What tumor cells cannot resist
Ebbing, E.A.

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GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Eva A. Ebbing
Chapter 9

**General discussion and Future perspectives**

Despite the advent of multimodality treatment strategies against esophageal adenocarcinoma (EAC), its outcome remains dismal.¹ This is mainly due to high therapy resistance and aggressive tumor behavior leading to early onset of metastatic disease.² ¹ In this thesis we aimed to generate an overview of currently available prognostic biomarkers and to unravel the mechanisms of therapy resistance in EAC. In search for such mechanisms we identified new drivers of therapy resistance, which were targetable by currently available drugs, allowing for the development of novel effective anti-cancer treatment regimens against EAC.

**Biomarkers in esophageal adenocarcinoma**

The clinical relevance of identifying biomarkers to complement new treatment options is nicely illustrated. For example, HER2 is found to be prognostic biomarkers in breast cancer and drugs targeting the HER2 receptor are currently successfully used in the clinic.⁴ ⁶ Moreover, expression of the HER2 receptor can be used to predict treatment response.⁷ To summarize the recent development of biomarkers in resectable EAC, we conducted a systematic search and meta-analysis (Chapter 2). Interestingly, one of the most statistically significant biomarkers identified was the PDL-1/PD-1 signaling axis, which is a pivotal immune checkpoint; PDL-1/PD-1 signaling is often activated in cancer and inhibits the T-cell mediated anti-tumor response.⁸ ⁹ This pathway can be targeted by humanized monoclonal antibodies, such as nivolumab and pembrolizumab to re-activate the anti-tumor activity of T-cells.¹⁰ ¹³ These FDA approved targeting agents have been successfully tested as treatment options for melanoma and non-small lung cancer¹⁴ ¹⁶ and their role in gastroesophageal adenocarcinoma is currently under investigation in various clinical trials,¹⁷ ¹⁹ including a phase II feasibility trial adding atezolizumab to the conventional chemo-radiation therapy (PERFECT study, NCT03087864). It has to be noted that patients with low expression of PD-L1 may still benefit from this new treatment option.²⁰ Other predictive markers, such as mutational load, or microsatellite instability (MSI), may be more accurate for selecting patients for PD-L1 targeting.²⁰ This emphasizes the importance of not only identifying (prognostic) biomarkers, but also to understand their function in tumor biology.

We also identified non-immune related targets as markers of disease outcome, including oncogenic c-MET, IGFBP7, and the stem cell-associated receptor LGR5. These markers are found in other cancer types as well and inhibition of these markers have shown effective anti-cancer activity in pre-clinical in vitro and in vivo studies, making them interesting candidates as future anti-cancer targets in EAC.²¹ ²⁴ This is underscored by current clinical trials in various cancer types that investigate the clinical relevance of the above mentioned biomarkers as targets, including LGR5 targeting for patients with metastatic colorectal cancer (NCT02726334), and c-MET targeting for patients with advanced stomach or esophageal cancer (NCT02344810). Together, our overview of the recently published biomarkers in EAC could yield interesting leads for the development of new treatment strategies against EAC.

**HER2 in esophageal adenocarcinoma**

The most extensively studied biomarker in EAC is ERBB2 (HER2). The reported prognostic value of HER2 is still undecided: some studies find that HER2 expression correlated with poor patient survival²⁵, whereas others report HER2-positivity to be significantly associated
with improved survival, or no difference in survival at all. Nevertheless, targeting HER2 has led to a significant survival benefit in advanced stage HER2-positive esophagogastric adenocarcinomas, as shown in the randomized phase III ToGA trial. Furthermore, HER2 positivity correlated with the anti-tumor efficacy of trastuzumab: HER2 expression on the cell surface of the tumor cells correlated with survival benefit from the addition of trastuzumab. HER2 status, however, may change during disease progression, changing the eligibility of patients for trastuzumab treatment. In Chapter 3 we examined HER2 discordance upon disease progression in pre-treatment biopsies, neoadjuvantly treated resection material, and metastasis biopsies. Discordance was observed in 2.1% of the cases between the pre-treatment biopsies and neoadjuvantly treated resection material and in 3.3% of the cases between the resection material and metastasis. In both cases the positive-to-negative and the negative-to-positive conversions were equally distributed. This observed discordance seems to occur in a minority of cases, however, it still implies that under- or overtreatment of patients with trastuzumab is a clinically relevant problem. Therefore, we suggest that the HER2 status is best determined on the tumor material corresponding to the tumor stage to be treated. However, the location of the cancerous tissue might be not accessible for sampling and there might even exist discordance between different metastatic sites or regions within the primary tumor. Therefore, as future perspective, it would be of great clinical value to develop a non-invasive detection method for HER2-positivity, for instance by using contrast-based MRI techniques by adding a specific dye recognizing the target marker. Imaging-based techniques may enable detection of metastases with different molecular makeups and allow for adjustment of the therapeutic strategy accordingly. Another non-invasive strategy might be, as reported in breast cancer, measuring the cleaved extracellular domain of HER2 in serum. However, a high amount of serum HER2 can be interpreted in two ways; (i) either it indicates treatment resistance or ineligibility by reporting no cell surface-bound HER2 for trastuzumab to bind to, or (ii) it indicates that the patient is in fact highly HER2 positive and therefore would benefit from trastuzumab treatment. How this cleaved HER2 relates to treatment outcome in EAC remains to be explored in future studies.

Studying mechanisms of therapy resistance

Identification of the mechanisms that drive therapy resistance is critical to the development of effective new anti-cancer strategies. For instance, the compensatory upregulation of the growth factor receptor family member HER3 following HER2 inhibition using trastuzumab in mammary carcinoma has urged the addition of pertuzumab, which inhibits the formation of HER2-HER3 heterodimers. This treatment strategy has shown a significant survival benefit for HER2-positive breast cancer. Nevertheless, the treatment efficacy varies widely between cancer types. For example, the survival benefit for HER2-positive advanced stage breast cancer treated with trastuzumab is larger than for advanced stage HER2-positive esophagogastric cancer. Moreover, even within the same tumor type, different mechanisms of drug resistance can be found towards the same drug between patients, which suggests inter-tumor heterogeneity. This highlights the importance of studying mechanism of therapy resistance per specific cancer types and using multiple models. For the research described in this thesis, we generated patient-derived xenograft (PDX) models for EAC, primary adherent and 3D organoid cultures of tumor cells (Chapter 4), and EAC-associated fibroblasts (Chapter 8). PDX models have the advantage over in vitro cell cultures that tumor cells grow in a complex
system containing tumor stroma, including fibroblasts and blood vessels that mimics the human setting. The human stroma, however, is replaced by mouse stroma, which is both an advantage and a disadvantage. This makes it easier to delineate the contributions of the tumor cells (human) and the tumor microenvironment (TME) (mouse) by molecular methods. However, some ligands are incapable of signaling through the receptor of another species (in this case human versus mouse), including IL6 and HGF. We, in fact, showed that such proteins are important stroma-derived signals that induce therapy resistance (Chapter 8). Also, like the in vitro cultures, these PDX models lack an active immune system. Nonetheless, these models can be expanded by the addition of human immune system components, to generate more advanced models.34-36 Still, compared to in vitro cultures methods, PDX-models are the most complete system and therefore may function as patient avatars to study treatment response. However, for implementation in the clinic, the time from obtaining tumor material to outgrowth in mice and assessment of treatment efficacy of a panel of therapeutics will need to be shortened; for primary grafted tumors, the time for a tumor to grow out ranges between 3-9 months. For 3D organoid cultures, this is somewhat shorter at 2-6 months, but still considerable. A good alternative might be to subject tissue slices of resected EAC tissue ex-vivo to various treatment regimens and determine the most beneficial treatment combination for the patient. However, cells in these tumor slices have a very limited survival time and cannot be expanded. Therefore, this technique is unfit for the assessment of subsequent treatment regimens, effects upon long-term treatment, or unraveling mechanisms of resistance. For this, the PDX models, 3D organoid, and primary cultures remain the optimal tools to complement the very limited number of available EAC cells lines for preclinical studies.

Mechanisms of resistance following targeted therapy

One of the reported mechanisms of trastuzumab resistance in breast cancer is the maintenance of growth factor receptor signaling via the upregulation of HER3.37-39 Similarly, we found the compensatory upregulation of HER3 to occur and drive resistance in EAC (Chapter 5). Although in breast cancer this mechanism often includes receptor hetero-dimerization with other growth factor receptor members,33, 40 in our model we only found proof for HER2-HER3 heterodimers, maintaining growth factor receptor signaling in spite of the presence of trastuzumab. This signaling was mediated by an increased amount of HER3, through its ligand NRG-1β, which we found was activated by metalloprotease ADAM10. Unfortunately, there is currently no FDA approved drug against ADAM10 and more importantly, the activity of ADAM10 is not restricted to the cleavage of NRG-1β on cancer cells, thus making it an unfavorable target.41, 42 However, ADAM10 does hold potential as a predictive marker for trastuzumab efficacy in EAC patients. Other ADAM-family members, including ADAM12 and ADAM17 have already shown their value as prognostic markers in various cancer types, including breast and ovarian cancer.43-45 Nevertheless, targeting HER3 would therefore be a more attractive approach.

In light of the previously identified trastuzumab-induced resistance, pertuzumab has been added to trastuzumab in current clinical trials for treatment of HER2-positive gastroesophageal cancer (TRAP; NCT02120911, JACOB; NCT01461057). However, this treatment combination has not previously been tested in EAC cells in vitro, nor in mouse models. The same holds true for trastuzumab treatment, which can be administered for longer time periods of 6 months up to several years in the clinic, while the consequences of such extensive treatments have not been assessed preclinically. Therefore, we investigated if the targeted HER3-NRG-1β signaling axis
was maintained during long-term trastuzumab treatment (Chapter 6). Intriguingly, we found HER3 to be down-regulated, which was accompanied by an activation of another mechanism of therapy resistance; epithelial-to-mesenchymal transition (EMT). Strikingly, combining trastuzumab and pertuzumab led to the downregulation of HER2 and HER3 in the tumor cells and in fact accelerated the induction of EMT. This mechanism could be rescued by releasing therapeutic pressure or by reactivation of the HER2-HER3 signaling axes by adding NRG-1β. This suggests that patients with acquired resistance towards HER2-HER3 targeting agents might benefit from a drug holiday. The clinical challenge will be to monitor this process. This could be done by developing contrast-based MRI or PET techniques by adding a specific tracer for either HER2 or HER3, as previously suggested in this discussion. Clearly, the true value of drug holidays needs to be proven and continuing treatment with a lower dose has previously been shown to be more effective than a drug holiday with respect to treatment outcome in for instance metastatic colorectal cancer. To allow for continued drug exposure, we aimed to investigate the possibility of adding yet another drug to trastuzumab and pertuzumab. In order to identify the driver of HER2-HER3 inhibition mediated EMT, we used gene set enrichment analysis (GSEA) on a publically available data set generated by The Cancer Genome Atlas (TCGA) containing gene expression data of 89 resected EAC specimens. The samples were dichotomized by HER3 (ERBB3) expression, given its well-defined association with an epithelial cell state, and by using a gene set correlated with EMT, we found TGFβ1 as the most significant differentially upregulated gene of this set. Thus, we identified TGFβ as the inducer of EMT in EAC, which was confirmed in in vitro experiments. Interestingly, studying the sequence of EMT induction, we found that a sub-population of the cells were instructed to undergo EMT and obtained a faster growth rate, thereby slowly replacing all cells with an epithelial morphology in the culture. Moreover, cells produced TGFβ that allowed for the instruction of EMT in a paracrine manner. This implies that even the cells that were not instructed to undergo EMT in the first place, eventually would obtain the mesenchymal phenotype in response to the TGFβ present in the tumor. Mesenchymal-like tumor cells are thought to contribute to tumor progression by their increased migratory capacity and resulting propensity for metastasis, however, recent work has shown a chemo-protective role for EMT rather than metastatic dissemination. In fact, we found proof for both hypotheses in our models of EAC. The resistance against all chemotherapeutics tested could be explained by the observed upregulation of cancer stem cell related markers. Cancer stem cells are known to be more clonogenic, resistant to a broad range of cytotoxic agents, and have previously been linked to EMT as well. Also, targeting the inducer of EMT resulted in a rescued, non-migratory, epithelial phenotype. This suggests that, when inhibiting TGFβ signaling, the tumor cells will regain sensitivity to cytotoxic drugs. This is an important aspect, as trastuzumab and pertuzumab are combined with conventional chemo-radiation therapy in the clinic. Due to its essential role in the induction of EMT in several tumor types, various drugs targeting TGFβ are under development and currently studied in clinical trials. Interestingly, the recently published outcome of the JACOB study was a negative result. We suggest that these disappointing results might be explained by TGFβ pathway activation. Given these results and the availability of FDA-approved agents against TGFβ signaling, we believe that a triple combination of trastuzumab, pertuzumab, and a TGFβ inhibitor in combination with conventional treatment is a promising new anti-cancer treatment strategy against EAC.
Mechanisms of resistance following chemoradiation therapy

Besides developing resistance towards targeting agents, patients can develop resistance against conventional chemo- or chemo-radiation therapy. In Chapter 7 we unraveled on the mechanisms of resistance following conventional chemo-radiation according to the CROSS regimen. Of the patients receiving the CROSS regimen, only 23% of EAC patients showed a complete pathological response. 64 We showed that following an in vitro approximation of the CROSS regimen by incubation with carboplatin, paclitaxel and fractionated radiation, EAC cell lines and primary cultures showed evidence of EMT. As we discovered TGFβ as the mediator of therapy resistance by inducing EMT in Chapter 6, we hypothesized that EAC cells activated the TGFβ pathway as a response to chemo-radiation as well. Indeed, following this triple modality treatment, EAC cells were found to produce TGFβ, which was accompanied by an enhanced migratory capacity. Inhibition of TGFβ signaling by using a specific neutralizing antibody restored the epithelial phenotype and resulted in decreased cell migration. Strikingly, 48% of EAC patients develop recurrent disease following the CROSS regimen of which 67% presents with distant metastasis. 65 These numbers makes it tempting to speculate that following chemo-radiation therapy, a subset of cells undergo EMT, which eventually lead to metastatic disease. Together with the findings in Chapter 6 we propose clinical trials to assess the efficacy of the addition of a TGFβ targeting agent to conventional chemo-radiation therapy, and in case of a HER2-positive EAC, combined with trastuzumab and pertuzumab. In such trials, patients most likely to respond favorably to the addition of TGFβ targeting agents might be determined by studying proxies for TGFβ pathway activation, for instance by measuring pSMAD2 levels in tumor tissue, or circulating markers.

Studies on mechanisms of therapy resistance have mainly focused on tumor cell-intrinsic properties, the relevance of which has also been shown in this thesis. However, it is increasingly evident that the tumor microenvironment (TME) contributes to therapy resistance as well. 66, 67 While in other tumor types the tumor-promoting functions of the TME have been defined, 68-70 surprisingly little is known for EAC. 71, 72 The aim of Chapter 8 of this thesis was to elucidate the role of the TME in mediating therapy resistance in EAC. Since cancer associated fibroblasts (CAFs) comprise the majority of the TME, 73 we first developed primary EAC associated-fibroblast cultures, either human tumor or PDX-derived. Using these cultures we found that the supernatant of these CAFs induced EMT via the secretion of IL-6, activating the STAT3 pathway in the tumor cells. This was accompanied by an increased migratory capacity, upregulation of stemness-associated markers, and downregulation of markers related to an epithelial cell state, including growth factor receptors. Most strikingly, IL-6-induced EMT conferred resistance against a broad range of clinically used chemotherapeutics in EAC cell lines and primary cultures. This is in agreement with our findings in Chapter 6. From this we derive two important conclusions; (i) growth factor receptor signaling functions to maintain an epithelial, non-migratory, phenotype in EAC and targeting this pathway leads to EMT. (ii) There are multiple pathways leading to EMT in EAC, which all induce a chemo-resistant phenotype. The latter highlights the dynamic nature of EAC cell morphology in response to the extrinsic factors that are relevant in EAC, including high therapeutic pressure and the abundance of TME. Moreover, it has been reported that tumors with a homogeneous HER2-positive staining pattern are more differentiated and thus more epithelial compared to tumors with a heterogeneous HER2 staining. 74 This, combined with our finding in Chapter 3 that patients with HER2-positive tumors show a better overall survival compared to patients
with HER2-negative EAC, provides further evidence for our theory that growth factor receptor signaling pathway maintains the epithelial cell state in EAC.

Importantly, the EMT induced by stroma-derived IL-6 could be reverted by inhibition of IL-6 signaling using a neutralizing antibody. This, together with our findings described in Chapter 6 and 7, indicates that EMT is an eminently targetable mechanism of therapy resistance in EAC. The identification of stroma derived IL-6 as an inducer of chemo-resistance could have great clinical impact, given the availability of FDA-approved IL-6 targeting agents. However, not all EACs have the same TME content, leading to heterogeneous IL-6 levels between patients. Moreover, given the involvement of IL-6 in inflammation, it is not likely to be a very specific marker. To distinguish patients who could benefit from IL-6 targeting, we investigated stroma-related genes which were differentially expressed between IL6 high and low expressing EAC tumors and identified ADAM12 as a soluble serum marker that predicts treatment outcome in patients receiving the CROSS regimen. Future work should focus on assessing the value of ADAM12 as a marker for TME content in EAC and possibly allowing selection of patients for targeting of stromal components. Moreover, ADAM12 serum levels may change following the course of disease progression or upon different treatment strategies, which may allow response monitoring. This should be addressed in future studies using a larger patient cohort.

Concluding Remarks
The results of this thesis shows that, upon therapeutic pressure, EAC cells are able to activate various mechanisms of resistance, making it challenging to treat EAC patients in a uniformly effective manner. Furthermore, limited treatment options are currently available for the treatment of EAC, which contributes to the poor overall survival of these patients. In this thesis we unravel therapy resistance mechanisms and identify biomarkers, hopefully enabling the development of more effective treatment options. Especially the identification of therapy-induced EMT is likely to open new avenues for treatment of EAC patients. The development of in vitro and in vivo EAC models, such as the PDX and novel primary organoid cultures described in this thesis provide excellent tools to further unravel mechanisms of drug resistance. Taken together, the new findings described in this thesis can guide the development of new anti-cancer treatment strategies directed against EAC and are -due to availability of FDA approved drugs for most of the targets identified- ready to be used in clinical trials.
References

28. Bang YJ, Van Cutsem E, Feyereislova A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal...
37. Lyu H, Yang XH, Edgerton SM, et al. The erbB3-...
36. Chung YS, Son JK, Choi B, et al. Transplantation of...
35. Li Y, Mention JJ, Court N, et al. A novel Flt3-deficient...
34. Lopez-Lastra S, Di Santo JP. Modeling Natural Killer...
33. Gu G, Dustin D, Fuqua SA. Targeted therapy for...
32. Baselga J, Cortes J, Kim SB, et al. Pertuzumab plus...
28. Moya-Horno I, Cortes J. The expanding role of...
15. Moya-Horno I, Cortes J. The expanding role of...


