Barrett's esophagus and esophageal adenocarcinoma: transcription factors and biomarkers
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DOI:
10.1016/j.dld.2014.09.014

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Summary, Discussion and Future perspectives
Summary and discussion

Esophageal adenocarcinoma (EAC) is thought to develop from its precursor lesion, Barrett’s esophagus (BE) through a metaplasia-dysplasia-carcinoma sequence. While persistent gastro-esophageal reflux disease (GERD) is the main risk factor for the development of BE, only a minority of GERD patients will develop BE during their lifetime. Patients with BE are enrolled in endoscopic surveillance programs aimed at early detection of BE malignant transformation, and early detection has been shown to significantly improve survival. However, BE is asymptomatic, and in the absence of BE biomarkers the majority of BE patients will remain undiagnosed and present with EAC symptoms, often at a disease stage that precludes curative treatment. Even in EAC patients undergoing treatment with curative intent consisting of neoadjuvant chemoradiotherapy (nCRT) followed by an esophagectomy the 5-year survival is only around 50%. The current management of BE and EAC thus has several challenges, some of which are addressed in this thesis. First, the underlying molecular mechanisms of BE development are unclear, and this limits the development of pharmacological treatment modalities. Second, the absence of a BE biomarker hampers the detection of BE patients. Third, a better understanding of EAC biology is required to further improve patient survival.

Understanding the signaling pathways that drive BE development could yield novel therapeutic targets. In chapter 1 we summarized the role of four main signaling pathways implicated in BE development and malignant progression: the Bone Morphogenetic Protein (BMP), Hedgehog (HH), Wingless-Type MMTV Integration Site Family (WNT) and Retinoic Acid (RA) signaling pathways. Activation of BMP, HH and RA signaling contributes to BE development, while high WNT and HH drive BE malignant transformation. Interestingly, these four signaling pathways also play a central role in the embryological development of the esophagus. Thus, studying the involvement of embryonic signaling pathways in the context of BE development and EAC carcinogenesis could serve as a potent approach to better understand the biology of BE and EAC.

The availability of representative experimental models are critical to the study of pathways driving BE and EAC. In chapter 2 we discuss several of the current BE and EAC models and describe their advantages and limitations. Traditional in vitro cell culture models are accessible and easy to use, but the number of representative BE and EAC cell lines is limited. Cell culture in the absence of a representative microenvironment could influence cell behavior and precludes the possibility to study epithelial-stromal interactions in EAC development. Surgical esophagojejunostomy animal models using mice and rats have been developed that incorporate a stromal component, but it is not clear whether they develop the same molecular alterations that are commonly found in human EAC cases. In addition, these models require the animal to be sacrificed in order
to take esophageal tissue samples, and do not allow biopsies at multiple time points. Two important novel developments have been the development of in vitro organotypic models of co-cultured stromal and epithelial cells that enable the study of stromal-epithelial interactions in vitro, and the use of computational models that simulate the dynamics of BE expansion and malignant transformation, that offer tractability and can be easily manipulated to reflect novel insights in BE and EAC.

In chapter 3 we studied the ability of RA to induce a columnar morphology in an organotypic model. We found that the addition of RA to the organotypic model caused loss of the multilayered squamous architecture and was associated with loss of the squamous cell-specific cytokeratin 13. When the organotypic model was used to culture a metaplastic BE cell line, goblet cell differentiation, a diagnostic feature of BE was observed that has not been seen in traditional cell culture methods. Dysplastic BE cell lines cultured in the organotypic model displayed invasion into the stromal compartment, suggesting that the model could also be used to study tissue invasion in EAC.

RA has previously been suggested to contribute to the development of the metaplastic columnar esophageal epithelium (1,2), but so far the transcription factors involved in this process were not identified. The aim of chapter 4 was to study the expression of squamous and columnar transcription factors during BE development using patient biopsies and examine the potential effect of RA on these transcription factors using an in vitro model of immortalized esophageal keratinocytes. We found that the squamous transcription factors p63 and SOX2 were expressed in the majority of cells in biopsies of squamous epithelium. In BE, p63 expression was lost completely while SOX2 expression persisted in a minority of BE biopsies. Both the columnar transcription factors GATA6 and CDX2 were expressed in a small minority of cells in squamous biopsies, but the percentage of positive cells increased significantly in biopsy samples of BE. While the percentage of GATA6-positive cells did not differ between BE biopsies containing goblet cells (intestinal type BE, IM) or without goblet cells (non-intestinal type BE, non-IM) we found that the percentage of CDX2-positive cells was significantly higher in IM biopsies compared to non-IM biopsies. In vitro, RA treatment significantly decreased protein expression of ΔNp63α, an isoform of the squamous transcription factor p63. However, RA treatment had no effect on the expression of SOX2. RA treatment also increased mRNA expression of the columnar transcription factors GATA6 and SOX9 but did not induce CDX2 mRNA expression. This can be explained by assuming that induction of CDX2 expression is a later event during BE development and associated with intestinal-type differentiation, but is not required for the differentiation of non-intestinalized columnar epithelium. These findings suggest that squamous epithelium and BE are each characterized by distinct patterns of transcription factor expression, and identify transcription factors whose expression is modulated by RA in squamous epithelium.
As described in chapter 4, the zinc finger transcription factor GATA6 is regulated by RA signaling. The aim of chapter 5 was to study GATA6 protein expression during BE development, malignant transformation and evaluate the prognostic significance of GATA6 protein expression in EAC. Using a series of 130 biopsy samples containing normal squamous epithelium, esophagitis, non-IM, IM and high-grade dysplasia we found that GATA6 expression is low in normal squamous epithelium, but progressively increased in metaplastic and dysplastic BE samples. Interestingly, GATA6 expression was already significantly increased in inflamed squamous epithelium due to GERD, indicating that inflammation-driven GATA6 induction could be an early event in BE development. While a previous study reported that GATA6 gene amplification was associated with a poor survival in EAC patients (3), we found no prognostic value of GATA6 protein expression in a cohort of 92 EAC resection specimens. This discrepancy may be related to protein expression levels being regulated by multiple post-transcriptional mechanisms and this could explain the discrepancy between the previously reported prognostic value of GATA6 gene amplification and our findings.

In Chapter 6 we set out to investigate the potential of circulating miRNA as biomarkers of BE, BE dysplasia and EAC. Using a Nanostring microarray we identified 6 miRNA's with a fold change >2 between sera of control patients and patients with either BE, dysplasia or EAC (miR-122-5p, miR-144-3p, miR-150-5p, miR-199a-3p, miR-233-3p and miR-320e). In an extended patient cohort the expression of miR-199a was significantly decreased in patients with BE compared with control patients, while miR-302e expression was significantly reduced in patients with BE and patients with dysplasia compared to control patients.

Despite the poor overall prognosis of EAC, there is considerable variation in survival between individual patients. This variation provides an opportunity to correlate biological characteristics with patient survival, thereby increasing the knowledge of EAC biology. In Chapter 7 the association of a panel of presumed Cancer Stem Cell (CSC) markers was studied in relation to patient survival in a cohort of 94 EAC patients that underwent a radical esophagectomy without neoadjuvant therapy. Protein expression of ALDH1, Axin2, BMI1, CD44 and SOX2 was measured semiquantitatively using immunohistochemistry and correlated with overall and disease-free survival. While no association was observed between ALDH1, AXIN2, BMI1 and patient survival, we found that loss of CD44 and SOX2 was associated with a worse prognosis. In a multivariate analysis, loss of both CD44 and SOX2 were significant prognostic factors for a shorter disease-free survival. Several explanations could account for this counter-intuitive finding. First, CD44 and SOX2 expression could not be valid markers of CSC in EAC, or the CSC compartment might be heterogeneous with CD44/SOX2-positive CSCs being primarily important in EAC tumor initiation, but less so in established EAC. The poor prognostic
value of CD44 and SOX2 loss in established EAC could then be explained by the other known functions of CD44 and SOX2. CD44 plays a role in adhesion to extracellular matrix and in stimulating inflammation (4,5) while SOX2 has been associated with autophagy and cellular senescence (6). A second explanation could be that CD44 and/or SOX2 could be valid markers for CSC *in vitro*, but not *in vivo*. Such an association has been recently described in non-small-cell lung cancer, where CD44+ cells were enriched for stem cell properties and SOX2 expression *in vitro*, but CD44 expression in patient tissues was associated with a favorable prognosis (7). The lack of functional validation of CD44 and SOX2 as CSC markers in EAC precludes a definitive answer, but the results presented in Chapter 7 highlight the need for functional validation of proposed CSC markers, even if a marker has previously been validated in other tumor types.

In Chapter 8 we explored the predictive and prognostic value of a panel of proteins that were previously associated with radiotherapy resistance in EAC patients treated with nCRT. The protein expression of SHH, CD44 and SOX2 was evaluated in two cohorts: 71 pre-treatment biopsies and 74 post-treatment resection specimens. In pre-treatment biopsies low SHH was predictive of poor disease-free survival and loss of SOX2 was associated with recurrence and poor disease-free survival in a univariate analysis, but it did not retain significance after multivariate analysis. While CD44 expression has previously been implicated in resistance to chemo-and radiotherapy (8-10), we found that loss of CD44 was associated with poor survival in the post-treatment resection specimens cohort. This finding is in concordance with our finding in chapter 7 that loss of CD44 is associated with a poor survival outcome in EAC patients treated with surgery only. Thus, the effect of CD44 loss appeared to be independent of treatment, suggesting that levels of CD44 expression reflect differences in cell behavior and not just resistance to treatment.

**Future perspectives**

*Understanding Barrett’s esophagus development*

Currently, no effective pharmacological treatment exists for BE. Targeting dysregulated signaling pathways could provide novel therapeutic targets for BE and EAC. Increased signaling through the BMP, HH and RA pathways contributes to the induction of columnar differentiation (Chapters 1-3) and antagonizing their activity could induce a regression of BE. Previous studies showed that Citral, a naturally occurring RA antagonist present in citrus fruits, could partially reverse RA-induced columnar epithelium towards a squamous epithelium in an *ex vivo* study (1) and cyclopamine, a HH antagonist, decreased the incidence of BE in an animal model (11). These preliminary results are encouraging, but several challenges need to be solved before this approach can be tested in humans. First, further progress requires better models of BE development and malignant progression.
An ideal experimental model would offer tractability, easy access at multiple time points and a representative microenvironment. Such a model is presently not available, but optimizing and combining existing models could significantly mitigate the limitations of current models. Organotypic models could be further developed to allow a crypt-based in vitro culture, while animal models that offer the possibility to trace cell fate and identity would enable elucidation of a hierarchy of BE cells. While BE is presumed to have a stem cell-based hierarchy analogous to columnar epithelium, the stem cell compartment has not been identified. Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) is a marker of stem cells in colonic crypts, and a recently developed BE mouse model has the option to trace LGR5+ cells in the cardia/esophagus region (12). A better understanding of the BE cell hierarchy could in turn be used to improve current computational models of EAC tumor development. Due to the small luminal diameter of the esophagus in small rodents biopsies at multiple time points are a challenging task, but advanced imaging techniques such as microPET/CT could allow for non-invasive monitoring of BE and EAC development and response to therapy and enable in vivo validation of data from in vitro models. Avoiding off-target effects is a second challenge, since each of the described pathways play an important role in intestinal homeostasis. Extensive validation using animal models of BE is therefore required before novel therapeutic therapies based on modulation of the BMP, HH and RA signaling pathways could be tested in humans. A better understanding of BE hierarchy would in turn improve computational in silico models, and these could simulate BE development in time periods that reflect human disease (years) instead of the months that are possible with in vitro or animal models.

Improving detection of Barrett’s esophagus and its malignant transformation

Despite a well-established pattern of EAC development through a metaplasia-dysplasia-carcinoma sequence, most patients with EAC are diagnosed at the stage of advanced disease. One of the main reasons for this is that BE itself is asymptomatic and therefore often not diagnosed. Several blood-based biomarkers of BE and EAC have been proposed, including serum proteomic patterns, serum leptin levels and cell-free circulating DNA (13-17). However, these potential biomarkers are limited by the need for complex equipment or variation due to other processes in the body (such as serum leptin levels).

In contrast, miRNA’s are highly tissue-specific and easily measured. Chapter 6 identifies two miRNA’s, miR-199a-3p and miR-320e with the potential to discriminate between subjects with and without BE and dysplasia. These results suggest the possibility to use circulating miRNA’s to further stratify members of an at-risk population for endoscopic screening. However, the findings of chapter 6 need to be replicated in other independent, larger cohorts. While using miRNA’s as biomarkers appears an attractive strategy, two major challenges need to be addressed in future studies. The first challenge is the current
lack of a reliable reference gene, preferably a miRNA the expression of which is not affected by gender, age or disease. The second challenge is the origin of miRNA’s found to be aberrantly expressed in serum of patients with disease. MiR-199a-3p is a known tumor suppressor miRNA in other solid malignancies, but was previously reported to be increased in EAC compared to squamous epithelium. In contrast, little is known about the function or tissue expression of miR-320e, making it difficult to functionally correlate circulating miRNA levels with tissue expression. Moreover, a recent paper reported that the majority of cell-free circulating miRNA’s previously reported as cancer biomarkers were highly expressed in blood cells (18), suggesting the possibility that changes in blood cell composition and/or integrity (e.g. hemolysis) can affect circulating miRNA levels. It has been recently shown that tumor-derived exosomes contain miRNA’s and are able to perform paracrine signaling (19). MiRNA’s from tumor-derived exosomes could provide a more reliable source of circulating miRNA’s, and should be studied further in the context of EAC.

**Correlating esophageal adenocarcinoma survival with biomarker expression; promises and challenges of the cancer stem cell model**

Loss of CD44 and SOX2 was found to be associated with poor survival in two independent cohorts, but the mechanism behind this finding is unknown. The results of chapter 7 and 8 raise two main questions for future studies: do CD44 and/or SOX2 mark subpopulations of EAC cells with stem cell-like properties, and what is the mechanism behind the observed adverse effect of low CD44 and SOX2 expression on survival? Stemness assays using (preferably) patient tumor samples could be used to investigate whether expression of CD44 and/or SOX2 is associated with characteristics of stem cells, such as self-renewal and enhanced colony formation in a limited dilution assay. Animal models implanted with CD44/SOX2 knockout EAC cell lines can be used to investigate the effect of CD44 and SOX2 loss on tumor growth and *in vivo* behavior, possibly explaining the mechanisms underlying the poor prognostic value of CD44 and SOX2 loss found in chapters 7 and 8.

Recently, two papers reported the identification of a circuit consisting of the WNT, BMP and GATA6 that regulates CSC expansion in colon cancer. WNT signaling promotes the expansion of colon CSC’s while BMP signaling induces CSC differentiation, antagonizing the oncogenic effect of WNT (20,21). GATA6 is a central mediator of this circuit and maintains stemness by stimulating WNT and antagonizing BMP signaling. Several findings support the notion that WNT/BMP/GATA6 circuit may also be a regulator of presumed CSC’s in EAC, and thus a potential treatment target. We describe a progressive increase in GATA6 expression during BE malignant transformation *in vivo* (chapter 5), while other studies described an increase in WNT signaling in BE and EAC (22,23) and a downregulation of the BMP4, a ligand of the BMP signaling pathway in EAC.
Animal models allow modulation of WNT and BMP signalling in the esophagus and can be used in future studies to validate the existence of the WNT/BMP/GATA6 circuit in BE and EAC. If this signaling axis also exists in EAC, it can be targeted through several strategies. First, as reviewed in Chapter 1, a number of WNT antagonists are currently undergoing trials in EAC, and results of their effect on tumor growth and potentiation of chemotherapy effect are eagerly awaited. A second approach to target EAC CSC could be the addition of BMP4. In colon cancer BMP4 suppletion antagonized WNT signaling induced cell death and sensitized colon cancer CSC to traditional chemotherapy (20), and a similar approach should also be tested in EAC models. However, while the CSC theory is an attractive conceptual approach to understand EAC biology and could yield novel therapies based on the selective targeting of the stem cell compartment, the absence of functionally validated CSC markers in EAC is an important limitation, and requires further research.

In conclusion, a better understanding of the molecular mechanisms of BE and EAC could provide new solutions for the current clinical challenges in BE and EAC management. In this thesis we show that aberrant activity of embryological signaling pathways, such as the RA pathway, could be a driver of BE development and this provides novel therapeutic opportunities. We found two circulating miRNA’s that can identify patients with BE and HGD. In addition we show that low expression of CD44 and SOX2 predicts a poor survival outcome in EAC patients. With further research and better models, the increased understanding of BE and EAC biology will likely translate into new surveillance options and therapeutic targets, and thus into improvement of preventive strategies, surveillance and treatment of our patients.
References


(15) Thompson OM, Beresford SA, Kirk EA, Bronner MP, Vaughan TL. Serum leptin and adiponectin levels and risk of Barrett’s esophagus and intestinal metaplasia of the gastroesophageal junction.
Obesity (Silver Spring) 2010 Nov;18(11):2204-2211.


