A search for molecular biomarkers in gastro-intestinal cancer
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chapter 2

ACTIVE MATRIX-METALLOPROTEASES ARE EXPRESSED EARLY ON AND ARE HIGH DURING THE BARRETT’S ESOPHAGUS MALIGNANCY SEQUENCE

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ABSTRACT

Objective: Molecular processes underlying Barrett’s malignant development are poorly understood. Matrix metalloproteases (MMPs) are enzymes involved in inflammation, tissue remodelling and malignant development. Therefore, active MMPs may have a role in early metaplasia development and Barrett’s esophagus’ malignant progression. We desired to gain more insight in the role of MMPs during the Barrett’s esophagus pathogenesis sequence and their potential as biomarkers.

Materials and Methods: In a surgical Barrett’s mouse model and in non-malignant Barrett’s and malignant esophageal cell lines, the activity of MMPs was investigated using a MMP activatable probe. MMP activity was further validated in Barrett’s esophagus and esophageal adenocarcinoma patient biopsies and differentiated by investigating MMP9 and MMP13 expression.

Results: The mouse model showed probe activation in stromal cells early on in the esophagitis and metaplasia stages. MMP probe activation was higher in the Barrett’s and cancer cell lines and biopsies as compared to normal cells and tissues. Co-immunostainings confirmed that at the tissue level the probe activation was mostly confined to CD45-positive stromal cells. MMP13 expression was highest in Barrett’s metaplasia while MMP9 was highest in the esophageal adenocarcinomas.

Conclusion: During the Barrett’s pathogenic process, MMP activity is increased early on in the inflamed esophagus and remains high in metaplasia and esophageal adenocarcinoma. However, there is a switch of MMP13 to MMP9 expression once neoplasia develops. In the future, detecting specific MMP subtypes could be used to distinguish non-malignant from neoplastic Barrett’s esophagus.
INTRODUCTION

Esophageal adenocarcinoma (EAC), a disease with a rather poor five-year survival rate of around 14%, has shown an increase in incidence in Western countries over the last few decades (1-3). A pre-malignant state that can give rise to EAC is Barrett’s esophagus (BE). BE is the metaplastic transformation of squamous esophageal epithelium into columnar, intestinal-type epithelium as a consequence of chronic gastroesophageal reflux disease (GERD). The intestinal-type of Barrett’s metaplasia (IM) is associated with an increased risk for the development of EAC, which occurs through a sequence of increasing tissue aberrancy from low grade dysplasia (LGD) to high grade dysplasia (HGD) to finally EAC. The number of patients with BE that actually progresses to EAC is rather small however. The incidence rates vary from 0.12 to 0.63% (4-6). This rate increases with increasing grade of dysplasia (5,7-9). Compared to the general population, patients with BE have an around 11-fold higher risk of developing cancer (5). Histopathology and endoscopic surveillance methods are currently the best diagnostic and predictive treatments available with respect to EAC detection in Barrett’s disease. These surveillance strategies are based on the grading of biopsies taken according to general guidelines during light endoscopy in 4 quadrants every two centimetres of the Barrett’s section (10,11). Presence of low grade dysplasia indicates that surveillance frequency should be increased, while high grade dysplasia and early EAC are grounds for endoscopic intervention (5,12). However, these lesions can be missed during endoscopy. Furthermore, a lack of inter-observer agreement has been shown with respect to histo-pathological staging of the endoscopy-acquired biopsies (13,14). As such research is being done into biomarkers as potential agents to improve upon the risk stratification and diagnostic powers of current endoscopic surveillance strategies. BE developments is associated with several risk factors such as GERD, obesity and smoking (15,16). Recently, a number of studies have shown that active reflux or poorly controlled GERD is an independent risk factor for developing EAC. A meta-analysis showed that individuals with GERD with daily symptoms compared to individuals with no or few symptoms had an odds ratio of 7.40 (95% CI = 4.94 - 11.1) for developing EAC (17). In comparison the standardized incidence ratio of EAC for BE patients was given as 11.3 (95% CI, 8.8 to 14.4) in a recent report (5). The chronic acid and bile reflux in GERD leads to a chronic inflammation of the esophagus, i.e. esophagitis (18-20). This chronic inflammation persists upon the development of BE and corresponds with the recruitment of inflammatory cells and the production of a number of chemokines, such as IL-6, IL-8, IL-1β and NF-κB by stromal as well as epithelial cells (21-25). Chronic inflammation is increasingly being viewed as one of the hallmarks of cancer and also in BE it is believed that inflammatory pathways contribute to malignant oncogenic transformation (26-28). Inflammatory factors thus could provide a novel source of biomarkers in BE.

An important group of molecules involved in inflammation are the matrix metalloproteinases (MMPs) (29). In healthy tissues these secreted or membrane bound enzymes play important roles in tissue remodelling through cleavage of the extracellular-matrix causing the release of a number of cytokines and chemokines. Through activation of several of these soluble mediators and cleavage of certain structural molecules such as E-cadherin, MMPs contribute to cell signalling cascades (30). There is also ample evidence for the production and activity of MMPs in cancer. In oncogenesis these enzymes, produced by epithelial and stromal cells, seem to
perform a variety of functions ranging from apoptosis resistance to angiogenesis (29,31-33). With regard to BE and related EAC, Salmela et al. showed that a large number of EAC biopsies show a ubiquitous expression of MMP7 mRNA. Showcasing a potential role for this protease early on in the pathogenesis of BE, also metaplasia samples showed expression of MMP7 mRNA while in contrast the normal esophagus was negative for the enzyme (34). Herszenyi et al. showed a gradual increase in MMP9 protein expression as detected by immunohistochemistry (IHC) along the BE pathogenic cascade from esophagitis to Barrett’s up to EAC (35).

Intimate connections between cancer, inflammation and the MMPs are factors that are all seemingly collected within Barrett’s pathogenesis. Furthermore, the enzymatic activity of the MMPs gives them the properties needed for potential real time imaging by applying activatable fluorescently labelled substrates. Therefore, we decided to evaluate if MMP activity could be useful for risk stratification and disease diagnosis in Barrett’s esophagus. To assess a broad range of MMP activity we opted to use the MMP-activatable fluorescent probe, MMPsense 680, for our experiments. This probe contains a peptide backbone with an attached quenched near infrared (NIR) fluorophore. Cleavage of MMP specific motifs within the backbone releases the quenched fluorophore and this leads to measurable fluorescence upon excitation with NIR light. This probe is cleaved by a number of MMPs such as MMP2,-3,-9 and -13, but is mainly activated by MMP9 and -13. In our study, we used a surgical mouse model to investigate and follow the gradual development of MMP activity during the reflux induced esophagitis and metaplasia sequence of BE. We also evaluated the expression of the MMPsense680 probe in non-malignant and malignant esophageal and Barrett’s cell lines, and in BE and EAC biopsies to assess the potential of MMP activity as a biomarker. This protease activity was further validated by searching for the main MMPsense 680 activating MMP proteins in the biopsies. By double stainings we explored the nature and origin of the probe-activating MMP activity in the tissues.

MATERIALS AND METHODS

BE mouse model. Twelve 4-6 weeks old C86F1 male mice, weighing 20-25 g and obtained from internal breeding at the Academic Medical Center (AMC) Amsterdam, were used for this pilot study. The mouse model was used for this pilot study after formal approval by the AMC animal ethical committee (project number: DIX102663). All surgery and imaging were performed under isoflurane inhalational anaesthesia to minimize suffering. Animals were housed in conventional (non-SPF) housing, with three animals per cage. In 9 of these animals an anastomosis between the esophagus and jejunum was created by implanting two 1.58 x 0.78 mm neodymium micro magnets. The first magnet was placed in the lower half of the esophagus and the second was placed via a separate jejunostomy in a post ligament of Treitz jejunal loop. Within several days the approximated magnets cause pressure necrosis between the jejunum and esophagus which leads to a fistula that allows reflux of bile into the distal esophagus. The magnets are excreted via the stools. In general 9 weeks after operation an average of 70% of the animals will have developed reflux induced inflammatory changes of the esophageal mucosa and up to 50% may already have developed metaplastic changes (Figure 1). For this study, animals were sacrificed in groups of three, 9, 12 and 16 weeks after the operation. Three animals remained un-operated
and were used as controls for each time point. The animals were injected with 150 µl of the MMPsense680 probe 24 hours before sacrifice. For each timepoint one operated animal was injected with 1x PBS buffer as an extra control. Sacrifice was performed with 100% CO2 gas, after induction with a 40/60 mixture of CO2/O2 rendered animals unconscious. After sacrifice tissues of the anastomosis site were snap frozen. These tissues were then sectioned and used for microscopic imaging, whereupon they were re-incubated with the probe.

**Patient biopsies.** The study was approved by the Academic Medical Center’s institutional medical ethical committee. All patients obtained written informed consent forms and gave written permission for taking extra biopsies for research purposes. Extra biopsies were taken from patients during either routine BE endoscopic surveillance or endoscopy for EAC in the period 2006-2011, to attain intestinal metaplasia, dysplasia, esophageal adenocarcinoma and coupled squamous tissue samples. Squamous samples were taken from endoscopically normal appearing mucosa at least 3 cm above the diseased mucosa. The tissues were immediately snap frozen in liquid nitrogen. Tissues were isolated from a total of 36 patients: 28 male and 8 female. Median age was 63 years (interquartile range = 58 - 72 years). All the included patients were on proton pump inhibition of 40 to 80 mg daily and endoscopically none had visible reflux esophagitis. Histological assessment was performed on correlating biopsies taken simultaneously for routine histo-pathological evaluation. Of the 36 samples, 14 biopsies were BE with intestinal metaplasia (IM), 7 were high grade dysplasia (HGD) and 15 were esophageal adenocarcinoma (EAC). For 2 of the HGD samples no squamous biopsies were available as controls. All other samples had coupled squamous samples.

**Near infrared MMP activity and fluorescent immunohistochemistry on tissues.** MMP activity was evaluated by microscopic imaging of the near infrared (NIR) fluorescence from the MMPsense 680 probe, as performed on 4 micrometer thick, snap frozen sections from the patient biopsies and mouse samples. Tissue sections were directly incubated with the MMPsense680 probe (PerkinElmer, Groningen, Netherlands), diluted in a 1mM Zn/CaCl2 solution at a 1:25 ratio, for 1h at 37 °C. After incubation, samples were counter-stained with DAPI (Life technologies, Bleijswijk, Netherlands) and visualized.

In case of stainings of MMP activity combined with fluorescent immunohistochemistry, after MMPsense 680 incubation, tissues were fixed in 4% formaldehyde in phosphate buffered saline (PBS). After fixation slides were incubated with the respective primary antibody of interest overnight at 4°C. Consequently, slides were incubated with fluorescently labeled secondary antibodies at room temperature for 1 hour. Primary immunoglobulins used were the mouse monoclonal anti-human MMP9(GE-213), MMP13(43-F09) and CD45 antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) and the mouse monoclonal E-cadherin (HECD1) (Abcam, Cambridge, United Kingdom) immunoglobulin. Secondary antibody was the FITC-labeled polyclonal anti-mouse immunoglobulin (Dako, Neverlee, Belgium).

**Cell culture and MMPsense680 incubation.** The human hTERT immortalized esophageal cell line, EPC2-hTERT, was a kind gift from Prof. A. Rustgi (University of Pennsylvania, PA, USA) (36). The Barrett cell line CP-A was kindly provided by Dr. R. Fitzgerald (University of
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Both cell lines were cultured in KSF medium (Life technologies, Bleijswijk, Netherlands) supplemented with human recombinant epithelium growth factor (EGF) and Bovine pituitary extract (Life technologies, Bleijswijk, Netherlands). The OE19 esophageal adenocarcinoma cell line was obtained from the ATCC and cultured in RPMI 1640 medium (Life technologies, Bleijswijk, Netherlands) supplemented with L-glutamine, penicillin/streptomycin and 10% fetal bovine serum (FBS) (Lonza, Basel, Switzerland). Cells were seeded at a density of 125*10^3 cells/ml in 24-well plates on coverslips. For real time detection of MMP activity, after culturing overnight, medium was removed and MMPsense680, diluted at a 1:25 ratio in 1mM Zn/CaCl2 solution was added per well to cover the cells. The plate was incubated in the dark for 1h at 37 °C. After incubation, coverslips were taken from the plate, counterstained with DAPI and placed on microscope slides to be visualized.

Image analysis. Fluorescent immuno-histochemistry pictures were taken with the Olympus BX51 microscope mounted with the Olympus XM10 monochrome camera (Olympus, Zoeterwoude, Netherlands). Pseudo-color mapping, brightness and contrast adjustments were done with Olympus Cell^F software. Pictures of Hematoxylin & Eosin stainings were taken with a DP70 Olympus digital camera mounted on a Olympus BX51 microscope (Olympus, Zoeterwoude, Netherlands).

To study the MMPsense680 and MMP co-localization the following protocol was used. For each sample two pictures were taken of separate parts of the tissue sections and used for a qualitative analysis of the probe signal and MMP protein localizations. The activated probe was visualized with a pink color by the Cell^F software, while the fluorescent MMP9 and MMP13 immunofluorescent stainings were given a green color. Co-localization of the two signals were visualized by the software as a red to yellow color gradient depending on the ratio of the overlapping intensities of the two fluorochromes. For the MMPsense 680 and E-cadherin or CD45 expression co-localization analysis, a similar approach was used. Here activated probe was again indicated by a pink color. CD45 and E-cadherin immunofluorescence were given a green color. Co-localization was indicated by a red to yellow color gradient.

Figure 1. H&E and MMPsense 680 staining of the Barrett’s mouse model at different time points. (a) H&E staining of frozen section of the esophago-jejunostomy in the surgical mouse model 9 weeks after operation. (b) H&E staining of the esophagus of a 9 weeks post-operation control mouse indicating mucosa (m), submucosa (sm) and lamina propria (p) of the normal esophagus. (c) Serial section of the same control mouse after incubation with the MMPsense680 probe. (d) H&E staining of the inflamed esophagus with submucosal infiltrates 9 weeks after operation. (e) Serial section of the same area shows probe activation (pink dots, see arrows) near the submucosal infiltrates. (f) H&E staining of the anastomosis site 9 weeks after the operation shows submucosal infiltrates at and near the junction (arrows). (g) Tissue section of an age matched operated mouse shows activation of the probe near infiltrate (arrows). (h) H&E staining of the anastomosis site 16 weeks after the operation shows an area with columnar metaplasia containing inflammatory cells (dashed circle) (i) Probe activation in a serial section of this metaplastic area (dashed circle).
**Immunoblotting.** Biopsies were lysed in MPER lysis buffer (Thermo Fisher Scientific, Etten-Leur, Netherlands) supplemented with protease and phosphatase inhibitors (Halt protease and phosphatase inhibitor cocktail, Thermo Fisher scientific, Etten-Leur, Netherlands). Lysates supplemented with sample buffer (125 mM Tris/HCl, pH 6.8; 4% SDS; 2% β-mercaptoethanol; 20% glycerol; 1 mg bromphenol blue) were loaded onto SDS-protein gels and subsequently transferred onto PVDF membranes (Millipore, Amsterdam, The Netherlands). The blots were incubated overnight at 4ºC with primary antibody, mouse monoclonal MMP9(GE-213) and MMP13(43-F09) or rabbit polyclonal anti-actin (Santa Cruz biotech, Heidelberg, Germany). Blots were consequently incubated for 1 hour at room temperature with the secondary antibody, anti-mouse and anti-rabbit HRP conjugated immunoglobulins (Dako, Heverlee, Belgium). Blots were developed in Lumilite plus (Boehringer-Mannheim, Mannheim, Germany) and chemiluminescence was detected using a Fuji LAS4000 illuminator (Fuji Film Medical Systems, Stamford, USA). Quantification of the blots was performed with the ImageJ software (version 1.44).

**Statistical analysis.** To determine significant differences in probe signal counts and relative protein levels, Mann-Whitney U test, t-test and ANOVA analysis were performed with GraphPad Prism 5 (GraphPad software, California, USA). Statistical significance was set at p < 0.05. The asterisk system for statistical significance used here was as follows: * is p ≤ 0.05. ** is p ≤ 0.01. *** is p ≤ 0.001.

**RESULTS**

**Probe activation in a surgical reflux induced esophagitis-metaplasia mouse model.** To investigate the development and localization of MMP activity during the initial steps of BE disease development, we applied MMPsense680 to a surgical reflux esophagitis-metaplasia mouse model. None of the 9 operated animals were lost as a consequence of the procedure. Three operated animals and 1 un-operated control animal were analysed for each of the three investigated time points. In the current study at 9 weeks, the H&E stained tissue sections showed that there is esophagitis with basal cell and papillary hyperplasia of the squamous epithelium with an influx of stromal cells in the submucosa (Figure 1d,f). Incubation of fresh frozen tissues showed activation of the MMPsense probe coinciding with these submucosal infiltrates, indicating that these cells express active MMPs (Figure 1e,g). At 16 weeks early non-intestinal type of columnar metaplasia is seen at the esophago-jejunal junction. In-between the metaplastic cells there is stromal infiltration (Figure 1h). Upon tissue incubation with the MMPsense680 probe, we found that MMP activity is highest in and near the junctional region. The metaplastic region also coincides with activation of the MMPsense probe, most likely due to MMP activity of the infiltrating cells (Figure 1i). In all control time-matched un-operated animals there was no activation of the MMPsense680 probe in the esophageal tissues (Figure 1c).

**Probe activation in cell lines of the Barrett’s pathogenic sequence.** To investigate the ability of the probe to detect MMP activity in epithelial cells and to differentiate between normal and BE tissues, we analysed three cell lines. Probe activation was investigated in the normal esophageal squamous, EPC2-hTERT, the non-dysplastic Barrett, CP-A, and the esophageal
adenocarcinoma, OE19 cell line. Upon incubation, EPC2-hTERT showed a low frequency (<5%) of cytoplasmic probe activation (Figure 2a). CP-A had a higher frequency (~70%) of cells with cytoplasmic activation (Figure 2b). Notwithstanding the larger cell density, also OE19 had a higher percentage (~25%) of cells showing cytoplasmic probe activation (Figure 2c) compared to EPC2-hTERT. In addition to the cytoplasmic staining, OE19 also showed extracellular activation of the probe, indicating that these cells also likely excrete the probe activating proteases.

**Probe activation and quantification in human BE tissues.** Next we analysed BE related ex vivo MMPsense680 activation, by incubating fresh frozen tissue sections from 14 IM, 7 HGD and 11 EAC patients, with the probe. Biopsies of normal coupled squamous epithelium were also included. In contrast to the diffuse cytoplasmic and extracellular staining as observed in the cell lines (Figure 2), in the tissues the activated probe was more intense and showed a ‘dotted’ appearance (Figure 3d). Squamous tissues showed little to none of this concentrated probe activation (Figure 3b). To assess the signal differences between the various tissue types

![Figure 2. MMPsense680 probe activity in esophageal cell lines. (a) Probe activity (arrows) in a normal esophageal cell line, (b) a Barrett’s cell line and (c) an esophageal adenocarcinoma cell line. DAPI was used as a nuclear counterstain (blue). Inserts show 2x enlargement of defined areas.](image)
Figure 3. H&E and MMPsense680 stainings of patient esophageal tissues. (a) H&E staining of a biopsy tissue section of squamous esophageal mucosa. Insert is a 2.4x enlargement of a mucosal area. (b) MMPsense680 probe activity in a serial section of the same biopsy. Insert is a 1.75x enlarged area with probe signals. (c) H&E staining of a biopsy of an early adenocarcinoma. (d) MMPsense680 probe activity in a serial section of the same lesion. DAPI was used as a nuclear counterstain (blue) in b and d. (e) Comparison of the mean discrete signal counts of the tissue types and corresponding normal squamous esophageal tissues. IM = intestinal metaplasia. HGD = high grade dysplasia. EAC = esophageal adenocarcinoma. Sq (IM) = squamous biopsies from patients with IM, Sq (HGD) = squamous from patient with high grade dysplasia, Sq (EAC) = squamous sample from patient with EAC. Statistical test results for Barrett’s tissues signal counts compared to the matched squamous tissues were for IM (paired t-test, ***p < 0.001), for HGD (Mann-Whitney U test,*p = 0.02) and for EAC (paired t-test, ***p < 0.001).
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We employed a semi-quantitative approach by determining the average activated probe signal count per tissue type. This was achieved by counting the discrete signals within a specified, constant region of interest. This region was centred over the area of the tissue section with the highest agglomeration of signals. There were rather large differences in signal count between the different samples per individual tissue type (Figure 3e). On average there was however no significant difference (one-way ANOVA, \( p = 0.97 \)) in level of signals between IM, HGD and EAC tissues (Figure 3e). However, all coupled squamous samples showed on average a lower amount of signals when compared to the respective coupled aberrant tissues (Figure 3e). This difference was significant for IM (Mann-whitney U test, "**" \( p < 0.001 \)), HGD (Mann-whitney U test, "*" \( p = 0.02 \)) and EAC (t-test, "***" \( p < 0.001 \)).

Figure 4. Matrix metalloprotease protein expression in patient tissues. (a) Immunoblot for MMP9 and MMP13 on normal squamous samples (Sq), intestinal metaplasia (IM) and esophageal adenocarcinoma (EAC). MMP9, molecular weight (MW) ~100 kDa, expression is highest in EAC. Expression of MMP13 (~48 kDa) is highest in IM. Actin (MW ~43 kDa) was used as a loading control. (b) Comparison of the relative MMP13 protein expression levels between Sq, IM and EAC samples. Significant differences were found between Sq-IM (t-test, "*" \( p < 0.02 \)) and IM-EAC (Mann-Whitney U test, "**" \( p = 0.005 \)). (c) Comparison of the relative MMP9 protein expression between Sq, IM and EAC samples. Differences were found between Sq-EAC (Mann-Whitney U test, "***" \( p < 0.001 \)) and IM-EAC (Mann-Whitney U test, "**" \( p = 0.003 \)).
Figure 5. MMPsense 680 co-localization with matrix metalloproteases and stromal and epithelial markers. (a) MMP13 protein expression (green color) detected by fluorescent immunohistochemistry on frozen sections of a biopsy with HGD co-localizes with activated MMPsense 680 probe (pink color). Co-localizing signals have a red to yellow color gradient (arrow). (b) Co-localization of MMP9 expression (green) and activated MMPsense 680 probe (pink) in a sample with HGD. (c,d) Fluorescent immunohistochemistry for E-cadherin (green arrow) and activated probe (red arrow) in a tissue section with HGD. (e,f) CD45 expression (green arrow) and activated probe (yellow arrow) in a biopsy with HGD. Co-localization is indicated by a red to yellow gradient.
Presence of activating proteases MMP9 and MMP13 in tissue samples. To evaluate the nature of the visualized and measured MMP activity in the BE tissues, protein lysates of 11 squamous esophageal, 6 IM and 8 EAC samples tissues were analysed for the presence of the two most prominent MMPsense680 activators, MMP9 and MMP13, by immunoblotting. Both MMPs were present in the BE tissues and upregulated when compared to the normal squamous tissues (Figure 4). Specifically, MMP13 expression was significantly higher in the IM tissues when compared to the normal squamous tissues (t-test, *p = 0.02) and EAC (Mann-Whitney test, **p = 0.005, Figure 4b). MMP9 was significantly higher expressed in the EAC tissues compared to both squamous and IM tissues (Mann-Whitney test, respectively ***p < 0.001 and **p = 0.003, Figure 4c).

Co-localization of the activated probes and activating proteases in BE tissues. To validate whether the probe signals actually correlate with protease activity we also investigated the co-localization of the main probe-activating proteases and the signals. To this end, tissue sections were first incubated with the probe and consequently immunohistochemistry was performed for the MMP9 and MMP13 proteins. MMP9 and MMPsense680 co-stainings were performed on 6 IM, 3 HGD, 4 EAC and 6 squamous esophageal tissues. MMP13 and MMPsense680 co-stainings were performed on 5 IM, one HGD, 3 EAC and 4 squamous esophageal tissue samples. We found that overall the signals of the two MMP-subtypes overlapped well with the activated probe signal as indicated by the red to yellow signals (Figure 5a,b). This supported the fact that the visualized signals are actually the probe activated by the MMPs.

Tissue localization of probe activation. To determine the origin of the observed MMP activity we investigated activation of the probe with respect to two cell type markers, namely E-cadherin and CD45. E-cadherin is a trans-membrane adhesion protein expressed by epithelial cells and was used here as an indicator of epithelial localization. CD45 is a membrane protein expressed by hematopoietic cells and was used as a marker for infiltrating cells in the stroma. E-cadherin/MMPsense680 double staining was performed for 7 IM, 4 HGD, 10 EAC and 3 squamous esophageal tissue section samples. CD45/MMPsense 680 double staining was performed on 8 IM, 4 HGD, 10 EAC and 3 squamous esophageal samples. Upon staining we observed that the activated probe signals co-localized more frequently with CD45 positive cells than with the E-cadherin expressing cells (Figure 5c,d,e,f). This co-localization implicates infiltrating stromal cells as the predominant activators of the probe in the tissue sections.

DISCUSSION
In this article we have described and explored the potential of the enzymatic activity of the matrix metallo-proteases to function as biomarkers in Barrett’s esophagus with the potential to be used for real time endoscopic imaging in the future. In our mouse model we could already observe MMP activity at the early stage of esophagitis and epithelial hyperplasia as a result of the bile influx into the esophagus, preceding the development of columnar metaplasia. Once metaplasia has developed, the MMP expressing cells are still present in the stroma in and near the metaplastic lesions. In other experiments we showed that MMP activity, as measured by
the MMPsense680 probe, gradually increased in the esophageal cell lines, from low probe activation in normal squamous esophageal cells to high activation in the esophageal metaplasia and adenocarcinoma cells. Probe activity in the tissue samples was significantly higher in all the transformed tissues of the Barrett’s pathogenic sequence, when compared to the normal squamous tissues. In these tissues, the visualized signals largely co-localized with two of the main probe activating enzymes, MMP9 and MMP13, which confirmed the nature of these signals as activated MMPs sense680 probe.

With respect to the biomarker potential of MMP activity as assessed by us, an interesting observation in our study was a switch in MMP subtype expression in the researched Barrett’s tissues. The expression of MMP13 was highest in IM, while MMP9 was mainly present in the cancer samples (Figure 4). Herszenyi et al, have demonstrated that the expression of MMP9 increases in the progressively aberrant tissues of the Barrett pathogenic sequence. The highest expression for MMP9 was seen in EAC, which is in line with our findings. Furthermore, even at the early stage of esophagitis they observed a significantly higher expression of the MMP9 protein compared to the control squamous tissues (35). This pattern indicates that this specific MMP could potentially function as a prognostic biomarker in BE as it is either up-regulated or selected for during Barrett’s pathogenesis. From a diagnostic point of view as a potential aid to standard white-light endoscopy for the real time in vivo discovery of aberrant lesions, one could also envision tailoring protease activated probes towards specific types of proteases for more specific targeting of dysplasia and early cancers. Also in this case MMP9 might be a good candidate. There are in fact currently studies ongoing to develop activatable probes geared towards specific proteases, including towards specific MMPs (38,39).

Another important observation with respect to the future refinement of MMP activity in Barrett’s esophagus is that when we more precisely analysed which cell types were predominantly activating the probe in situ, we found that in the patient tissues the probe signals were mostly co-localizing with CD45 positive inflammatory cells. This predominantly stromal localization of MMP activity was in line with the observations in our mouse model. To better distinguish which MMPs might better serve as disease markers and predictors it might be useful to distinguish those subtypes predominantly produced by stromal and inflammatory cells.

Besides pointing to the main source of MMP activity in BE the data we collected on the predominantly stromal localization during the development of the disease also offers interesting mechanistic insights. In a recent article it was shown that solely the overexpression of the pro-inflammatory cytokine IL-1β in the esophagus and fore stomach of mice lead to the development of columnar metaplasia at the age of 12-15 months (28). IL-1β expression had prior been shown to correlate with the presence of Barrett’s tissues and increasing BE tissue aberrancy (22,25). Furthermore, IL-1β lies upstream of the IL-6 cytokine which has also been shown to be more highly expressed in Barrett’s tissues compared to squamous esophageal tissues. IL-6 expression is believed to be involved in disease progression in BE by contributing to the activation of anti-apoptotic genes in the diseased tissues (21,40). Interestingly, IL-1β has been shown to be activated and degraded in vitro by a number of MMPs, such as MMP2, -3 and -9 (41,42). Conversely, IL-1β can also lead to the expression of MMPs (43,44). Our data showed an early rise in MMP activity during the process of Barrett’s pathogenesis in our mouse model.
MMPs could thus possibly contribute to the transformation process through the activation of IL-1β and/or might be activated early on by this cytokine and contribute to the pathogenic process through the activation of other soluble factors (29,30,33). In this respect the search for the best MMP biomarker could thus also reveal important and possibly useful early targets to prevent the development of metaplasia.

In summary, we were able to detect high MMP activity as an early and consistently present entity in the process of Barrett’s pathogenesis. This might indicate a role for these proteases in the onset and development of the disease. The predominantly stromal localization of the observed MMP activity falls in line with the theory of an important role for inflammation in BE as a risk factor for developing EAC (26-28). Our findings indicate that MMP activity could be a valuable target for performing real time imaging in vivo to detect hidden non erosive inflammation in BE for better risk stratification of patients. In this respect once the current MMPsense 680 probe becomes approved for clinical applications it could be further evaluated in an endoscopic setting.

**REFERENCE LIST**


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