New insights in optimizing treatment and the role of cancer stem cells in esophageal cancer
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Chapter 8

Summary, discussion and future directions
SUMMARY

The incidence of esophageal cancer (EC) is still increasing and the overall 5-year survival at the time of diagnosis is 15-20%. In the Netherlands an increase of esophageal adenocarcinoma (EAC) is seen, while esophageal squamous cell carcinoma (ESCC) more often occurs in Asian countries. Barrett’s esophagus (BE) is a known precursor lesion of EAC. BE is a metaplastic condition in which the normal esophageal squamous epithelium is replaced by a columnar epithelium, that can progress to dysplasia and eventually in EAC. However only a small percentage of BE patients progress towards EAC and since we have no markers that predict progression, patients are offered regular endoscopic surveillance. The standard treatment for EC patients without distant disease consists of neoadjuvant chemoradiotherapy (CRT) followed by radical surgery. However, despite many efforts the 5-year survival with optimal treatment is still around 50%, and therefore further improvement of EC therapy is urgently needed.

The high recurrence rate in patients undergoing optimal therapy indicates a failure to eradicate all tumor cells. Even patients with a pathological complete response (pCR) to neoadjuvant CRT, without detectable vital tumor cells neither in the primary tumor nor in regional nodes of the resection specimen, can relapse shortly after treatment. This implies that low numbers of tumor cells already have metastasized even before treatment at an early stage of disease, illustrating their aggressive properties. Tumor progression, chemoresistance and the ability to metastasize early are characteristics that have been attributed to cancer stem cells (CSCs). CSCs are a subset of cancer cells with stem cell characteristics, such as the potential to self-renew and multilineage differentiation capacity. CSCs are more tumorigenic than bulk tumor cells and may sustain current treatment, leading to rapid relapse, as seen in some EC patients. A subset of CSCs have also been proposed to have features of epithelial to mesenchymal transition (EMT), that lead to a more mesenchymal state enabling CSCs to invade and metastasize (1). The elucidation of a possible CSC subpopulation in esophageal cancer is important as it could provide new directions for treatment. CSCs were initially discovered in leukemia, and later on also in solid tumors, including breast and colon cancer. The Wnt pathway is particularly known to be essential in maintaining both normal stem cell (SC) and CSC properties in the intestine (2,3). The Wnt pathway and its downstream targets, including the membrane proteins CD44 and Lgr5, were found to be suitable markers for detecting and isolating CSCs in colorectal cancer (4,5). In esophageal cancer, studies to identify CSCs are limited and most studies have been performed in ESCC. In EAC,
the presence of a CSC subpopulation has not been convincingly demonstrated, which is related to a current lack of specific CSC markers in esophageal cancer.

In order to improve outcome of EC patients, this thesis describes options to optimize current treatment modalities and the use of EAC cell lines as a potential model to study CSCs. Moreover, it provides new insight in prognostic markers for survival and malignant progression in esophageal cancer.

In chapter 2 and 3 the optimization of current treatment in esophageal cancer was investigated by determining the optimal definition of a tumor free circumferential resection margin (CRM) related to outcome and by comparing survival and toxicity in two definitive CRT regimens.

Involvement of the CRM has previously been associated with a poor prognosis but the optimal cut-off was not yet defined (6). In chapter 2 the optimal cut-off for the CRM and its prognostic significance was determined. In this study we defined the optimal cut-off at a CRM ≤1mm and showed that a tumor positive CRM, is an independent prognostic factor for recurrence and survival. Moreover, patients with a positive CRM had a similar prognosis as patients with a positive longitudinal resection margin (R1-resection). Therefore a positive CRM should be clinically considered as a R1-resection.

When patients are not eligible for surgery, either due to technical irresectability of the tumor or because of a relative high comorbidity, definitive chemoradiation (dCRT) is often the curative treatment of choice. Cisplatinum/5-FU is the current used standard chemotherapy according to European and American guidelines, but is known to have a relative high toxicity profile (7,8). Carboplatin/paclitaxel is generally preferred in case of cardiovascular and/or pulmonary comorbidity because it has fewer side effects. This regimen is also used as neoadjuvant CRT scheme and was applied in the CROSS trial. The aim of chapter 3 was to retrospectively analyze the differences in survival and toxicity between the two above mentioned dCRT regimens in five centers in the Northeast Netherlands. No differences were found in overall survival (OS) and disease free survival (DFS) between both regimens. However, toxicity rates were significantly lower in the carboplatin/paclitaxel group and treatment compliance was significantly higher compared to the cisplatinum / 5-FU group. This suggests that carboplatin/paclitaxel could be considered as a good alternative for cisplatinum / 5-FU as dCRT in esophageal cancer patients.

In chapter 4, 5, 6 and 7 the aim was to gain a better understanding of esophageal
carcinogenesis by investigating the presence of CSCs in a cell line model, and by investigating several new possible prognostic markers. The CSC model has been investigated in various cancers, and might also hold true for esophageal cancer.

In chapter 4 we investigated the potential of CSC enrichment in an in vitro three dimensional spheroid cell culture model. Previously, these culture conditions have been reported to induce de-differentiation and facilitate growth of CSCs in other tumor types (9). The EAC cell line OE19, when cultured as spheroids under serum-free conditions, has enhanced CSC properties as determined by spheroid formation and chemoresistance assays in vitro and by increased tumor growth in vivo. This enrichment for CSC characteristics was not seen in another spheroid-cultured EAC cell line, OE33S, when compared to monolayer OE33 cells. However, following implantation in NOD/SCID mice mainly spheroid-cultured OE19S, but also spheroid-cultured OE33S cells displayed enhanced tumor growth compared to monolayer-derived xenografts. We proceeded by studying differences in mRNA expression in spheroid and monolayer cultured OE19 and OE33 cells, as well as in the derived tumorgrafts, using an Illumina array. Particularly, two genes were found to be altered in both OE19 and OE33 models, KLF2 and C-FOS. Furthermore, Gene Set Enrichment analysis showed downregulation of DNA regulation and cell adhesion pathways. Monolayer and spheroid-derived xenografts did not show significant differences, in contrast to the in vitro findings, what may be caused by prolonged exposure to a similar mouse microenvironment. This study indicates that the OE19 spheroid model possesses CSC characteristics, such as chemoresistance and enhanced tumor growth in vivo, and might be used to study CSCs in EAC. Further characterization of the OE19 spheroid model should include limited dilution transplantation assays in vivo to investigate its propagation potential.

GATA6 is a transcription factor, important in intestinal development and a suggested oncogene in esophageal cancer (10,11). GATA6 has also been implicated in Wnt signalling in pancreas and colon cancer (12,13). In esophageal cancer, GATA6 gene amplification has been associated with the progression of BE towards EAC and has been implicated as a prognostic factor for EAC patients (11,14). In chapter 5 GATA6 was analyzed during the sequence of malignant progression from normal squamous cell epithelium, towards the metaplastic precursor lesion BE and EAC. GATA6 protein expression was upregulated during the progression from the normal squamous cell epithelium towards BE, low grade dysplasia (LGD) and finally high grade dysplasia (HGD) and EAC. No correlation was found between GATA6 expression and survival of EAC patients. However, the role of GATA6 in malignant transformation of BE
towards EAC deserves further investigation.

In **chapter 6** we hypothesized that if CSCs are responsible as well for tumor relapse, CSC markers might serve as prognostic markers. Therefore the expression of several potential CSC markers was examined. Expression of Axin2 and CD44, both targets of the Wnt pathway, and the expression of other proposed CSC markers; ALDH1, Bmi-1 and SOX2 were determined in 94 EAC patients, treated with only surgery alone. Both, loss of CD44 and of SOX2 showed to be a prognostic factor for a poor survival in EAC patients (HR 1.73 CI 1.00-2.96 P=0.046 and HR 2.06 CI 1.14-3.70 P=0.016). ALDH1 and Axin2 were also inversely correlated with outcome in a univariate analysis but did not remain significant in a multivariate analysis. Nowadays patients are treated with neoadjuvant chemotherapy, however, prognostic and predictive markers for response to therapy are lacking for this group. Therefore in **chapter 7**, the expression of CD44, SOX2 and the Hedgehog ligand Sonic Hedgehog (SHH) was investigated in relation with prognosis and response to therapy in EC patients treated with neoadjuvant CRT followed by surgery. SHH has been previously suggested to have a role in radiotherapy resistance in esophageal cancer (15). While no relation was observed between these markers and response to neoadjuvant CRT, loss of CD44, SHH and SOX2 was associated with either a poor survival or with recurrence in EC patients. In our cohort loss of CD44 expression in post-treatment material of non-complete responders, was an independent prognostic factor for poor disease free survival (DFS) and cancer specific survival (CSS) (DFS: HR 2.81 CI 1.45-5.45 P=0.002 and CSS: HR 3.48 CI 1.70-7.16 P=0.002). Loss of SHH in pre-treatment biopsies was also an independent prognostic factor for poor survival (HR 2.27 1.05-4.89 P=0.036). Loss of SOX2 in pre-treatment biopsies was related to recurrence (P=0.029) and showed a trend for a worse survival (DFS: HR 1.99 1.04-4.89 P=0.039 and CSS: HR 2.16 1.08- 4.34 P= 0.030). However, this was not significant in a multivariate analysis, possibly due to the lower patient number in this cohort. The potential of these markers to be used for prognosis is of interest and should be further validated in different cohorts and with functional assays.

**DISCUSSION AND FUTURE DIRECTIONS**

Given the poor prognosis of EC patients, extensive research is required to improve the survival of patients. Even with current curative intended treatment, consisting of neoadjuvant CRT with radical surgery, only half of EC patients will survive longer than 5 years. One important approach to improve survival is through better understanding of EC oncogenesis and disease progression in order to develop novel targeted
therapies. Besides focussing on new treatment modalities, optimization of current treatment is also an important approach to improve outcome. For several decades, surgery alone (through a transthoracic esophagectomy) was the only curative treatment option, until recent studies including the Dutch CROSS trial showed a significant survival benefit of neoadjuvant CRT (16). By increasing the 5-year survival from 40% to around 50%, this approach is nowadays considered standard of care. Optimizing current CRT regimens for both neoadjuvant and definitive treatment modalities may lead to better outcome and reduced toxicity for EC patients. This can be achieved by more accurate radiotherapy and by improving chemotherapy or adding new targeted therapy. In this thesis a retrospective study of two different dCRT regimens showed that the carboplatin/paclitaxel scheme has a significantly lower toxicity with equal survival compared to the current standard cisplatinum/5-FU regimen. Although preferable this alternative treatment has to be evaluated in a randomized clinical trial. However in case of small patient numbers such as in dCRT in esophageal cancer, retrospective studies also could give sufficient insights in the tolerability and adequacy of current regimens. Consequently, as carboplatin/ paclitaxel gives a reduction in toxicity without influencing survival outcome this regimen should be considered as a better alternative.

Stratifying patients in risk groups that might benefit from additional treatment could also lead to improved survival. However adjuvant therapy after neoadjuvant CRT is not applied in ongoing clinical trials in EC patients. Patients with pathologically positive CRM or nodal involvement, as shown in this thesis, have a bad prognosis and perhaps adjuvant treatment would be beneficial in these high-risk groups.

Characterizing CSCs in esophageal cancer
A better understanding of the CSC model in esophageal oncogenesis and disease progression may provide new leads for targeted therapy. While several markers have been reported to associate with esophageal CSCs, a definitive characterization of CSCs in EC has not yet been clearly established. CD44, CD90 and p75NTR have been suggested to enrich for CSC-like cells in esophageal cancer (17-19). However, a broad panel of possible CSC markers, including CD44 and CD24, were tested by Grotenhuis et al. using primary patient material, but neither of these markers enriched for cells with enhanced tumorigenicity in vivo (20). Besides the use of markers, functional assays can also enrich for CSCs. The side-population assay, based on Hoechst exclusion assay, enriched for EC cells with CSC-characteristics demonstrated by enhanced tumorigenicity in vivo (21). In this thesis, three-dimensional spheroid culturing of the EAC cell line OE19 in serum-deprived medium
enhanced CSC characteristics. Therefore this model may be useful to further explore the CSC model in EAC, although further characterization of the OE19 spheroid model is required, including limited dilution assays in vivo. In addition, we found upregulation of C-FOS and KLF2 in the spheroid model and RNA interference-based knockdown of these genes and subsequent testing the effect on spheroid formation in vitro and tumor forming ability in mice, will establish their possible involvement in these CSC characteristics. However, we observed that spheroid growth does not always induce CSC characteristics, because spheroids grown from the OE33 cell line did not show significant enhancement of such characteristics in vitro. Perhaps medium-induced reprogramming will not be possible in all cancer cells, or cells may already have a CSC phenotype that cannot be enhanced further. Therefore caution should be taken when using spheroid models as CSC models and each spheroid model should be characterized independently. To further explore the CSC model in EC, the use of primary tumor cells derived from fresh patient material might provide a better model system. However, the use of neoadjuvant CRT has strongly reduced the presence of vital tumor cells in resected specimens, while the number of cells obtained from pre-treatment biopsies is often limited, complicating the generation of novel primary cell culture models. An alternative approach to study CSCs in EAC could involve mouse models that mimic EAC development. In a transgenic BE mouse model overexpressing interleukin-1-β (IL-1-β) and crossed with a Lgr5-Cre-ERT/Rosa-LacZ reporter mouse, Lgr5 positive cells were shown to migrate from the cardia and were found in the metaplastic Barrett’s epithelium (22). The combination of lineage tracing of labelled stem or progenitor cells in the normal squamous epithelium together with an inducer of malignant progression (such as IL-1-β overexpression), could be another approach to identify potential mechanisms in CSCs driving EAC development.

**Wnt target genes as favourable prognostic factor**

Markers and pathways that are involved in CSCs and EMT, such as the Wnt and Hh pathway, could be potential novel therapeutic targets in several cancer types, including esophageal cancer. Since activated Wnt signalling has been reported to play a role in the progression of dysplasia towards EAC (20,23), and is also well known for regulating CSCs properties in other cancers, particularly colon cancer, we hypothesized that expression of Wnt target genes such as CD44 and Axin2 might associate with a worse prognosis in EC patients. However, in this thesis, we observed in two independent patient cohorts that low expression of CD44 was related with a worse prognosis. This finding may be explained by several reasons. First, it might be that these particular Wnt target genes are not associated with CSCs in EAC. As
mentioned, Grotenhuis et al. did not find a relation between, amongst others, CD44 expression and higher tumor propagating potential in vivo (20). Second, epigenetic mechanism might play a role in the expression of Wnt-target genes. In colorectal cancer, high Wnt activity correlates with CSCs in preclinical models, while in patient material high Wnt target gene expression was correlated with a good survival (24). To unravel this apparent discrepancy de Sousa et al. found that Wnt target genes that are part of a CSC gene signature in colorectal cancer cells are silenced by methylation during disease progression, and this is associated with the acquisition of an overall more immature stem cell phenotype of the tumor cells (24). Interestingly, use of a demethylating agent in vitro and in xenografts led to reduced colorectal cancer tumor growth that could be explained by restored expression of previously methylated Wnt target genes, particularly those known to have an inhibitory feedback function on Wnt signalling, such as Axin2 (24). Hypermethylation of Wnt-inhibitory proteins in esophageal cancer has also been described in several cell lines and in patient material (25,26). Whether the methylation status of Wnt target genes is also predictive for survival in EAC remains to be investigated. Other types of epigenetic regulation, in particular microRNA's may also be involved in determining Wnt activity. For example, the transcription factor GATA6 could directly regulate Lgr5, and GATA6 expression on its turn was epigenetically regulated by microRNA-363 (12). Also microRNA-145 showed to regulate both GATA6 and BMP4 expression in BE (27). Third, the CSC compartment might be heterogeneous, encompassing different subsets of CSCs and these subsets could possibly be driven by different pathways. Wnt target genes might be related to CSCs that initiate tumor growth, whereas other CSCs driven by different pathways are involved in tumor progression and metastatic disease. In the esophagus, the Wnt pathway is important both during embryological development as well in the malignant progression from Barrett’s metaplasia towards dysplasia and EAC (20,23). While membranous β-catenin is present in both the normal esophageal squamous epithelium and in Barrett’s metaplasia, activation of the canonical Wnt pathway as determined by nuclear translocation of β-catenin only occurs during the malignant transformation of BE towards low-grade dysplasia (LGD) and high-grade dysplasia (HGD), with no further increase of nuclear translocation of β-catenin in EAC (20,23). A similar peak in expression at HGD was observed for other Wnt targets such as Cyclin D1 and SOX9 (23). The activation of the Wnt pathway could partly be explained by downregulation of Wnt antagonist such as the Wnt-inhibitory protein 1, and via upregulation of Wnt agonists such as the Wnt ligand Wnt2 (25,28). In contrast to colorectal cancers, where APC mutations are an important cause of dysregulated Wnt signalling, such mutations are rare in EAC (29,30), suggesting a different mechanism of activation of Wnt signalling. Similar as in colon cancer,
in EC the Wnt pathway might play a role in tumor initiation but perhaps another pathway driving a different subset of CSCs may lead to progression or recurrence, hence that are not characterized by Wnt signalling and high expression of Wnt target genes such as CD44. In the migratory CSC (MCSC) concept the heterogeneity within the tumor is explained by a subset of CSC cells that is able to metastasize (31). To investigate a possible dual role of the Wnt pathway in EC it would be an interesting approach to use the Wnt reporter system TCF/LEF promoter coupled to green fluourescent protein, which was used for identifying Wnt driven CSCs in colon cancer (32). Such an approach would allow the comparison between Wnt-high and Wnt-low fractions with regard to tumor propagating potential in vivo as well as their metastatic capacities. Fourth, cell lineage specific differences may affect the outcome of activated Wnt signalling through β-catenin. For instance, in melanoma β-catenin could act as a suppressor of invasion by activation of a melanoma specific protein (33), and interestingly loss of β-catenin expression being part of a seven-marker signature was a negative predictor of survival in melanoma patients (34). In conclusion, the possible favourable prognostic role of Wnt target genes in EC might be explained by several mechanisms, such as epigenetic modulation of target genes, CSC heterogeneity within the tumor or cell lineage specific variation in signalling outcome, which should be further explored.

GATA6 in malignant progression
The role of GATA6 in malignant progression of EAC is of particular interest, since a recent study showed that GATA6 could directly regulate Wnt signalling by binding to the Lgr5 promoter and also repress bone morphogenetic protein 4 (BMP4) expression, thereby regulating the expansion of the stem cell compartment in colon adenoma's (35). In this thesis an upregulation of the expression of the transcription factor GATA6 during malignant progression towards EAC was found. GATA6 is known to be important for the proliferation and differentiation of intestinal cells in mice (10). In colon adenoma's, GATA6 deletion was found to reduce adenoma formation in a mouse colon adenoma model leading to increased survival of the mice (35). Analyses of the underlying mechanism revealed that GATA6 competes with the β-catenin/TCF complex for binding the BMP4 promoter, causing reduced BMP4 expression and thereby expansion of the tumour stem cell compartment (35). In concordance with these results, in this thesis GATA6 expression was observed in the bottom of crypts that expanded in the progression towards dysplasia. Perhaps upregulation of GATA6 could play a role in decreased BMP4 signalling in progression towards dysplasia. A decrease in BMP4 expression has been observed in EAC compared to BE (36), but the interaction of BMP4 and GATA6 in malignant progression of BE remains to
be elucidated. Interestingly in this context, we observed an upregulation of GATA6 in esophagitis in chapter 5. Although speculative, GATA6 could perhaps be induced in the stem cell compartment of the normal esophageal epithelium by inflammation and play a role in the development of BE and subsequently lead to enhanced Wnt-signalling seen later during malignant progression of BE towards dysplasia.

**Therapeutic targeting of Wnt and Hh pathways**

Clarifying the role of Wnt in BE and esophageal cancer is also of importance with respect to the use of Wnt signalling inhibitors as a therapeutic strategy. Several inhibitors have been developed and tested in preclinical in vitro and in vivo models, and moreover, are currently tested in clinical trials. Porcupine is an enzyme necessary for secretion of the Wnt ligands and its inhibitor LGK974 was shown to effectively target the Wnt pathway and led to reduced tumor volumes of Wnt-driven tumors in vivo (37). This drug is currently tested in a phase I trial for malignancies depending on Wnt ligands (38). A second Wnt antagonist that competes with Wnt-ligands for binding to the Frizzled receptor is OMP-54F28 (FZD8-Fc), currently undergoing phase 1 testing (39). In a follow-up on the preclinical work with a demethylation agent in colon cancer by de Sousa et al., a clinical study is currently ongoing in colon cancer patients that are preoperatively treated with the demethylation agent decitabine to investigate if Wnt target gene expression is increased in resected tumors compared to preoperative biopsies (40). Whether these agents may have efficacy in EC remains to be investigated.

The Hh pathway might also hold promise as a new target for treatment in EC, since Hh pathway activity has been associated with CSCs EC as was mentioned earlier above. Inhibition of Hh signalling by cyclopamine, a natural SMO-inhibitor, led to a reduction of CSCs in several tumor types, such as glioblastoma and pancreatic cancer (41-43). In the normal esophageal epithelium both absence and presence of Hh ligands as SHH have been described (44-46). In addition, several studies describe a clear upregulation of Hh-target gene expression in ESCC and EAC patients (45,46). In ESCC and EAC cell lines treatment with cyclopamine and the oral Smo-antagonist, BMS-833923, reduced cell growth and induced apoptosis (15,45,47). Furthermore, Hh inhibition sensitized for radiotherapy in EAC cells, suggesting that Hh signalling might have a role in therapy resistance (15). A phase I trial with BMS-833923 in combination with cisplatinum and capecitabine in inoperable EAC patients has been performed and publication of the results is awaited (48). In this thesis the potential of the Hh-ligand SHH as predictive and prognostic marker in patients treated with neoadjuvant CRT was evaluated. SHH was not a predictive
marker for neoadjuvant chemoradiotherapy in our cohort, but low SHH expression did correlate with a worse patient survival. The mechanisms underlying this finding are currently unclear, however, similar mechanism may be involved as mentioned earlier for the Wnt pathway, including cell lineage dependent differences in function and heterogeneity in the CSC population. Better insight in the role of the Hh pathway in esophageal cancer development and disease progression is therefore required.

Conclusions
Optimization of current treatments has shown to improve prognosis and outcome for esophageal cancer patients. An accurate defined CRM had a significant impact on prognosis, while carboplatin/paclitaxel, as dCRT regimen appeared to be a better tolerated regimen with similar survival outcome compared to cisplatinum/5-FU. In the future, prognostic factors as loss of CD44 or SOX2 might be of help in stratifying patients eligible for adjuvant therapy. Moreover, the OE19 spheroid model has some CSC characteristics and further validation of this model is therefore of interest. Identification and the role of CSCs in EAC is a promising strategy to further improve survival in EAC patients. In particular the possible role of the Wnt and Hh pathway in relation to CSCs in esophageal cancer should be clarified which may lead to novel therapeutic strategies.
REFERENCES

(9) Lee J, Kotliarova S, Kotliarov Y, Li A, Su Q, Donin NM, et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 2006 May;9(5):391-403.


