Chapter I

Introduction
1.1 Natural course of Dukes’ C colon cancer: the scope of the problem

Colon cancer is one of the most common malignant diseases in the western world. The incidence of colorectal cancer ranges widely from 3.8/100,000 in Jordan to 53/100,000 inhabitants in Sweden.\(^1\)\(^2\) This disease affected 7993 Dutch patients in 1995.\(^3\) According to American data 70% of these patients have resectable disease and 30% will have advanced disease at time of presentation.\(^4\) Patients who present with advanced disease have a poor prognosis with a near 0% 5-year survival rate if the tumor and metastases cannot be resected by surgery. Of the patients with resectable disease 45% will indeed be cured by resection of the tumor. Although survival rates for colon cancer increased from 41% in the 1950s to 54% in the 1980s still a large percentage of patients will die from this disease.\(^4\) For rectal cancer, the increase in survival is slightly lower compared to colon cancer, 40% in 1950 to 51.5% in 1985.\(^5\) For many decades the biological behaviour of colorectal cancer is insufficiently understood but due to the expanding genetic knowledge of cancer in general, and colon cancer in particular, it is considered that colon tumors are a conglomerate of tumors with different types of biological behavior necessitating an individual treatment plan considering surgical, chemotherapeutic, immunological and genetic aspects. For years we know that different histological characteristics identify patients with aggressive or more indolent types of tumors. For example a patient with a good differentiated tumor without perineural growth or vascular invasion has a better survival than a patient with a moderately differentiated tumor with extensive mucinous aspects, perineural invasion and tumor positive lymph nodes. These histological prognostic parameters are included in several staging systems.\(^6\)\(^-\)\(^10\) The main purpose of these staging systems is to identify subgroups of patients with colorectal cancer to estimate the behavior of the disease. Current treatment of patients with colon cancer is based on these classification systems. Dukes published one of the first colorectal cancer classifications.\(^8\) He divided patients with rectal cancer in patients with infiltrative cancer limited to the submucosa and muscularis (Dukes A), cancer that invades the bowel wall through the muscular layer but without lymph node metastases (Dukes B) and patients with lymph node metastases (Dukes C). Patients with advanced disease were later categorized as Dukes D. Later classifications of colon cancer have been published, for example the Astler Coller\(^7\) and the TNM classification\(^6\), but the Dukes classification has gained widespread clinical use because of its simplicity and did no worse when compared to other staging
These classification systems have been very helpful in selecting patients with low, intermediate and high risk for recurrent cancer. A major disadvantage of these classification systems is the lack of individualization. For example a patient with a Dukes’ C colon cancer will nowadays be treated with adjuvant chemotherapy with an overall survival gain of approximately 10-15%. Unfortunately, due to lack of individualization the 10-15% of patients that benefit from this therapy cannot be identified on beforehand, and therefore all patients have to be treated.

Patients with a colon tumor involving the (sub) mucosa only will have a near 100% survival rate after surgical resection. Survival drops to approximately 70-80% when the tumor has infiltrated the bowel wall without lymph node metastases. Surgical resection is currently the only curative option for these patients. Colon cancer patients with lymph node metastases but without distant metastases are treated with surgical resection and have an estimated 5-year survival of 35-40%. The number of tumor positive lymph nodes involved is the major determent of recurrent disease. Patients with more than four positive lymph nodes have a significantly lower survival than patients with less than four positive lymph nodes. For these patients with a high risk for recurrent cancer effective treatment of microscopic disease can be helpful in reducing the number of treatment failures. When developing (chemo- or immunological) therapies to reduce major cancer death, attention should primarily be focused on this group of patients because of the high rate of cancer recurrence and the gain that can be achieved by adjuvant therapy. To identify effective adjuvant treatment in patients with Dukes B or even Dukes A colon tumors, many thousands of patients are needed in randomized controlled trials because of the low incidence of treatment failures. Thus far no effective adjuvant treatment has been identified for patients with Dukes B colon tumors. For patients with advanced colon tumors survival is poor but during the last decade efforts are made to improve survival, primarily by extensive surgery. Although a great deal of research has been done, effective chemotherapy curing all patients has yet to be discovered.

**1.2 The role of surgery in the treatment of Dukes C colon cancer**

Patients with colon tumors are usually treated with resection of the diseased colon and without doubt this treatment will continue to be the most important part of treatment in the future. In the majority of patients with a colorectal tumor, a curative resection can be
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performed. But 30% of patients have distant metastases at the time of presentation and frequently a palliative resection will be the only treatment option.\(^4\)

The surgical resection requires resection of the tumor including a sufficient amount of normal colon. Although a colon tumor rarely spreads more than 2 cm longitudinally, and therefore a limited bowel resection could be justified, the resection has to include the draining lymph nodes as well. The extent of lymph node dissection remains a matter of debate, but good results have been obtained with “high ligation” by which all paracolic, intermediate and high lymph nodes are resected.\(^12\)

Although, surgical technique seems to be more important for rectal cancer (Total Mesorectal Excision) than for colon tumors this aspect is still matter of debate and subject of large trials. The discussion of these techniques is out of the scope of this thesis and the reader is referred to relevant literature on this subject. Patients with recurrent disease or synchronic metastases are offered palliative chemotherapeutic regimes containing various types of cytotoxic drugs. During the last decade metastatic resection of liver and lung, peritoneal resection with or without chemo/radio therapy is offered more and more to patients with advanced disease. Especially treatment of liver metastases (surgical, cryo-therapy or high frequency) is justified because of low morbidity and promising survival percentages. Of those patients not amenable to surgical salvage therapy, chemotherapy can be advised, but only 20% of patients will have a (temporary) remission and most patients will die within two years.

Seventy percent of patients with a colorectal tumor will have a curative resection and after histo-pathological examination of the resected colon the Dukes stage can be determined. If positive lymph nodes are found surgical adjuvant chemotherapy can be initiated.\(^13\)

Most patients staged after a curative resection have either a Dukes B or a Dukes C colon tumor. The 5-year survival of patients with a Dukes B colon tumor is approximately 70-80% while patients with positive lymph nodes (Dukes C) have a much lower percentage of survival, as stated earlier. Patients with a tumor limited to the mucosa have a very good survival after a curative resection approaching 100% while tumors’ penetrating the muscular wall decreases 5-year survival to 60%.\(^14\)

Overall, 50% of the patients diagnosed with a colon tumor will die within 5 years. It is this group of patients with the grim prospect of dying from this tumor that is primarily the object of study in this thesis and many of the chapters now following will be focused on questions how to improve treatment, patient selection and follow-up. Surgery will remain the cornerstone of colon cancer treatment but the integration
of surgery with immunological, chemotherapeutic and genetic aspects can offer better survival options for these patients.

1.3 Chemotherapy in the adjuvant treatment of Dukes’C colon cancer: the answer to the problem?

Heidelberger et al discovered more than 40 years ago 5-Fluorouracil (5-FU). Till this day this fluorinated pyrimidine remains the most effective drug in the treatment of colon cancer. Numerous studies have been published with a consistent inhibitory effect on disease progression in patients with advanced colon cancer. Unfortunately chemotherapy had only a marginal effect on survival. In 1990 Moertel et al presented a large prospective randomized, multicenter study in which patients with Dukes B or Dukes C colon cancer were treated with either 5FU/Levamisole, Levamisole or no adjuvant treatment, after a curative resected colon cancer without any indications for gross macroscopic residual disease. In case of microscopic residual disease, 5FU/Levamisole proved to be the most effective treatment regime, especially in patients with Dukes C colon cancer, resulting in a survival increase of approximately 15%. In patients with Dukes B colon cancer, 5-FU/ Levamisole had only a small beneficial effect on disease free and overall survival. The Moertel study initiated numerous other studies, some focused on shorter and less intensive treatment regimes while others focused on more effective treatment regimes. Especially the addition of folonic-acid (Leucovorin) and the (controversial) addition of Levamisole are subject of study.

1.4 Immunological treatment strategies

The Immunological treatment of colon cancer patients dates back to the early seventies when Tripodi stated that a non-specific stimulation of the cellular T-cell system by Levamisole, an anti-helmintic drug primarily used in veterinary medicine, reversed the anergic stated of patients with advanced colon cancer. This observation was done by Di-Nitro-Chloro-Benzene skin tests. Normal individuals with intact cellular defense mechanisms usually have a strong skin reaction to DNCB, but patients with advanced (colon) cancer do not usually have such a strong reaction. Treated with different doses of Levamisole, initially absent skin
reactions, became positive after time. But skin reactions disappeared during disease progression, suggesting that decreased cellular immunity in case of progressive disease is a reversible process. Although the precise function of Levamisole is not known it is thought that Levamisole decreases the expression of the Major Histocompatibility Complex (MHC I) on colon tumor cells. Prior studies have indicated that natural killer (NK) cells are more efficient in attacking cells with lowered or no MHC I expression. An MHC I deficiency induced by Levamisole would augment NK-mediated immune surveillance. Many studies were initiated to study the effect of immune modulating agents such as Levamisole in patients with cancer, and colon cancer in particularly, but failed to demonstrate any beneficial effect on survival. So, in the end of the seventies interest in the immunological treatment of (colon) cancer faded away and treatment was focused on modifications of chemotherapy. Moertel and his co-workers initiated renewed interest in the use of Levamisole in combination with chemotherapy (5-Fluorouracil). The results of a pilot study published in 1986 were sufficiently to initiate a large-scale multicenter randomized trial, which was published in 1990 and was the starting point of this thesis. A major concern in the evaluation of Moertel’s study is the large number of patients who did not profit of this immunochemotherapy. The natural course of Dukes C colon cancer is characterized by a 5-year survival of 35-45% as stated in Chapter 1.1. 5-FU/Levamisole as adjuvant treatment can increase this percentage by approximately 10 to 15%, leaving 40 to 50% of the patients without any substantial effect on survival. This group, however, does have to experience an intensive treatment during one year. Identification of this subgroup of patients by immunological methods could be beneficial in selecting patients for further adjuvant immunochemotherapy. Developing reliable methods to test the T-cell system might therefore be important in the selection of colon cancer patients. This subject will be further addressed in Chapter II.

1.5 Genetic blueprint for patients with Dukes’ C colon cancer. An alternative for histological prognostic factors?

Colon tumors are one of the tumors best studied for their carcinogenesis. The development from polyp to invasive cancer is characterized by many changes in the genome. A combination of environmental, dietary and hereditary factors, acting either alone or in combination, cause changes in vital regulatory genes leading to cellular dysfunction. The
initiating genetic changes are followed by other genetic changes that will ultimately lead to the phenotypic behavior of the tumor. In colon cancer mutations occur in at least three different kinds of genes: oncogenes, tumor suppressor genes and mismatch repair genes. We are now beginning to understand that (combinations) of mutations in (pro)oncogenes, suppressor genes and mismatch repair genes are not only of influence on initiating carcinogenesis but also on the phenotypic behavior of the tumor and the reaction to treatment we give them. A better understanding of the precise genetic changes and ultimately the phenotypic behavior will aid the clinician in treating these patients in a way that will not expose these patients to an inappropriate type of treatment.

**Oncogenes**

Normal regulatory genes called proto-oncogenes can be mutated and are then called: oncogenes. Most proto-oncogenes regulate cell proliferation and when mutations in these genes lead to an altered protein the altered cellular function can lead to uncontrolled growth. Mutation in a single allele is sufficient to cause an altered protein, in contrast to tumor suppressor genes that displays a recessive mode of action, which means that both alleles have to be inactivated by mutation or gene loss before they lose their function. Of the more then 70 oncogenes KRAS is one of the best-studied gene families in colon cancer. The ras family encodes proteins related to G-proteins; a membrane bound protein, which is involved in signal transduction. KRAS (Kirsten ras) is located on the long arm of chromosome 12. Mutations in this gene are usually located on only three codons: 12, 13 and 61 (a codon consist of three base-pairs and encodes for a single aminoacid). All of these mutations allow the translation of a full-length ras protein. This mutated gene product is usually in a permanent state of activation leading to uncontrolled cell growth.

**Tumor suppressor genes**

Tumor suppressor genes are normal genes necessary to maintain cellular homeostasis. The proteins repress cell proliferation and promote cell differentiation. The APC gene located on chromosome 5 and the p53 gene located on chromosome 17 are the two most studied genes in colorectal cancer. The APC gene on chromosome 5 (“FAP” gene) is considered an important step in carcinogenesis. Mutation of both alleles leads to an inactivated gene, which leads to loss of control and uninhibited cell growth. In Familial Polyposis Colon (FAP) cancer patients
mutation of the APC gene is largely elucidated and predisposes to an abnormal apoptosis (programmed cell death) leading to early onset colorectal cancer. The only way these patients can be “cured” is to excise the entire colon and restore continuity (preferably) with an ileoanal pouch procedure. But not only in the FAP syndrome APC gene mutations lead to cancer but also in sporadic colon cancer this gene plays a vital role as gatekeeper of normal colonic cell proliferation. The APC gene is altered by point mutations in the germ line of both FAP and Human Non Polyposis Colon Cancer (HNPCC) patients and in tumors from sporadic colorectal cancer patients. These data suggest that more than one gene on chromosome 5q21 may contribute to colorectal carcinogenesis and that mutations at the APC gene can cause precancerous polyps in HNPCC also. One of the most well-known and best studied tumor suppressor genes is TP53. This gene, located on chromosome 17, is the most frequently mutated gene in human cancer and it plays a central role in malignant transformation. Whenever DNA damage is imposed on a cell this leads to activation of the TP53 protein, which in turn activates genes, involved in cell cycle arrest (p21 gene). This arrest between the G1 and S phases of the cell cycle allows the cell to repair the damaged DNA. If the damage is too large, apoptosis will result. The protein product of the TP53 gene has been used in many studies by way of immuno-histochemical staining of the tumor. This semi-quantitative measurement is not always reliable in predicting whether or not there is a mutated gene. Gene analysis is a far better way to know if a mutation is present or not. It is obvious that mutations of the p53 gene will lead to uncontrolled cell growth after DNA damage and the appearance of a neoplastic cell population.

Mismatch repair genes.

DNA mismatch repair genes encodes for enzymes involved in the recognition and repair of mispaired bases during DNA replication. These genes play an important role in patients with the HNPCC syndrome but will not be discussed further here.

KRAS and TP53 analysis

The oncogene KRAS and the tumor-suppressor gene TP53 are further analysed in this thesis. A reliable, efficient and user-friendly detection system for mutations in these genes is necessary for future evaluation of phenotypic behaviour of colorectal cancer patients. This phenotypic behaviour can be determined by the integration of clinical survival data and gene
analysis. Because of the difficulty to obtain survival data in cancer patients (usually more than 5 years) mutation analysis on paraffin embedded tumor material is one of the solutions to this problem. In this thesis mutation detection-systems using Denaturing Gradient Gel Electrophoreses (DGGE) are primarily used in the analysis of paraffin embedded colon tumors belonging to patients with adequate clinical follow-up data. The improvement of mutation analysis of TP53 and KRAS genes is subject of Chapter 3 and 4.

Tumors, and colon tumors in particular, are still treated based on histological, clinical and radiological parameters. In this thesis aspects of genotyping of colon tumors will be presented and may serve as a starting point for future research to identify the precise phenotypic behaviour of every colorectal tumor. Knowledge of the precise phenotypic behaviour of colon tumors is the key to better risk assessment and treatment of these patients.

1.6 Questions addressed in this thesis

The treatment of patients with colon cancer, and patients with high-risk colon cancer in particular, is challenging considering the high incidence of recurrent disease and death due to metastatic disease. Efforts should be taken to treat patients with adjuvant chemotherapy only when they have a maximum chance for cure. Case selection is necessary to prevent treatment to patients who are not likely to benefit from intensive forms of adjuvant treatment.

In chapter II we examine if DNCB (Di-Nitro-Chloro-Benzene) skin testing correlates with plasma levels of sIL-2r and sCD8 and secondly if the application of DNCB has any influence on the production of sIL-2r and sCD8.

In chapter III a comprehensive mutation detection assay is presented for the entire coding region and all splice site junctions of the KRAS oncogene. This assay is based on denaturing gradient gel electrophoresis and tested on archival paraffin-embedded tumor material from 35 Dukes C colon tumors.

In chapter IV, a comprehensive mutation detection assay for the entire coding region and all splice site junctions of TP53 is presented. The assay, also based on DGGE, can be applied to DNA extracted from fresh or frozen samples or to DNA extracted from paraffin-embedded tissues. This assay is tested on DNA from cultured lung-cancer cell lines and from paraffin-embedded Dukes C colorectal carcinomas.

In chapter V mutations in K-RAS and TP53 genes are considered to affect biological behavior and might therefore influence chemotherapy susceptibility. In this chapter we investigate
whether the survival of Dukes C colon cancer patients treated with adjuvant chemotherapy is influenced by KRAS and TP53 mutations.

In chapter VI, we investigate whether KRAS and TP53 mutations influenced survival in left and right-sided Dukes C colon cancer.

In chapter VII the results of the IKN90-11 colontrial are presented. This prospective randomised, multicenter trial was initiated in 1991 to assess the effect of modulation of 5-fluorouracil/levamisole by low-dose Leucovorin on the survival of patients with Dukes C colon cancer.

In chapter VIII we investigate the clinical value and costs of different diagnostic tools applied in the IKN 90-11 colon trial to identify patients with potentially curable recurrent disease after adjuvant chemotherapy for curatively resected Dukes C colon cancer.

References


Chapter I


