What tumor cells cannot resist
Ebbing, E.A.

Citation for published version (APA):

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GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

Eva A. Ebbing
Chapter 1

**Introduction to esophageal cancer**

As the first section of the GI tract, the esophagus encounters all the food that is consumed, and it is in close contact with the acidic environment of the stomach. Although robust biological barriers against these influences exist including the lower esophageal sphincter, the normal homeostasis of the esophagus can be disrupted. For example, under influence of excessive alcohol intake or acid reflux from the stomach cells may transform, which may lead to the development of esophageal squamous cell carcinoma (ESCC) at the more proximal section, or esophageal adenocarcinoma (EAC) at the more distal part of the esophagus. These two histologically distinct subtypes cover 98% of all esophageal cancers (EC). Based on histology, a clear geographical difference can be observed in prevalence; in Asian countries 90-95% of the esophageal cancer cases are ESCC, in the Western world 90-95% of the EC patients present with EAC. Moreover, whereas the incidence of ESCC is decreasing, an alarming increase of EAC is observed in Western countries. The work in this thesis will mainly focus on the EAC subtype of EC.

**Esophageal adenocarcinoma**

The incidence of EAC has increased six-fold during the last three decades. More than 2,100 new patients are diagnosed with EAC in The Netherlands each year. The mechanisms that drive EAC development are largely unclear although it is known that a vitamin-poor diet, smoking, increased body mass index (BMI), the duration of reflux symptoms, and presence of a hiatal hernia are risk factors. Also, approximately 85% of the cancer patients is male which might be due to the abovementioned risk factors being more prevalent in males compared to females.

One of the most clearly defined risk factors for EAC is gastroesophageal reflux disease (GERD), which leads to Barrett’s esophagus, a premalignant condition for the development of EAC, in approximately 5-15% of cases. This metaplastic condition, in which esophageal squamous cells are replaced by intestinal-type columnar cells, is thought to be a protective response to chronic tissue inflammation induced by the acidic environment as a result of GERD, and affects 1-6% of the general population. Patients suffering from Barrett’s esophagus have an estimated annual transition rate to EAC between 0.12 and 0.5%, and for that reason there is intense discussion about the (cost) effectiveness of a Barrett’s surveillance program. Dysphagia is the most common (late) symptom of EAC, however, in most patients the development of the disease in its first stages is asymptomatic, resulting in an advanced disease stage at diagnosis. Only approximately 30% of the patients are diagnosed with regional disease (T1-4 without positive lymph nodes or metastasis) (Figure 1). Despite the benefits of recent multimodality treatments the overall survival of these patients remains dismal with a 5-year survival of 5-40%, depending on tumor stage. These percentages indicate an urgent need to improve treatment strategies. Biomarker research can contribute to this in two ways, (i) by patient stratification based on (bio)marker expression, thereby selecting patients for a specific treatment that is likely to be effective, and (ii) by identifying potential new targets which may contribute to the development of new anti-cancer treatment options. Therefore, in Chapter 2 we conduct a systematic search to generate an overview of promising existing biomarkers in resectable EAC, potentially contributing to patient stratification and the development of new...
General introduction and outline of the thesis

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Figure 1. A schematic overview of the TNM stage in esophageal adenocarcinoma. HGD = high grade dysplasia, T = tumor, N = Nodes, M = Metastasis.

anti-cancer targets.

TREATMENT OF EAC PATIENTS

The treatment strategy of EAC varies according to stage.\textsuperscript{11, 12} For HGD or T1 tumors smaller or equal to 2 cm, endoscopic mucosal or submucosal resection and/or ablation is preferred.\textsuperscript{13}

Patients with T1 tumors with node-positive disease, and T2-4a tumors without distant metastasis are suitable for chemo-radiation treatment followed by esophagectomy, if the patient’s condition allows. Given the positive results of the CROSS trial (chemo-radiation-therapy for esophageal cancer followed by surgery),\textsuperscript{14, 15} which improved the median overall survival by 24 months, this regimen has become standard of care for resectable EAC patients in several countries. The CROSS regimen consists of five cycles of carboplatin and paclitaxel administered weekly, combined with fractionated radiation (daily 1.8 Gy on weekdays totaling 41.4 Gy), after which an esophageal resection is performed. The surgical procedure and the post-surgical consequences are intense; patients are restricted to eating small portions of food, avoid or limit certain types of food (fatty, acidic, alcohol), and are required to sleep at an angle of 45 degrees to avoid heartburn or reflux.\textsuperscript{16-18} Multiple (neo)adjuvant/perioperative treatment strategies are currently tested in clinical trials involving chemotherapeutics such as cisplatin, 5-Fluorouracil (5-FU), capecitabine, oxaliplatin, cisplatin, docetaxel, epirubicine.\textsuperscript{12, 19}

Patients with unresectable, non-metastatic disease (T4b) are eligible for definitive chemoradiation, which is a combination of 5-FU, cisplatin, paclitaxel, and radiation of a total dose of 50.4 Gy,\textsuperscript{20, 21} or the CROSS regimen extended with one week of treatment.\textsuperscript{22, 23}

Patients diagnosed with synchronous metastatic disease, or with metachronous metastasis
Chapter 1

following curative treatment, receive palliative care. The goal of palliative systemic treatment is to extend lifespan but also to improve or maintain the quality of life of patients by controlling dysphagia or other symptoms of disease. Various treatment options are currently available, but the optimal treatment strategy for first-line systemic palliative therapy is under debate and will most likely vary widely between EAC patients. Fluoropyrimidine and platinum combinations can be regarded as standard of care.24, 25 Beside the conventional cytotoxic agents, targeted therapy has become pivotal in the treatment of numerous cancer types. Unfortunately, compared to other cancer types, EAC is lagging behind in the development of targeted therapies.26 Thus far, only ramucirumab, a humanized antibody against VEGFR2 as a single agent or in combination with paclitaxel, is approved for second-line palliative systemic treatment and trastuzumab, a humanized antibody against HER2, is standard of care in combination with chemotherapy for first-line palliative systemic treatment of HER2-positive esophagogastric adenocarcinomas.28 The clinical value of the addition of trastuzumab to the trimodality CROSS regimen for treatment of HER2-positive EAC is currently tested in a phase III trial (NCT01196390).

HER2 (ERBB2, Neu) is part of the HER/ERBB-family of receptor tyrosine kinases (RTKs). Other members include the epidermal growth factor receptor (EGFR or HER1), HER3, and HER4.29, 30 Approximately 21-31% of EAC patients have HER2-positive tumors.31-33 The HER2 status of EAC is typically determined by the consensus method;34, 35 EAC tumor biopsies with strong staining of more than 10% of the tumor cells are defined as HER2 positive; IHC 3+. For EAC resection specimens more than 30% of the tumor cells have to be positive. In addition, all specimens scoring IHC 2+ (i.e. weaker staining than IHC 3+) are further examined by in situ hybridization to determine ERBB2 gene amplification status. IHC 2+ tumor cells with >6.0 copies of the ERBB2 gene are confirmed as HER2 positive cases, making these patients eligible for trastuzumab treatment. However, in the course of disease progression HER2 status may change, resulting in discordance in HER2 status between the primary tumor and metastases, thereby changing patient eligibility for trastuzumab treatment. Also, HER2 status may change under the pressure of chemo-radiation treatment, causing discordance between the biopsy at diagnosis and the resected tumor specimen. Therefore, in Chapter 3 we aim to examine HER2 discordance upon disease progression and therapeutic pressure by assessing HER2 expression in pre-treatment tumor biopsies, resection specimens, and metastases derived from EAC patients.

Models to study therapy resistance mechanisms in EAC

Despite recent advances in therapeutic strategies, including the CROSS regimen and the addition of trastuzumab for HER2-positive EAC, the survival benefit remains modest. This indicates an urgent need to study the underlying mechanisms of therapy resistance. To do so, adequate EAC models are required. Cancer cell lines are the most frequently used in vitro models in cancer research. For in vivo experiments, either mutation-driven mouse models or patient derived xenograft (PDX) models can be used. In EAC, however, only very limited tumor cell lines exist and due to the absence of a clear driver mutation sequence, genetic EAC mouse models are lacking.36 Moreover, due to inter-tumor heterogeneity the induced mechanisms of therapy resistance might differ between EAC’s pointing out the need to study these mechanisms in multiple models derived from different EAC patients. Therefore, in this
thesis we set out to develop new pre-clinical models for EAC. In Chapter 4 we show how we generate PDX models and primary cultures for malignancies of the upper GI tract, which are a valuable addition to the few currently available EAC cell lines to study treatment response and to identify mechanisms of drug resistance.

To overcome the limitations of two-dimensional cancer cell lines that adhere to and grow on plastic, more physiologically relevant three-dimensional in vitro culture models have recently been developed, such as the organoid culture system. Although this culture method is well established for various cancer types like colon, lung, and pancreatic cancer, organoids for EAC have not been described. Therefore, in Chapter 8 we aim to develop a 3D culture method for EAC and describe the establishment of primary EAC organoid cultures and use this system to identify and model mechanisms of therapy resistance.

**MECHANISMS OF THERAPY RESISTANCE**

Identification of the mechanisms that induce or mediate drug resistance is pivotal for the development of new and more effective anti-cancer treatment strategies. In this thesis we investigate three different mechanisms of therapy resistance; (i) compensatory signaling via upregulation of receptor family members, (ii) the induction of epithelial-to-mesenchymal transition (EMT), and (iii) resistance mediated by signals from the tumor microenvironment.

**Compensatory signaling by upregulation of receptor family members**

Activation of the HER-signaling pathway leads to enhanced cell viability and proliferation, contributing to the development and progression of various epithelial cancer types. HER-pathway activation is mediated via receptor phosphorylation, which is initiated by homo- or heterodimerization of receptors upon ligand binding to one of the receptors (Figure 2). Although no activating ligand is known for HER2, its most potent signaling is mediated by HER2-HER3 heterodimers following neuregulin-1β (NRG-1β) ligand binding to HER3, resulting in downstream AKT activation. Trastuzumab targets HER2 by mediating internalization of the receptor, hence preventing receptor dimerization. Clinical studies in breast cancer have shown the additive anti-tumor effect of trastuzumab combined with chemotherapy than chemotherapy alone. However, various mechanisms of trastuzumab induced resistance are reported in breast cancer including: (i) Truncated HER2 (p95HER2) which lacks its extra cellular domain and thereby preventing binding of trastuzumab to the HER2 receptor. (ii) Inactivating-mutations in the down-stream tumor suppressor gene PTEN (PTEN loss) which is reported in 36% of the HER2 positive breast cancer patients and results in a constitutively active HER2 signaling. (iii) Overexpression of MET or IGF-1R which are other tyrosine kinase receptors, to compensate for the by trastuzumab inactivated HER2.
Interestingly, when NRG-1β is bound to HER3, trastuzumab cannot prevent the formation of HER2-HER3 heterodimers. Moreover, despite the observed significant survival benefit in the ToGA trial, combining trastuzumab with standard chemotherapy in advanced-stage HER2-positive esophagogastric adenocarcinoma patients demonstrated modest survival benefit. Although an initial response was observed, patients ultimately all showed progressive disease. Since mutations in HER2 or its downstream signaling components are not common in EAE, we hypothesize that EAC tumor cells activate compensatory signaling by the upregulation of growth factor receptors other than HER2 following trastuzumab treatment, contributing to the observed progressive disease. In Chapter 5 we explore this mechanism and identify that resistance against trastuzumab is mediated by the upregulation of both the HER3 receptor and its ligand NRG-1β.

Induction of epithelial-to-mesenchymal transition as a mechanism of therapy resistance
The transition from an epithelial to a mesenchymal phenotype is characterized by an enhanced migratory capacity of cancer cells, resulting in the development of metastasis and thereby contributing to poor disease outcome. EMT has previously been found to be involved in trastuzumab-induced resistance in HER2-positive breast and gastric cancer. The mechanisms that induce EMT and its consequences may vary widely between and within tumor types. Its role in EAC has remained unexplored. We hypothesize that when relatively intense treatments are applied, such as trastuzumab combined with pertuzumab, or a triple treatment modality existing of carboplatin, paclitaxel and radiation therapy, EAC cells undergo EMT. In Chapter 6 we explore the effect of combined trastuzumab and pertuzumab treatment. In Chapter 7, we explore the mechanism of resistance following conventional chemo-radiation therapy and find that esophageal adenocarcinoma cells undergo EMT in response to both treatment regimens. Thus far, no clinically feasible treatment strategy has been identified to circumvent these mechanisms of acquired resistance. Therefore, our next aim is to identify the mediator of EMT and to investigate the possibility to target the inducer of EMT (Chapters 6 and 7).
Resistance mediated by signals from the tumor microenvironment

The mechanisms of therapy resistance as identified in Chapter 5-7 are mediated by the tumor cells themselves in a cell-intrinsic manner. However, increasing evidence exists for an important role for the tumor microenvironment (TME) in cancer development and progression.56 Cancer associated fibroblasts (CAFs) are the main constituent of the TME, and have been found to exert tumor-promoting activities by driving tumor invasion, angiogenesis, and drug resistance by their mechanical contributions to the stroma and by the secretion of cytokines.57 CAFs can be detected by the expression of smooth muscle actin (α-SMA) and their presence is associated with poor survival and disease progression in several cancer types including colon, breast and esophagus.58-61 The tumor-promoting activities of these cells vary between malignancies and heavily depend on the cytokines secreted.62 We hypothesized a role for the EAC TME in the development of therapy resistance. In Chapter 8 we aim to identify the cytokines produced by EAC associated fibroblasts by the isolation of primary EAC associated fibroblasts and explore their contribution to treatment resistance mechanisms in EAC.

In summary, the objective of the research as described in this thesis aims to identify and target the mechanisms of therapy resistance in EAC. For this purpose, we conduct a systematic review and meta-analyses to generate an overview of existing biomarkers in EAC. Using our developed PDX mouse models and primary cultures we identify three different mechanism of therapy resistance and their drivers. By targeting these drivers, we show an effective anti-tumor response in our models. Our findings can contribute to the development of more effective anti-cancer treatment regimen.