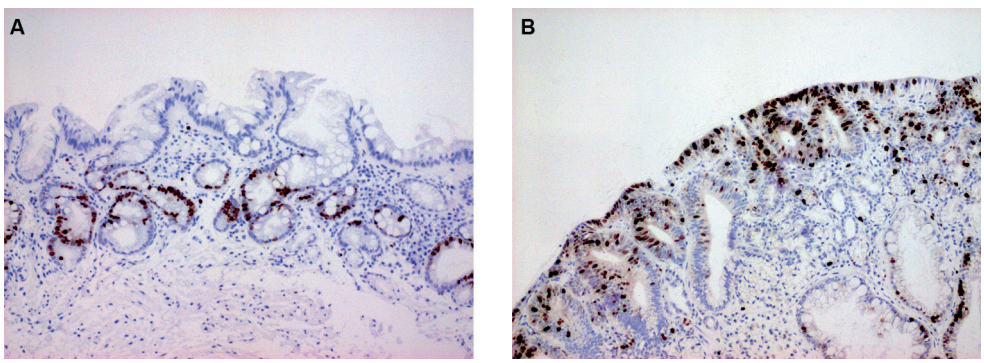


Figure 6. Ki67 immunohistochemical pattern: A) Normal proliferation-zone in non-dysplastic columnar metaplasia B) Abnormal proliferation-zone in high-grade dysplastic columnar metaplasia.

AMACR

AMACR (a-Methylacyl-CoA racemase), also called P504S, is a cytoplasmic enzyme that plays an essential role in the oxidation of fatty acid-chains and the biosynthesis of bile-acids. AMACR is normally expressed in hepatocytes, renal tubular cells, gall bladder epithelium and bronchial epithelium. AMACR is a well-known, clinically established biomarker for prostate high-grade intraepithelial neoplasia and cancer.

In 2006 Dorer & Odze tested a monoclonal AMACR antibody on CM tissue

material and found no expression in non-dysplastic CM, but increasing expression in LGD, HGD and EAC, reporting 100% specificity and 38% and 81% sensitivity, respectively, for LGD and HGD, suggesting that AMACR may be a good biomarker also for CM related dysplasia.[136] These findings have largely been confirmed in later studies.[137, 138] The most recent Phase 3 study reported AMACR expression in 49% of non-dysplastic CM biopsies, and 63%, 91% and 71% in LGD, HGD and EAC respectively. Inter-observer agreement was only moderate (kappa-value 0.44), and the authors concluded that AMACR does not have enough power to be used as a single biomarker for neoplastic progression.[139] Indeed evaluation of cytoplasmic immunoexpression is usually more problematic than evaluation of nuclear immunoexpression.

TP53

The TP53 gene is located on the short arm of the human chromosome 17 (17p13). Loss of heterozygosity as well as biallelic mutation of TP53 has been found in a wide variety of tumor tissues, and it has been demonstrated that over 50% of human tumours carry TP53 mutations. TP53, also called "The guardian of the genome", is regarded as a tumour suppressor gene. Its main function is to encode for the nuclear protein p53 that plays a key role in cell-cycle control by arresting it at the G1/S regulation point as a response to damage, and inducing DNA repair proteins, and also in induction of apoptosis, thereby preventing replication of damaged cells. Under normal conditions p53 is short-lived and expressed at a very low level, but in case of cellular stress it becomes

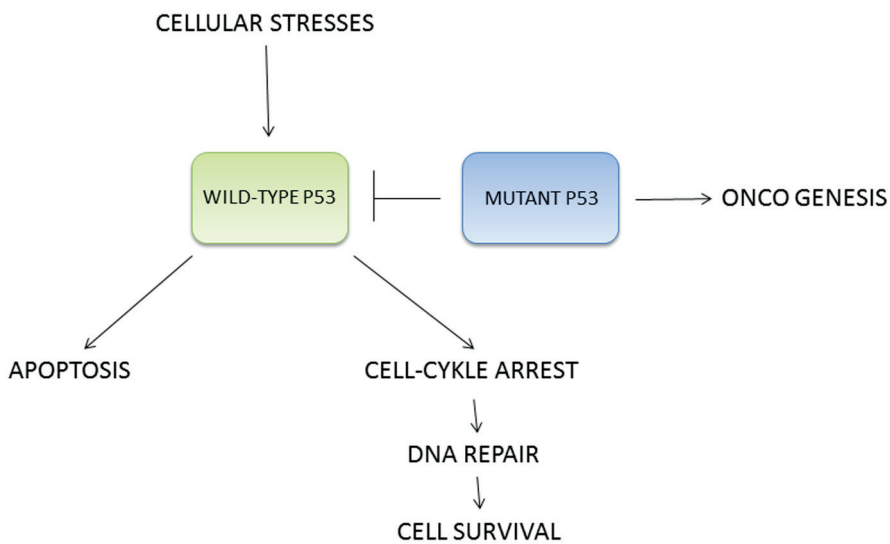


Figure 7. Dominant-negative effect of mutant p53 on wild-type p53. In response to cellular stresses, wild-type p53 is activated and induces cell cycle arrest and/or apoptotic cell death. Mutant p53 binds to wild-type p53 and inhibits its tumor suppressor function, thereby promoting tumor formation.

stabilized and therefore accumulates rapidly in the cell nucleus. Mutant forms of p53 have a longer half-live and display a dominant-negative behavior to wild-type p53.[140, 141] (Figure 7)

Up to 57% of esophageal adenocarcinomas harbor mutations of TP53, and mutations are detected significantly more frequently in HGD than in LGD lesions.[142] For this reason TP53 mutations have long been suggested as a biomarker for dysplasia in CM.[143] Its clinical implementation has been hampered for several reasons. Genetic analyses of tissues, even if more feasible lately, are expensive and complicated to handle in everyday clinical practice. Immunohistochemistry (IHC), on the other hand, is a quick and cheap technique available in all pathology laboratories, and p53 nuclear protein accumulation can easily be detected by this technique. It is however well-known that there is discordance between TP53 mutation and p53 protein expression as detected by IHC.[142, 144] Approximately 30% of EAC carry nonsense mutations that appear as false negatives using IHC.[145] Furthermore, not all IHC positive cases correlate with TP53 mutations, thus giving false- positive results for mutational status.[146] Nevertheless, it can be argued that IHC-detectable presence of p53 can be considered as a biomarker in its own right since other mechanisms leading to accumulation of the protein, such as induction of wild-type p53 in response to DNA damage or alteration in other genes, may represent increased risk for disease progression.[147] In fact, there is strong evidence for p53 as a biomarker for prevalent dysplasia and risk of neoplastic progression in CM, with good diagnostic reproducibility.[130, 147, 148, 149, 150, 151, 152] The British Society of Gastroenterology states in its 2014 guidelines that p53 immunostain should be considered as an adjunct to routine clinical diagnosis of dysplasia in CM.[153] (Figure 8 A-B)

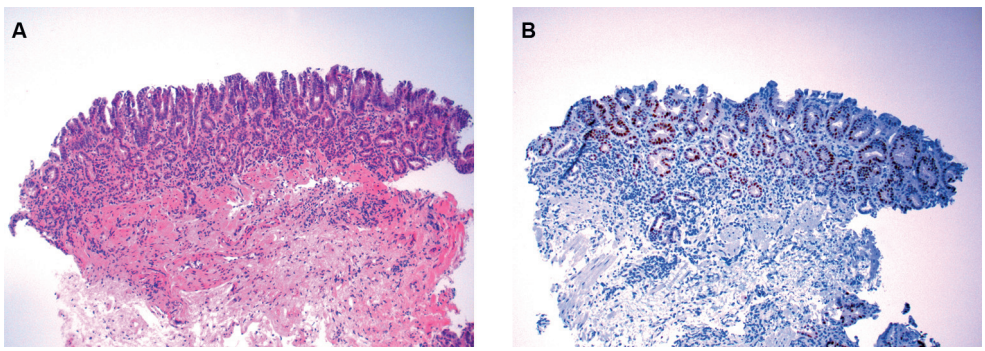


Figure 8. A) Columnar metaplasia with high-grade dysplasia. B) Immunohistochemical detection of overexpression of p53 in columnar metaplasia with high-grade dysplasia.

Aims of the thesis

The aims of this thesis were to study methods for assessment of dysplasia in the columnar lined esophagus and to explore the relationship between columnar metaplasia and the esophageal submucosal glands.

The following topics were addressed:

- To devise clinically applicable methods for assessing p53 and Ki67 immunohistochemical expression in columnar lined esophagus and to compare the interobserver agreement between these methods and routine hematoxylin and eosin evaluation.
- To examine the distribution of the esophageal submucosal glands in relation to the type of overlying epithelium in patients with columnar lined esophagus.
- To evaluate the presence of multilayered epithelium and to compare the distribution, size and morphology of the esophageal submucosal glands beneath metaplastic columnar mucosa with those beneath normal squamous epithelium in patients with columnar lined esophagus.
- To characterize the immunophenotypic relationship between the squamous and the glandular compartments in the esophagus of patients with columnar lined esophagus.

Materials

Figure 9. One of the seven esopageal resection specimens with columnar metaplasia, opened, pinned on a cork plate and fixated.

Study I

For this study 115 esophageal biopsy cases were randomly selected from the files of the Department of Clinical Pathology and Cytology, University Hospital Malmö. Of these 47 were originally diagnosed as NFD, 5 as IFD, 50 as LGD and 13 as HGD, between 1 July 1999 and 31 December 2000.

Studies II-III

In these studies seven esophageal resection specimens were collected prospectively, from patients (5 male, 2 female) who underwent resection for preoperatively untreated adenocarcinoma of the distal esophagus at Lund University Hospital 2007. The surgical resection included the proximal part of the stomach and the vast majority of the intrathoracic part of the esophagus. All specimens had segments with columnar metaplasia in the esophagus. (Figure 9)

In study III one additional specimen was included and used as a control. This resection specimen was from a female patient with preoperatively untreated adenocarcinoma confined to the gastric part of the GEJ, without any columnar metaplasia in the esophagus.

Study IV

In this study eight tissue blocks were chosen for immunohistochemical analysis. They originated from three of the seven CM containing specimens included in studies II-III, and were selected because they met our criteria of having SMG and two or more of the other tissue components.

Methods

Histopathology

In study I, the consensus diagnoses on the H&E sections between the three participating pathologists, were achieved after a discussion based on criteria for assessment of dysplasia described by Haggitt (Haggitt RC. Barrett's esophagus, dysplasia and adenocarcinoma. Hum. Pathol. 1994; 25;982–993.) (Table 1)

Table 1. Diagnostic criteria for dysplasia according to Haggitt	
Low-grade dysplasia	
	Preserved crypt architecture
	Stratified nuclei occurs but do not reach the apical surfaces
	Nuclei are enlarged, crowded and hyperchromatic
	Mitotic figures may be present in the upper portion of crypts
	Mucus is usually diminished or absent
	The abnormalities extend to the mucosal surface
High-grade dysplasia	
	Distortion of crypt architecture with branching, lateral budding or cribriform pattern
	Stratified nuclei reaches the crypt luminal surface and polarity might be lost
	Nuclear abnormalities as in low-grade dysplasia but there is bigger variation among the nuclei
	Mucus is usually absent
	The abnormalities extend to the mucosal surface

In studies II and III, the surgical resection specimens were examined histologically in toto. Assessments were made by light microscope and measurements with an ocular microscale and an eyepiece graticule.

In study II, the transformation-zones (TZ) between squamous and columnar mucosa were defined as extending 0.25 mm proximal and distal from the exact point of transition, giving each TZ a length of 0.5 mm. Each circumscribed collection of submucosal acini was regarded as one SMG. Each circular structure with a central lumen

found in the submucosa or mucosa, coated by cuboidal or squamous or multilayered epithelium, was also designated as one SMG.

In study III, the most proximal transition between squamous and columnar mucosa in each longitudinal section of the esophageal resection specimen was defined as the neosquamocolumnar junction (NSCJ). The length of this junction was defined as extending 5 mm proximal and distal from the exact point of transition, giving each NSCJ a length of 10 mm. Each circumscribed collection of mucinous acini in the submucosa was regarded as one SMG, provided it was larger than 0.18 mm^2. Each rounded or longitudinally cut structure, lined by a cuboidal, non-mucinous epithelium, located in the SMG, was regarded as an intraglandular duct and was measured separately. The SMG excretory ducts in the submucosa and mucosa were not counted in study III.

In study IV, the tissue components that were assessed, included gastric mucosa, esophageal metaplastic columnar mucosa with or without goblet cells, SMG acinar cells, SMG myoepithelial cells, adenocarcinoma, SMG intra-glandular ductuli, SMG excretory ducts and esophageal squamous epithelium.

Immunohistochemistry

In study I, newly prepared sections were immunostained by the peroxidase-anti-peroxidase method in DAKO TechMate 500/100 according to the manufacturer´s recommendations. For quantification of p53 expression, the area with the most prominent p53 IHC positivity was identified at low power magnification. All intensely dark brown columnar epithelial nuclei in one to five high-power fields (HPF) (X400 magnification) in this area were calculated, and the mean number of positive nuclei was reported. For quantification of Ki67 expression, a similar "hot spot" method was used to, if possible, identify an area with an aberrant proliferation zone. Only areas with well-oriented mucosa could be assessed. Two HPFs had to be used to cover the lower and the upper halves of the mucosa. All intensely dark brown columnar epithelial nuclei in these two HPFs were counted, and the quotient between the number of positive nuclei in the upper and the lower halves of the mucosa reported. The H&E consensus diagnoses were used as gold standard for comparison between H&E and IHC evaluations. **In study IV,** newly prepared sections were immunostained by the EnVision method in DAKO AutostainerPlus according to the manufacturer´s recommendations. The expression pattern of CK20, CDX2, MUC2, CK14, CK17, CK4 and lysozyme was evaluated independently by two pathologists. The staining was classified as positive when a specific, brown, intracytoplasmic or nuclear stain was identified in more than 1% of the cells belonging to a particular morphologic structure.

Statistics

In **study I**, Kappa statistics were used to calculate the chance-corrected proportional agreement between the pathologist´s assessments of dysplasia for H&E, p53 and Ki67 IHC. The Spearman bivariate correlation analysis was performed to investigate possible correlation between the gold standard and the p53 and Ki67 IHC assessments. The statistical analyses were performed using SPSS version 11.0.

In **studies II –IV**, the distribution of the data was tested using the Kolmogorov-Smirnov test of normality. As the data were not normally distributed, comparisons were made using nonparametric tests and the results were reported as medians and interquartile ranges unless otherwise stated. For continuous data, the Mann-Whitney U test was used for comparisons between groups and the Wilcoxon's signed ranks test was used for two related samples. Friedman´s analysis of variance was used for comparisons of more than two related samples. Analysis of relationship between 2 variables was made using linear regression analysis. Statistical analysis was performed using the software SPSS 15.0 and SPSS 21.0.

Results

Study I

The results of the assessments of the H&E stained esophageal biopsy sections are shown in Table 2. The chance corrected proportional agreement was only fair, with a mean κ value of 0.240, while the mean κ values for the assessments of Ki67 IHC and p53 IHC were 0.520 and 0.715, indicating moderate and good agreement respectively.

Table 2. Consensus diagnoses, individual assessments and original diagnoses of the studied cases in H&E

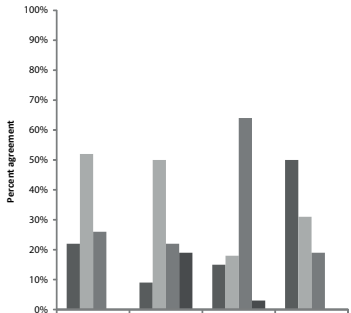
	Not BE	NFD	IFD	LGD	HGD
Consensus diagnoses	11 (9.6%)	23 (20.0%)	32 (28.7%)	33 (28.7)	16 (13.9%)
Observer 1 diagnoses	10 (8.7%)	33 (28.7%)	21 (18.3%)	41 (35.6%)	10 (8.7%)
Observer 2 diagnoses	14 (12.2%)	7 (6.1%)	44 (38.2%)	27 (23.5%)	23 (20.0%)
Observer 3 diagnoses	5 (4.3%)	59 (51.3%)	25 (21.7%)	12 (10.5%)	14 (12.2%)
Original diagnoses	0 (0.0%)	47 (40.9%)	5 (4.3%)	50 (43.5%)	13 (11.3%)

Number of cases (percentages) assessed as not Barrett´s esophagus, (Not BE), negative for dysplasia (NFD), indefinite for dysplasia (IFD), low-grade dysplasia (LGD) and high-grade dysplasia (HGD).

The greatest disagreement was found among the IFD and LGD cases in assessment of H&E, where no agreement were achieved in 19% and 3% of the cases, respectively. The highest value for agreement was found among the NFD and IFD cases in assessment of p53 IHC, where total agreement between the three observers were found in 100% and 91% of the cases, respectively. (Figure 10)

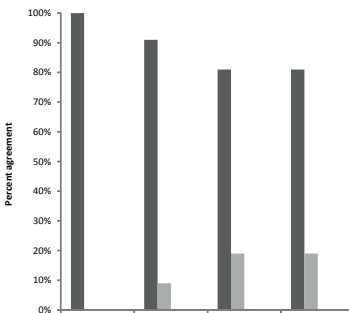
Comparison between two methods of assessing IHC yielded excellent correlation for both p53 IHC ($r = 0.998$, $p = 0.01$) and Ki67 IHC ($r = 0.973$, $p = 0.01$). One of the methods was more laborious requiring all columnar cells to be counted, while the other was easier, with counting of only IHC-positive cells.

A



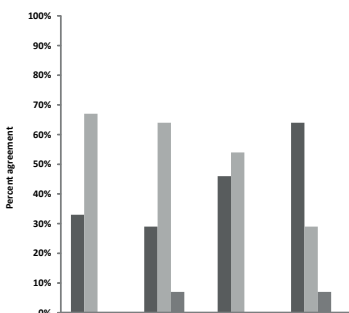
	23 cases NFD	32 cases IFD	33 cases LGD	16 cases HGD
Total agreement	22%	9%	15%	50%
Two observers agree	52%	50%	18%	31%
According two one observer	26%	22%	64%	19%
No agreement	0%	19%	3%	0%

B



	20 cases NFD	32 cases IFD	32 cases LGD	16 cases HGD
Total agreement	100%	91%	81%	81%
Two observers agree	0%	9%	19%	19%
According to one observer	0%	0%	0%	0%
No agreement	0%	0%	0%	0%

C



	18 cases NFD	28 cases IFD	28 cases LGD	14 cases HGD
Total agreement	33%	29%	46%	64%
Two observers agree	67%	64%	54%	29%
According to one observer	0%	7%	0%	7%
No agreement	0%	0%	0%	0%

Figure 10. Illustration of the distribution of the percentage agreement of assessments among observers in the groups Negative for dysplasia (NFD), Indefinite for dysplasia (IFD), Low grade dysplasia (LGD) and High grade dysplasia (HGD) using (A) Hamatoxylin&Eosin staining and immunohistochemistry with (B) p53 and with (C) Ki67.

Study II

The cumulative extent of the different surface epithelial types and the number of SMG observed in all sections of the seven specimens are presented in Table 3. The median frequency of SMG was similar in the metaplastic segments (0.12 SMG/mm) and the normal squamous segments (0.10 SMG/mm) (p = 0.620).

Further sub-classification of the metaplastic segments revealed an uneven distribution of the SMG, with an accumulation of SMG beneath the transition zones between columnar metaplasia and squamous islands, as well as a relative accumulation beneath the squamous islands, compared to beneath columnar metaplasia. (Table 4) The median frequency of SMG in the TZ, beneath SI and beneath CLO was 0.53 SMG/mm, 0.22 SMG/mm and 0.08 SMG/mm, respectively. The difference in frequency of SMG in the TZ, SI and CLO was significant (p = 0.001 and p = 0.002).

Table 3. The cumulative extent of the different sur-face epithelial types and the number of submucosal glands observed in the seven resection specimens.

Pat 1	377.3	60	306.1	49
Pat 2	46.5	2	671.9	12
Pat 3	0.9	4	377.8	14
Pat 4	242.9	11	518.5	51
Pat 5	1088.6	11	167.1	10
Pat 6	398.0	90	257.2	43
Pat 7	574.2	58	80.3	13
Total	2728.4	236	2378.9	192

SS, proximal squamous esophageal segment; SMG, sub-mucosal gland units; MS, metaplastic esophageal segment.

Table 4. The cumulative extent of the different surface epithelial types and the number of SMG observed in the metaplastic segments.

	SI (mm)	SMG in SI	CL (mm)	SMG in CL	TZ (mm)	SMG in TZ
Pat 1	43.3	10	262.8	39	24.0	22
Pat 2	0.0	0	671.9	12	4.0	1
Pat 3	1.0	0	376.8	14	1.5	2
Pat 4	44.9	14	473.6	37	18.5	22
Pat 5	34.7	7	132.5	3	16.5	7
Pat 6	106.1	22	151.1	21	15.0	8
Pat 7	13.3	3	67.0	10	8.5	4
Total	243.3	56	2135.7	136	88.0	66

SI, Squamous islands in the metaplastic segments; SMG, submucosal gland units; CL, columnar lined parts of the metaplastic segments; TZ, transition-zones in the metaplastic segments.

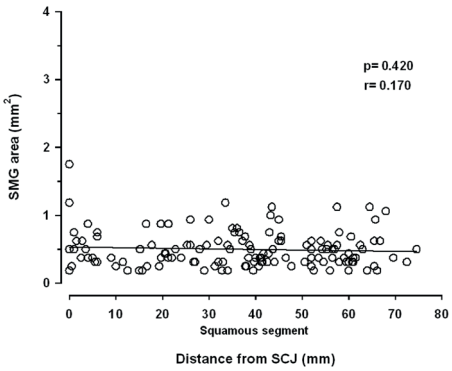


Figure 11. Linear regression analysis showing the relationship between the area of the submucosal glands (SMG) and the distance to the squamocolumnar junction (SCJ) in the esophageal specimen without columnar-lined esophagus.

Of the total 339 SMG larger than 0.18 mm found in the eight specimens, 135 (40%) belonged to the control case without columnar metaplasia.

In the control case, the distribution and size of SMG was even. (Figure 11) In the seven cases with columnar metaplasia, the SMG were larger and showed an accumulation in the neosquamocolumnar junction (NSCJ). (Figure 12)

There was a significant difference in the size of the SMG between the metaplastic columnar segments (0.81 mm^2) and the SMG in the normal squamous segments (0.56 mm^2) in the cases with columnar metaplasia (p = 0.001), and also between the size of the SMG in the metaplastic columnar segments and the control case (0.44 mm^2) (p =0.003). The median frequency of SMG in the NSCJ was significantly higher (0.080 SMG/mm) than that of the squamous (0.013 SMG/mm) and metaplastic segments (0.031 SMG/mm) (p = 0.022).

The proportion of the area occupied by intraglandular ductuli in the SMG (area IGD/area SMG) was similar in the metaplastic segments (0.20) and the normal squamous segments (0.25) in the seven cases with columnar metaplasia (p = 0.258).

In the cases with columnar metaplasia, multilayered epithelium was found predominantly in the excretory ducts of the SMG, and occurred in both the metaplastic segments (65%) and the squamous segments (35%). The frequency of ME was significantly higher in the metaplastic segments (1/158 mm) than in the squamous segments (1/341 mm) (p = 0.028).

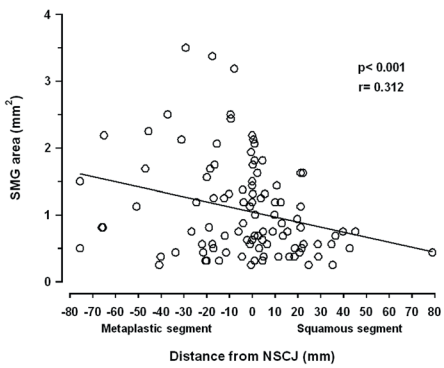


Figure 12. Linear regression analysis showing the relationship between the area of submucosal glands (SMG) and the distance to the neosquamocolumnar junction (NSCJ) in the esophageal specimens with columnar metaplasia.

Study IV

The numbers and proportions of observed immunoexpression per assessed tissue compartment in parts of the metaplastic esophageal segments are shown in Table 5.

Table 5. Immunohistochemical characteristics of the tissue compartments in the columnar lined esophagus.

Tissue Compartment	Lysozyme+	CK20+	CDX2+	MUC2+	CK14+	CK4+	CK17+
Gastric mucosa	2/2(100%)	2/2(100%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
Columnar metaplasia	6/6(100%)	4/6(66%)	4/6(66%)	5/6 (83%)	0/6 (0%)	3/6 (50%)	2/6 (33%)
SMG acini	8/8(100%)	0/8(0%)	0/8(0%)	0/8 (0%)	0/8 (0%)	1/8 (12%)	0/8 (0%)
Tumour	2/5(40%)	3/5(60%)	2/5(40%)	2/5 (40%)	1/5 (20%)	1/5 (20%)	3/5 (60%)
SMG myoepithelium	0/8(0%)	0/8(0%)	0/8(0%)	0/8 (0%)	8/8 (100%)	0/8 (0%)	8/8 (100%)
Intra-glandular ducts	0/8(0%)	0/8(0%)	0/8(0%)	0/8(0%)	8/8 (100%)	7/8 (87%)	8/8 (100%)
Excretory ducts	1/5(20%)	0/6(0%)	0/6(0%)	0/6 (0%)	6/6 (100%)	6/6(100%)	4/6 (66%)
Squamous epithelium	0/5(0%)	0/5(0%)	0/5(0%)	0/5(0%)	5/5 (100%)	5/5(100%)	4/5 (80%)

Number and proportion of observed immuno-expression per assessed tissue compartment.

The intestinal markers, CK20, CDX2 and MUC2, were all expressed in the anticipated pattern in columnar metaplastic mucosa, with no expression in SMG or in squamous epithelium

Of the squamous markers CK14, CK4 and CK17, an unexpected expression of CK 4 and CK17 was also found in metaplastic columnar cells. Furthermore, CK4 was expressed in the apical columnar cells of multilayered epithelium, and CK17 was expressed in squamous cells in the transformation zones between squamous and metaplastic columnar mucosa, as well as between squamous mucosa and the excretory ducts of the SMG. (Figure 13A and B)

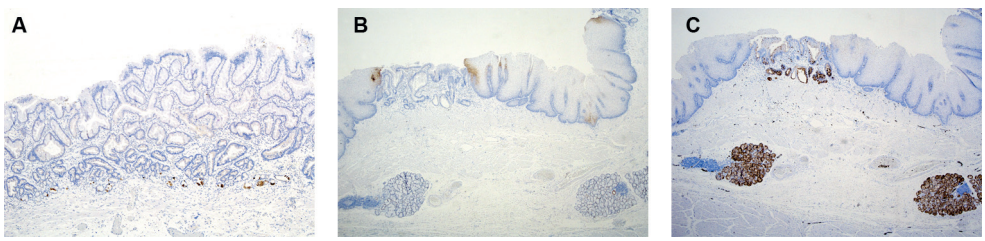


Figure 13. A) Expression of CK4 in columnar cells with basal location in the metaplastic mucosa. B) Expression of CK17 in the transformation-zones. C) Expression of lysozyme in the acinar cells of the submucosal glands, as well as in the metaplastic mucosa.

The cuboidal cells of the intraglandular and excretory ducts of the SMG had a similar expression pattern for the squamous markers.

Lysozyme was expressed both in the acinar cells of the SMG and in the metaplastic columnar cells, in the latter mostly in cells with basal location in the mucosa. (Figure 13C)

In adenocarcinoma cells, expression of CK17 and CK20 dominated.

Discussion

Study I

Against the background of the well-known inter-observer variation in assessment of dysplasia in biopsy material from columnar lined esophagus, we devised and tested two methods for immunohistochemical assessment of p53 and Ki67 as surrogate dysplasia markers.

The inter-observer agreement on all categories of H&E stained sections was somewhat worse (κ 0.240) than previously reported by Montgomery E et al (κ 0.43).[112] Our results probably reflected the heterogeneous composition, and limited number of observers in our study, with one resident, one general and one experienced GI pathologist. In Montgomery's group the skill base was more homogenous, with all 12 observers being experienced GI pathologists. The more varied experience level in our study-group may also have influenced our final consensus diagnoses used as gold standard. However, our lowest inter-observer agreement was in the categories IFD and LGD, as in Montgomery's group, indicating that the distribution of disagreement between categories was valid.

The inter-observer agreements improved substantially for the assessments of the IHC markers Ki67 (κ 0.520) and p53 (κ 0.715) compared to the assessment of H&E stained sections. These results were in accordance with earlier reports and confirmed that light microscopy assessment of these IHC markers is well reproducible among pathologists.[150, 154]

At the time we published study I, there was emerging international research activity in the field of "Barrett´s esophagus", focusing on the possible prognostic value of p53 and cellular proliferation.[142, 149, 155, 156, 157, 158] The quantification methods described were often elaborate, demanding special technical equipment, unsuited to routine clinical practice.[158, 159, 160] Our aim therefore was to devise and test more easily applicable methods.

For the evaluation of Ki67 IHC, we developed a method that could both describe and quantify the mucosal expression pattern. The ratio between the number of positive

cells in the upper and the lower parts of mucosa was chosen as an objective measure of the upwards shift in the proliferation-zone that has previously been described in dysplasia.[132] A ratio of more than 0.7 corresponded to HGD in 12 of 14 cases. Interestingly a moderate shift in the proliferation-zone was more common in the IFD than the LGD category (6 of 28 versus 2 of 28 cases). The increased cellular proliferation in IFD might be a response to inflammatory activity or indicate development of dysplasia in this indefinite category. In recent studies, no significant differences have been found in progression rates between patients initially diagnosed with NFD or IFD, however patients with IFD carry a significant risk of harboring prevalent metaplasia and patients with multifocal IFD are more likely to progress to HGD/EAC than the NFD or IFD groups.[161, 162] This highlights the need for explicit endoscopy biopsy protocols to aid the assessment of prevalent dysplasia and multifocal IFD.

In a phase 4 prospective, long-term follow-up study, no association was seen between total Ki67-positive proliferative fractions in diploid CM biopsies and progression to EAC, as measured by flow cytometry.[131] However flow cytometry doesn't capture crucial information on the topographic distribution of the epithelial-cell proliferation. Indeed, the major weakness of our chosen method for evaluating Ki67 IHC is that it demands well-orientated sections to discriminate the topographic distribution of cellular proliferation. In our material 15% (16/104) of the cases had to be excluded from Ki67 IHC assessment. It is generally recognized that, biopsy material from the GI tract must be orientated for adequate assessment, and biopsies from the esophagus are no exception to this requirement. Thus high-quality laboratory work can minimize the limitation of the chosen method.

For the evaluation of p53 IHC, we designed a method based on identifying a "hot-spot" at low-power magnification, and quantification of p53-positive epithelial cells in a few high-power fields within this hot-spot. Previous studies have used various quantification methods , ranging from counting positive cells among 1000 epithelial cells through to considering a cut–off of more than 10% p53 positive epithelial cells as indicative of aberrant expression.[133, 142, 149, 159] During the past ten years no definitive international consensus has been reached on how to assess p53 IHC over-expression. The trend during this period has gone from detailed, laborious methods to a simplified, binary approach determining either positivity or negativity. Kastelein et al have been the most detailed, defining p53 IHC aberrant expression as one or more glands with complete loss or over-expression of p53.[105, 129, 130,151, 152, 163, 164]

In our study we found a significant over-expression of p53 in 10 of 100 p53 IHC cases, but in only 44% (7 of 16) of the HGD cases. This differs strongly from previous and more recent reports, where the proportion of p53 IHC over-expression in HGD cases has ranged from 77% to 89%.[129, 133, 142, 151, 152] Our assessment of p53 IHC was made in a blinded manner without access to the hematoxylin&eosin slides, and we made no attempt to correlate morphologic findings with IHC findings. Hence, it is

probable that the areas of interest for HGD were not represented in a subset of the p53 IHC slides, especially since the HGD cases were presumably more elaborately sectioned in the clinical work-up before our study.

There was a significant correlation between the consensus diagnoses and the p53 IHC and Ki67 IHC assessments (p=0.01), which we interpret as support for the use of these biomarkers as adjunct in dysplasia assessment of esophageal CM biopsies, especially when used in combination.

In addition to the two methods noted above, we also evaluated a more laborious method, analogous to previous reports, where all epithelial cells in the investigated areas were counted and the IHC positive cells were assessed as a proportion of all these epithelial cells. There was a strong correlation (p=0.01) between counting only IHC positive cells and counting proportions of IHC positive cells, indicating that the easier methods were equally good.

In summary, we have devised easy and reproducible methods for measuring p53 and Ki67 IHC to grade esophageal CM biopsies. These surrogate markers correlate well with dysplasia and are associated with less inter-observer variation than morphologic dysplasia grading.

Studies II, III and IV

The question of the origin of columnar metaplasia in esophagus is essential in understanding metaplasia development and in designing adequate treatments. The esophageal submucosal glands and their excretory ducts have been proposed as providing a stem cell source for both neosquamous islands and columnar metaplasia (with the latter via an intermediate step of multilayered epithelium). However, studies of the human SMG in healthy individuals or in patients with esophageal disease are rare. The distribution and morphology of the SMG, and their possible relationship with CM in patients with CM, have so far remained largely unclear. In studies II–IV we have addressed these issues by conducting comparative and descriptive analyses of data collected from a total of eight esophagectomy specimens embedded in their entirety.

In our studies the numbers of SMG were similar in the normal squamous segments (SS) and the metaplastic segments (MS) but differed significantly from the control case, which had 4 times as many SMG than the median SMG of the seven other cases. The data in this field, although limited, strongly points to a real and large variation in the number of SMG per individual. In 1910 Goetsch E found 741, 62 and 140 SMG in three esophagi, and in 1988 Medeiros LJ found a variation between 0 and 24 SMG in 16 systematically taken sections per case from 20 esophagi.[165, 166] Since we have found a range from 3 to 135 SMG per esophagus, we feel that our data clearly confirm earlier

findings and that no certain conclusion can be drawn on the difference in numbers of SMG between the control case and the seven cases with CLO. As it has been reported that patients with reflux esophagitis have an impaired esophageal mucin secretion, it is worth considering the possibility that patients with CLO might either be born with fewer SMG, and a resulting decreased defense against reflux, or have lost a part of their SMG, for example in a reflux-induced atrophic process.[167] Our results do not support the latter assumption.

We used different methods in studies II and III, resulting in discrepancies between the absolute numbers of assessed SMG and between frequency figures (SMG/mm) among the studies. In study III a total of 224 SMG smaller than 0.18 mm^2 as well as excretory ducts were excluded. This might have had an influence on our findings, excluding possible atrophy of the SMG.

As with the findings on the numbers of SMG, in study II we found that the frequencies of SMG were also similar in the SS and the MS (calculated as total SMG found, divided by total mm examined esophageal tissue). Interestingly, when we conducted a more detailed analysis of the MS, we found that the distribution of SMG varied considerably, with twice as high a frequency of SMG under the squamous islands (SI) and a five-fold increase in the transformation-zones (TZ) between columnar metaplasia and the squamous islands, compared with the frequency in the SS. In study III there was a non-significant difference in the frequency of the SMG between SS and MS, suggesting that the change of methodology may have affected SS more than MS. However, in both SS and MS, there was a strong accumulation of SMG in the neosquamocolumnar junction (NSCJ) between the segments, amounting to an eight-fold increase in frequency, compared with SS. These findings confirm the importance of SMG in esophageal epithelial defense, identifying the SI as remains of squamous epithelium sheltered by SMG. They also illustrate the neosquamocolumnar junction as a line of demarcation of where the concentration of SMG is enough to prevent further propagation of the metaplastic segment.

The SMG are considered equivalent to oral minor salivary glands and can be expected to share similar reaction patterns and pathology with them. Different kinds of hyperplasia of the oral glands exist. For example, adenomatoid acinar hyperplasia of the minor salivary glands is a rare disease entity described as a focal reactive process of idiopathic nature.[168,169,170] Furthermore, extensive compensatory hyperplasia of the minor salivary glands have been reported after adjuvant external radiotherapy to the face and neck region.[171] However, salivary glands also have a general plastic capacity and can recover after injury by inducing a rapid regeneration process in which the number of acinar cells increases.[172] Since the size of the SMG (mm^2) in our study, was significantly larger in the MS than in both the SS and control case, and since there were no signs of hypertrophy of acinar cells or acini, our finding is consistent with hyperplasia of the SMG in the esophageal metaplastic segment. It also constitutes morphologic evidence

that the SMG are affected by and react to the same stimuli that causes CM.

We hypothesized that reflux-related injury of the SMG could lead to atrophy, which, by analogy with other salivary glands, would be characterized by a diminished amount of acini and an increased amount of intra-glandular duct-like structures.[172, 173] To investigate the eventual atrophy of the SMG we calculated the proportion of the area occupied by intra-glandular ductuli (IGD) in each SMG (IGD mm^2 / SMG mm^2). This proved to be similar in the MS and the SS, thus no evidence for reflux-related atrophy was found with our assessment method. Braxton DR et al. have recently described two new metaplastic changes within the SMG, named oncocytic glandular metaplasia (OGM) and necrotizing sialometaplasia-like change (NSMLC).[174] Both OGD and NSMLC are of non-mucinous ductal epithelial origin and have therefore in our study been assessed as IGD. Braxton DR et al found that Barrett´s esophagus (BE) was independently associated with NSMLC, without relationship to treatment history. In their study, patients with BE were compared with patients without BE, and NSMLC was seen in SMG in all parts of the esophagus, thus the relation between NSMLC and exposure for reflux remains uncertain. Furthermore, they made no attempt to assess the degree of atrophy of the acinar compartment in the SMG. Their conclusion that NSMLC may be an explanation for the impaired mucosal secretory capacity found in GERD patients is interesting and could imply that the SMG hyperplasia we have found are of a compensatory nature.

It has now been established that it is mainly eccrine sweat glands, but also pilosebaceous units, that are the sources of re-epithelialization in human skin wounds. Three days after wounding, epidermal outgrowths appear above each appendage and continue to grow until they merge with each other.[175] Esophagus is devoid of these adnexal structures, as are all human non-keratinizing squamous mucosa and submucosa. From this, it seems logical to view the SMG as the esophageal counterparts of skin adnexa and the source of re-epithelialization in this organ. Such a conclusion is supported by reports of the development of neosquamous epithelial islands after acid suppression therapy, laser ablation and anti-reflux surgery in patients with CM.[176, 177, 178] Our findings of an accumulation of SMG under squamous islands in the metaplastic segment can thus be interpreted both as evidence that these SMG protect remnants of the original squamous epithelium and that the squamous islands are newly developed from the SMG units. The low frequency of SMG beneath columnar metaplasia in the metaplastic segments would then imply that these areas have relative lower capacity for squamous re-epithelialization.

The concentration of SMG units in the transformation-zones (TZ) between squamous islands and columnar metaplasia in the metaplastic segments, together with the observation of a high frequency of multilayered epithelium in the SMG excretory ducts in metaplastic segments, provides strong morphologic evidence for an origin in the SMG unit of both neosquamous islands and columnar metaplasia. Further support for

this theory can be found in previously reported genetic analyses which show clonal relationships between excretory ducts, neosquamous islands and columnar metaplasia.[65] The exact location of the putative stem cells for CM remains to be found, but there is evidence of several stem cell populations existing in the same SMG unit.[64]

Our finding in study IV of overlapping immunophenotypes between the SMG unit, the columnar metaplasia and the transformation-zones is in agreement with, and further supports the theory of the origin of CM in the SMG unit.

One of our chosen markers, CK17, is normally expressed in appendages of the skin, in skin wound-healing and is also proposed to be a marker of bi-potential cells in the squamocolumnar transition-zone of the uterine cervix.[179, 180] Interestingly, we found CK17 expression not only in the SMG intra-glandular and excretory ducts, but also in the transformation-zones in the metaplastic segments. This shows that the SMG ducts are immunophenotypically related to skin adnexa and that cells involved in wound-healing in skin are related to cells found in the transformation-zones where development of both neosquamous islands and columnar metaplasia takes place. This indicates that CK17 may indeed also be a marker of bi-potential cells in the transformation zones in the esophagus. In our limited material, it seems that cells with CK17 expression are frequent in CM-related adenocarcinoma of the esophagus. This observation needs to be addressed in future studies.

Another of our chosen markers, CK4, is normally expressed in supra-basal cells of non-keratinizing stratified squamous epithelia. Intriguingly, we found expression of CK4 not only in the SMG intra-glandular and excretory ducts, but also in multilayered epithelium and in the most basally located glands in the metaplastic columnar mucosa. We interpret this finding as an evidence of an immunohistochemically detectable relationship between the SMG ducts, ME and the basal glandular layer in CM. It may indicate a stage in the epithelial conversion from a squamous to a columnar phenotype.

The antibody for lysozyme was the third of our chosen markers that was expressed in both the SMG unit and in the metaplastic columnar mucosa. Lysozyme is an enzyme with antibacterial properties.[181] The development of CM is considered to be a reactive and adaptive response to chronic reflux since columnar epithelium is better suited to withstand the components of the refluxate than squamous epithelium.[3, 182] Since it has been shown that there is an altered microbiotic environment in esophagi with CM, compared to normal esophagi, it might be deduced that the expression of lysozyme in CM is a part of the adaptive response to this changed environment.[183, 184, 185] In our antibody panel, lysozyme was the only marker with a strong, consistent expression in the SMG acinar cells. It was also the only one showing a highly specific immunophenotypic connection between SMG acini and columnar metaplasia, implying that as well as the SMG intra-glandular ducts and excretory ducts, the SMG acini also play a part in the development of CM.

In conclusion we have provided morphologic evidence for the importance of SMG in the esophageal epithelial defence, and shown that the SMG beneath metaplastic columnar mucosa are hyperplastic and thus seem to respond to the same stimuli that cause the metaplasia. We propose that the SMG represents the esophageal counterpart to skin adnexa in their function as sources of re-epithelialization. We have provided morphologic and immunohistochemical evidence for the origin of both neosquamous islands and columnar metaplasia in the SMG units. However, in patients with esophageal columnar metaplasia there are large individual variations in parameters as severity and composition of reflux, function of the reflux-barrier and the intrinsic defense mechanisms including number and function of the SMG, leading to a complex spectrum of esophageal injury. It is therefore likely that several mechanisms for adaptation and repair can be activated simultaneously.

Conclusions and future perspectives

- Clinically applicable, well-reproducible methods have been devised for assessing esophageal CM biopsies by use of IHC of p53 and Ki67.

  Development of automated laboratory equipment for orientation and paraffine-embedding of biopsy material would improve diagnostics in GI pathology in general, and especially facilitate the interpretation of localized IHC expression, as for Ki67 and p53.

  Digitalization of slides, meaning a switch from light-microscopy to computer-based assessments, will enable automated cell counting. Further studies are needed to develop and validate digital methods for IHC assessement.

  A national audit on assessment of p53 and Ki67 IHC in esophageal biopsies from metaplastic columnar mucosa is likely to have a valuable standardizing effect.

- The esophageal submucosal glands are important in epithelial defence and undergo hyperplasia in those parts of the esophagus showing columnar metaplasia.

  Studies on how different compositions of the gastroesophageal refluxate influence SMG functions and how these might be stimulated by medication will offer opportunities for innovative new treatments for patients with esophagitis and /or columnar metaplasia in esophagus.

- The SMG units are the esophageal counterparts of skin adnexa in their function as sources of re-epithelialization, and can give rise to growing neosquamous islands, as well as to columnar metaplasia.

  Development of techniques for leaving non-mutated SMG units and neosquamous islands intact in ablative therapies could lead to more efficient eradication of dysplasia and faster wound-healing.

Development of techniques for transplantation of non-mutated SMG or minor salivary glands into areas of dysplastic columnar metaplasia might be a gateway to a new treatment modality.

Studies focusing on the role of the SMG in the development of squamous dysplasia and squamous carcinoma would be of great interest for a better understanding of the different pathogenesis behind squamous carcinoma and adenocarcinoma of esophagus.

Populärvetenskaplig sammanfattning

Matstrupen är ett tubformat organ som förbinder munhålan med magsäcken. Den har en principiell anatomisk uppbyggnad som resten av mag-tarm- kanalen men avviker genom att slemhinnan, precis som i munnen, utgörs av ett icke-förhornande skivepitel. Resterande del av mag-tarm- kanalen utkläds av körtelförande slemhinna. Förhornande skivepitel utgör vad vi i dagligt tal kallar hud. I hudens djupare delar finns svettkörtlar och hårsäckar. I munnen och matstrupen finns inte sådana, men där finns det spottkörtlar. De under slemhinnan befintliga slemproducerande körtlarna (SMG) i matstrupen anses vara besläktade med munnens små spottkörtlar. De utsöndrar bl.a. syraneutraliserande vätska och utgör en del av matstrupens försvar mot den tunntarmssaft och magsaft som kan rinna tillbaka i matstrupen.

Människor kan drabbas av gastroesofageal refluxsjukdom (GERD), ett tillstånd där tunntarms- respektive magsaft åker upp i matstrupen onormalt länge och i onormalt stora mängder. GERD har visat sig leda till skador i matstrupens slemhinna orsakande inflammation och sårbildning. Vissa individer med GERD kan också drabbas av att slemhinnan i matstrupen ställvis omvandlas till slemhinna av körtelförande typ. Detta kallas metaplasi. Förekomst av körtelmetaplasi innebär en högre risk att drabbas av körtelcancer i matstrupen, en form av cancer som förr var mycket ovanlig men som de senaste decennierna ökat kraftigt och nu i Sverige blivit vanligare än skivepitelcancer i matstrupen.

Utvecklingen från CM till cancer går via, i ljusmikroskop, iakttagbara förändringar i körtelcellerna, vilka indelas i låggradiga förändringar (LGD), obestämbara, möjligen inflammationsbetingade förändringar (IFD) och höggradig dysplasi (HGD). Från de individer som har besvär av GERD och som genomgår endoskopisk undersökning tas vävnadsprov, s.k. biopsier, om CM ses. Proverna skickas till patologen för analys. Om LGD/IFD eller värre förändring föreligger erbjuds patienten att ingå i ett kontrollprogram med återkommande endoskopier. Om precancerösa förändringar påvisas så kan de tas bort med endoskopisk teknik, men om cancer utvecklats måste matstrupen om möjligt opereras bort.

Det har länge varit känt att den enskilde patologens bedömning av rutinfärgade biopsier överensstämmer dåligt med patientens prognos och också med den bedömning grupper av inom området specialutbildade patologer gör. Om mer än en specialutbildad patolog är överens om diagnosen så ökar sannolikheten starkt för att diagnosen är

riktig. Man har under decennier sökt efter bättre metoder för bedömning av cancerförstadier i körtelmetaplasi. Immunhistokemi är en vanlig laboratoriemetod där färgmärkta antikroppar mot ett visst protein binder in till de celler som uttrycker proteinet och därmed synliggör det. Intensiv forskning inriktad på att leta proteiner som kan användas som markörer för LGD, IFD och HGD har lett till en uppsjö av förslag på sådana, men endast ett fåtal markörer har hållit måttet i jämförande, framåtblickande långtidsstudier. En av dem är p53, ett protein som normalt uttrycks i cellkärnor och som deltar i regleringen av celldelning och celldöd. Vid mutationer i TP53 genen kan proteinet bli icke-fungerande och därmed hämning av celldelning utebli, vilket kan leda till cancerutveckling. Mutation kan indirekt påvisas genom att proteinet ansamlas i cellkärnan och blir möjligt att upptäcka med immunhistokemi. En annan markör är Ki67, ett protein som uttrycks i cellkärnan hos celler som är i delningsfas. I tumörer och förstadier till tumörer är celldelningen ökad och den normala celldelningszonen förändrad, vilket kan synliggöras med immunhistokemi.

I studie I har vi utformat och testat metoder för bedömning av p53 och Ki67 immunhistokemi på biopsimaterial från patienter med körtelmetaplasi i matstrupen. Först gjordes bedömning på rutinfärgat material av tre patologer. Därefter bestämdes den korrekta diagnosen för varje biopsi vid en gemensam genomgång. Denna korrekta diagnos användes sedan som facit vid jämförelse med de individuella bedömningarna av de p53 och Ki67 immunhistokemiska färgningarna. Skillnaderna i överensstämmelse mellan de olika patologernas bedömning minskade avsevärt vid användande av p53 och Ki67 jämfört med vid rutinfärgning. Det fanns också ett samband mellan grad av precancerös förändring, grad av p53 överyttryck och grad av förskjutning av celldelningszonen i slemhinnan. Jämförelse mellan en mer arbetskrävande bedömningsmetod och en lättare visade att de var likvärdiga avseende resultat. Våra resultat visar att föreslagna metoder är reproducerbara, kliniskt användbara och behäftade med mindre bedömningsavvikelse mellan patologer än sedvanlig bedömning av rutinfärgat material. Immunhistokemisk färgning för p53 och Ki67 är användbara, kompletterande, prognostiska metoder för bedömning av biopsimaterial från körtelmetaplasi i matstrupen.

Det finns flera teorier om varifrån körtelmetaplasin i matstrupen har sitt ursprung. Man har föreslagit utväxt av körtelslemhinna från magsäcken upp i matstrupen, direkt omvandling av skivepitel till körtelepitel i matstrupen och även att körtelepitelet växer ut från de under slemhinnan belägna slemkörtlarna i matstrupen. Frågan om körtelmetaplasins ursprung är viktig, dels för förståelse av metaplasiutveckling generellt och dels för utformning av nya, bättre behandlingar.

I studie II-IV har vi utfört jämförande och beskrivande undersökningar av fördelningen och den histologiska samt immunhistokemiska karaktären av slemkörtlarna i matstrupen hos patienter med körtelmetaplasi. För detta ändamål har vi använt operationspreparat bestående av matstrupen och delar av magsäcken från sju patienter med körtelmetaplasi och ett kontrollfall utan körtelmetaplasi. Andelen SMG var lika stor

i den metaplastiska delen av matstrupen som i den normala delen men antalet SMG hos de olika individerna varierade mellan 3 och 135 SMG. Högsta antalet fanns hos kontrollfallet. Fördelningen av SMG (antal SMG per millimeter matstrupe) var också likartad vid jämförelse mellan metaplastisk och icke-metaplastisk matstrupe, men var ojämn inom de metaplastiska delarna. Där sågs ansamlingar av SMG under övergångszonerna mellan skivepitel och körtelepitel och minskat antal SMG under körtelepitelet. Hos kontrollfallet sågs ingen sådan ansamling utan SMG var jämnt fördelade i hela matstrupen. Slemkörtlarnas medianstorlek var signifikant större i metaplastiska delen av matstrupen jämfört med såväl den normala delen som med kontrollfallet. Ingen förtvining av SMG påvisades i matstrupens olika delar. Immunhistokemisk undersökning visade ett överlappande infärgningsmönster mellan slemkörtlarna, körtelmetaplasi och övergångszonerna för tre antikroppar. En av dessa markerar ett protein, CK17, som normalt uttrycks bland annat i hudens svett- och talgkörtlar, i hud i samband med sårläkning och i övergångszonen mellan skivepitel och körtelepitel i livmoderhalsen. Den andra markerar ett protein, CK4, som normalt uttrycks i icke-förhornande skivepitel. Den tredje markerar ett antibakteriellt enzym, lysozyme, som normalt uttrycks i bland annat i spottkörtlar och i de s.k. Brunnerska körtlarna i tunntarmen.

Dessa resultat utgör, tillsammans med andra tidigare studier inom området, bevis för slemkörtlarnas betydelse för försvaret av matstrupens slemhinna och visar att slemkörtlarna under metaplastisk slemhinna förstoras, vilket visar att de svarar på samma stimuli som de som orsakar metaplasin. Ansamlingen av SMG i övergångszoner och under bevarade eller nybildade skivepitelöar, tillsammans med fynd av övergångsepitel i slemkörtlarnas utförsgångar talar starkt för att såväl körtelmetaplasi som skivepitelöar har sitt ursprung i SMG. I analogi med hudens sårläkning, vilken sker fläckvis och utgår från svettkörtlarna, föreslår vi att matstrupens slemkörtlar motsvarar hudens svettkörtlar i deras funktion som stamcellsresurs. Detta resonemang stöds bl.a. av tidigare studier som visat nybildning av små skivepitelöar i den metaplastiska delen av matstrupen efter behandling av GERD, samt också av det i vår studie visade uttrycket av CK17.

Våra resultat utesluter inte att andra samtidiga system för anpassning och reparation av slemhinnan är i funktion hos patienter med körtelmetaplasi i matstrupen.

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Study I

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Ki67 and p53 immunohistochemistry reduces interobserver variation in assessment of Barrett's oesophagus

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Ki67 and p53 immunohistochemistry reduces interobserver variation in assessment of Barrett's oesophagus

Aims: To devise clinically applicable methods for assessing p53 and Ki67 immunohistochemical (IHC) reactivity in Barrett's oesophagus (BE) and to compare the interobserver agreement between these methods and routine haematoxylin and eosin (H&E) evaluation.
Methods and results: One hundred and fifteen biopsies diagnosed as BE, selected from the files of the University Hospital MAS, Malmö, were re-evaluated for dysplasia by three pathologists. For IHC analysis areas with the most prominent positivity were evaluated. The mean of p53+ epithelial nuclei/high-power field (HPF) was obtained by counting between 1 and 5 HPFs/biopsy. A proliferation quotient (PQ) was obtained by dividing the number of Ki67+ epithelial nuclei in the upper half by the lower half of the mucosa, using two HPFs. Mean κ values were 0.24, 0.71 and 0.52 for H&E, p53 and Ki67 evaluations, respectively. There was a correlation between increasing severity of dysplasia, IHC measurable overexpression of p53 and shift of the mucosal proliferation zone towards the surface, measured as PQ.
Conclusions: The described methods for p53 and Ki67 evaluation are more reproducible than routine H&E evaluation of BE. Furthermore, the IHC methods correlate with the severity of dysplasia and are useful supplementary prognostic markers.

Keywords: Barrett's oesophagus, interobserver agreement, Ki67, p53, proliferation

Abbreviations: BE, Barrett's oesophagus; HGD, high-grade dysplasia; HPF, high-power field; IFD, indefinite for dysplasia; IHC, immunohistochemistry, immunohistochemical; LGD, low-grade dysplasia; NFD, negative for dysplasia; PQ, proliferation quotient

Introduction

Barrett's oesophagus (BE), where columnar epithelium with intestinal metaplasia replaces the squamous epithelium in the oesophagus, is considered to be a precancerous condition in which the development of adenocarcinoma can be followed through various grades of epithelial dysplasia.[1–3] Surveillance programmes for BE patients rely on histopathological assessments of oesophageal biopsies. However, the interpretation of changes leading to the classification of dysplasia in haematoxylin and eosin (H&E)-stained sections is subjective and suffers from considerable interobserver variation.[4–8] Some studies report improvement with higher κ values when immunohistochemistry (IHC) is used.[9,10] Two of the most thoroughly studied BE dysplasia-related markers, p53 and Ki67, have still not been standardized for routine pathological examination, partly due to disagreements in interpretation.[11–21] Hence the aim of this study was to devise an easy and reproducible method for assessing the IHC reactivity of p53 and Ki67 in BE.

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Materials and methods

From all oesophageal biopsies diagnosed as BE between 1 July 1999 and 31 December 2000, 47 cases originally diagnosed as negative for dysplasia (NFD), five as indefinite for dysplasia (IFD) and 50 as low-grade dysplasia (LGD) were randomly selected from the files of the Department of Clinical Pathology and Cytology, University Hospital MAS, Malmö. Furthermore, all 13 cases diagnosed as high-grade dysplasia (HGD) during this time period were also selected. For the purpose of our study, newly prepared serial sections were stained with p53 antibody (Dako, Glostrup, Denmark; clone DO-7; dilution 1 : 400) and with Ki67 antibody (Dako; clone MIB-1; dilution 1 : 200) by the peroxidase–antiperoxidase method, according to the manufacturer's recommendations, in DAKO TechMate 500/100®.

All the original H&E sections were coded and examined in a blinded fashion by three pathologists: one with a special interest in gastrointestinal pathology, one resident from the university department and one pathologist from a county hospital. No calibrating meeting was held in advance to reflect the daily, routine approach. After the individual assessments, we held a consensus meeting where all biopsies for which there was disagreement were reviewed and the final diagnoses were determined following a discussion based on criteria described by Haggitt,[1] referred to here as 'consensus diagnoses'.

EVALUATION OF THE H&E-STAINED SECTIONS

Of the original 115 cases, 11 cases were excluded because of erroneous diagnoses of BE or incorrect codings. Dysplasia was graded according to the recommendations of Haggitt[1] (Table 1) into four categories: NFD, LGD, HGD and IFD defined as features of LGD but with significant occurrence of neutrophils and/or ulceration.

EVALUATION OF THE p53 AND Ki67 IHC-STAINED SECTIONS

The IHC evaluations were performed in a blinded manner without access to the H&E slides, so as not to influence the observers. The quality of the haematoxylin counterstaining of the immunostained sections did not allow reliable assessment of morphological dysplasia, especially not IFD and LGD. For the assessment of p53 IHC, four of the 104 cases were excluded due to technical errors identified when at least one observer judged the section as suboptimal for evaluation. For quantification of p53 expression the following method was devised: the area with the most prominent p53 IHC positivity was identified at low-power magnification. All intensely dark brown/black columnar epithelial nuclei in one to five high-power fields (HPFs) within this area were counted. The result of each assessment was given as the mean number of positive columnar epithelial nuclei/HPF. The HPFs were obtained at 10×40 magnification, diameter 0.44 mm. For the assessment of Ki67 IHC, 16 cases of the 104 cases were excluded due to technical error, including loss of tissue. The Ki67 expression pattern was determined as follows: an area with positive nuclei in the surface epithelium and/or upper halves of the crypts was chosen at low-power magnification. In this area, counting of intensely dark brown/black columnar epithelial nuclei was performed in the two mucosal compartments: (i) the surface epithelium together with the upper halves of the crypts, and (ii) the lower halves of the crypts together with the deeper located glands. Two HPFs were used to cover the two mucosal compartments. The pattern of epithelial proliferation was given as a quotient between the number of positive cells in the upper and the lower part of the mucosa (PQ). The higher the value of the quotient, the stronger the shift of the proliferation zone from its normal basal location towards the surface. To compare the described

Table 1. Diagnostic criteria for dysplasia

Low-grade dysplasia
Preserved crypt architecture
Stratified nuclei occurs but do not reach the apical surfaces
Nuclei are enlarged, crowded and hyperchromatic
Mitotic figures may be present in the upper portion of crypts
Mucus is usually diminished or absent
The abnormalities extend to the mucosal surface
High-grade dysplasia
Distortion of crypt architecture with branching, lateral budding or cribriform pattern
Stratified nuclei reaches the crypt luminal surface and polarity might be lost
Nuclear abnormalities as in low-grade dysplasia but there is bigger variation among the nuclei
Mucus is usually absent
The abnormalities extend to the mucosal surface

methods of IHC quantification and the more labourious but previously reported[16,18] method of calculating the exact proportion of positive cells, one of us also counted all IHC-negative columnar epithelial cells in the same HPFs. The proportion was then calculated by dividing the number of IHC-positive cells by the sum of positive and negative cells. To compare H&E and IHC staining evaluations, the consensus diagnoses were used as 'gold standard'.

STATISTICAL METHODS

Kappa statistics and Spearman bivariate correlation analyses were performed using SPSS 11.0 for Windows (Chicago, IL, USA).

Results

INTER-OBSERVER AGREEMENT IN THE H&E ASSESSMENTS

The results of the individual evaluations by the three pathologists, the consensus diagnoses and the original diagnoses are shown in Table 2. The chance corrected proportional agreement between the three pathologists was only fair, indicated by a mean κ value of 0.240.

Comparison between individual assessments and consensus diagnoses is also presented as the percentage of total agreement in the different dysplasia categories (Figure 1a). Total agreement was only 21% when all BE dysplasia classes were considered. Best agreement was achieved in the HGD class (50%), although this increased to 74% and 81% in the NFD and HGD classes, respectively, if one combines the rates of agreement of two out of three and all three pathologists.

INTEROBSERVER AGREEMENT IN THE p53 IHC ASSESSMENTS

The range of mean positive nuclei/HPF was 0–210. Based upon the mean of the results of our p53 IHC assessments in the different dysplasia classes according to the consensus diagnoses, three intervals could be delineated arbitrarily: 0 to one positive nucleus/HPF corresponding to the margin of error, > 1–5 positive nuclei interpreted as p53 overexpression with unknown significance, and > 5 positive nuclei interpreted as definite p53 overexpression (Table 3 and Figure 2). Using these intervals the mean κ value was 0.715. The Spearman correlation coefficient was 0.329, significant at the 0.01 level, between consensus diagnoses and p53 IHC assessments. The percentage total agreement versus disagreement is shown in Figure 1b. Total agreement was high in all four dysplasia classes, ranging between 81% and 100%. When all classes were considered, total agreement was 88%.

INTEROBSERVER AGREEMENT IN THE Ki67 IHC ASSESSMENTS

The range of PQ values was 0.03–3.36. As with the p53 IHC calculations, we delineated three PQ intervals: 0–0.3, > 0.3–0.7 and > 0.7 representing an increasing shift of proliferation towards the surface epithelium (Table 4 and Figure 3). The mean κ value for the Ki67 IHC assessments, measured as PQ intervals, was 0.520. The Spearman correlation coefficient was 0.278 between consensus diagnoses and Ki67 IHC assessments and 0.366 between p53 and Ki67 IHC assessments, both significant at the 0.01 level. The distribution of IHC assessments versus consensus diagnoses is presented in Figure 1c. The total agreement for

Table 2. Consensus diagnoses, individual assessments and original diagnoses of the studied cases in haematoxylin and eosin staining

	Not BE	NFD	IFD	LGD	HGD
Consensus diagnoses	11 (9.6%)	23 (20.0%)	32 (28.7%)	33 (28.7%)	16 (13.9%)
Observer 1 diagnoses	10 (8.7%)	33 (28.7%)	21 (18.3%)	41 (35.6%)	10 (8.7%)
Observer 2 diagnoses	14 (12.2%)	7 (6.1%)	44 (38.2%)	27 (23.5%)	23 (20.0%)
Observer 3 diagnoses	5 (4.3%)	59 (51.3%)	25 (21.7%)	12 (10.5%)	14 (12.2%)
Original diagnoses	0 (0.0%)	47 (40.9%)	5 (4.3%)	50 (43.5%)	13 (11.3%)

Number of cases (percentages) assessed as not Barrett's oesophagus (Not BE), negative for dysplasia (NFD), indefinite for dysplasia (IFD), low-grade dysplasia (LGD) and high-grade dysplasia (HGD).

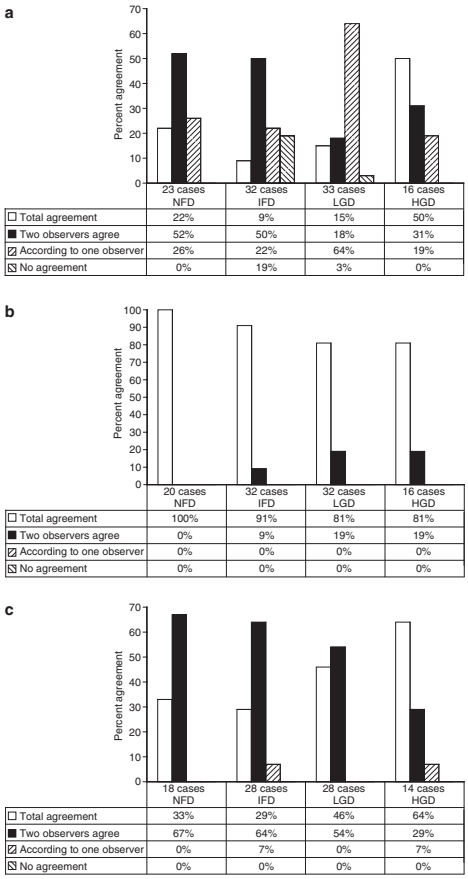

Figure 1. Distribution of percentage agreement of assessments among observers in the group negative for dysplasia (NFD), indefinite for dysplasia (IFD), low-grade dysplasia (LGD), and high-grade dysplasia (HGD) using **a**, H&E; **b**, p53 IHC; **c**, Ki67 IHC.

Ki67 was 20% higher than in the H&E assessments but 47% lower than in the p53 assessments, when all dysplasia classes were taken together.

COMPARISON BETWEEN COUNTING IHC-POSITIVE COLUMNAR EPITHELIAL CELLS AND COUNTING PROPORTIONS OF IHC-POSITIVE CELLS AMONG ALL COLUMNAR EPITHELIAL CELLS

The outcome of p53 IHC evaluation comparing the two methods is presented in Table 5.

Table 3. The distribution of cases in groups with mean p53+ nuclei 0–1, >1–5 and >5 compared with consensus diagnoses

	p53 0–1	p53 >1–5	p53 >5	Total no. of cases
NFD	20	0	0	20
IFD	31	0	1	32
LGD	27	3	2	32
HGD	9	0	7	16
Total no. of cases	87	3	10	100

NFD, Negative for dysplasia; IFD, indefinite for dysplasia; LGD, low-grade dysplasia; HGD, high-grade dysplasia.

The Spearman correlation coefficient was 0.998 ($P = 0.01$) between p53+ cell counts and the proportion of p53+ cells among all epithelial cells. The same calculation for Ki67 PQ gave a correlation of 0.973 ($P = 0.01$).

Discussion

Histopathological diagnosis of dysplasia in BE is essential in the follow-up and treatment of patients, but it is subjective and suffers from considerable interobserver variation. It seems that the diagnosis of HGD is the least problematic, while the delineation of LGD/IFD from NFD is more difficult. Over-diagnosis of LGD results in more frequent monitoring of the patients, which has both psychological and economic consequences. Recent reports describe improvement of the diagnostic accuracy when p53 and/or Ki67 IHC are used and also especially in identifying LGD at risk for progression to HGD/cancer.[9,10,12,15,18,21,24]

The main function of the tumour suppressor gene *p53* is to participate in the control of cell division through its protein product p53. Mutation of this gene can result in accumulation of non-functional p53 detectable by IHC. IHC staining of p53 suffers from both false-negative and false-positive results, compared with *p53* gene sequencing,[18] so introduction of an additional parameter can be expected to increase precision. The Ki67 protein is present within the nucleus in all active phases of the cell cycle, and is a well-known proliferation marker. Hence, use of p53 and Ki67 antibodies allows the assessment of abnormalities due to genetic changes involved in carcinogenesis as the result of abrogation of antigrowth signalling and avoidance of apoptosis, reflected in increased proliferation.[22]

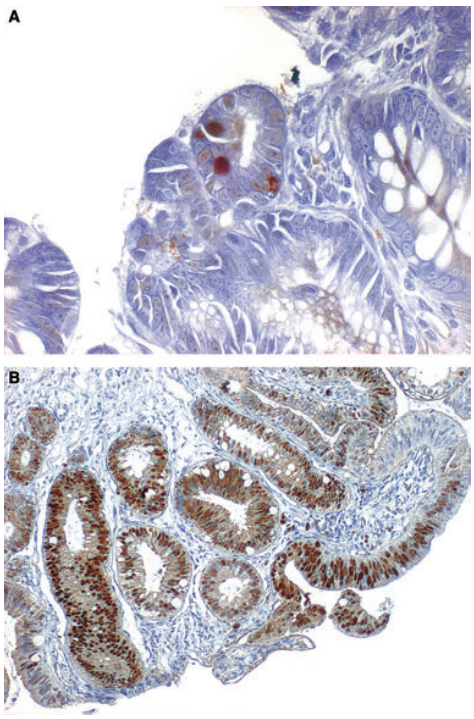

Figure 2. p53IHC. **A,** LGD > 1–5 positive nuclei/HPF. **B,** HGD > 5 positive nuclei/HPF.

Table 4. The distribution of cases in groups with proliferation quotient 0–0.3, > 0.3–0.7 and > 0.7 compared with consensus diagnoses

	Ki67 0–0.3	Ki67 > 0.3–0.7	Ki67 > 0.7	Total no. of cases
NFD	18	0	0	18
IFD	21	6	1	28
LGD	26	2	0	28
HGD	2	0	12	14
Total no. of cases	67	8	13	88

NFD, Negative for dysplasia; IFD, indefinite for dysplasia; LGD, low-grade dysplasia; HGD, high-grade dysplasia.

In our study we used easily applicable methods for the quantification of p53 and Ki67 IHC showing increased interobserver agreement, compared with

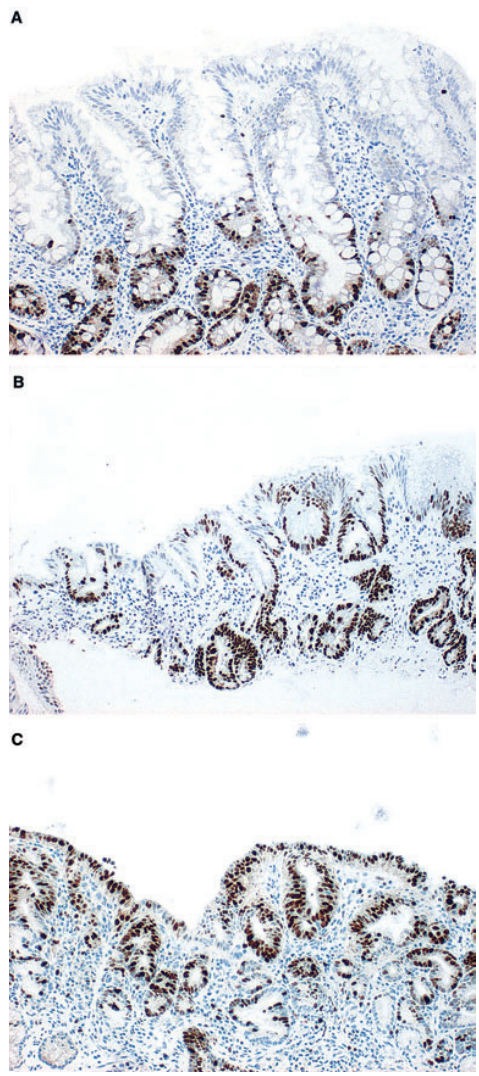

Figure 3. Ki67 IHC. **A,** NFD, PQ = 0–0.3. **B,** LGD, PQ > 0.3–0.7. **C,** HGD, PQ > 0.7.

H&E dysplasia grading. Using p53, the increase in total agreement between the observers was 82% in the IFD class and 66% in the LGD class. Even agreement in the diagnosis of HGD was improved by 31%. Using Ki67, the improvement was less with

Table 5. Comparison between method A = counting mean number of p53+ epithelial cells and method B = calculating percentage of p53+ epithelial cells, when cut-off for p53 positivity is > 5/high-power field (HPF) in method A and > 10%/HPF in method B

	NFD		IFD		LGD		HGD	
	Method A	Method B	Method A	Method B	Method A	Method B	Method A	Method B
Positive (*n*)	0	0	1	1	3	1	7	6
Negative (*n*)	20	20	31	31	29	31	9	10

a 31% increase in agreement in the LGD class and 20% increase in the IFD class. Calculating the chance-corrected proportional agreement, i.e. the κ value, gives an improvement from fair (0.240) in the H&E assessment to moderate (0.520) in the Ki67 IHC assessment and good (0.715) in the p53 IHC assessment. Our study was designed to mimic everyday clinical practice and to mirror average experience in the field among surgical pathologists, in contrast to previous studies[4,7] and we also believe that this might explain the high interobserver variation in our H&E assessments. Our results confirm the recommendation that a diagnosis of HGD/cancer in BE requires the unanimous agreement of at least two pathologists, especially in view of the possible serious consequences for the patient.

The methods used for IHC quantification are simple and easy, although the Ki67 assessment is limited by the need for well-oriented sections to determine the border between the upper and lower halves of the crypts. Rioux-Leclercq *et al.*,[13] Whittles *et al.*[14] and Halm *et al.*[16] have described more elaborate methods, excellent for research but unsuited to clinical practice or demanding investment in expensive equipment.[15,17] The very strong correlation between counting only the IHC-positive cells and counting both positive and negative cells (which implies counting more then 500 cells per section) indicates that the less labourious method is equally good (data not shown).

Our findings agree with those of Kahn *et al.*[9] and Skacel *et al.*,[10] who showed increased κ values when p53 IHC was used compared with histological interpretation in BE dysplasia grading. Regarding the proliferation pattern in BE, visualized by Ki67 IHC, Hong *et al.*,[19] among others,[13,14,23] have found topographical differences in proliferation between LGD and HGD in accordance with our findings of PQ over 0.7 in HGD compared with around 0.3 in LGD, IFD and NFD dysplasia classes. Van Sandick *et al.*[15] used computerized morphometry and immunoquantification and concluded that quantitative analysis of p53, Ki67 and a stratification index can have additional value to subjective grading of BE biopsy samples. They pointed out that in clinical practice every fifth biopsy would be unsuitable for assessment with their method due to limiting inclusion and exclusion criteria. In our study we had to exclude at most 15% (16 of 104) of the biopsies, which is slightly less.

The rather few examples of p53 overexpression and proliferation pattern change in the IFD and LGD classes might suggest that such alterations are late events in dysplasia development in BE and that IHC markers for early events remain to be discovered. In the NFD class there were no cases outside the lowest intervals. In the IFD class there was one case with strong p53 expression (mean 43.33 positive nuclei/HPF) and a definite shift of proliferation towards the surface (mean PQ 1.33). In the LGD class there were two cases with strong p53 expression (5.53 and 71.21 mean positive nuclei/HPF), but unfortunately without corresponding Ki67 assessment due to technical error. One additional case in this class had a high p53 value (35.20 mean positive nuclei/HPF) but a normal proliferation pattern (mean PQ 0.22). These four cases might represent LGD in transition to HGD and show that cases with precancerous cell cycle regulation changes that ought to be under careful clinical surveillance[9,10,12,15,18,21,24] can be correctly identified by measuring p53 overexpression and the Ki67 proliferation quotient. In the HGD class there was one case with no p53 expression and a normal proliferation pattern (mean PQ 0.14), possibly representing a neoplastic proliferation following a different carcinogenic pathway.

Our aim was to perform a methodological study and no follow-up data are available. The methods used should be validated in a prospective study; furthermore, IHC technology is beset with laboratory-specific variables, consequently new methods must be tested locally before clinical application.

In conclusion, we report that: (i) classical morphological grading of dysplasia in BE biopsies is accompanied by high interobserver variation. Better accuracy of diagnosis is achieved when more then one pathologist agrees; (ii) counting p53+ nuclei in 5 HPFs and Ki67+ nuclei in upper and lower parts of the mucosa are simple, clinically applicable methods for supplementary BE assessment to identify cases with precancerous cell cycle regulation changes; (iii) using these methods, immunohistochemical assessments of p53 and Ki67 have low and moderate interobserver variation, respectively. Combined use reduces the risk of wrong interpretation caused by false-positive or -negative IHC; and (iv) there is a correlation between increasing severity of dysplasia in BE biopsies, measurable IHC overexpression of p53 and shift of the mucosal proliferation zone towards the surface, measured as IHC Ki67 expression.

Acknowledgement

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Study II

Unfortunately minor errors appeared in two of the tables in the publication of Study II in Histopathology (2012). The correct versions are presented here in "Results" page 41.

Histopathology

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Submucosal glands in the columnar-lined oesophagus: evidence of an association with metaplasia and neosquamous epithelium

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Submucosal glands in the columnar-lined oesophagus: evidence of an association with metaplasia and neosquamous epithelium

Aim: A multipotential stem cell, possibly located in the submucosal gland ducts, has been suggested as the origin of metaplastic mucosa in the oesophagus. The topographic distribution of these glands and their excretory ducts (SMG) within the columnar lined oesophagus (CLO) is largely unknown. The aim of this study was to examine the distribution of SMG in relation to the type of overlying epithelium in patients with CLO.

Methods and results: Seven oesophageal resection specimens were examined histologically *in toto*. The median frequency of SMG was similar in the metaplastic segments (0.12 SMG/mm) and the normal squamous segments (0.10 SMG/mm). Within the metaplastic segments, the median frequency of SMG beneath the squamous islands was significantly higher than that observed under the columnar lined parts (0.22 versus 0.08 SMG/mm, $P = 0.046$), There was a strong accumulation of SMG at the squamo-columnar transition zones (0.53 SMG/mm), which was significantly greater than that found in the columnar and squamous parts ($P = 0.001$ and 0.002, respectively).

Conclusions: The relative accumulation of SMG beneath squamous islands and the squamo-columnar junctions within the metaplastic segment supports the hypothesis that both metaplastic columnar mucosa and neosquamous epithelium originate from a progenitor in the SMG.

Keywords: Barrett's oesophagus, columnar lined oesophagus, stem cell, submucosal gland

Abbreviations: CLO, columnar lined oesophagus; SMG, submucosal glands and their excretory ducts; TZ, transition zone

Introduction

The cellular and molecular mechanisms involved in the development of Barrett's mucosa are poorly understood, although there are several different hypotheses as to which cells give rise to the metaplastic tissue. Increasing interest is being generated by the hypothesis that metaplastic Barrett mucosa results from change in the differentiation of multipotent stem cells, which are induced to differentiate into a columnar epithelium as a result of continuous exposure to injury from refluxed gastric juice. The stem cell theory is attractive, as it provides an explanation for the variety of cellular phenotypes found in Barrett's oesophagus. Further, it explains how regeneration of squamous epithelium is possible, and it correlates well with evidence that the cell of origin is intrinsic to the oesophagus.[1] The location of such a stem cell is under debate, but it has been proposed to be located in the epithelial basal layer or the submucosal gland ducts.[2,3]

Detailed information on the topographic distribution of submucosal glands in healthy individuals and

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patients with columnar lined oesophagus (CLO) is sparse; however, results from thorough autopsy studies have not shown significant accumulation to any specific part of the oesophageal tube.[4–7] Endoscopically obtained oesophageal biopsies often reveal the ostia of the gland ducts, but rarely catch the submucosal glands because of their deep location. Consequently, histological studies of the relative distribution of submucosal glands require surgical resection specimens or autopsy specimens. In 2005 Coad *et al.*[8] used parts of oesophageal resection specimens, and extensive serial cutting enabled them to show a close spatial localization between submucosal gland duct structures and CLO as well as with squamous islands surrounded by CLO.

In an attempt to shed light into the importance of the submucosal gland unit in the pathogenesis of oesophageal metaplastic mucosa, the aim of the present study was to evaluate the topographic distribution of submucosal glands and their excretory ducts (SMG) in the entire oesophagus and in relation to the type of overlaying epithelium in patients with a CLO. This was conducted in prospectively collected oesophageal resection specimens examined *in toto* from patients with high-grade dysplasia or adenocarcinoma within a CLO.

Materials and methods

STUDY POPULATION

Seven patients (five male, two female) who underwent oesophageal resection for adenocarcinoma of the distal oesophagus at Lund University Hospital 2007 were included into the study. The Ethics Committee of Lund University approved the study (LU 400/2007).

The median age of the patients at the time of resection was 74 years (range 62–87) years. All patients were diagnosed with CLO on the pre-operative endoscopic examination. None of the patients had been subjected to endoscopic mucosal resection or other ablative therapy and no neoadjuvant radio- and/or chemotherapy had been given. The surgical resection included the proximal part of the stomach and the vast majority of the intrathoracic part of the oesophagus.

HISTOLOGICAL EVALUATION

All resected gastro-oesophageal specimens were fixed and dissected in a standardized manner by the same gastrointestinal pathologist (E.L.). Prior to fixation, all specimens were opened along the curvatura major and pinned on a cork plate utilizing a modest stretching. After fixation the entire oesophageal segments of all patients were cut longitudinally into a total of 80 approximately 5-mm-thick slices, resulting in a total of 300 tissue pieces embedded in 71 full mount and 128 standard paraffin blocks. Sample sections (4 μm) were stained with haematoxylin and eosin. Assessment by light microscope and measurement with ocular microscale were performed by the same pathologist (E.L.) using the same microscope.

The length of the proximal squamous segment was measured in each longitudinal slice of oesophagus. The extent of the metaplastic segment of each longitudinal slice of the oesophagus was also measured. The metaplastic segment was defined to extend from the point where the most proximal metaplastic columnar mucosa was found to the point where the proximal part of the invasive tumour or oxyntic type mucosa from the stomach was found. The areas at the transition between squamous and columnar mucosa was defined as transition zones (TZ). For the purpose of this study, these areas were defined as extending 0.25 mm proximal and distal from the exact point of transition, giving each TZ a total length of 0.5 mm (Figure 1).

The number of SMG was assessed in relation to the type of surface epithelium observed perpendicular to the main part of the gland or duct. We designated each circumscribed collection of acini as one SMG. The normal tortuous route of the SMG imply that in a two-dimensional section transversely cut duct structures can represent parts of one duct as well as parts from several ducts. Each transversely visualized, circular structure, coated by cuboidal or squamous epithelium, with a central lumen, found in the submucosa or mucosa was therefore also designated as one SMG.

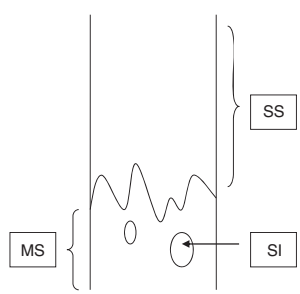

Figure 1. Schematic illustration of a columnar lined oesophagus. SS, squamous segment; MS, metaplastic segment; SI, squamous islands.

STATISTICAL ANALYSIS

Data are presented as median and interquartile ranges (IQR) unless stated otherwise. Analyses of continuous data were made using the Mann–Whitney *U*-test for comparisons between groups. Statistical analyses were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

Results

The cumulative extent of the different surface epithelial types and the number of SMG observed in all sections of the seven resection specimens are presented in Table 1. The proximal squamous segments constituted 53% and the metaplastic segments 47% of the accumulated length of all oesophageal sections. Fifty-five per cent of the SMG were located in the squamous segment and 45% in the metaplastic segment. The median frequency of SMG was similar in the metaplastic segments (0.12 SMG/mm) and the normal squamous segments (0.10 SMG/mm) ($P = 0.620$).

The metaplastic segments were subclassified further based on the type of the overlaying epithelium. In the longitudinally cut sections, the metaplastic section appeared as a row of alternating islands of columnar and squamous mucosa, ending with a relatively long columnar part which merged into tumour or oxyntic gastric mucosa. The characteristics of the metaplastic segments are presented in Table 2. The columnar lined parts constituted 90% and the squamous parts constituted 10% of the cumulative extent of the metaplastic segment. The total number of CLO islands and squamous islands constituting the metaplastic segment was similar (62 versus 61) and the length ranged from 0.05 to 34.0 mm and from 0.12 to 26.1 mm, respectively. Also the number of small islands (≤1.0 mm) of columnar and squamous mucosa was similar (14 versus 12).

The median frequency of SMG beneath the squamous islands (0.22 SMG/mm) was significantly higher than that observed in the columnar lined parts of the metaplastic segment (0.08 SMG/mm) ($P = 0.046$). The highest median frequency of SMG was found in the TZ. In these junctional areas composed of 0.5 mm of equal parts of squamous and columnar mucosa, the median frequency of SMG was 0.53 per mm, which was significantly higher than that found in the columnar and squamous parts of the metaplastic segments ($P = 0.001$ and 0.002, respectively).

Table 1. The cumulative extent of the different surface epthelial typesand the number of submucosal glands (SMG) observed in the seven resection specimens

	SS (mm)	SMG in SS	MS (mm)	SMG in MS
Pat 1	377.3	50	306.1	49
Pat 2	46.5	2	671.9	12
Pat 3	0.9	4	377.8	14
Pat 4	242.9	11	518.5	51
Pat 5	1088.6	11	167.1	10
Pat 6	398.0	90	257.2	43
Pat 7	574.2	53	80.3	13
Total	2729.4	236	2378.9	192

SS, Proximal squamous oesophageal segment; SMG, submucosal gland units; MS, metaplastic oesophageal segment.

Table 2. The cumulative extent of the different surface epthelial types and the number of SMG observed in the metaplastic segments

	SI (mm)	SMG in SI	CL (mm)	SMG in CL	TZ (mm)	SMG in TZ
Pat 1	43.3	10	262.8	39	24.0	22
Pat 3	0.0	0	671.9	12	4.0	1
Pat 3	10	0	376.8	14	1.5	2
Pat 4	44.9	14	473.6	37	18.5	22
Pat 5	34.7	7	132.5	3	16.5	7
Pat 6	105.1	22	151.1	21	15.0	8
Pat 7	13.3	3	67.0	10	8.5	4
Total	243.3	56	2135.7	136	88.0	66

SI, Squamous islands in the metaplastic segments; SMG, submucosal gland units; CL, columnar lined parts of the metaplastic segments; TZ, transition zones in the metaplastic segments.

Discussion

Seven oesophagectomy specimens from patients with adenocarcinoma within segments of metaplastic mucosa were examined *in toto* and analysed with regard to the frequency of the submucosal gland units in relation to the type of luminal oesophageal mucosa. There was no difference in the frequency of SMG between the normal squamous segments and the metaplastic segments. However, within the metaplastic segments there was a significant concentration of SMG beneath the squamous islands and an even stronger concentration

of SMG beneath the small mucosal compartments with dual differentiation defined as TZ. These observations provide new morphological evidence of an association between the oesophageal submucosal gland unit and maintenance or renewal of squamous epithelium, and also point towards an association with development of metaplastic columnar mucosa. Gastroeoesophageal reflux disease is known to be a major risk factor for the development of CLO and oesophageal adenocarcinoma, and the therapeutic focus has been on preventing reflux through surgical and pharmacological intervention.[9,10] However, for an understanding of the mechanism behind the development of CLO, we believe that the intrinsic oesophageal defence against reflux which is composed, in part, of secretion of bicarbonate from the SMG needs to be explored. Theoretically, regeneration of damaged oesophageal mucosa may arise from a pluripotent cell within the oesophagus, which has the ability to differentiate into squamous as well as metaplastic columnar mucosa depending on environmental and/or genetic factors. It has been proposed that the SMG may function as a cellular reservoir from which restoration of damaged oesophageal epithelium occurs.[8] In order to assess the association between SMG and metaplasia we evaluated the spatial distribution of SMG in the proximal squamous segment and the reflux exposed metaplastic segment of the distal oesophagus. It is commonly believed that in the normal oesophagus, SMG are scattered throughout the entire oesophagus with a relative accumulation in the proximal and distal regions.[11,12] However, there is sparse information on the distribution of SMG in the normal human oesophagus, and well-designed studies supporting this hypothesis are lacking. Available reports include the thorough examination of three oesophagi by Goetsch in 1910.[5] He conducted detailed studies of longitudinal strips of macroscopically normal oesophageal specimens and concluded that there was a strong tendency for the glands to be arranged in longitudinal rows and that there were considerable individual variations both with regard to the number and to the distribution of the oesophageal glands. More recently van Nieuwenhove *et al.*,[13] in their study of 15 necropsy specimens, showed that there might be a small but not statistically significant difference in distribution of the submucosal glands between the upper, middle and lower third of the oesophagus. Based on these observations it is unlikely that the submucosal glands have a significantly uneven distribution within the normal oesophagus. In our study, the frequency of SMG in the proximal oesophagus lined by squamous epithelium was similar to that found in the distal metaplastic segments. These observations support the earlier studies, in that there is a large individual variation in the distribution of SMG without a tendency for accumulation in any specific segment of the oesophagus.

Within the longitudinally cut sections, the luminal surface partly appeared as a row of alternating islands of columnar and squamous epithelium. Interestingly, we found that within the metaplastic segments the median frequency of SMG was significantly higher beneath squamous epithelium compared with that observed under columnar mucosa. As the relative distribution of SMG was similar in the normal squamous and the metaplastic segments of the oesophageal specimens, this finding clearly establishes a significant association between the squamous islands and the submucosal glands and their ducts. Squamous epithelium that is completely surrounded by columnar mucosa can potentially arise in two different ways. First, it may be a remnant of the original squamous epithelium. Theoretically, it is possible that mucous from the gland ducts provide local protection from the injurious effects of refluxed gastric juice in the proximity of the duct ostiae, thus leaving the squamous epithelium in this area intact. Secondly, and most probably, squamous islands represent areas of neosquamous epithelium that develop in metaplastic columnar mucosa in which reflux of gastric juice and its injurious effects has been markedly reduced or abolished. This hypothesis is supported by the fact that squamous islands are rare in patients with untreated Barrett's oesophagus but develop frequently with time, following effective acid suppression therapy and antireflux surgery.[14,15] The accumulation of SMG observed in our study suggests that these areas of regenerated squamous epithelium may have their cellular origin within the SMG. Morphological evidence of the relationship between oesophageal gland ducts and squamous islands has been provided by Coad *et al.*[8] who, in their study based on extensive serial cutting of samples from oesophagi with Barrett's epithelium, showed a direct continuity between the gland duct epithelium and squamous islands in all 15 of their cases.

The finding of a strong and significant accumulation of SMG in the TZ between the squamous epithelium and columnar mucosa within the metaplastic segments of the oesophageal resection specimens was unexpected. One possible explanation for this intriguing observation may be that the same gland duct unit may have the capability to give rise to both metaplastic columnar and neosquamous epithelium. In support of this hypothesis, Leedham *et al.*[16] has been able to demonstrate the presence of an identical p16 point mutation in gland duct tissue and in an adjacent BE

crypt and also a wild-type squamous island, emerging from a wild-type gland duct surrounded by a p53 mutated CLO field, suggesting that both CLO and squamous islands can arise from gland ducts. The more complex meshwork of epithelial interrelations of the metaplastic mucosa was demonstrated further by evidence that the clonal heterogeneity in CLO arises from multiple independent clones rather than one clone bifurcation from a single progenitor, as believed earlier. Their findings can be interpreted as a picture of growing individual clonal epithelial islands, wild-type or mutated, originating from gland ducts scattered throughout the metaplastic segments. Morphological evidence supporting the hypothesis that one single SMG can give rise to both epithelial types is provided by the observations of Glickman *et al.*,[17] who in 2001 described a multilayered epithelium composed of both basal squamous cells and superficial columnar cells, with a morphological and an immunohistochemical phenotype similar to the phenotype of the submucosal gland duct epithelium. In one of their cases the multilayered epithelium was contiguous with the superficial aspect of the mucosal gland duct epithelium. This is an observation that we confirm as a frequent phenomenon in our material, although it has not been the main subject in our study and therefore not specifically quantified (Figure 2).

We believe that the accumulation of SMG in the TZ between squamous and glandular epithelium is consistent with the idea that the metaplastic columnar mucosa may arise from SMG. However, if this hypothesis holds true, it is disturbing that we did not find an

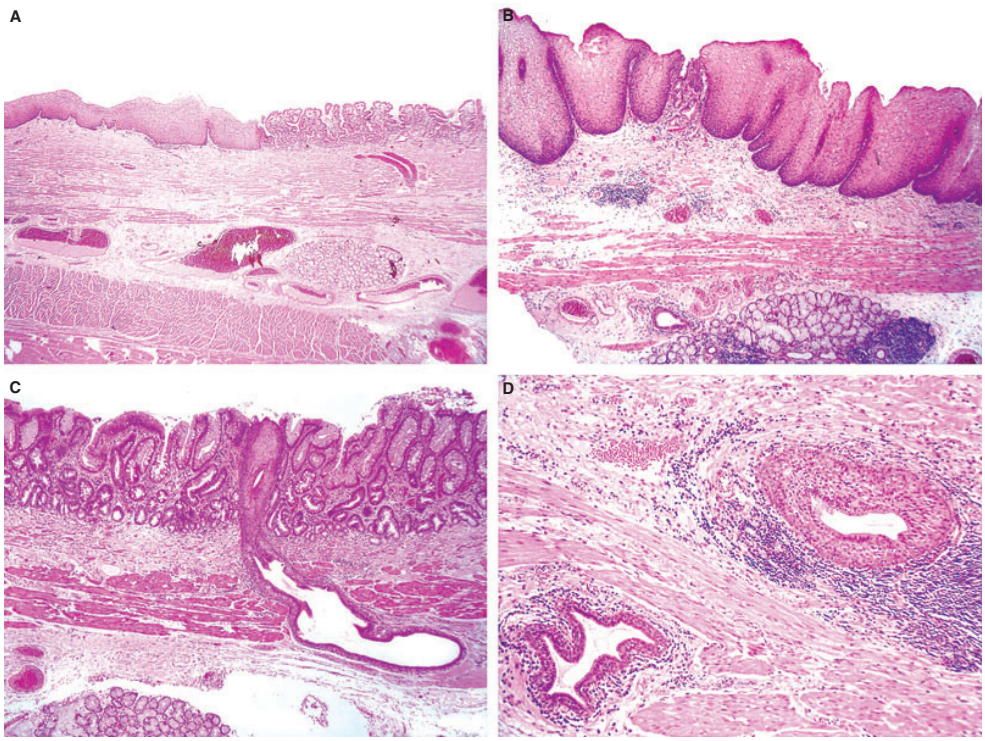

Figure 2. **A**, Low-power view of a submucosal gland in the transformation zone between the squamous segment and the metaplastic segment. **B**, Low-power view of a submucosal gland in the transformation zone between columnar and squamous lined parts of oesophagus in the metaplastic segment. **C**, Focus with direct connection between a submucosal gland duct and a small squamous island. **D**, High-power view of multilayered epithelium in a submucosal gland duct.

increase of SMG beneath the CLO islands. A reasonable explanation for the relative lack of accumulation of SMG under columnar mucosa in comparison with the squamous islands is that squamous islands develop from the SMG of metaplastic mucosa of the distal oesophagus when acid reflux and its injurious effects on the mucosa is markedly reduced. In patients with untreated reflux disease, the metaplastic mucosa of the distal oesophagus is most commonly uniform without significant visible islands of squamous epithelium. With effective treatment of reflux, islands of squamous epithelium appear. As these islands of neosquamous epithelium seem to develop from SMG within the columnar lined segment, this would result in a relative accumulation of the SMG beneath the regenerated squamous islands that is not observed in the remaining parts of the metaplastic segment.

The question of origin of the metaplastic epithelium in CLO is important, not only for a better understanding of the results of present treatment modalities but also for the development of new surgical and pharmacological treatments and preventions. We conclude that in patients with a CLO, the occurrence of oesophageal submucosal glands was similar beneath the proximal normal squamous epithelium and the metaplastic segment, suggesting that there is no difference in the distribution of SMG in the distal compared to the proximal oesophagus. It further indicates that acid reflux and the subsequent development of metaplasia does not significantly affect the spatial distribution of SMG. Our findings of a significant accumulation of SMG beneath the inlet squamous islands and a strong concentration beneath the TZ with dual differentiation suggest that both metaplastic columnar mucosa and neosquamous epithelium may originate from a progenitor located in the submucosal gland duct unit. Further studies using progenitor cell markers are needed to validate the accumulating evidence for the importance of the oesophageal gland duct unit as a source of precursor cells in the development of CLO and the regeneration of squamous epithelium.

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Study III

Histopathology

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Hyperplasia of the submucosal glands of the columnar-lined oesophagus

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Hyperplasia of the submucosal glands of the columnar-lined oesophagus

Aim: To evaluate the presence of multi-layered epithelium (ME) and to compare the distribution, size and morphology of the oesophageal submucosal glands (SMG) beneath reflux exposed metaplastic columnar mucosa with those of normal squamous epithelium in patients with columnar-lined oesophagus (CLO).

Methods and results: In eight oesophageal resection specimens, the SMG of the metaplastic segments were significantly larger than those in the squamous segments of patients with CLO (0.81 versus 0.56 mm^2, $P = <0.001$). There was an accumulation of SMG close to the neosquamocolumnar junction (NSCJ), as indicated by a higher median frequency of SMG (0.080 SMG/mm) compared with that of the squamous (0.013 SMG/mm) and metaplastic segments (0.031 SMG/mm) ($P = 0.022$). The frequency of ME was significantly higher in the metaplastic compared with the normal squamous segments (1/158 mm and 1/341 mm, respectively, $P = 0.028$) and ME was found almost exclusively (96%) in direct connection with the excretory ducts of SMG.

Conclusions: Hyperplasia of SMG in the metaplastic segment, accumulation of SMG near the NSCJ, the presence of ME in connection with the excretory ducts of SMG and metaplasia are all reflux-induced morphological changes, possibly induced by stimulation of progenitors in the excretory ducts of the SMG.

Keywords: barrett's oesophagus, columnar-lined oesophagus, multi-layered epithelium, progenitor cell, reflux, submucosal gland

Introduction

Metaplastic gland-forming columnar mucosa within the oesophagus, which frequently includes intestinal differentiation with goblet cells, is a complication in gastro-oesophageal reflux and the main risk factor for oesophageal adenocarcinoma.[1,2]

It has been suggested that injury from reflux of gastric contents stimulates the development of metaplasia through an intermediate morphological step characterized by a multi-layered epithelium (ME) with features of both squamous and columnar mucosa.[3] With time and continued reflux, intestinalization of the metaplastic columnar mucosa may occur as a second step in the development of Barrett's oesophagus.[4,5] The cell of origin for metaplasia is unknown, but according to current hypotheses possible stem cell niches for oesophageal metaplasia may be located in the basal layer of either the squamous epithelium or the excretory ducts of the oesophageal submucosal glands (SMG).[6]

Studies of the human SMG are rare and little is known about the distribution, morphology and the function of the SMG in healthy individuals and in patients with oesophageal disease. It is possible that in severe reflux disease, reflux of gastroduodenal juice may reach and injure the excretory ducts of the SMG, and in response to this stimulation morphological as

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well as functional changes may appear. Theoretically, acid-related injury of the SMG could lead to morphological alterations such as atrophy, which would be characterized by glands that are occupied mainly by duct-like structures and with diminished numbers of acini. Alternatively, stimulation by excessive acid exposure may increase the demand for mucosal protection and, as a consequence of this adaptation, hyperplasia of the SMG may occur.

In this study we evaluated the effect of severe and long-standing acid reflux of the human SMG and their excretory ducts by evaluating and comparing the distribution, size and the morphology of the SMG underlying metaplastic columnar mucosa with those found beneath the normal squamous epithelium in prospectively collected oesophageal resection specimens from patients with CLO and associated neoplasia.

Materials and methods

STUDY POPULATION

Prospectively collected oesophageal resection specimens from seven patients (five male, two female) were examined. These patients all underwent oesophageal resection for pre-operatively untreated adenocarcinoma of the distal oesophagus at Lund University Hospital in 2007. Their median age was 74 years (range 62–87 years). All were diagnosed with CLO on pre-operative endoscopic examination. One additional female patient aged 54 years with adenocarcinoma confined to the gastric part of the gastro-oesophageal junction without CLO was used as a control.

HISTOLOGICAL EVALUATION

All gastro-oesophageal resection specimens were formalin-fixed and dissected in a standardized manner. Prior to fixation, all specimens were opened along the greater curvature of the stomach and pinned onto a cork plate under moderate stretching. After fixation, the entire oesophagus of each patient was sliced longitudinally into a total of 80 slices approximately 5 mm thick, resulting in a total of 341 tissue pieces and 5878 mm of available oesophageal tissue. Sample sections (4 μm) were stained with haematoxylin and eosin and assessed using a light microscope with an eyepiece graticule. The metaplastic and squamous segments were defined and measured in each longitudinal slice of oesophagus, as described previously.[7] The most proximal transition between squamous and columnar mucosa in each longitudinal section was defined as the neosquamocolumnar junction (NSCJ). For the purpose of this study, this area was defined as extending 5 mm proximal and distal from the exact point of transition, giving each NSCJ a total length of 10 mm.

The area of each SMG was measured at ×4 magnification where each square represents an area of 0.0625 mm^2. The amount of squares that were partly or totally filled with SMG acini and intraglandular ductuli (IGD) was used for the assessment of the area of each individual SMG. Each circumscribed collection of mucinous acini was regarded as one SMG, provided that it was larger than 0.18 mm^2. Each rounded or longitudinally cut structure lined by a cuboidal, non-mucinous epithelium, located between the mucinous acini of the SMG, was regarded as an intraglandular duct and was measured separately. To assess the degree of atrophy, the proportion of the area of the SMG that was occupied by IGD was calculated. A size limit of the SMG was chosen to avoid misrepresentation of the relation between the area of the SMG and the area of the IGD by any cuts through the peripheral parts of SMG. The number of SMG and the distance between each SMG and the NSCJ was measured in the same longitudinal section, and the type of surface epithelium located perpendicularly to the centre of the SMG was noted.

The presence of ME, which was defined as a hybrid epithelium with features of both squamous and columnar epithelia, characterized by squamoid cells in its basal layers and columnar mucous cells in its superficial layers,[8] was assessed in the resection specimens of the seven patients with CLO.

STATISTICAL ANALYSIS

The data distribution was tested using the Kolmogorov–Smirnov test of normality. As the data were not distributed normally, comparisons were made using non-parametric tests and the results were reported as medians and interquartile ranges, unless stated otherwise. Continuous data were compared using Wilcoxon's test for paired data and the Mann–Whitney *U*-test was used for comparisons between groups. Friedman's analysis of variance was used for comparisons between more than two related samples. Analysis of the relationship between two variables was made using linear regression analysis.

Statistical analysis was performed using SPSS version 21.0. The Ethics Committee of Lund University approved the study (LU 400/2007).

Results

Detailed histological evaluation of the eight oesophageal resection specimens revealed a total number of 339 SMG that were larger than 0.18 mm^2. One hundred and thirty-five of these were found in the control patient without CLO. The median number of SMG observed in the resection specimens of the seven patients with CLO was 35 (ranging from 3 to 71). The median length of squamous and metaplastic segments in the longitudinal sections was 40 and 24.3 mm, respectively. The median area of the SMG was 0.44 mm^2 in the control case, which was slightly smaller numerically than those of the normal squamous segments of the cases with metaplasia. In the oesophageal resection specimens with CLO, the median area of the SMG found beneath metaplastic columnar mucosa was significantly larger (0.81 mm^2) than that of the normal squamous epithelium (0.56 mm^2) (P = <0.001) and that of the SMG found in the control case (P = 0.003) (Figure 1A,B).

In the control case without metaplasia, the SMG were distributed evenly along the entire length of the specimen, with no accumulation to any specific part of the oesophagus (Figure 2). Further, the size of the SMG was similar throughout the whole oesophagus. In sharp contrast, there was a marked accumulation of SMG in the proximity of the NSCJ in the patients with CLO (Figure 3). The apparent accumulation of SMG around the NSCJ was confirmed by the observation that the median frequency of SMG in the area of

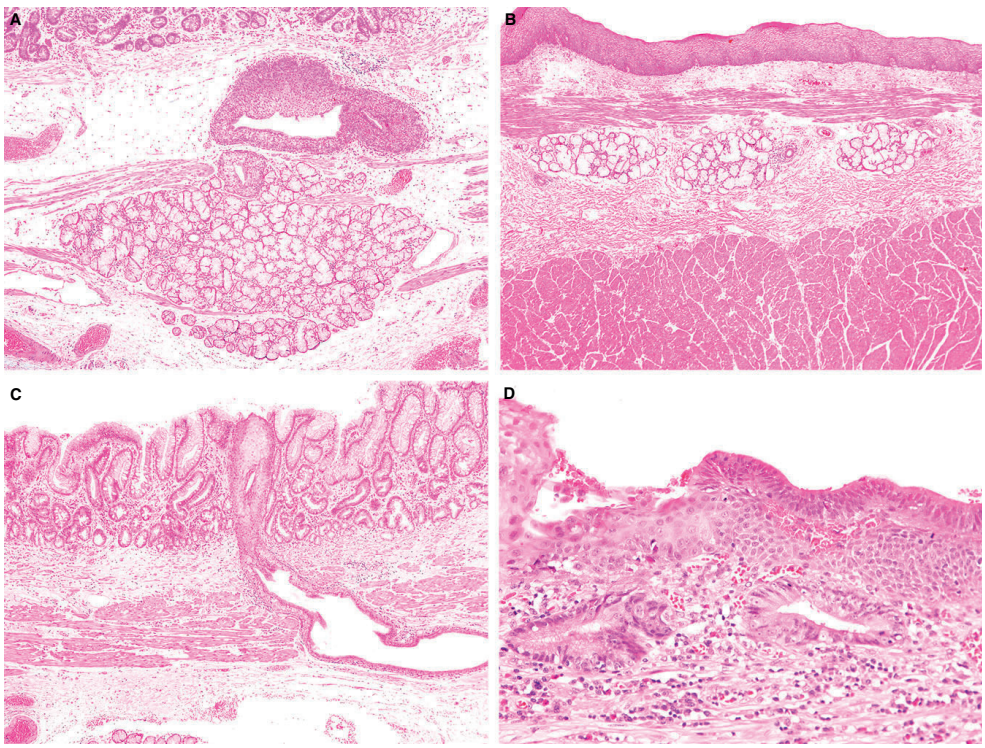

Figure 1. **A**, A hyperplastic submucosal gland with multi-layered epithelium in the excretory duct in the metaplastic segment 9 mm below the neosquamous junction [haematoxylin and eosin (H&E)]. **B**, Submucosal glands 73 mm above the gastro-oesophageal junction in the case without columnar-lined oesophagus (H&E). **C**, An excretory duct with multi-layered epithelium in direct connection with a squamous island in the metaplastic segment (H&E). **D**, Erosion and multi-layered epithelium forming a bridge between a squamous island and columnar epithelium in the metaplastic segment (H&E).

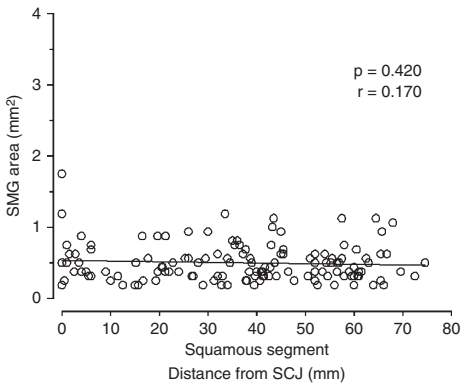

Figure 2. Linear regression analysis showing the relationship between the area of the submucosal glands (SMG) and the distance to the squamocolumnar junction (SCJ) in the oesophageal specimen without columnar-lined oesophagus. The line denotes the regression line.

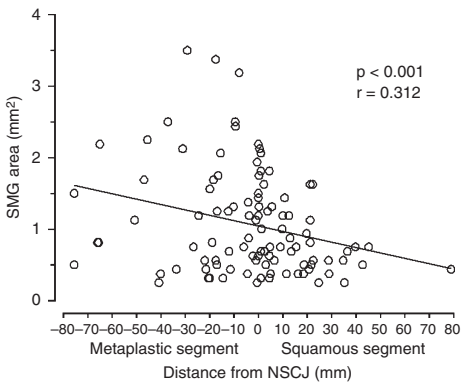

Figure 3. Linear regression analysis showing the relationship between the area of the submucosal glands (SMG) and the distance to the neosquamocolumnar junction (NCSJ) in the oesophageal specimens with oesphogeal columnar metaplasia. The line denotes the regression line.

the NSCJ (0.080 SMG/mm) was significantly higher than that of the squamous (0.013) and metaplastic segments (0.031) ($P = 0.022$).

The proportion of the area of the SMG that was occupied by IGD was measured in an attempt to assess objectively the degree of atrophy of the SMG in the seven specimens with CLO. The relation between these areas was similar in the metaplastic segments and the normal squamous segments (0.20 and 0.25, respectively, $P = 0.258$).

In the seven cases with CLO, 15 foci of ME were identified in metaplastic mucosa and eight foci were found in the squamous segment. The frequency of ME was more than two times higher in the metaplastic compared with the normal squamous segments (1/158 mm and 1/341 mm, respectively, $P = 0.028$). The distance between foci of ME and the NSCJ ranged from 129 mm oral to 53 mm aboral to the NSCJ and there was a small but not significant accumulation of ME close to the NSCJ. All but one of the foci of ME (96%) was observed in SMG excretory ducts, and one of these was in direct conjunction with a small squamous island surrounded by metaplastic columnar mucosa (Figure 1C). The single focus of ME without a connection to the excretory ducts of SMG was seen forming a bridge between squamous and columnar epithelium (Figure 1D). There was a significant correlation between the number of SMG in a resection specimen and the observation of a focus of ME ($r^2 = 0.884$, $P = 0.002$).

Discussion

Detailed information on the topographic distribution of submucosal glands in healthy individuals and patients with columnar-lined oesophagus is sparse, and available reports are conflicting. Based on autopsy studies, it is commonly believed that there is a tendency for the glands to be arranged in longitudinal rows and that there are considerable individual variations both with regard to the number and to the spatial distribution of the oesophageal glands.[9] Niewenhove *et al.* reported that there might be a small but not statistically significant difference in the distribution of the submucosal glands between the upper, middle and lower third of the oesophagus.[10] Using a double-contrast barium oesophagogram in patients with intramural pseudodiverticulosis, Mahajan *et al.* demonstrated that the SMG are more numerous and distributed more uniformly along the entire oesophagus than suggested by previous autopsy studies.[11] In the current study, the number of SMG was highest in the specimen without CLO and there was a marked variation in the number of SMG in the seven cases with CLO. Interestingly, the SMG of the control patient without metaplasia were distributed evenly along the entire length of the specimen, with no accumulation to any specific part of the oesophagus. Although this observation was limited to one specimen, we also found that the distribution of SMG was

relatively uniform in the seven cases with CLO. This conclusion is supported by the observation that the frequency of SMG was similar in the metaplastic and normal squamous segments (0.031 and 0.013 SMG/mm, respectively, $P = 0.208$). Our observations confirm that there is a considerable individual variation in the number of SMG, but suggest that they are distributed relatively homogeneously in the human oesophagus.

The linear regression analysis showed that there was an accumulation of SMG in the area close to the NSCJ in the patients with CLO. It could be argued that the apparent concentration of SMG around the NSCJ observed in the linear regression analysis may be an artefact due to over-representation of short longitudinal sections. However, we do not believe that this finding is a result of a disproportionately high proportion of such short segments, as long longitudinal sections strongly dominated the material. Further, the accumulation of SMG was confirmed objectively by the significant difference in the frequency of SMG beneath the NSCJ compared with that of SS and MS. The marked connection between the location of the NSCJ and SMG provides strong evidence for the hypothesis that metaplasia originates from the SMG unit. As the SMG appear to have a relative uniform distribution throughout the normal oesophagus, we do not believe that there was a true accumulation of SMG at a certain level of the oesophagus. The accumulation of SMG was rather confined to the NSCJ itself which, due to its irregular shape, is located at different levels of the oesophagus in different parts of its circumference. Metaplasia is likely to be an adaptive form of defence, because it provides greater protection against acid injury than does normal squamous epithelium. We hypothesize that when reflux-induced stimulation of the SMG exceeds a certain threshold, stem cells in the ducts of the SMG generate metaplasia as a defence mechanism against injury. With increasing degrees of acid reflux, metaplasia extends from the ducts of the SMG and expands into the oesophagus, replacing the normal squamous epithelium. It is well known that the length of the metaplastic segment is determined by the degree and extent of injurious acid reflux.[12] The accumulation of SMG close to the NSCJ may be explained by the balance between acid reflux and oesophageal defence mechanisms at this level. The lack of stimuli from acid reflux prevents the metaplastic mucosa from expanding and, as a result, the NSCJ remains close to the SMG.

The proportion of IGD within the SMG was measured to assess objectively possible acid reflux-related atrophy of the SMG. However, as the proportion of IGD in SMG was similar in the metaplastic and the normal squamous segments, we found no evidence of atrophy of the SMG in response to reflux-induced injury. In sharp contrast, the SMG of the metaplastic segment were significantly larger than those in the squamous segments of patients with CLO and also larger than the SMG of the control specimen. The size of the SMG of the normal human oesophagus is unknown, but based on the observation from the control specimen, the area of SMG appears to be similar throughout the entire oesophagus. The larger size of the SMG in the metaplastic segment is due most probably to hyperplasia, as there was no sign of hypertrophy of the acini or the acinar cells, although the size of the latter cells was not measured specifically. The finding supports our hypothesis, and suggests that excessive acid reflux may lead to morphological changes of the SMG. The conclusion that the larger SMG in the metaplastic segment may be a result of reflux-induced hyperplasia is supported by observations in animal experiments, where gastro-oesophageal reflux triggered a proliferative response that was 10 times larger in the SMG and their excretory ducts than in the oesophageal squamous epithelium.[13] This shows clearly that the SMG and their excretory ducts are susceptible to stimulation from injurious acid reflux and thus capable of responding with proliferation.

To our knowledge, this is the first study to assess the presence of ME and its association with SMG in oesophageal resection specimens, as previous studies have been based on endoscopic biopsies of the SCJ only.[3,8,14] The concept that ME and possibly metaplasia originates from the ducts of the SMG is supported strongly by our finding of ME almost exclusively in connection with the SMG excretory gland duct epithelia. Interestingly, ME was found relatively distant from the NSCJ and also in the normal squamous epithelium. This implies that ME may be a normal physiological phenomenon, but the observation that the frequency of ME was significantly higher in the metaplastic segment suggests that it may also develop in response to a reflux-induced injury. Our finding of ME from an excretory duct in direct connection with a squamous island and ME forming a bridge between columnar and squamous epithelia suggests that a multi-potent progenitor cell in the gland duct has the capability to give rise to both metaplastic columnar and neosquamous epithelium.

Although the small number of oesophageal resection specimens is a limitation of this study, we believe that the extensive sampling and detailed histological examination of the specimens provide sufficient

material for making valid observations. In the future, detailed studies of pre-operatively untreated oesophageal specimens will be difficult to perform, as patients with high-grade dysplasia are now treated increasingly with endoscopic mucosal resection and/or radiofrequency ablation and manifest cancer with neoadjuvant chemo- and/or radiotherapy.

We conclude that the SMG in the metaplastic segments were larger than those in the normal squamous epithelium, suggesting that severe reflux induces hyperplasia of the SMG. The accumulation of SMG in the area close to the NSCJ, in combination with the observation of ME almost exclusively in connection with the excretory ducts of SMG, suggests strongly that ME and metaplasia may originate and expand from the SMG unit. Hyperplasia of the SMG and the development of ME and metaplasia are all morphological evidence of a physiological adaptation to protect the oesophageal mucosa from reflux, possibly mediated through reflux-induced stimulation of stem cells in the excretory ducts of the SMG.

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Conflicts of interest

Ester Lörinc and Stefan Öberg declare no conflicts of interest.

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Study IV

Immunohistochemical Characterization of the Submucosal Glands of the Columnar lined Oesophagus

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Manuscript title: Immunohistochemical Characterization of the Submucosal Glands of the Columnar lined Oesophagus

Running title: Immunohistochemistry in columnar lined oesophagus

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Abstract

Aims: To characterize the immunophenotypic relationship between the squamous and the glandular compartments in the oeophagus of patients with columnar lined oesophagus.

Methods and results: Eight tissue blocks from three oesophageal resection specimens from patients who underwent oesophagectomy due to adenocarcinoma of the oesophagus were selected for immunohistochemical analysis. The markers of intestinal differentiation (CK20, CDX2 and MUC2) were all expressed in the anticipated pattern, solely in the glandular compartment of the resection specimens. CK4, CK17 and Lysozyme were expressed in both the glandular and the squamous compartments. In addition, CK 17 expression was found on both the squamous and glandular margins of the squamocolumnar transformation zones and in the SMG intraglandular and excretory ducts.

Conclusions: There is an immunophenotypic connection between the squamous and the glandular compartments in columnar lined oesophagus with expression of lysozyme, CK4 and CK17 in both squamous and columnar cells. The observation of CK4 and CK17 in the ducts of the SMG as well as in the metaplastic columnar mucosa and squamous epithelium suggest that metaplasia and regeneration of squamous epithelium originates from progenitor cells within the ductular structures of the SMG unit.

Keywords: Columnar-lined oesophagus, Barrett´s oesophagus, submucosal gland, CK17, CK4, metaplasia, progenitor.

Introduction

The human submucosal oesophageal glands (SMG) produce mucus containing bicarbonate that buffer residual acid after episodes of reflux of gastric contents. [1, 2] The buffering effect of the SMG works in concert with the lower oesophageal sphincter, the diaphragmatic pinchcock and oesophageal peristalsis to protect the oesophageal epithelium from the injurious effects of gastro-oesophageal reflux. Failure of these mechanisms may lead to erosive esophagitis and sometimes to development of columnar cell metaplasia, where the normal squamous epithelium is replaced by metaplastic gland-forming columnar epithelium. [3, 4] Metaplasia is the main risk factor for oesophageal adenocarcinoma. [5] The cell of origin for metaplasia is unknown but it is generally believed that the metaplastic columnar cells arise from within the oesophagus. According to current hypothesis, possible stem cell niches for metaplasia may be located in the basal layer of either the squamous epithelium or the excretory ducts of the oesophageal submucosal glands. [6]

In our previous studies of the relationship between the SMG and the type of overlying mucosa in patients with columnar lined oesophagus (CLO), the finding of an accumulation of SMG and excretory ducts under the squamocolumnar transition zones and the squamous islands suggests that both neosquamous epithelium and columnar metaplasia derive from SMG-related structures. [7] The aim of the present study was to further test the hypothesis that metaplasia originates from the SMG unit by studying the immunohistochemical expression pattern of the SMG and their excretory ducts and to compare it with that of other morphologic structures in oesophageal resection specimens from patients with CLO and high-grade dysplasia and/or adenocarcinoma.

Methods

Prospectively collected oesophageal resection specimens from three patients were examined in toto. The patients were all diagnosed with CLO on preoperative endoscopic examination and underwent oesophageal resection for adenocarcinoma of the distal oesophagus at Lund University Hospital 2007. None of the patients had been subjected to neoadjuvant radio- and/or chemotherapy, endoscopic mucosal resection or other ablative therapy. All resected gastro-oesophageal specimens were formalin-fixed and dissected in a standardized manner, as previously reported. [7]

In order to study the immunophenotypic relationship between different tissue components within the CLO, we characterized and compared the immunohistochemical expression pattern in the squamous and the glandular compartments of the oesophagus. The tissue components in the squamous compartment included oesophageal squamous epithelium, SMG intraglandular ductuli (IGD) and the SMG excretory ducts (ED). The tissue components of the glandular compartments included gastric mucosa, metaplastic columnar epithelium with or without goblet cells, SMG acinar cells (AC), and adenocarcinoma. For the immunohistochemical analysis, we selected eight tissue blocks that included SMG

and two or more of the other tissue components chosen for the study. For each block, 4 µm thick serial-sections were cut, mounted and stained in Dako´s AutostainerPlus according to the manufacturer´s recommendations. The antibodies used were chosen to illustrate intestinal columnar differentiation, squamous differentiation and lysozyme expression (Table I). Tissue sections containing colonic mucosa (CK20, CDX2, MUC2), squamous oesophageal epithelium (CK14, CK17, CK4), and duodenal Brunner´s glands containing specimens (lysozyme) were used as positive controls. Two pathologists with a special interest in gastrointestinal pathology independently evaluated the immunohistochemical stainings. The stainings were classified as positive when a specific, brown intracytoplasmatic or nuclear stain was identified in more than 1 % of the cells belonging to a particular morphologic structure, and negative if there was a general absence of staining in those cells. There were only minor discrepancies in evaluation between the pathologists, mainly concerning focal positivity, and consensus was easily reached by discussion during joint microscopic reevaluation.
The Ethics Committee of Lund University approved the study (LU 400/2007).

Results

Table 2 shows the proportion of observed immunoexpression in each of the morphologic structures included in this study. CK20 was expressed in the foveolar cells of the gastric mucosa and the superficial layer of the metaplastic columnar mucosa, but there was no staining of acinar cells, intraglandular ducts, excretory ducts or squamous epithelium. CDX2, and MUC2, were both expressed in cells of the metaplastic columnar mucosa, however most MUC2 staining was found in goblet cells. There was no expression of CDX2 or MUC2 in squamous epithelium, acinar cells, intraglandular ducts or excretory ducts (Figure 1 A-C).

CK14 was consistently expressed in the basal cells in squamous epithelium, in intraglandular and excretory ducts as well as in myoepithelium. CK4 was expressed in squamous epithelium above the basal cell layer, in the apical cells of the excretory ducts and with an even but weak intensity in the intraglandular ductuli. There was no CK4 staining of myoepithelial cells or gastric mucosal cells. Interestingly, there was focal expression of the squamous marker CK4 in the deep parts of the metaplastic columnar mucosa (Figure 2 A-D). In one of eight observations there was also a weak staining of a few SMG acinar cells.

CK17 was focally expressed in excretory ducts, in intraglandular ductuli and in myoepithelium. In the transition zone between squamous and metaplastic columnar epithelium, there was a consistent weak but specific cytoplasmatic CK17 staining observed predominantly in suprabasal squamous epithelial cells, but also in adjacent metaplastic columnar cells. There was a similar expression of CK17 in suprabasal squamous cells in the vicinity of the orifices of the excretory ducts (Figure 3 A, C-D).

There was a strong cytoplasmatic expression of lysozyme in all acinar SMG cells and in the lower half of the metaplastic columnar mucosa, predominantly in the

deepest located mucinous glands and in all goblet cells (Figure 1 D-F). Lysozyme staining was also found in the apical cells of excretory ducts and in the foveolar cells of gastric mucosa.

Tumor glands were observed in five of the ten tissue blocks. The tumor cells stained focally positive for all antibodies used in this study, although the intestinal immunophenotype and CK17 expression dominated the pattern (Figure 3B).

Discussion

This is the first study that compares the immunohistochemical expression patterns of the oesophageal submucosal glands with that of other tissue components in oesophageal resection specimens from patients with CLO. We found that three of the antibodies in our panel (CK4, CK17 and lysozyme) were expressed in both squamous and columnar cells, suggesting an immunophenotypic relationship between these tissue compartments.

The CK20, CDX2 and MUC2 antibodies, markers of intestinal columnar cells, of an intestine-specific transcription factor expressed in the nuclei of epithelial cells and of a cytoplasmatic glycoprotein, mainly expressed in intestinal goblet cells and enterocytes, respectively, were chosen to illustrate the intestinal phenotype. [8, 9, 10] They were all expressed in the anticipated pattern in the glandular compartment but there was no expression of these markers in the squamous epithelium or the SMG intraglandular or excretory ducts. Although we found no intestinal differentiation of the ducts of the SMG, other investigators have found evidence for non-intestinal columnar differentiation in the intraglandular and excretory ducts using a broader immuno-panel. [11, 12] The observation of non-intestinalized columnar differentiation of the ducts of the SMG is important because this means that they have the same columnar phenotype as non-intestinalized metaplastic columnar mucosa in the oesophagus. Thus, the excretory ducts of the SMG may be the source of oesophageal columnar metaplasia and it can be hypothesized that when reflux induced stimulation of the SMG exceeds a certain threshold, stem cells in the ducts of the SMG generate metaplasia as a defense mechanism against injury. With increasing degree of acid reflux, metaplasia extends from the ducts of the SMG and expands into the oesophagus, replacing the normal squamous epithelium. The absence of intestinal differentiation of the SMG excretory ducts in our study corresponds well with the commonly accepted assumption that the development of Barrett's oesophagus starts with the expansion of cardiac type mucosa and that intestinalization occurs with time as a second step in its pathogenesis. [13, 14, 15]

A previous report of immunohistochemical expression patterns in the oesophagus of pigs have found CK20 staining of SMG acinar cells. [16] This observation led the authors to suggest that, since CK20 is expressed in the metaplastic columnar glands, metaplasia might originate from the SMG. In sharp contrast to this report, all SMG in our study were completely negative for staining of

CK20. Our observations indicate that there may be substantial differences in cytokeratin expressions between the SMGs of humans and pigs. This also implies that there are differences in differentiation and function of SMG between the species and highlights the need for precaution when analyzing results from animal models of human disease.

The CK14, CK4 and CK17 antibodies, markers of basal keratinocytes, of suprabasal cells in nonkeratinizing stratified squamous epithelium and of basal cells of complex epithelium, were chosen to illustrate the squamous phenotype and to identify early squamous differentiation. [17, 18, 19] In our study, CK14 and CK4 were expressed in the anticipated pattern in the squamous epithelium, the excretory and the intraglandular ducts. It has previously been suggested that there are CK4 positive serous cells, often also termed demi-lunes or subsidiary cells, within the acini of the SMG. [2, 16] However, we did not find any serous cells and no CK4 expression of the acini, except for in one SMG where a few acinar cells with mucous morphology showed weak cytoplasmatic CK4 staining. We interpret this rare observation as an accidental focal finding and an artifact due to sectioning of the transition between acinar cells and the CK4 positive intraglandular ductular tructures. Based on the absence of morphological evidence of serous cells and the lack of CK4 staining of the acini, we as well as others, question the existence of serous cells in human SMG. [20, 21] Multilayered epitheilium (ME) is a hybrid epithelium with features of both squamous and columnar epithelia and is characterized by squamoid cells in its basal layers and columnar mucous cells in its superficial layers. [12, 22, 23, 24] ME is seen predominantly in or adjacent to the esophageal gland ducts and is more frequently found in individuals with erosive esophagitis and CLO than in controls. [25, 26] The association between CLO and ME has led to the hypothesis that the development of metaplasia occurs with ME as an intermediate morphological step. [12, 22, 23, 24, 26] Although not specifically assessed in this study, one focus of multilayered epithelium was observed in an excretory duct with CK4 expression focally both in the apical columnar layer as well as in the basal squamous layer. CK4 expression has previously been described in multilayered epithelium in human oesophageal tissue but not in metaplastic columnar mucosa, which makes our finding of small clusters of CK4 expressing cells in glands in the basal part of the metaplastic columnar epithelium intriguing. [22, 27] A possible reason for the absence of CK4 expression in previous studies may be that they have missed the CK4 expressing cells in the deeper parts of the metaplastic mucosa as they have relied on biopsy material only. It is possible that the observed clusters of CK4 expressing cells are remains of excretory ducts with multilayered epithelium in a conceivable transformation to differentiated columnar metaplasia . [24]

CK17 is normally expressed in the epidermal appendages, transitional epithelium, myoepithelium and the basal cells of complex epithelium. [28] Expression of CK17 has been described in a variety of premalignant states and carcinomas, but this is the first report of CK17 expression in CLO related adenocarcinoma. [29, 30, 31, 32] The significance of CK17 in malignant transformation of metaplastic columnar mucosa is unknown and needs to be addressed in future studies.

Our observation of CK17 expressing cells localized to both sides of the squamocolumnar transition zones is a novel finding and especially interesting as such cells have been proposed to be bipotential epidermal stem cells and progenitor cells of both squamous and columnar epithelium at the squamocolumnar junction of the uterine cervix. [33, 34] If CK17 expressing cells in the squamocolumnar transition zones and the SMG excretory ducts of the oesophagus have the same inherent properties they may also be markers of stem cells and therefore important in the formation of metaplastic columnar mucosa as well as neosquamous epithelium. Further, CK17 expression has been reported to be induced in the suprabasal cells close to epidermal wound edges. [35] As metaplastic columnar mucosa is believed to develop as a consequence of healing of erosive esophagitis, it is possible that our finding of CK 17 expressing cells in the transition zones represent a similar healing process and that these cells are activated in the development of metaplasia.

As CK17 expression was consistently observed on both sides of the squamocolumnar transition zones, we believe that the corresponding expression of CK17 at the orifices of the excretory ducts means that also these represent transition zones. Alternatively, the CK17 expression at the squamocolumnar transition zones may actually be remains of the orifices of excretory ducts. The latter assumption is supported by our previous finding of an accumulation of SMG excretory ducts in the area of the transition zones. [7] These observations and the fact that CK4 and CK17 were consistently expressed in the SMG intraglandular and excretory ducts as well as in both metaplastic columnar mucosa and squamous epithelium shows that there is an immunophenotypical relationship between these tissues and that metaplasia originates from stem cells located in the ducts of the oesophageal submucosal glands.

Lysozyme is an enzyme with antibacterial properties characterized by the ability to break down bacterial cell walls. [36] Lysozyme was the only marker that was expressed in the metaplastic columnar mucosa, in the SMG acinar cells as well as focally in the apical parts of the excretory ducts. In a recent report of lysozyme expression in metaplastic columnar mucosa it has been suggested that lysozyme helps to protect the metaplastic segment from a pathogenic biota present in the luminal microenvironment. [37] In fact, there is evidence for an altered composition of the bacterial flora in patients with reflux disease. Patients with erosive esophagitis and CLO have been shown to have a decrease in total bacterial counts, and an increase in the presence of *Campylobacter* in the oesophagus compared with patients with a normal oesophagus. [38, 39] It is possible that an altered microbiotic environment, in combination with reflux, is a triggering signal for putative stem cells in the SMG excretory ducts to switch from squamous to a columnar differentiation, and that this initiates metaplastic columnar gland formation in the oesophageal mucosa.

We conclude that there is an immunophenotypic relationship between the squamous and the glandular compartments of the CLO with expression of lysozyme, CK4 and CK17 in both squamous and columnar cells. The consistent expression of CK4 and CK17 in the cells lining the SMG intraglandular and excretory ducts as well as in both metaplastic columnar mucosa and in squamous epithelium suggest that

metaplasia and regeneration of squamous epithelium originate from progenitor cells within the ductular structures of the SMG unit.

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Title Table 1 List of antibodies, manufacturers and dilutions

Antibody	Manufacturer	Dilution
CK4	Abcam ab9004	1:1000
CK14	Novocastra LL002	1:25
CK17	DAKO M7046	1:50
CK20	DAKO M7019	1:25
MUC2	Abcam ab76774	1:500
CDX2	Abcam ab15258	1:50
Lysozyme	Novus Biologicals EPR 2994	1:5000

Title Table 2 Immunohistochemical characteristics of the tissue compartments in the columnar lined oesophagus

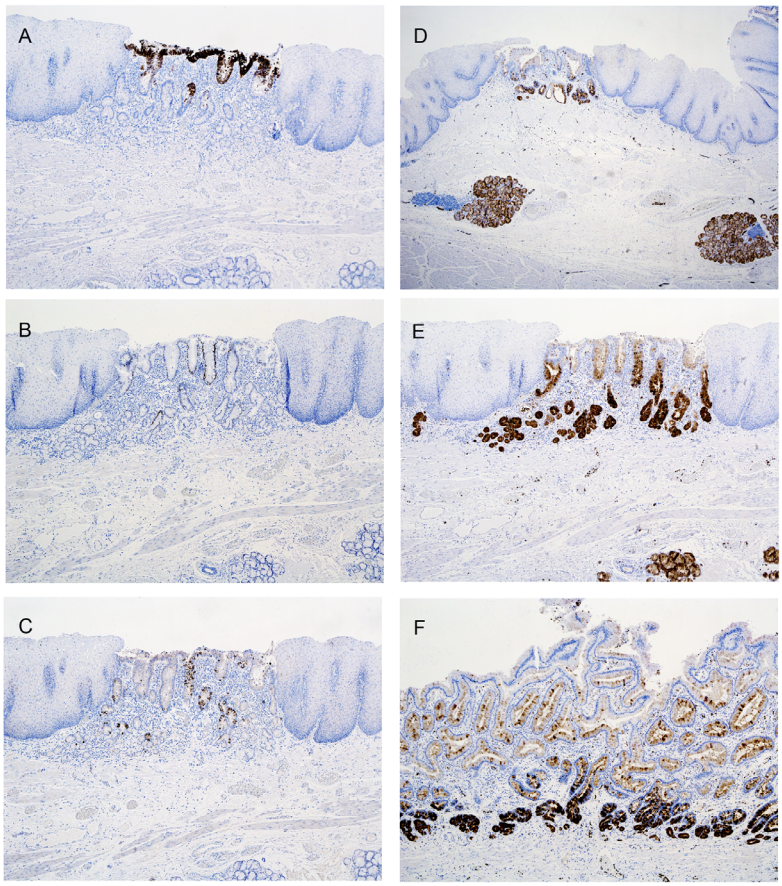
Tissue Compartment	Lysozyme+	CK20+	CDX2+	MUC2+	CK14+	CK4+	CK17+
Gastric mucosa	2/2 (100%)	2/2 (100%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
Columnar metaplasia	6/6 (100%)	4/6 (66%)	4/6 (66%)	5/6 (83%)	0/6 (0%)	3/6 (50%)	2/6 (33%)
SMG acini	8/8 (100%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	1/8 (12%)	0/8 (0%)
Tumour	2/5 (40%)	3/5 (60%)	2/5 (40%)	2/5 (40%)	1/5 (20%)	1/5 (20%)	3/5 (60%)
SMG myoepithelium	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	8/8 (100%)	0/8 (0%)	8/8 (100%)
Intra-glandular ducts	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	8/8 (100%)	7/8 (87%)	8/8 (100%)
Excretory ducts	1/5 (20%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	6/6 (100%)	6/6 (100%)	4/6 (66%)
Squamous epithelium	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	5/5 (100%)	5/5 (100%)	4/5 (80%)

Explanatory note Table 2

Number and proportion of observed immuno-expression per assessed tissue compartment

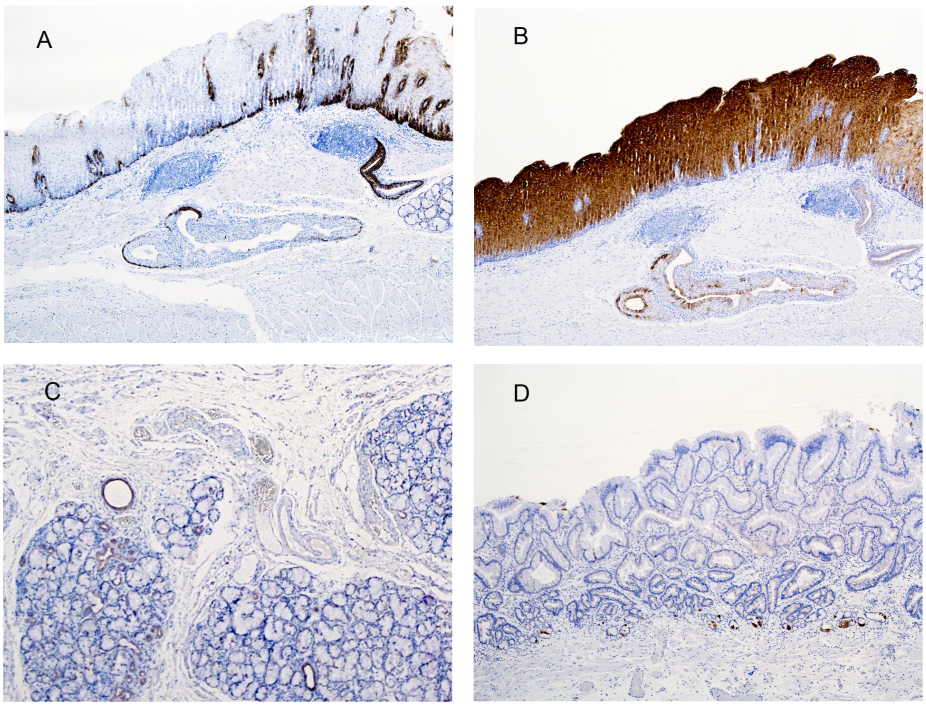
Legend for Figure 1

Figure 1: The immunohistochemical staining pattern for intestinal markers (A-C) and lysozyme (D-F) in columnar lined oesophagus. **A**: CK20 expression in the superficial parts of metaplastic columnar mucosa. **B**: CDX2 nuclear expression in a few columnar cells. **C**: MUC2 expression mainly in goblet-cells in the metaplastic columnar mucosa. **D**: strong expression of lysozyme in all acinar cells of the submucosal glands and in the columnar cells in the deeper half of the metaplastic columnar mucosa. **E** and **F**: lysozyme expression is seen predominantly in the deepest located mucinous glands and in goblet-cells.



Legend for Figure 2

Figure 2: The immunohistochemical staining pattern for CK14 (A) and CK4 (B-D) in different tissue compartments of the columnar lined oesophagus. **A**: CK14 expression in the basal cells of squamous epithelium and in basal cells of the submucosal glands excretory ducts (ED), including multilayered epithelium (ME). **B**: CK4 expression in supra-basal cells of squamous epithelium. In ME CK4 expression is seen in both the superficial and basal cell layers (focally in the latter). **C**: CK4 expression in the cells of the extraglandular and intraglandular ducts of the submucosal glands. **D**: CK4 expression in the deepest located glands in the metaplastic columnar mucosa.



Legend for Figure 3

Figure 3: The immunohistochemical staining pattern for CK17 in columnar lined oesophagus and related adenocarcinoma. **A**: CK17 expression in the intraglandular ductuli and the dilated excretory ducts of the submucosal glands. CK17 expression is also seen in a few columnar cells in the inflamed, metaplastic columnar mucosa. **B**: CK 17 expression in tumor glands. **C**: CK17 expression in suprabasal cells in the transition zones between squamous epithelium and metaplastic columnar mucosa. Corresponding CK 17 expression is also seen around the basal attachment zone of a presumed submucosal gland excretory duct. **D**: CK 17 expression in suprabasal squamous cells and in a few columnar cells in the transition zones.