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RESEARCH

A Relation Between Cell Cycle and Intestinal Metaplasia in Oesophageal Biopsies Using Optical and Digital Microscopy

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Abstract Protein expression changes in relation to cell cycles provide important information, and it may represent a new method for an early diagnosis of metaplasia – dysplasia – adenocarcinoma sequence. We investigated potential changes in cell cycle genes such as protooncogenes (PCNA, EGFR), tumour suppressor gene (p53), apoptotic TUNNEL (Tdt mediated dUTP nick and labelling) gene, as well as small intestinal mucus antigen (SIMA) and large intestinal mucus antigen (LIMA), which accumulates in metaplastic epithelium due to the inflammatory process in routine oesophageal biopsies using immunohistochemistry. Oesophageal biopsies were taken from patients with Barrett's oesophagus (n=30), reflux oesophagitis (n=30), healthy oesophagus (n=30) and healthy cardia (n=10). Immunohistochemical signalling was carried out by Streptavidin-Biotin-AEC (aminoetil-carbazol). Expression of PCNA was statistically significantly lower in healthy oesophagus ($p<0.05$) versus reflux oesophagitis and Barrett's oesophagus. However, no significant change was detected in the expression of SIMA and LIMA in intestinal metaplasia. Further, EGFR, p53 and TUNNEL levels were significantly different in healthy versus Barrett's oesophagus. Manual counting using virtual microscopy was comparable with the result using conventional light microscopy, but the former is significantly quicker. There was no difference between manual and automated cell counting ($p>0.05$).

Keywords Cell cycle · Intestinal metaplasia · Optical and digital microscopy

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Introduction

Barrett's oesophagus is a premalignant condition, which develops as a complication of chronic gastrooesophageal reflux disease (GORD) and increases the risk for oesophageal adenocarcinoma. 1–3 % of the population is diagnosed with GORD in the Western countries [1]. Epidemiological studies demonstrated that the risk for developing oesophageal adenocarcinoma is increased by 30–125 times if Barrett's oesophagus is present [2]. Follow-up of patients with Barrett's oesophagus showed that adenocarcinoma developes in the metaplastic epithelium in 0.5–1 % in a year, 5–10 % in 10 years and 18–36 % over 20 years [3]. However, it is still unclear what determines changes towards carcinogenesis and what are the main characteristics of those.

Barrett's oesophagus is a useful model to investigate in vivo human carcinogenesis, since multi-step metaplasia – dysplasia – adenocarcinoma process can be well observed. It has been shown previously that development of dysplasia and adenocarcinoma is relatively rare after metaplasia. This may indicate that other factors (genetic, environmental, nutritional) may play important roles in the initiation of malignant transformation [4]. Despite a technical revolution in endoscopic techniques and strict follow-up protocols with oesophageal biopsies there are cases always which are detected relatively late. Further development in molecular genetic research will probably reveal new details in tumour biology as well as provide a chance for an earlier diagnosis.

Recently, numerous studies were published on cell proliferation markers including PCNA (proliferating cell nuclear antigén) and Ki67 [5]. PCNA is excellent indicator of cell proliferation in G1/S phases. Increased expressions of both markers were detected by immunohistochemistry and flow cytometry when metaplastic cells are transformed to dysplastic [6]. Cell proliferation can be demonstrated by Ki67 levels

using immunohistochemistry [7, 8]. Furthermore, Ki67 is an excellent biomarker of apoptosis, as well [9]. During apoptosis the activation of endonucleases lead a fragmentation of DNA, which can be demonstrated by TUNNEL (Terminal deoxynucleotid transferase- mediated dUTP Nick and Labelling) assay [10].

Protooncogenes enhance cellular multiplication and encode proteins of growth factor signalling. EGFR (Epidemialis Growth Factor Receptor)—or c-erbB1-gene—can be found on 7p12-13 locus. Increased expression of this protooncogene was detected in late stages of oesophageal cancer, when the disease process is usually associated with nodal metastases [11].

Tumour suppressor genes encode proteins responsible for blocking cell division. One of the most important tumour suppressor genes is TP53 (17p13), which encodes p53 protein. In case this gene is disabled, cell cycle arrests in G1 phase until the fault is repaired by DNA repair enzymes. If the fault cannot be corrected, p53 will drive the cell towards to apoptosis [12]. Since p53 regulates numerous genes and proteins intracellularly, any fault in the gene may contribute to tumour development. p53 mutations were relatively frequently observed during metaplasia-dysplasia-adenocarcinoma sequence. The half-life of the improperly functioning p53 gene increases and the gene accumulates in such an extent that it is detectable intracellularly using immunohistochemistry [13]. Lack of p53 expression correlates with poor prognosis of patients with Barrett's adenocarcinoma [14].

Picture 1.: Virtual microscopy

Fig. 1 Virtual microscopy

Picture 2: Light microscyopy

Fig. 2 Light microscyopy

Detection of calyceal cells in the distal esophagus is a definite diagnosis for Barrett's oesophagus [15]. Pluripotent stem cells, which form Barrett epithelium finally, originate from the papillae of the inflammed epithelial layer or colonize the oesophageal epithelium from the crypts [8, 16]. Numerous studies have been published on the origin of stem cells and some argue that the oesophageal metaplastic cells originate from the bone marrow actually [17]. The mucin of the columnar and calyceal cells can be demonstrated by immunohistochemistry and in situ hybridization [18]. Further, a gradual increase in mucin antigen 1 (MUC1) expression was demonstrated during metaplasia-dysplasia-adenocarcinoma sequence [19]. MUC1 peptide can adhere to ICAM1 (intercellular adhaesion molecule-1) and, therefore, it may play an important role in the development of metastases [20]. Small intestinal mucus antigen (SIMA) and large intestinal mucus antigen (LIMA) accumulate in metaplastic epithelium due to the inflammatory process in routine oesophageal biopsies.

Table 1 Comparison of light and virtual microscopy cell counts

Examined parameters	Light / Virtual microscopy (Spearmann R^2)
PCNA	0.98
p53	0.94
SIMA	0.87
LIMA	0.92
EGFR	0.82
TUNEL	0.97

Table 2 PCNA, EGFR, TUNEL, SIMA. LIMA and p53 expressions in normal oesophageal and cardiac biopsies, compared to GORD and Barrett's oesophagus

Labelling index±SD	PCNA	SIMA	LIMA	P53	Tunel	EGFR
Healthy	0.62+0.15	0.65+0.14	0.64+0.14	0.65+0.14	0.56+0.16	0.51+0.16
GERD	0.83+0.06	0.67+0.14	0.67+0.12	0.71+0.13	0.46+0.18	0.52+0.18
Barrett	0.72+0.12	0.66+0.17	0.66+0.11	0.72+0.11	0.40+0.12	0.43+0.12
Significance	<0.05			<0.05	<0.05	<0.05

In our study, therefore, we investigated the epithelial expression of PCNA, EGFR, TUNEL, SIMA, LIMA and p53 expressions in oesophageal (and cardiac) biopsies to understand better the pathophysiological changes in metaplastic transformation in Barrett's oesophagus.

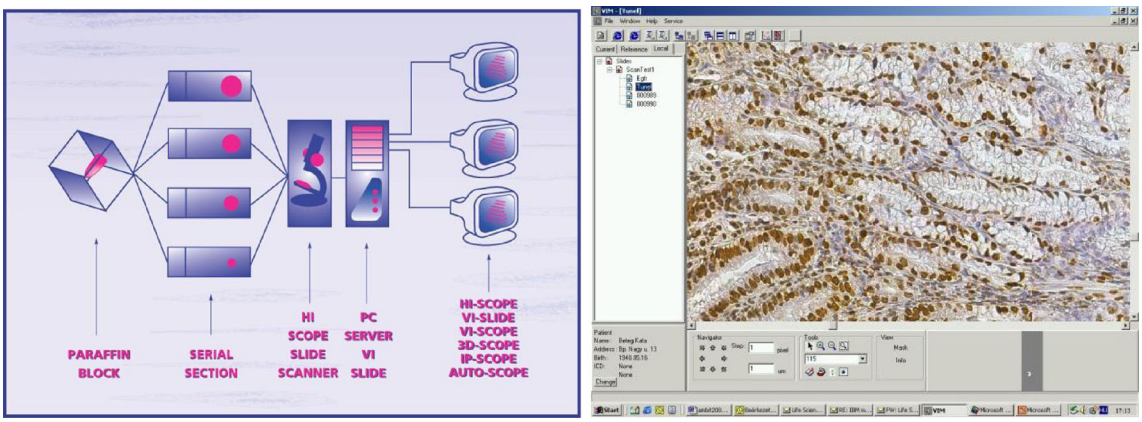
Material and Methods

Oesophageal biopsy samples were taken during oesophago-gastro-duodenoscopy. Patients were selected based on the pathological examination of the biopsies as opposed to endoscopic features. In group 1, 30 patients were enrolled, whose previous endoscopical biopsies revealed Barrett's oesophagus characterized by intestinal metaplasia but not dysplasia. In group 2 (n=30), patients with previous endoscopical biopsies showing oesophagitis only without metaplasia were used. In group 3 (n=30), patients with macroscopically and histologically normal oesophagus were enrolled. Group 4 (n=10) contained patients with biopsies from healthy gastric cardia, as a negative control. PCNA, EGFR, TUNNEL, SIMA. LIMA and p53 expressions were investigated by immunohistochemistry using Streptavidine–Biotin–Aminoethil–Carbazol method. Altogether 600 slides were examined by light microscopy first and then virtual microscopy after digitalization. AEC positive cell nuclei were counted in at least 1000 cell in every slide. Conventional light microscopic cell counting is relatively time consuming and less exact as opposed to digital counting developed in our laboratory. In the automated digitalized slides epithelial layer and intestinal gland structures were separated first followed by identification and counting of immunohistochemistry positive cells. For comparison, we also counted the cell nuclei on the digitalized slides after automated counting. Statistical analysis was performed using a programme and a test was applied to calculate significant differences. A difference was considered statistically significant if p value was less than 0.05 (Figs. 1 and 2).

Results

Counting of 1000 cells on conventional light microscopy takes approximately 90–120 min on one slide. However, the same can be done on digitalized slides in 25–30 min only. There was no statistically significant difference found on comparison between the results of light and virtual microscopy in terms of cell counts (Table 1).

We found a significantly lower expression of PCNA in biopsies taken from healthy oesophagus and cardia compared to reflux oesophagitis (p<0.05). Furthermore, p53 expression was significantly higher in Barrett's oesophagus as opposed to normal oesophageal and cardiac epithelium (p<0.05). Similarly, significantly lower TUNEL level was found in GORD in comparison with normal mucosa (p<0.05). Likewise,



Picture 3.: Virtual microscopy technique

Fig. 3 Virtual microscopy technique

TUNEL was lower in Barrett's oesophagus than in GORD ($p<0.05$). Finally, EGFR expression was also significantly lower when compared to expressions in normal oesophageal or cardiac mucosa ($p<0.05$) (Table 2). In contrast, no difference was found in the expressions of SIMA and LIMA in between normal, GORD and Barrett's epithelial biopsies (Table 2) (Fig. 3).

Discussion

Since the incidence of oesophageal adenocarcinoma has been rising continuously in Europe as well as in the United States, early detection of Barrett's oesophagus, as a premalignant condition, is highly important. While 5-year survival of patients with early stage Barrett's oesophageal cancer is 83–90 %, the same for later stages is 10–15 % only [21]. However, detection of Barrett's metaplasia in patients without preceding GORD symptoms is very challenging. There are no data published on prevalence of Barrett's disease in patients without typical reflux syndromes. Supposedly, patients at potential risk for developing of oesophageal adenocarcinoma are not only the 5–10 % of those with typical reflux. This is supported by the fact that 40 % of patient with Barrett's metaplasia never developed typical symptoms suggesting preceding GORD [22]. Hence, early recognition and follow-up of reflux disease will not provide safety for all patients at risk for developing Barrett's metaplasia and consequent oesophageal cancer. In addition to acid and bilious reflux, genetic testing of patients in the risk group may provide further help in early identification of prognostic factors, which play an important role in the development of metaplasia –dysplasia-adenocarcinoma sequence. Currently, obese (BMI>30) males between 50 and 70 years with reflux symptoms more than 10 years are considered to be at risk [23]. In these patients, the routine follow-up in completion with genetic biomarker screening may lead to an even earlier detection of Barrett's cancer. Since numerous genetic alterations are usually necessary to the development of the disease, it is unlikely that screening of a few biomarkers only can provide adequate tool for earlier diagnosis [24]. It is quite likely, that further development in molecular genetics research will provide more insight in tumour biology as well as lead to application of new biomarkers in the routine practice. Despite numerous clinical studies have been published using various diagnostic panels of molecular biological biomarkers, there is not any simple, quick and validated test available in the routine clinical practice [7]. Our study implicates that significant differences in biomarker levels can be found in early phases such as epithelial inflammation and metaplasia yet, which could be potentially applied in the clinical practice for early diagnosis. Routine application of these can be facilitated by automatic evaluation of digitalized pathological slides, as we demonstrated.

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