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Increased PXR and suppressed T-cell signaling are associated with malignant degeneration of Barrett’s esophagus

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Abbreviations:

Barrett’s esophagus (BE)
esophageal adenocarcinoma (EAC)
gastro-esophageal reflux disease (GERD)
high grade dysplasia (HGD)
immunohistochemistry (IHC)
Inter Quartile Range (IQR)
median circumferential BE segment length (C)
non-dysplastic Barrett’s esophagus (NDBE)
proton pump inhibitors (PPI)
standard deviation (SD)

Contributions


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Ethical Statement

The corresponding author, on behalf of all authors, jointly and severally, certifies that their institution has approved the protocol for any investigation involving humans or animals and that all experimentation was conducted in conformity with ethical and humane principles of
ABSTRACT

Background and Aims

Barrett’s esophagus (BE) is the precursor lesion for esophageal adenocarcinoma (EAC). To detect EAC in early stage, patients with BE undergo endoscopic surveillance. Surveillance cohorts largely consist of non-dysplastic Barrett’s esophagus (NDBE) patients with a low annual progression risk (<0.5%). Predictive biomarkers for malignant progression of NDBE could improve efficacy of surveillance. Biomarker research has mostly focused on aberrant protein expression on BE epithelial cells. Moreover, insight in cell signaling driving malignant transformation is unknown. This study uses a data-driven approach to analyze tumor-stroma interaction in NDBE which progressed to high grade dysplasia (HGD) or EAC.

Methods

In this case control study, we performed RNA sequencing analysis on index NDBE biopsies from six patients who during long term follow-up progressed and seven who not progressed to HGD/EAC. For control samples, squamous and duodenum tissues from BE patients were analysed. For validation we used qPCR.

Results

Significant differences in BE transcriptomic profiles between progressors and non-progressors were found by principal component and differential expression analyses. Ingenuity pathway analysis indicated that eight cell signaling pathways were significantly upregulated in the progressors and 14 pathways were significantly downregulated. The most interesting finding
was the upregulation of the Xenobiotic Metabolism PXR Signaling pathway in the progressor cohort, while of the downregulated pathways in progressors several were related to the immune system.

Conclusion

These novel transcriptomic insights are fundamental for developing (chemo-)preventive therapies. These could be therapies, which protect against toxins, including biles, responsible for PXR activation, or which enhance protective immune mechanisms. The identified RNA markers are promising biomarkers for improving risk stratification in surveillance programs.

**Keywords:** biomarkers, Barrett’s esophagus, predictive medicine, RNA-seq, PXR, Immune signals

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**INTRODUCTION**

Barrett’s esophagus (BE) is the precursor lesion for esophageal adenocarcinoma (EAC). In BE the normal esophageal squamous lining is replaced by abnormal intestinal-like columnar
mucosa, as a result of gastro-esophageal reflux disease (GERD). Patients with BE undergo endoscopic surveillance to detect and treat malignancies in early stage. The vast majority of BE surveillance cohorts consist of patients with non-dysplastic Barrett’s esophagus (NDBE). Patients with NDBE have a relatively low risk to progress to high grade dysplasia (HGD) or EAC. The annual frequency of malignant progression of NDBE is between 0.9-1.0% in endoscopic surveillance series, but much lower in series form national registries (1). As a result, the cost-effectiveness of endoscopic surveillance programs for NDBE patients is debated (2). Biomarkers that could predict malignant progression of NDBE are therefore urgently required. In the past, hypothesis-based rather than data-driven research has been conducted to identify candidate biomarkers in BE at the gene expression/protein level (3-5). The most important biomarker reported so far by cohort and case-control studies is dysregulated expression of the tumor suppressor P53, which is due to P53 gene mutations or gene loss. Currently, overexpression of mutated p53 or complete loss of p53 gene expression due to allelic loss assessed by immunohistochemistry (IHC) is the only biomarker used to risk stratify BE patients in clinical practice. The problem is that current biomarkers solely based on epithelial aberrations seem not to be able to accurately predict progression of NDBE. P53 mutations occur at relatively late stage during malignant progression, and mostly appear in dysplastic BE or shortly before dysplasia or cancer occurs (6). P53 mutation and overexpression is generally not observed in NDBE patients and seems to have limited prognostic value in this subgroup of BE patients. This group of patients has a low frequency of progression in combination with long progression intervals.

Until now, most biomarker research in BE has focused on alterations at the level of the epithelial BE cells. Several studies also demonstrated that stromal factors excreted by non-epithelial cells within the BE mucosa may play a role during the malignant progression of BE (7). Therefore the role of the microenvironment of BE epithelium for predicting disease
outcome deserves to be further investigated. Here, we hypothesized that analysis of factors expressed by both epithelial and stromal cells within the BE mucosa at the gene expression level could yield important information with respect to the malignant progression of BE. Such analysis may lead to the identification of biomarkers and the discovery of critical signaling pathways. Although the sequential genetic mutations in the DNA of epithelial BE cells conferring a biological advantage to a subset of cells play a role in malignant transformation in BE (8, 9), insight in earliest biological mechanisms and pathways which drive this process are greatly unknown. It is very likely that crosstalk between epithelial cells and the surrounding stroma, including fibroblasts, vasculature and immune cells, has critical roles in the onset of the malignant progression of BE. Similar crosstalk plays a critical role in the development of other cancer types (10) (8).

In a study of Owen et al., RNA sequencing profiles of bulk BE tissues and single BE cells showed the existence of distinct cell populations. Interestingly, the RNA sequencing profiles which characterized BE epithelial cells proved to overlap with esophageal submucosal gland cells, and were marked by expression of LEFTY1 and OLFM4 (11). These transcriptomic analyses also showed that SPINK4 and ITLN1 are markers for goblet cells, and their presence might be involved in the development of BE (11). However, transcriptomic markers related to disease progression to cancer were not identified in this study.

In this study our goal was to increase the insight into the pathophysiology predisposing to carcinogenesis in NDBE. We hypothesized that the microenvironment surrounding the epithelial cells is an integral part of the pre-cancer biology, and dysregulation of specific signaling pathways within both the epithelium and the microenvironment is involved early on during the malignant degeneration of BE. The aim of this study was to elucidate which
specific pathways associated with malignant progression are dysregulated in epithelial cells and the surrounding stroma in the non-dysplastic stage. In this case control study, RNA sequencing analysis of NDBE biopsies was performed to quantify large numbers of genes in both the epithelial and stromal compartments. The pathways identified in this study may offer new candidate biomarkers, but also potential targets for preventive therapies in order to reduce patient risk on developing cancer.

RESULTS

Patient characteristics

13 BE tissue biopsies from unique patients who were in surveillance programs, of whom 6 were defined as long term progressors and 7 as long term non-progressors, were analysed in the study. None of the patients had any visible signs of reflux during endoscopy at time the biopsies were taken. The majority of patients was male (83.3% and 85.7% in progressors and non-progressors, respectively) with a mean age of 60 years (standard deviation (SD) 10.4) for progressors and 50 years (SD 8.4) for non-progressors.

The median circumferential BE segment length (C) (5.0 cm (Inter Quartile Range (IQR) 1.0) and 3.0 cm (IQR 2.3)) was significantly different between the progressors and the non-progressors (p=0.048). In the progressor group, the mean time between date of the index biopsy and the date of progression was 5 years (IQR 6). A graphical view of times between index biopsies, surveillance endoscopies and progression is shown in figure 1. For the non-progressors the mean time between date of biopsy and last date of follow-up was 8 years (IQR 3) (table 1). The patient characteristics are shown in table 1. Squamous and duodenal tissues served as controls and were also sequenced.
Principal Component Analysis

After RNA sequencing data reduction of all protein-coding genes was performed by obtaining a set of principal components, a clear difference between BE tissue from progressors and non-progressors was observed when samples were plotted on principal components 3 and 4 (figure 2), but not on principal components 1 and 2 (supplementary figure 1). This suggests that clear differences in transcriptomic profiles between the two groups exist. The duodenal tissues, which were basically used for control purposes from progressors and non-progressors showed significant overlap on PC1, PC2, PC3 and PC4, suggesting they have similar transcriptomic profiles. Similar results as for the duodenal tissues were seen for the squamous tissues.

Differentially expressed genes between progressors and non-progressors

Differential expression analysis showed that 1446 genes were differentially expressed of which 751 genes were upregulated in BE biopsies from progressors versus non-progressors. As suggested by the principal component analyses, there were minimal transcriptional differences between duodenal respectively squamous tissues from progressors versus non-progressors. Between progressors and non-progressors a total of only 5 differentially expressed genes were found for the duodenal biopsies and between the squamous samples only 81 were differentially expressed. The differentially expressed genes between BE biopsies from progressors and non-progressors are visualized in a log ratio-mean average plot (figure 3.A) and a heatmap (figure 3.B).

Dysregulated pathways determined by Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (Qiagen) is a tool that enables pathway analysis on lists of differentially expressed genes resulting from comparing gene profiles between groups. IPA analysis was applied to interrogate more specifically which biological processes were
differentially regulated between the two groups (results are listed in supplementary table 1). Eight cell signaling pathways were significantly upregulated in the progressors. These upregulated pathways in the progressors were involved in cell metabolism. These pathways included “super pathway of melatonin degradation“, “nicotine degradation III“, “fatty acid oxidation“, “serotonin degradation” and “xenobiotix metabolism PXR signaling pathway” (figure 4). PXR signaling is involved in transport of toxic agents including bile acids (figure 5). Increased expression of PXR has been reported earlier in BE and EAC (12). Of interest were also those pathways that were downregulated in the progressors. Six of the 14 pathways that were significantly downregulated in progressors, were immune pathways, including “Role of NFAT in regulation of the Immune Response“ and various signaling pathways important for T Lymphocytes, IL-8 signaling and “IL-15 production“. Surprisingly, the pathway “Regulation of the epithelial mesenchymal transition by growth factors pathways“ was lower in progressors than in non-progressors.

**Validation of PXR expression by qPCR**

QPCR analysis to validate gene expression values of PXR as found by RNA sequencing was performed for 10 BE samples (4 progressors, 6 non-progressors) with sufficient RNA left after RNA seq. Gene expression quantification of PXR by RNA sequencing and qPCR on the same samples did highly correlate (Wilcoxon signed rank test p=0.004). Gene expression values of PXR by qPCR was significantly different between progressors and non-progressors (independent 2-group Mann-Whitney U Test p=0.02), with higher delta cp values thus lower gene expression of PXR in non-progressors (figure 6).

**Estimation of different types of immune cells using CIBERSORT**
The finding of dysregulation in inflammatory/immune signals prompted us to further interrogate the data in order to identify the different populations of cells within the BE biopsy specimens. CIBERSORT was applied to estimate the different types of immune cells. Estimated scores of abundancies showed that plasma cells (p=0.051) and activated dendritic cells (p=0.07) tended to be higher in non-progressors and resting T cells, CD4 memory cells tended to be higher in biopsies from progressors (p=0.07).

DISCUSSION
Surveillance cohorts of BE patients largely consist of NDBE patients which carry relatively low progression risk and generally progress after many years of follow-up. Management of this patient group requires a more advanced approach aimed at improving risk stratification and more efficient preventive and surveillance management.

In the current case control study, we used RNA sequencing analyses and an unbiased approach to elucidate which specific pathways are up- or downregulated in the non-dysplastic stage of BE which after long periods of follow up would or would not progress to HGD or EAC. These pathways might provide insight in the background pathophysiology which early on predisposes for the malignant progression of patients with NDBE. These pathways may potentially unveil biomarkers to improve risk stratification and/or targets to improve preventive strategies.

Our most interesting finding was the upregulation of the Xenobiotic Metabolism PXR Signaling pathway in the progressor cohort. This increased expression as observed by RNA sequencing, was validated by qPCR. PXR Signaling is known for its regulation of detoxification of foreign substances. Bile acids are important ligands for this nuclear receptor. The activated state of PXR signaling in potential progressors is most likely related to the fact that these NDBE patients suffer from the presence of harmful chemicals as a result of gastro-esophageal reflux disease, leading to mucosal insults and DNA damage (13, 14).
Finding increased metabolic activity in the same progressors samples pointing to cell renewal and increased proliferation is in line with the upregulated PXR signaling. There are several factors that can explain the active PXR signaling in the progressors group. The most plausible reason is an incomplete control of bile reflux, despite there were no signs of active reflux during endoscopy and all patients in this study were on long term high dose PPI (proton pump inhibitors). In these cases there seems to be acid control through use of PPI, while exposure to bile acids may have persist. Refluxates of BE patients contain more bile acids than healthy subjects and bile acids have been demonstrated to lead to DNA damage even at neutral pH (15-18). Moreover, our group has shown direct effects from bile acids on development of intestinal type of metaplasia which resembles human BE mucosa in a mouse model (19). More extensive research is required to understand the exact mechanisms and bile receptors that are involved. In one older study exposure of BE cells to bile acids induced translocation of PXR to the nucleus but did not cause increased PXR mRNA levels (12). In another study on Crohn’s disease by another group, it has been shown that attenuation of bile acid composition leads to differential expression of FXR and PXR (20). If the same association exists in BE has to be investigated.

Our findings indicate that patients with active PXR signaling, still have insufficient protection against chemicals and mucosal insults by bile acids. It is possible that this subgroup requires extra measures to prevent such damage. These measures could include better monitoring of bile reflux in NDBE patients and in case of high exposure, to provide extra protective measures by combining PPI with mucosa protective agents or through changing the ‘aggressiveness’ of the bile pool for instance by using urso-deoxycholic acid. This would be an interesting topic of future research.

Previous research showed that patients with BE have an altered immune response compared
to patients with GERD without BE. BE is characterized by an anti-inflammatory Th2-like response, rather than the pro-inflammatory cell-mediated cytokine profile seen in GERD (21, 22). In general, Th2-mediated immunity is associated with promotion of angiogenesis (23, 24) and inhibition of cell-mediated Th1 immunity and subsequent tumor cell killing (25). In the current study, we found that several immune pathways, including iCOS-iCOSL signaling in T Helper Cells and IL-15 expression, were downregulated in NDBE patients that progressed to HGD or EAC compared to patients without progression. Therefore, we conclude that a subset of patients with NDBE show disrupted immune signaling, potentially related to decreased immune surveillance (26).

Our observation, which indicates an association between downregulation of specific immune pathways in NDBE and progression to HGD or EAC is novel and until now received little attention. Previously, a higher risk of malignant progression has been associated with upregulated inflammatory pathways, which potentially can be suppressed by PPI and aspirin. The only clinically used pharmacological treatment to avert progression in BE is lifetime treatment with PPI, which suppresses the amount of reflux and as such decreases reflux esophagitis and potential DNA damage directly caused by acid and indirectly by bile acids (19). Moreover, aspirin add-on to high dose PPI improves outcomes in patients with BE (2) and protects for both EAC and esophageal squamous cell carcinoma (27). The antitumor activity of aspirin is thought to be based on COX-dependent and COX-independent mechanisms. Inhibition of COX-2 and COX-2-derived prostaglandin E-2 results in inhibition of inflammation-related carcinogenesis through NF-κB and MAPK pathways (28, 29) and alteration of proliferation and apoptosis cancer pathways including MAPK, PI3K and cAMP/PK pathways, respectively (30). We did not see any difference between these pathways in progressor and non-progressors. The downregulation of chemokine signaling and
T-cell signaling pathways, which we identified in the progressors (who were using high dose of PPI), indicates that these findings are independent from PPI use.

A few notes are to be made on the methodology of our paper. The number of patients analysed by RNA sequencing is low, because availability of fresh frozen tissues required to perform high quality RNA sequencing, from patients with NDBE before progression occurred is limited. We decided not to use paraffin embedded tissue for RNA expression analyses, because although this type of patient material is easier to obtain it has important limitations with regard to the quality and amount of data that can be generated.

It is known that DNA methylation and its epigenetic regulatory effects on transcription alters with age (31). However, this potential confounder could not be the case as we found no significant difference in age between progressors and non-progressors and in RNA expression between profiles from control tissues of duodenum and squamous tissue from progressors and non-progressors.

In summary, we showed upregulation of PXR signaling and downregulation of immune pathways important for T cell regulation in BE patients that progress to EAC. These insights opens the potential for preventive therapies that protect against the toxins including biles responsible for PXR activation and therapies that can boost immunosurveillance to prevent progression of NDBE to EAC. Moreover, these stromal derived RNA markers are promising markers for further assessment of their ability to select the small group of NDBE patients that might benefit from intensified surveillance and treatment, while others may not need surveillance.
Figures and tables

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Progressors (n=6)</th>
<th>HGD/EAC (n=6)</th>
<th>Non-Progressors (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex fraction</td>
<td>83.3% (n=5)</td>
<td>85.7% (n=6)</td>
<td>1*</td>
</tr>
<tr>
<td>Mean age (±SD)</td>
<td>59.7 (10.4)</td>
<td>49.7 (8.4)</td>
<td>0.09§</td>
</tr>
<tr>
<td>Median Circumferential BE segment length (C) in cm</td>
<td>5.0 (1.0)</td>
<td>3.0 (2.3)</td>
<td>0.048#</td>
</tr>
<tr>
<td>Median maximum BE length (M) in cm (IQR)</td>
<td>4.5 (2.5)</td>
<td>3.0 (0.5)</td>
<td>0.22#</td>
</tr>
<tr>
<td>Biopsy level 1, 2, 3 (n)</td>
<td>(4, 1, 1)</td>
<td>(7, 0, 0)</td>
<td>0.25 §§</td>
</tr>
<tr>
<td>Mean BMI (± SD)</td>
<td>23.6 ±2.0</td>
<td>23.3 ±1.8</td>
<td>0.81§</td>
</tr>
<tr>
<td>Use of proton pump inhibitors</td>
<td>100% (n=6)</td>
<td>85.7% (n=6)</td>
<td>1*</td>
</tr>
<tr>
<td>Family history of BE</td>
<td>16.7% (n=1)</td>
<td>42.9% (n=3)</td>
<td>0.56*</td>
</tr>
<tr>
<td>Family history of esophageal cancer</td>
<td>33.3% (n=2)</td>
<td>14.3% (n=1)</td>
<td>0.56*</td>
</tr>
<tr>
<td>Smoking</td>
<td>83.3% (n=5)</td>
<td>57.1% (n=4)</td>
<td>0.56*</td>
</tr>
<tr>
<td>Median time between biopsy and progression/last time no progression years (IQR)</td>
<td>5(6)</td>
<td>8(3)</td>
<td>0.8#</td>
</tr>
</tbody>
</table>

*Fisher’s exact test (two-sided)  
§ Welch Two sample T-test (two-sided) (parametric)  
 §§ Pearson’s Chi-squared test  
# Wilcoxon rank sum test with continuity correction (non-parametric)

BE Barrett’s Esophagus; SD Standard Deviation; IQR Inter Quartile Range

Figure 1. Time intervals between index endoscopy, surveillance endoscopies of progressors and non-progressors. (HGD = High Grade Dysplasia, IMCA=Intra Mucosal esophageal adenoCarcinoma).

Figure 2. The Principal component analysis of BE, duodenal and squamous biopsies of progressors and non-progressors on PC3 (x-axis) and PC4 (y-axis)

Figure 3A Log ratio-mean average plots with each gene visualized as a dot (grey) and differentially expressed genes depicted by the red dots. BE tissue from progressors compared to non-progressors have 1446 differentially expressed genes, suggesting underlying biological differences between these samples.

Figure 3B Gene expression in progressors (right side, in pink) and non-progressors (left side,
in blue). The rows depict the differentially expressed genes, the columns depict the samples. Samples are shown in order of their tissue type (shown by the colored bar at the top). Genes are shown in order of their fold change from differential expression analysis.

Figure 4. Ingenuity Pathway Analysis indicate upregulated pathways (red) and downregulated pathways (blue) in progressors versus non-progressors.

Figure 5. Graphical view of the Xenobiotic metabolism PXR signaling pathway.

Figure 6. Violin plots showing the median (black horizontal line in white box) for delta cp by qPCR for PXR for Barrett’s non-progressors n=6 (left) and Barrett’s progressors n=4 (right), p=0.02.

REFERENCES
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