Targeting cancer stem cells: Modulating apoptosis and stemness
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Chapter 1

General introduction
Introduction to CRC

There are more than 200 different types of cancer and those that originate in colon, rectum or small intestine are collectively called colorectal cancer (CRC) \(^1\). CRC is the third most common cancer worldwide with nearly 1.4 million new cases in 2012 \(^2\). Fearon and Vogelstein suggested already 25 years ago that sequential accumulation of mutations leads to CRC \(^3\). The so called Vogelgram summarizes mutations and epigenetic changes that frequently occur at different phases of tumor development in CRC, including mutation in \(APC\), \(KRAS\) and \(TP53\) (Figure 1). It is important to mention that this proposed order of mutations is useful but not observed in all CRCs as there is considerable variation in the number of mutations and also the order in which mutations occur can differ between patients \(^4\), \(^5\).

**Figure 1: Vogelgram summarizing (epi-)genetic changes leading to transition from normal tissue to carcinoma.**

Mutations in *adenomatosis polyposis coli* (\(APC\)) is seen in approximately 80% of the CRC patients and is thought to be the first step in CRC tumorigenesis in some patients \(^6\), \(^7\). This tumor suppressor gene is a key player in the WNT signaling pathway. In the absence of any WNT ligands \(APC\) forms, together with glycogen synthase kinase \(3\beta\) (GSK\(3\beta\)), Axin and casein kinase \(1\alpha\) (CKI\(\alpha\)), a destruction complex that tightly regulates protein levels of the transcriptional co-activator \(\beta\)-catenin. The destruction complex phosphorylates \(\beta\)-catenin, which directs binding to the E3 ubiquitin ligase \(\beta\)-TrCP resulting in ubiquitination and degradation of \(\beta\)-catenin. However, when WNT ligands are present, these interact with their cognate receptors Frizzled and LRP5 or LRP6 inducing Frizzled-LRP5/6 complex formation. Conformational changes in these receptors allow LRP phosphorylation followed by inhibition of GSK3\(\beta\) by Dishevelled and binding to Axin. The resulting inhibition of the destruction complex, allows for accumulation of cytoplasmic \(\beta\)-catenin due to a failure to phosphorylate and subsequent
translocation to the nucleus. Here nuclear β-catenin interacts with T-cell factor (TCF) / lymphoid enhancer-binding factor (LEF) transcription factors, and induces transcription of target genes including LGR5, Cyclin D1, and C-MYC (Figure 2). Mutations in the APC gene lead to decreased activity of the APC tumor suppressor gene and result in activation of WNT signaling pathway in the absence of ligands, leading to increased cell proliferation. Therefore APC mutations contribute to the transition of normal epithelium to early adenomas (Figure 1). In a recent study it was shown that restoring expression of APC in an APC knock down mice results in regression of tumors even when additional mutations are present. This provides evidence that APC mutations are not only important for tumor initiation but also for CRC maintenance.

**Figure 2: WNT signaling pathway.** WNT ligands induce accumulation of β-catenin and thereby increased transcription of target genes including LGR5. See texts for detailed information. Abbreviations: Dsh: Dishevelled, β-cat: β-catenin.
The Vogelgram indicates that \textit{APC} mutations are followed by activating mutations in the \textit{Kirsten Rat Sarcoma (KRAS)}. \textit{KRAS} mutations contribute to the transition from early to late adenoma and is reported in 30-50\% of CRC patients \(^7\), \(^12\), \(^13\). \textit{KRAS} is a one of the key players in the EGF pathway. This pathway is activated when a ligand like epidermal growth factor (EGF) binds to its receptor e.g. EGFR. This pathway ultimately leads to activation of ERK and ELK1 that stimulate cell proliferation \(^14\). The relevance of \textit{KRAS} mutations in CRC is recently studied in mouse intestine by inducing \textit{KRAS} mutations. Activating \textit{KRAS} mutations give mutated stem cells a competitive advantage over their wild-type (WT) counterparts, making the chance that WT cells are replaced by \textit{KRAS} mutant cells higher \(^15\). In line with this it was also shown that activating \textit{KRAS} mutations in stem cells increase crypt fission, spreading the mutation throughout the intestinal lining. This process makes it possible that \textit{KRAS} mutant cells can expand beyond the crypt border \(^16\). These studies provide evidence that \textit{KRAS} mutations are advantageous during CRC development.

In the Vogelgram \textit{KRAS} mutations are followed by mutations in \textit{TP53} gene. Bi-allelic inactivation of \textit{TP53} contributes to late stage adenoma to malignant carcinoma development \(^12\). In CRC \textit{TP53} is often inactivated by a loss of heterozygosity (LOH) of the chromosome 17p and a somatic mutation in the remaining allele. \textit{TP53} mutations are found in approximately 60\% of CRC patients \(^7\). Under physiological conditions, the p53 protein is pivotal in maintaining genome integrity and in inducing apoptosis or senescence in cells damaged beyond repair. In response to DNA damage p53 is stabilized by ATM and regulates the expression of several genes involved in cell cycle control that lead to G1 or G2/M cell cycle arrest. If the DNA cannot be repaired p53 will induce a stable cell cycle arrest (senescence) or apoptosis in these cells \(^17\), \(^18\). In \textit{TP53} mutant cells this tumor suppression mechanism is impaired and despite mutations cells will progress through the cell cycle leading to aneuploidy and ultimately to more advanced disease \(^19\). The above mentioned \textit{APC}, \textit{KRAS}, and \textit{TP53} mutations contribute to the progression from normal epithelium to adenoma to carcinoma (Figure 1).

In addition to the classical \textit{APC-KRAS-TP53} CRC progression sequence discussed above CRC is also typified by epigenetic changes. These changes reversibly affect gene activity and the best described mechanism in CRC is CpG island methylation. During the process of methylation a methyl group (CH\(_3\)) is added by DNA methyltransferases on cytosine residues. Methylation of promoters occurs at sites were cytosine (C) is followed by a guanine (G), hence the name CpG methylation \(^20\). This methylation occurs in so called CpG islands and results in a closed chromatin structure that is correlated with decreased transcriptional activity \(^21\). In CRC CpG methylation of genes has
been shown to induce tumor progression and in fact certain CRCs are characterized by methylation of a wide variety of CpG islands, the so called CpG island methylator phenotype (CIMP). Tumors that belong to this group have been suggested to develop along a separate tumorigenic pathway, but strong evidence for this is still lacking. Nonetheless, there are several distinct pathways described leading to development of CRC. In one of these pathways the $MLH1$ gene is silenced by methylation. This gene encodes for a DNA mismatch repair protein and silencing gives rise to tumors with high mutation rate. These patients are classified as microsatellite instable (MSI) and tumors have different features compared to non-MSI tumors including high T-cell infiltration and a more mucinous presentation \(^{22-24}\). Importantly, also this subset of CRCs contains a hereditary form called Lynch syndrome in which patients carry germline mutations in these DNA repair genes.

**Inter- and intra-tumoral heterogeneity in CRC**

The Vogelgram describes that progression is associated with an accumulation of mutations \(^3\). However, when patients present with CRC in clinical practice, most of these mutations will already have occurred and the actual staging is performed based on macroscopic and microscopic assessments of the tumor. Patients of which the cancers have not invaded beyond the submucosa or muscularis propria are classified as stage I. In stage II cancer cells invade through muscularis propria into pericolorectal tissues. In contrast to stage I and stage II in stage III lymph node metastasis are observed. In stage IV distant metastasis are present in for instance liver, lung, ovary, or nonregional nodes \(^25\). Staging is crucial for therapy choice and directly correlates with 5-years survival. For examples, stage I patients have a 5-years survival of approximately 90%, which is less than 10% in stage IV patients. Despite the fact that stage II patients have a localized disease approximately 20% of these patients will eventually progress upon successful surgery either showing local recurrences or distant metastases \(^26\). There is a big effort to identify these poor prognosis patients and prognostic tests have been developed that use gene expression profiles including ColoPrint and the Oncotype Dx. Both platforms are able to discriminate patients with high risk for recurrence from patients that have good prognosis \(^27,28\). It is worth to mention that these tests can be useful for prognosis but not for prediction of therapy response. In addition to these two gene expression platforms in chapter 3 we show another method to discriminate high and low risk stage II CRC patients \(^29\). Not only between patients, but also within patients tumor heterogeneity is observed. In a recent study intratumoral heterogeneity in CRC was studied by Sottoriva et al.
They isolated glands that are clusters of cells, from various locations within 15 different CRCs. High intratumoral heterogeneity in these CRCs were observed that was caused by mutations that occur early in CRC development. The authors claim that besides mutation in APC, KRAS, and TP53 stringent selection is absent in CRC development and therefore not the fittest mutations but the time when a mutation occurs is important for CRC. Interestingly, they also report that glands far from each other show similar mutations suggesting that mutations occur early on and spread throughout the tumor 30.

Furthermore, there is also another type of heterogeneity that is dependent on hierarchy. In chapter 2 we discuss this form of intratumoral heterogeneity in detail. Many cancers including CRC are hierarchical organized with cancer stem cells at the top of this hierarchy. This is similar to non-neoplastic tissues and organs including colon. In the colon proliferation of stem cells generate progenitor cells that differentiate into adsorptive enterocyte cells, secretory goblet and entero-endocrine cells. This hierarchy is also present in CRC and the cancer counterpart of the colon stem cells, the so called colon- cancer stem cells (colon-CSCs), drive tumor growth and have similar to their normal counterpart self-renewal capacity and can spin off progeny that will differentiate into more differentiated tumor cells 31.

Hierarchy in tissues or tumors is in part regulated by the microenvironment. A solid tumor consists not only of tumor cells but also infiltrating cells and stromal cells. This so called micro-environment plays an important role in tumor progression and consists of fibroblasts, infiltrating immune cells and the tumor vasculature 32. To illustrate, in CRC myofibroblasts have a major impact on the hierarchical organization of tumors by supporting CSCs. Colon-CSCs display high WNT signaling and stemness can be supported by the tumor microenvironment 33. In chapter 5 we extend on these original observations and confirm that myofibroblasts can support colon-CSCs and even induce stemness in more differentiated cells. More importantly, we show that the microenvironment plays a role in therapy resistance as it induces anti-apoptotic mechanisms in differentiated tumor cells 34.

Treatment of CRC patients

The stage at presentation is crucial in treatment decision making. Surgery is the main treatment of stage I CRC patients. No adjuvant therapy is offered to these patients. However in the Netherlands patients diagnosed as stage III are, after surgery, adjuvantly treated with FOLFOX or CAPOX. In case of contraindication for these severe chemotherapeutic regimens these patients can be treated with 5FU with leucovorin.
or capecitabine monotherapy. Stage IV patients can be treated with above mentioned FOLFOX, CAPOX or FOLFIRI regimens combined with or without targeted therapy. For stage II disease the reality is a bit more complicated. Despite the fact that 20% of the patients will recur the benefit of adjuvant therapy is relatively limited. Therefore only a subset of the stage II patients are treated similar to stage III patients. These so called high risk patients present with at least one of the following features; poorly differentiated tumor, presence of bowel obstruction or perforation, less than 12 lymph nodes evaluated (in Netherlands less than 10), tumor penetration into visceral peritoneum or vascular, lymphatic or perineural invasion of tumor cells.

FOLFOX regime consists of the chemotherapeutics fluorouracil, leucovorin, and oxaliplatin. Fluorouracil (5FU) is an antimetabolite and is a pyrimidine analogue. 5FU irreversibly inhibits thymidylate synthase (TS), an enzyme that is important in thymidine biosynthesis. Inhibition of TS by 5FU leads to thymidine starvation, which negatively affects DNA replication and subsequently leads to apoptosis. Leucovorin also called folinic acid is closely related to folic acid (vitamin B9). Leucovorin is anabolized to CH2-tetrahydrofolate and is required for optimal binding of TS to its substrate during thymidine biosynthesis. This binding last for a longer period of time because of leucovorin and thereby it boosts the effect of 5FU. Oxaliplatin is a third-generation platinum analog and it binds to the nitrogen atom of guanine. Covalent interaction of oxaliplatin with two guanines results in intra-strand crosslinks that lead to DNA lesions and cell death. CAPOX regime consists of capecitabine and oxaliplatin combination treatments. Capecitabine is a prodrug that is metabolized into 5FU and as discussed above mechanism of action of 5FU is via inhibition of TS. In the FOLFIRI regime oxaliplatin is replaced by the type I topoisomerase inhibitor irinotecan. Topoisomerases are enzymes that relax overwinding of DNA by cleavage of the DNA, which is a process needed during DNA replication and transcription. Irinotecan binds and stabilizes topoisomerase I - DNA complexes and thereby prevents re-ligation. This results in double strand breaks and subsequently to cell death. Stage IV CRC patients can be treated with the regimens discussed above in combination with angiogenesis inhibitor or RTK inhibitors. Patients can additionally be treated with monoclonal antibodies (mAb) inhibiting VEGF (e.g. Bevacizumab) or if they have a WT KRAS tumor with mAb inhibition EGF-receptor (e.g. Cetuximab and Panitumumab). Combining chemotherapy regimens with RTK inhibition increases overall survival of CRC patients.
Therapy resistance and apoptotic signaling

Therapy resistance is a major concern of cancer treatment. In chapter 2 we describe mechanisms that are reported to induce therapy resistance, including enhanced DNA damage response, enhanced expression of drug efflux pumps, and apoptotic blockade. In this chapter I want to discuss in more detail the apoptotic signaling and its role in therapy resistance in CRC. In normal healthy cells pro- and anti-apoptotic signals are tightly regulated. Anti-apoptotic BCL2 family members, consisting of BCL2, BCLXL, BCLW, MCL1, A1/BFL1, and BCLB bind to pro-apoptotic molecules like BAX and BAK and inhibit their function. However, when cells are stressed, for instance after exposure to chemotherapy, pro-apoptotic BH3 molecules get activated (Figure 3). These proteins are able to inhibit anti-apoptotic BCL2 family proteins like BCLXL. Inactive BCLXL is not able to inhibit BAX, which subsequently shows enhanced relocation to the mitochondria where it oligomerizes and forms pores. It is believed that some of the BH3 molecules including BID and BIM are able to directly activate BAX or BAK. Pore formation by BAX and BAK in the outer mitochondrial membrane leads to release of various apoptotic molecules from the mitochondrial inter-membrane space to the cytosol. One of the most important pro-apoptotic molecules that leaks out of the mitochondria is cytochrome c. In the cytosol cytochrome c can catalyze formation of APAF-1 / caspase-9 complex. In this apoptosome complex caspase-9 becomes activated and is able to cleave and activate caspase-3 and -7, which are effector caspases that can cleave many targets. Poly ADP ribose polymerase (PARP) and inhibitor of caspase activated DNAse (ICAD) are only two of

Figure 3: Apoptotic signaling pathway. Apoptotic stimuli can activate BH3 molecules. These molecules can inhibit anti-apoptotic BCL2 family members and thereby indirectly activate BAX and BAK. In contrast to e.g. NOXA some BH3 molecules including BIM and BID are able to directly activate BAX and BAK. Activation of BAX and/or BAK triggers cytochrome-c (Cyt-C) release into the cytosol. This initiates a caspase cascade whereby caspase-9 (Casp9) gets activated that can activate caspase-3 (Casp3). The latter is able to cleave many proteins and induce cell death. Pro- and anti-apoptotic molecules are highlighted in red and green, respectively.
the many targets of these caspases. ICAD cleavage directly impedes on the activity of caspase-activated DNAse, which is released and results in fragmentation of DNA 52.

There are many mechanisms reported in cancer that can tip the balance between pro- and anti-apoptotic signaling in favour of anti-apoptotic signals. Decreased and defective pro-apoptotic signaling occurs for example when TP53 is mutated. WT TP53 induces upon stress, expression of pro-apoptotic molecules PUMA, NOXA, BAX, and BID 53, which upon TP53 mutation no longer occurs as effectively leading to a defective apoptotic response to stress. In addition to TP53 also PTEN mutations occur in patients with CRC. This leads to hyperactivation of PKB/AKT, which is a kinase that amongst others phosphorylates pro-apoptotic molecule BAD, resulting in its sequestrating by 14-3-3 and inactivation 54. This shows that mutations found in CRC can regulate apoptotic signaling and thereby tips the apoptotic balance in favour of anti-apoptotic signaling. Moreover, in some MSI CRC patients the pro-apoptotic molecule BAX can be mutated. There are homozygous deletions or inactivating mutations in BAX identified in CRC patients. The human BAX gene contains a microsatellite repeat and instability leads to a frameshift 55, 56. In absence of BAX protein apoptosis is partially blocked. Next to decreased pro-apoptotic signaling, many CRCs have increased anti-apoptotic signaling. Anti-apoptotic BCL2 family member BCLXL is highly expressed in cancer compared to normal tissue 57, 58 and knocking down BCLXL makes colon cancer derived cell lines more sensitive to chemotherapy 59. In chapter 5 we further discuss the importance of BCLXL in CRC. We show that ectopic overexpression of this protein makes CRC cells more resistant to chemotherapy and that inhibition of this protein with an inhibitor can sensitize CRC cells to chemotherapy. Together showing that this molecule is important for CRC cell survival and therapy response 60.
Outline of this thesis

This thesis is an overview of the research that I performed to understand therapy resistance in CRC and to identify novel treatments to improve therapy. First, in chapter 2 we summarize the findings that CSCs are resistant to tumor therapy and I also discuss the effort that has been taken to target these therapy resistant cells. In chapter 3 we generate a colon-CSCs signature and we use this set of genes to predict outcome in stage II CRC patients. We show that not the numbers of CSCs in tumors but the methylation of a set of WNT target genes can stratify stage II CRC patients with good or bad prognosis. In the general introduction of this thesis (chapter 1) we therefore address heterogeneity in CRC that is determined by genetic and epigenetic alterations. In chapter 4 and the following chapters we study therapy resistance in colon-CSCs. In chapter 4 we transduced colon-CSCs with an inducible caspase-9. We show that activation of caspase-9 induces cell death in colon-CSCs, indicating that downstream activation of the apoptotic signalling is possible in colon-CSCs. To reliably measure cell death in colon-CSCs we subsequently developed an FACS based assay that allowed us to measure cell death in colon-CSCs and differentiated tumor cells simultaneously. Using this assay we showed that colon-CSCs are more resistant to various chemotherapeutics compared to more differentiated tumor cells (chapter 5). Further study showed that this therapy resistance is because the apoptotic balance is tipped in favour of anti-apoptotic molecules. Colon-CSCs are dependent on the anti-apoptotic protein BCLXL and inhibition of BCLXL sensitizes colon-CSCs towards chemotherapy (chapter 5). In chapter 6 we show that factors released by the microenvironment can induce stemness in differentiated tumor cells. As a consequence, these cells become resistant to chemotherapy. Similar to colon-CSCs, in de-differentiated cells therapy resistance can be overcome by inhibition of anti-apoptotic BCL2 family members. Chapter 7 shows our effort to find pathway inhibitors that sensitize colon-CSCs towards chemotherapy. From a small compound screening we identified histone deacetylase (HDAC) inhibitors as compounds that sensitize colon-CSCs towards chemotherapy. These compounds differentiate colon-CSCs and thereby sensitize them towards chemotherapy (chapter 7). Besides treatment with HDAC inhibitors, in chapter 8 we show that induction of ER-stress also differentiates colon-CSCs. We show that ER-stress induced differentiation sensitizes colon-CSCs towards chemotherapy. In chapter 9 we discuss critically our findings described in this thesis and try to put forward the implication of these findings.
Chapter 1

References


Chapter 1


