

# *Chapter 9*

*Summary, conclusions and  
future perspectives*

## Summary, conclusions and future perspectives

Colorectal cancer (CRC) is the second cause of cancer related deaths in the western world. Although improvements have been made in surgical techniques and the development of new chemotherapeutic modalities, 5-year survival rates are still around 60 %<sup>1</sup>, leaving ample room for improvement. Nevertheless, progress is being made in various fields of investigation. Before summarising the studies performed in this thesis, some recent developments in colorectal cancer research are discussed below, which may help to put things in perspective.

One area of research is dedicated to improve early detection of colorectal cancer or its precursor lesion, the adenoma, for example by screening the general population and surveillance of high-risk populations. The latter include subjects with the CRC predisposition syndromes Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC), subjects with a family history of CRC, patients with previous adenomas or CRC or patients with longstanding inflammatory bowel disease. Efforts are made to develop strategies to better identify subjects at risk for HNPCC. An early diagnosis of patients with HNPCC is particularly important since periodic colonoscopic screening has been shown to improve mortality in HNPCC patients.

With respect to current screening procedures, several options are available, including faecal occult blood testing, flexible sigmoidoscopy and colonoscopy, each of which has its advantages and disadvantages. Potential future screening modalities include virtual colonoscopy, serum proteomics and the detection of molecular abnormalities in stool samples<sup>1</sup>. A related area of study is further improvement in endoscopic detection techniques of early neoplasia, such as high-magnification chromoscopic colonoscopy.

Next, the potential of chemopreventive measures is increasingly being recognised, again especially in high-risk populations. Chemoprevention is the use of pharmacological or natural agents to prevent the development of cancer or precancerous lesions. Although there is evidence that certain non-steroidal anti-inflammatory drugs (NSAIDs), calcium and ursodeoxycholic acid reduce the risk of adenoma or carcinoma development, the effects are incomplete and there is a need for regimens with better efficacy. A promising strategy lies in combining different chemopreventive agents. Such an approach in the animal model of FAP, using the combination of a NSAID with an epidermal growth factor receptor (EGFR) kinase inhibitor, showed encouraging results<sup>2</sup>. The efficacy of similar combination regimens, combining NSAIDs with other anticancer drugs, needs further exploration.

Further, efforts are made to increase the efficacy of chemotherapy. Chemotherapy in CRC is used in the adjuvant setting in patients with stage III colon tumours and subject of study in stage II/III rectal cancer. In the palliative setting, chemotherapy is given in patients with stage IV colorectal cancer. The benefit of adjuvant chemotherapy in stage II colon cancer patients is controversial, but there appears to be a high-risk subpopulation that may benefit. Attempts are being made to identify genetic or protein markers in stage II colon cancer patients that predict recurrence<sup>3</sup> and in stage II/III patients to predict sensitivity to

chemotherapy<sup>4</sup>. It is expected that newly developed genomic and proteomic approaches may provide more sensitive markers or indicators of response than current biochemical, immunohistochemical or morphological methods. Built on an improved understanding of the molecular biology of the disease, advancement in the treatment of CRC is also being made by the identification of several molecular targets in colon tumours, potentially amenable for directed specific therapies. It can be expected that such a strategy results in direct interference with the cellular pathway involved, with less toxicity compared to the toxicity associated with the less specific effect of chemotherapy. Recently, the first of such compounds have been introduced into clinical practice, in particular the management of advanced CRC, namely antibodies directed against EGFR and the vascular endothelial growth factor respectively<sup>5,6</sup>. Increasing knowledge of the molecular events characterising colorectal carcinogenesis will allow the development of novel therapies aiming at specific molecular targets.

It is known that the development of CRC is characterised by a sequence of events during which normal colonic epithelium gradually transforms to carcinoma, in most cases via the development of colorectal adenomas, the so-called adenoma-carcinoma sequence. The adenoma-carcinoma sequence is driven by an accumulation of molecular (epi)genetic alterations causing progressive disorders in cell growth, differentiation and apoptosis. Apoptosis, or programmed cell death, plays an important role in the development and maintenance of tissue homeostasis. Multicellular organisms also use apoptosis to eliminate potentially dangerous cells, such as genetically damaged cells, including tumour cells. Abnormalities in apoptotic function have been identified as contributing events in the pathogenesis of colorectal cancer. Furthermore, resistance to apoptosis induction by chemotherapeutic drugs or radiotherapy frequently hampers its efficacy in the treatment of established tumours.

During apoptosis, a complex death program is initiated that ultimately leads to the fragmentation of the cell. The death program can be induced by two major apoptosis signalling pathways namely the 'extrinsic' and the 'intrinsic' pathway. The extrinsic pathway is initiated by triggering cell death receptors on the cell surface, leading to activation of the intracellular apoptotic machinery. The intrinsic pathway of apoptosis is initiated via the mitochondria by cellular stress, for example following DNA damage caused by chemotherapeutic drugs and radiation. Mitochondria induce apoptosis by releasing cytochrome-c into the cytosol, which causes the assembly of a multiprotein caspase-activating complex, known as the apoptosome. Among the regulators of the intrinsic pathway are Bcl-2 protein family members and inhibitors of apoptosis proteins (IAPs). The elucidation of the molecular mechanisms regulating these processes is of primary interest as this may guide specific targeted therapies.

Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a transmembrane protein belonging to the TNF superfamily. TRAIL triggers the extrinsic apoptotic pathway by binding to its membrane-bound death receptors DR4 and DR5, which transmit an apoptotic signal via their intracellular death domains. TRAIL can also bind to decoy receptors DcR1 and DcR2 that are unable to initiate an apoptotic signal. Although

the physiological role of TRAIL is not exactly known, there are data suggesting a role in immunomodulation. *In vitro*, TRAIL induces apoptosis in a wide variety of cancer cell lines but not in normal cells. Preclinical data in mice and nonhuman primates have shown that recombinant TRAIL induces tumour regression without serious systemic toxicity. Therefore, TRAIL is considered a promising new anti-cancer agent.

The aims of this thesis were to clarify the changes occurring in the degree of apoptosis during the gradual development of colorectal cancer. Furthermore, the potential of TRAIL as a therapeutic agent was investigated in colorectal adenomas and carcinomas. Tumours were studied from patients with a sporadic background as well as from FAP and HNPCC patients. Finally, mechanisms behind the chemopreventive action of the NSAID sulindac on colorectal epithelial cells were studied, as recent data suggested a mechanism involving TRAIL mediated apoptosis.

Following a brief introduction and outline of this thesis in **Chapter 1**, current knowledge of mechanisms of apoptosis and changes in the degree of apoptosis in the development of colorectal cancer was reviewed in **Chapter 2**. Although there is a multitude of studies available on apoptosis in colon tissue at different stages of colorectal cancer development, results from these studies are contradictory. Considerable controversy exists as to whether the frequency of apoptosis increases or decreases during the adenoma-carcinoma sequence. Therefore, a systematic review of all available studies, which addressed changes in frequency, and distribution of apoptosis in colon epithelium was performed. Studies were included if either normal or neoplastic colonic epithelium, or both, was examined. A PUBMED search revealed 53 articles. Several aspects from these articles were studied such as methods used to determine apoptotic cell death, frequency of apoptosis and locoregional distribution of apoptotic cells in normal mucosa, adenomas and carcinomas, correlations between the degree of apoptosis and proliferation and the prognostic significance of the degree of apoptosis in colorectal cancer.

There was a wide variety in the methods used to determine apoptotic cells in the reviewed studies. Most studies had used terminal deoxyribonucleotidyl transferase-mediated nick end labelling (TUNEL, n = 29). Other methods used were *in situ* end labelling (ISEL, n = 7), morphology by light microscopy (n = 7), TUNEL or ISEL in conjunction with morphology by light microscopy (n = 9), ISEL in conjunction with morphology by electron microscopy (n = 1), morphology by light microscopy in conjunction with electron microscopy (n = 2) and the recently developed method of detection of cleaved cytokeratin 18 by immunohistochemistry (n = 2). Caspase-cleaved cytokeratin 18 is an early marker of apoptosis that can be recognised in epithelial cells using an antibody (M30) directed against this neo-epitope. Percentages of apoptotic cells (apoptotic indices) reported with these methods varied widely. This variation is probably partly due to a major limitation of the TUNEL and ISEL methods, i.e. false positive marking of necrotic cells in addition to apoptotic cells. Others have already seriously questioned the applicability of the TUNEL method to evaluate apoptotic cell death in the gastrointestinal tract. Although recently new methods of identifying apoptotic cells with higher specificity have been developed, such as M30 immunoreactivity, few studies have used these techniques.

Concerning the degree of apoptosis in normal mucosa, reported apoptotic indices varied between 0.11 and 11 %. It was noted that in most studies sections of normal colonic mucosa were in fact retrieved from macroscopic normal tissue adjacent to adenomas or carcinomas and one could argue whether this represents truly normal epithelium. One study revealed that apoptosis in normal mucosa obtained from resection margins indeed differed from genuinely normal mucosa, which warrants caution when interpreting studies on apoptosis in normal colonic mucosa. When studying the transition from normal colonic mucosa to adenomas, most studies indicated a shift towards higher apoptotic indices. The real controversy centres on the transition from adenoma to carcinoma. Most studies reported that the frequency of apoptotic cell death in carcinomas was higher than in adenomas. However, in other studies, no differences or even opposite results were found. Concerning the distribution of apoptotic cells during the adenoma-carcinoma sequence, there was general agreement that in adenomas apoptosis predominates at the base of the crypt, with proliferative activity prevailing at the luminal surface. This is a complete reversal of the pattern seen in normal epithelium. With respect to the correlation between rates of apoptosis and proliferation in adenomas and carcinomas, again conflicting results were obtained. Some studies indicated a correlation between the two, whereas others did not. Regarding the prognostic value of apoptotic indices it was found that most studies suggest that colorectal tumours with high apoptotic indices were associated with better prognosis and survival.

Given the limitations mentioned earlier of the techniques most often used for the detection of apoptotic cells, TUNEL and ISEL, a recently developed technique to investigate apoptosis in colorectal tissue was studied in **Chapter 3**. The method used, assessing M30 immunoreactivity, is based on recognition in epithelial cells of caspase-cleaved cytokeratin 18 which is an early and specific marker of apoptosis. The technique was validated against the gold standard of identification of apoptotic cells. i.e. by morphological criteria. Following the observation from Chapter 2 that most studies on apoptosis in normal colorectal epithelium were in fact performed in macroscopic normal tissue adjacent to adenomas or carcinomas, it was investigated whether apoptotic indices differ between normal mucosa obtained from resection margins and genuinely normal mucosa. Paraffin sections of normal colonic mucosa (n = 30), normal mucosa obtained from resection margins from carcinomas (n=30), colorectal adenomas (n = 84) and carcinomas (n = 40) were studied. Apoptosis of epithelial cells was assessed by M30 immunoreactivity as well as by morphological criteria and expressed as a proportion of the total number of cells counted (apoptotic index).

Mean apoptotic indices assessed by M30 immunoreactivity were  $0.18 \pm 0.04$  % in normal mucosa,  $0.42 \pm 0.04$  % in adenomas and  $1.97 \pm 0.24$  % in carcinomas. Apoptotic indices determined by morphological criteria were  $0.23 \pm 0.03$  %,  $0.62 \pm 0.06$  % and  $1.78 \pm 0.19$  %, respectively. Apoptotic counts were higher in normal mucosa obtained from resection margins than in genuinely normal mucosa ( $p < 0.0001$ ). There was a good correlation between apoptotic indices obtained by M30 immunoreactivity and morphological criteria ( $r = 0.71$ ,  $p < 0.01$ ). These findings support the use of the M30 method in the study of apoptosis in colorectal tissues.

To investigate the potential use of TRAIL or TRAIL receptor agonists as therapeutic agents in colorectal neoplasms, the expression of the four membrane-bound TRAIL receptors DR4, DR5, DcR1 and DcR2 was studied in colon tissue sections of different stages of colon cancer development, i.e. normal colon, adenomas and carcinomas. The expression and localisation of TRAIL and its receptors were investigated by immunohistochemistry in normal mucosa (n = 10), adenomas (n = 19) and carcinomas (n = 21). Tissues were obtained from patients with a sporadic background of their disease. In addition, correlations between expression of TRAIL and its receptors and the degree of apoptosis (assessed by M30 immunoreactivity) and histopathological characteristics were explored. The results are described in **Chapter 4**.

In normal mucosal epithelial cells, TRAIL and TRAIL receptor expression was observed. In adenomas and carcinomas, expression of all four receptors was seen. However, TRAIL expression was lost in a subset of colorectal tumours in comparison to adjacent normal mucosa. This occurred more frequently in carcinomas than in adenomas ( $p < 0.05$ ). Intensity of DR4 and DR5 staining was stronger in neoplastic cells compared to adjacent normal epithelial cells, and was accompanied by a higher degree of apoptosis. No differences were found between neoplastic and normal cells regarding DcR1 and DcR2 expression. No correlations were found between TRAIL or TRAIL receptor expression and histopathological characteristics. The stronger expression of DR4 and DR5 in neoplastic cells as opposed to normal cells holds promise for the future use of TRAIL or TRAIL receptor agonists as therapeutic agents in colon neoplasms.

Given these encouraging results in patients with sporadic disease, the investigations were expanded to tissues obtained from patients with FAP and HNPCC, described in **Chapter 5**. Expression of DR4 and DR5 was studied using immunohistochemistry in colorectal adenomas and carcinomas from patients with sporadic disease (n = 74 and 56 respectively), FAP (n = 41 and 4 respectively) and HNPCC (n = 50 and 21 respectively). In addition, sporadic tumours with microsatellite instability (MSI) were studied, to examine the role of BAX gene mutations. BAX was reported to play a role in sensitivity to TRAIL-mediated apoptosis *in vitro* and colorectal tumours with MSI are prone for mutations in the BAX gene. The presence of BAX mutations in MSI positive tumours could potentially limit the use of TRAIL or TRAIL receptor agonists. Carcinomas with high frequency MSI (MSI-H, n = 42, of which 27 sporadic and 15 HNPCC-associated) were analysed for apoptotic cell death, assessed by M30 immunoreactivity, and BAX mutations.

Immunohistochemistry revealed that most adenomas from all three patient groups expressed DR4 and DR5. Most carcinomas expressed DR4, except for six cases, all with mucinous histology. All carcinomas, including those with mucinous histology, showed DR5 expression. BAX mutations were found in 6 out of 42 MSI-H cancers, with similar apoptotic indices and expression of DR4, DR5 and TRAIL in BAX mutant and BAX wild-type cases. These data do not support the presence of BAX inactivation as a critical mechanism *in vivo* to evade apoptosis in MSI-H tumours. Taken together, these results indicate that selectively targeting TRAIL death receptors in sporadic and hereditary settings has potential as prevention or treatment strategy.

It is important to identify prognostic factors that can help to develop new patient-tailored treatment strategies for CRC patients. Resistance of tumour cells to induction of apoptosis is a limiting factor in the efficacy of chemotherapeutic treatment of colon cancer. In vitro, chemotherapy and recombinant human (rh) TRAIL were shown to act synergistically on induction of apoptosis in colon cancer cells. The prognostic significance of expression of TRAIL, DR4 and DR5 on overall and disease free survival and disease recurrence was studied and described in **Chapter 6**. Tissue microarrays were constructed containing tumour tissue of 376 stage III colon cancer patients who had participated in an adjuvant chemotherapy study in the past <sup>7</sup>. These arrays were analysed by immunohistochemistry for TRAIL, DR4 and DR5 expression. Median follow-up was 43 months. Kaplan-Meier survival analysis and Cox proportional hazard analysis with adjustment for treatment arm, age and gender was performed.

Most colon cancers showed high expression of TRAIL (82.8 % of cases), DR4 (91.7 %) and DR5 (87.1 %). High DR4 expression was associated with worse overall survival {odds ratio (OR; 95 % CI) = 2.22 (1.03-4.81);  $p = 0.04$ } and worse disease free survival {OR = 2.19 (1.06-4.53);  $p = 0.03$ }, compared to low DR4 expression. High DR5 expression was associated with better survival with borderline significance {OR = 0.65 (0.41-1.03);  $p = 0.07$ }. Within the group of patients with recurrent disease, the time to recurrence was longer for those with high DR5 expression ( $p = 0.03$ ) or high TRAIL expression ( $p = 0.007$ ) compared to those with low DR5 or TRAIL expression levels. The finding that most tumours showed high DR4 and DR5 expression indicates that rhTRAIL may potentially be used as an adjunct to adjuvant chemotherapeutic treatment of stage III colon cancer patients.

Recently, two randomised placebo-controlled trials were published showing a chemopreventive effect of aspirin in patients with previous colorectal adenomas or carcinomas <sup>8,9</sup>. These studies were discussed in **Chapter 7**, and mechanisms behind the chemopreventive action against colorectal cancer development of NSAIDs were briefly reviewed. Both studies indicated a moderate chemopreventive effect of aspirin in these populations. The anticancer properties of NSAIDs have been demonstrated in vitro as well as in vivo (animal studies, epidemiological reports, and intervention studies). Several mechanisms through which NSAIDs alter colorectal carcinogenesis have been elucidated, including the induction of apoptosis in neoplastic cells, via mechanisms dependent and independent of cyclo-oxygenase. The exact mechanisms involved in apoptosis induction are unknown but recent evidence suggests that sulindac-induced apoptosis involves DR5.

There is also evidence suggesting that NSAIDs work by modulating the Wnt-pathway. The formation of adenomas in the intestine is thought to be initiated by mutational events occurring in either APC or the gene for  $\beta$ -catenin, both operating in the Wnt-signalling pathway. In normal cells,  $\beta$ -catenin is membrane bound, with cytoplasmic  $\beta$ -catenin being degraded by the APC/axin/GSK3 $\beta$ -complex. Loss of functional APC or mutations in the  $\beta$ -catenin gene lead to the nuclear accumulation of  $\beta$ -catenin, which binds and activates the transcription factor T-cell factor 4 (TCF4). Consequently, activated TCF4 activates a genetic programme that is presumed to be responsible for early adenoma formation. The importance of the Wnt-pathway in which  $\beta$ -catenin/TCF4 activity acts as a switch controlling

proliferation versus differentiation in the intestinal epithelium seems critical in the early development of colorectal neoplasia. It has been shown that activated Wnt-signalling decreases concentrations of the cell cycle regulating protein p21, thereby allowing cells to proliferate. Recent evidence suggests that part of the chemopreventive effect of sulindac is mediated via p21. For example, microarray data of rectal biopsy specimens and colonic cells in culture showed that sulindac induced expression of p21. Furthermore, homozygous inactivation of p21 in APC<sup>min</sup> mice (the mouse model of FAP) eliminated the ability of sulindac to reduce the number of small intestinal tumours in these mice. So, p21 could be the link between the Wnt pathway and the chemopreventive effect of sulindac on intestinal adenoma formation.

These data served as the background for studying the effects of sulindac on apoptosis and expression of DR4 and DR5,  $\beta$ -catenin and p21 in normal appearing colon mucosa. The results are described in **Chapter 8**. Biopsies obtained before and after sulindac treatment during two chemoprevention studies<sup>10,11</sup> were collected. Patients (n = 18) with HNPCC had received 150 mg sulindac twice daily for 4 weeks in a placebo-controlled cross-over design. Patients (n = 6) with FAP had received 150 mg sulindac twice daily for 6 months. Apoptosis was assessed by M30 immunoreactivity and expression patterns of DR4, DR5,  $\beta$ -catenin and p21 were studied by immunohistochemistry.

In HNPCC patients, apoptotic indices were similar following placebo and sulindac. Also in FAP patients, apoptotic indices were not different after sulindac compared to pre-treatment values. Expression of DR4 and DR5 was observed in all samples with no consistent differences following sulindac versus placebo/baseline. Intensity of membranous  $\beta$ -catenin staining was lower in HNPCC samples following sulindac compared to placebo ( $p < 0.001$ ). Similar results were obtained in FAP samples ( $p < 0.01$ ). p21 expression before and after sulindac treatment was similar, in both patient groups. In conclusion, sulindac inhibits  $\beta$ -catenin expression in normal colorectal epithelium from HNPCC and FAP patients without affecting apoptotic indices and DR4, DR5 and p21 expression. An inhibiting effect of sulindac on  $\beta$ -catenin expression has also been observed in APC<sup>Min</sup> mice as well as in human adenomas. Whether this phenomenon is important in the chemopreventive effects of sulindac remains to be investigated.

## Conclusions and future perspectives

Several genes that regulate apoptosis are inappropriately expressed or mutated in colorectal cancer. Although the idea of decreased apoptosis during colorectal cancer development is conceptually attractive, the available evidence from in vivo material suggests that the proportion of apoptotic cells increases gradually during the adenoma-carcinoma sequence. In vitro, tumour progression is usually associated with resistance to apoptosis induction. Although these data seem contradictory at first sight, and potentially confusing, they are not conflicting. The contradiction resolves when one realises that in vivo studies describe spontaneous apoptosis whereas in vitro studies describe induced apoptosis, whether by chemotherapy or other agents. Considering the limitations of the current methods of in

situ end labelling of DNA fragments, used in the vast majority of *in vivo* studies, improved diagnostic criteria for the identification of apoptotic cells are needed and can be expected in the near future. The results from this thesis using the method of M30 immunoreactivity support its use in the study of apoptosis in colorectal tissues.

Successful nonsurgical elimination of cancer cells ultimately involves apoptosis. Essentially all cytotoxic drugs work by induction of apoptosis, usually not only of malignant cells but also of normal cells, manifested by toxicity. Over the last years, knowledge has increased of mediators of apoptosis in colon cells, which are dysregulated in tumour cells, such as Bcl-2 family members and IAPs<sup>12</sup>. These suppressors of apoptosis have become targets for drug development aimed at restoring apoptosis sensitivity of tumour cells. Alternatively, one can imagine strategies aimed at direct activation of agonists of apoptosis, via the extrinsic pathway. Unlike its family members TNF and FasL, TRAIL is not associated with induction of sepsis and hepatotoxicity respectively. Based on preclinical toxicity and activity data and the results described in this thesis showing broad expression of the pro-apoptotic TRAIL receptors in colorectal neoplasms, recombinant human (rh) TRAIL is considered of great interest for clinical use. Agonistic TRAIL receptor antibodies, directed towards the death receptors DR4 and DR5 have shown to exert the same effects as rhTRAIL<sup>13,14</sup>. The first phase-I trials using rhTRAIL and agonistic TRAIL receptor antibodies are underway and results from these studies will indicate the value of these agents as anticancer therapeutics.

Furthermore, combination regimens including rhTRAIL should be explored. Findings from several reports indicate that the combination of rhTRAIL with certain chemotherapeutic drugs, NSAIDs, proteasome inhibitors and interferon- $\gamma$  all induce apoptosis in an additive or synergistic fashion<sup>15</sup>. Another intriguing approach is the development of bi-specific antibodies. Bi-specific antibodies combine immune cell activation with tumour cell recognition, as a result of which pre-defined effector cells kill tumour cells. Recently, a technique that combines EGFR-signalling inhibition with target cell-restricted apoptosis induction using a TRAIL fusion protein with engineered specificity for EGFR showed promising results<sup>16</sup>. Analogously, a TRAIL fusion protein with specificity for the carcinoma-associated antigen EGP2 has been developed<sup>17</sup>. Such combination strategies potentially reduce the dose of TRAIL required for anti-tumour activity and should be further explored.

Apart from the possible future application of TRAIL in colorectal cancer, there may also be a potential role in treatment or prevention of adenomas. Although adenomas are generally removed during endoscopy, there are situations in which this cannot be accomplished. For example, large, flat sessile adenomas sometimes cannot be removed for technical reasons. Another example is the vast number of adenomas that is usually encountered in FAP patients, which precludes endoscopic removal. Recent data show that TRAIL also has apoptosis-inducing effects on colon adenoma cells<sup>18</sup>. This has been confirmed in a study in which adenoma cell lines and *ex vivo* cultures of human adenomas were examined<sup>19</sup>. The finding in Chapters 4 and 5 of this thesis of expression of TRAIL receptors DR4 and DR5 in colorectal adenomas supports further investigation of rhTRAIL or other TRAIL receptor agonistic agents in the treatment of adenomas.

In conclusion, new treatment options for CRC have been developed based on insights into mechanisms involved in the regulation of apoptosis. The potential clinical application of rhTRAIL or other TRAIL receptor agonistic agents in the treatment of colorectal adenomas and carcinomas, not only in sporadic cases but even more so in the setting of hereditary predisposition syndromes, should be further explored. Results from the first phase-I studies with rhTRAIL and TRAIL receptor agonistic antibodies are eagerly awaited and should guide future therapeutic strategies. The key to further improvement of prevention and therapeutic strategies for colorectal neoplasms is probably not to search for the Holy Grail: one ideal anticancer agent. Rather, it is the hunt for defining the optimal combination of agents, targeting different intracellular pathways that will ultimately result in killing neoplastic cells.

## References

1. Weitz J, Koch M, Debus J, Höhler T, Galle PR, Büchler MW. Colorectal cancer. *Lancet* 2005;365:153-165.
2. Torrance CJ, Jackson PE, Montgomery E, et al. Combinatorial chemoprevention of intestinal neoplasia. *Nat Med* 2000;6:1024-1028.
3. Wang Y, Jatko T, Zhang Y, et al. Gene expression profiles and molecular markers to predict recurrence of Dukes' B colon cancer. *J Clin Oncol* 2004;22:1564-1571.
4. Ribic CM, Sargent DJ, Moore MJ, et al. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. *N Engl J Med* 2003;349:247-257.
5. Cunningham D, Humblet Y, Siena S, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* 2004;351:337-345.
6. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004;350:2335-2342.
7. Bleeker WA, Mulder NH, Hermans J, Otter R, Plukker JT. The addition of low-dose leucovorin to the combination of 5-fluorouracil-levamisole does not improve survival in the adjuvant treatment of Dukes' C colon cancer. IKN Colon Trial Group. *Ann Oncol* 2000;11: 547-552.
8. Sandler RS, Halabi S, Baron JA, et al. A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. *N Engl J Med* 2003;348: 883-890.
9. Baron JA, Cole BF, Sandler RS, et al. A randomized trial of aspirin to prevent colorectal adenomas. *N Engl J Med* 2003; 348: 891-899.
10. Rijcken FE, Hollema H, van der Sluis T, Boersma-van Ek W, Kleibeuker JH. Sulindac increases epithelial cell proliferative activity in the proximal colon of HNPCC patients. *Eur J Gastroenterol Hepatol* 2005;17: A56-57.
11. Giardiello FM, Hamilton SR, Krush AJ et al. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N Engl J Med* 1993;328:1313-1316.
12. Reed JC. Apoptosis-targeted therapies of cancer. *Cancer Cell* 2003;3:17-22
13. Pukac L, Kanakaraj, Humphreys R, et al. HGS-ETRI, a fully human TRAIL-receptor 1 monoclonal antibody, induces cell death in multiple tumour types in vitro and in vivo. *Br J Cancer* 2005;92:1430-1441.
14. Motoki K, Mori E, Matsumoto A, et al. Enhanced apoptosis and tumor regression induced by a direct agonist antibody to tumor necrosis factor-related apoptosis-inducing ligand receptor 2. *Clin Cancer Res* 2005;11:3126-3135.
15. Van Geelen CM, de Vries EG, de Jong S. Lessons from TRAIL-resistance mechanisms in colorectal cancer cells: paving the road to patient-tailored therapy. *Drug Resist Updat* 2004;7:345-358.
16. Bremer E, Samplonius DF, van Genne L, et al. Simultaneous inhibition of epidermal growth factor receptor (EGFR) signaling and enhanced activation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-mediated apoptosis induction by an scFv:sTRAIL fusion protein with specificity for human EGFR. *J Biol Chem* 2005;280:10025-10033.
17. Bremer E, Kuijlen J, Samplonius D, Walczak H, de Leij L, Helfrich W. Target cell-restricted and enhanced apoptosis induction by a scFv:sTRAIL fusion protein with specificity for the pancarcinoma-associated antigen EGP2. *Int J Cancer* 2004;109:281-290.
18. Hague A, Hicks DJ, Hasan F, Smartt H, Cohen GM, Paraskeva C, MacFarlane M. Increased sensitivity to TRAIL-induced apoptosis occurs during the adenoma to carcinoma transition of colorectal carcinogenesis. *Br J Cancer* 2005;92:736-742.
19. Jalving M, Koornstra JJ, de Jong S, et al. TRAIL induces apoptosis in colorectal adenoma cell lines and ex vivo adenoma cultures. *Gastroenterology* 2005;128(Suppl2):A437.

