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CYP2W1 in colorectal cancer

– aspects of risk, prognosis and future treatment options

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Live as if you were going to die tomorrow,

Learn as if you were going to live forever.

Mahatma Gandhi

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ABSTRACT

Colorectal cancer (CRC) is a common disease and a major cause of cancer related death globally. Prognosis is rather good in early stages but worse in cases with disseminated disease. There is a constant need of developing all treatment modalities, which also has been done over the last decades. There are needs to find predictive markers in order to assess who will and who will not benefit of chemotherapy, just as there are needs to develop drugs targeting novel pathways and proteins prevalent in primary tumors and metastases. CYP2W1 is a member of the cytochrome P450 superfamily of enzymes with unknown physiological functions but found to have the capacity to metabolize both carcinogens and various other xenobiotic substances. It is expressed in fetal rat colonic tissue and in human colorectal tumors, but not to our knowledge in any adult normal human tissue. CYP2W1 expression, both mRNA and protein, has previously been studied in a material consisting of about 50 colorectal tumor samples.

We wanted to investigate the extent of CYP2W1 expression in a larger tumor material using immunohistochemistry. We found it also interesting to see if CYP2W1 expression affects prognosis. Another aim of the thesis was to assess the association between polymorphism in the *CYP2W1* gene and risk to develop CRC. A last aim was to evaluate the CYP2W1 expression in metastases.

For the first aim, we used three different patient cohorts, two of which were derived from a randomized Nordic trial aiming to compare no adjuvant versus adjuvant treatment in patients with stage II and III CRC. These cohorts consisted of 162 and 235 patients respectively. The third patient cohort consisted of 96 patients being resected for liver metastases from CRC. All tumor manifestations in these patients were investigated with immunohistochemistry, addressing both the first and the last aim, and the findings indicate that CYP2W1 is expressed at high levels in between 26-36% of the primary tumors. It is expressed in about one third of lymph node metastases and almost half of the liver metastases.

We performed two investigations regarding CYP2W1 as a prognostic factor using the two cohorts from the Nordic study (n=162 and n=235). In the first study, high CYP2W1 expression was of independent prognostic value for poor survival together with stage. In the second study aiming to reproduce this, the result was not as clear-cut, CYP2W1 was of prognostic value only in multivariate analysis but not in univariate analysis. In the subgroup of patients with stage III CRC (n=132), CYP2W1 expression was of independent prognostic value.

The third aim was addressed using a material of DNA from individuals in a large case-control study aiming to investigate various polymorphisms and their relation to CRC risk. DNA from 1785 CRC patients and 1761 control subject was analyzed regarding

three *CYP2W1* variants. We also experimentally assessed enzymatic activity of the gene products of the variants studied. No difference was seen, neither in CRC risk between cases and controls, nor in enzymatic activity between the three variant proteins.

In conclusion, *CYP2W1* seems to be expressed in about one third of primary CRC and half of the liver metastases. The association with prognosis in CRC requires further studies to be elucidated. Genetic polymorphism in the *CYP2W1* gene does not seem to have any impact on CRC risk.

The tumor specific expression of a catalytic enzyme in CRC and metastases is interesting in the aspect of future targeted anti-cancer therapies.

LIST OF ABBREVIATIONS

AA	Arachidonic acid
AhR	Aryl hydrocarbon receptor
Ala	Alanine
APC	Adenomatous polyposis coli
ARNT	Aryl hydrocarbon receptor nuclear translocator
BMP	Bone morphogenetic protein
COX	Cyclooxygenase
CRC	Colorectal cancer
CRP	C-reactive protein
CYP	Cytochrome P450
EGFR	Epidermal growth factor receptor
EMT	Epithelial – mesenchymal transition
FAP	Familial adenomatous polyposis
GWAS	Genome-wide association studies
HCA	Heterocyclic amine
HIF1 β	Hypoxia inducible factor 1 β
HNPCC	Hereditary non polyposis colon cancer
HPLC	High performance liquid chromatography
IHC	Immunohistochemistry
IGF	Insulin-like growth factor
IL	Interleukin
KRAS	Kirsten rat sarcoma viral oncogene homologue
Leu	Leucine
LOI	Loss of imprinting
miRNA	micro RNA
NF κ B	Nuclear factor κ B
NOC	N-nitroso compounds
OR	Odds ratio
PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol -3-OH kinase
Pro	Proline
RR	Relative risk
SNP	Single nucleotide polymorphism
STAT	Signal transducer and transcription factor
TGF- β	Transforming growth factor β
Thr	Threonine
TNM	Tumor – Node - Metastasis
TP53	Tumor protein 53
95% CI	95% Confidence interval

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

- I **The expression of the novel CYP2W1 enzyme is an independent prognostic factor in colorectal cancer – A pilot study**
D. Edler, K. Stenstedt, K. Öhrling, M. Hallström, M. Karlgren, M. Ingelman-Sundberg, P. Ragnhammar. *European Journal of Cancer* 45(2009) 705-712.
- II **The expression of CYP2W1: a prognostic marker in colon cancer**
K. Stenstedt, M. Hallström, I. Johansson, M. Ingelman-Sundberg, P. Ragnhammar, D. Edler, *Anticancer Research* 32:3869-3874 (2012).
- III **Cytochrome P450 2W1 polymorphism: functional aspects and relation to risk for colorectal cancer**
K. Stenstedt, S. Travica, G. Jia, I. Barragan, K. Pors, L. Patterson, D. Edler, S. Mkrтчian, I. Johansson, M. Ingelman-Sundberg, *Pharmacogenomics* (2013), 14 (13), 1615-1622.
- IV **The expression of CYP2W1 in colorectal primary tumors, corresponding lymph node metastases and liver metastases**
K. Stenstedt, M. Hallström, F. Lédel, P. Ragnhammar, M. Ingelman-Sundberg, I. Johansson, D. Edler, Submitted.

THESIS AT A GLANCE

	Question	Patients and methods	Results	Conclusion
I	What is the expression pattern of CYP2W1 like in colorectal cancer? Is CYP2W1 expression associated with survival?	162 patients with stage II and III colorectal cancer (117 colon and 45 rectum) treated between 1991 and 1996. Tumor samples analyzed with immunohistochemistry.	Strong expression of CYP2W1 was seen in 36% of patients. Strong expression correlated independently with poor 5-year survival.	CYP2W1 is strongly expressed in about one third of colorectal tumors, and it seems to be an independent prognostic factor.
II	Are the results of study I reproducible in another cohort of patients? Is CYP2W1 expression similar in two different samples from the same tumor?	235 patients with stage II and III colon cancer treated between 1991 and 1996. Tumor samples analyzed with immunohistochemistry.	Strong expression of CYP2W1 was detected in 30% of tumor samples. There was good correlation between two slices from the same tumor. Independent prognostic value only in multivariate analysis, not in univariate analysis.	CYP2W1 is expressed in about one third of colorectal tumors with similar expression in different slices of the same tumor. The question of prognostic significance needs further investigation.
III	Is polymorphism in the <i>CYP2W1</i> gene associated with altered risk of developing colorectal cancer? Does genetic polymorphism alter the function of the enzyme?	DNA from 1785 patients and 1761 control subjects was analyzed with PCR regarding polymorphisms <i>CYP2W1</i> *2 and <i>CYP2W1</i> *6. Genetic constructs of these polymorphisms were inserted into SW480 colon cancer cell line and enzymatic activity of the gene products were assessed.	No difference was seen in genotype between cases and controls. No difference in functional activity was detected between the variant enzymes.	Genetic polymorphism in the <i>CYP2W1</i> gene does not alter the functional activity of the enzyme. Nor does it affect risk to develop colorectal cancer.
IV	Does the expression of CYP2W1 change in the individual patient between primary tumor, lymph node metastases and liver metastases? What is the expression pattern of CYP2W1 like in liver metastases?	Material from primary colorectal tumors, lymph node metastases and liver metastases from 96 patients, analyzed with immunohistochemistry.	High CYP2W1 expression was detected in 26% of primary tumors, 31% of lymph node metastases and in 48% of liver metastases. The difference between primary tumors and liver metastases was statistically significant.	The amount of tumors with high expression of CYP2W1 seems to increase between primary tumors and liver metastases. This finding needs validation with another method.

INTRODUCTION

Colorectal cancer – epidemiology

Colorectal cancer (CRC) is a significant global health problem being the third most common cancer in men and the second most common cancer in women, and causing about 600.000 deaths per year (1). There is a great variation in incidence between different countries with the highest rates seen in Australia/New Zealand and Western Europe, and the lowest in Africa and South-Central Asia (2). In Sweden, 3256 men and 2924 women were diagnosed with CRC in 2009 (3).

Mortality rates in CRC have declined in Sweden and in other western countries as well, during the past decades. This is most probably due to improved surgical techniques, more potent chemotherapeutics and the development of techniques to resect metastatic disease, but also to early detection of the tumor (4-6). In the United States, the falling incidence rates and decreasing mortality rates are believed to be strongly related to early detection in screening programs (1, 2). In Sweden, an organized screening program started in the Stockholm-Gotland region in 2008, and a national prospective study run by the regional centers of epidemiological oncology is planned to start in autumn 2013 in order to evaluate the effect of population based CRC screening in Sweden.

In spite of all improvements in treatment regimens and early detection programs, CRC remains to be a major cause of cancer related deaths with a global mortality of 600.000 deaths 2008 (1).

Staging and survival

Survival in CRC is highly dependent on stage at diagnosis of the tumor. In Sweden, the staging procedure involves endoscopy of the colon and rectum, computed tomography of the thorax and abdomen, in rectal cancer also an investigation with magnetic resonance imaging, and may in selected cases include a positron emission tomography examination. All information from the examinations is put together to assign a stage according to the Tumor – Node – Metastasis (TNM) classification system. This TNM stage can be translated into a simplified stage where stages I and II means tumor growth in the organ of origin only, stage III means spread to regional lymph nodes and stage IV means distant spread to other organs. After the examinations, all patients are discussed in a multi-disciplinary team conference where the final decision on treatment is made.

In a recent study of all Swedish patients diagnosed with CRC between 2000 and 2007, 47% were stages I or II, almost 30% were stage III and 23% were stage IV (5). Liver metastases are present in 15-20% of CRC patients at time for diagnosis, and approximately another 10-30% will develop liver metastases during the following years, according to a couple of fairly recent, population based studies (7, 8).

A recently published national follow-up of five-year survival after treatment for CRC in Sweden shows survival rates in stage I of 90-100%, stage II of 75-90%, stage III of 45-60%, and stage IV of about 10% (9, 10).

About 20% of the patients with liver metastases from CRC are eligible for liver resections (11). Complete resection of all metastases in combination with modern chemotherapy radically improves survival in those patients with five-year survival rates reaching as high as 45-50% (11-14).

Treatment of CRC – a task for the multidisciplinary team

Survival in CRC has increased due to improvements in all treatment modalities. In order to cure the patient, the tumor must be completely removed along with its regional lymphatic drainage and blood vessel supply. In addition to that, large or locally advanced tumors must be shrunken to resectable size using neoadjuvant treatment which may be radiotherapy, chemotherapy or a combination. In addition to that, suspected microscopic spread must be treated with chemotherapy.

Survival in both colon and rectal cancer is significantly better when applying the above mentioned surgical principles developed by Heald in 1979 and Hoheberger in the late 1990s (15-17). Heald developed the total mesorectal excision method in rectal cancer surgery and Hohenberger suggested a similar approach in colonic cancer surgery. Evaluations of these methods have supported the view that an intact mesorectal or mesocolic fascia is of great importance for survival outcome. With this complete mesorectal or mesocolic removal follows the removal of primarily draining lymph nodes which is also important for outcome. The increasing awareness of risk for intraoperative tumor spread also has improved survival by developing a more cautious technique in cancer surgery where the tumor should ideally be covered with surrounding tissue, and care must be taken not to damage this tissue (18-20).

Preoperative radiation therapy has led to better survival in rectal cancer (21-23). Chemotherapy is used preoperatively in locally advanced CRC, and postoperatively in stage III colonic cancer or stage II with certain risk factors. This also has contributed to the improved outcome (24-26). Regarding metastatic CRC, stage IV, survival has improved because of both the development of more efficient chemotherapeutic agents and targeted treatments with for example antibodies (27), and the increasing use of resections for localized metastases in e.g. liver, as mentioned above.

This complex picture with many different treatment modalities tailored differently to different patients, makes the multidisciplinary team the central hub in the treatment planning and implementation for CRC patients. There is an increasing amount of evidence favoring this way of working with cancer patients (28).

In spite of all the good progress being made in the treatment of CRC, there is always a need for new treatment options, not least because of the side effects associated with radical surgery, radiation and chemotherapy, but also because of development of tumor cell clones resistant to chemotherapy. In stage IV CRC patients, only about 20% are eligible for resection of the metastases, the remaining 80% would also benefit from having a larger number of available treatment options. There is also a need to find ways to identify both patients who will and those who will not benefit of chemotherapy and antibody based biological treatments.

CRC risk – inherited factors

The pathogenesis of CRC is believed to be multifactorial in most cases, but there are a couple of well described inherited syndromes which almost inevitably result in CRC: hereditary non polyposis colon cancer (HNPCC or Lynch Syndrome), familial adenomatous polyposis (FAP) and the rarest form hamartomatous polyposis syndromes. They account for only about 4% of all CRC cases altogether (29, 30).

Between 10-30% of patients with CRC seem to have a familial clustering where more complex genetic interactions are important. In a Swedish population based study from the Västmanland region, 11% of CRC patients had at least one first degree relative with CRC (31). Twin studies have also been made in order to evaluate the contribution of hereditary factors to what is often referred to as sporadic CRC. In a Scandinavian twin study, Lichtenstein et al estimated that 35% of the CRC risk is due to heritable factors while 60% is attributed to shared environmental factors and 5% non-shared environmental factors(32).

The majority of cases – 70-80%- are believed to be caused mainly by environmental factors. This hypothesis is supported by migration studies where for instance Japanese immigrants to the United States have been found to develop a more western like disease pattern, including CRC (33). There might also be cases where low penetrance genes are more or less important contributors to the development of CRC by their way of interacting differently to environmental factors. Support for this view came initially from genome-wide association studies (GWAS) where DNA from CRC cases and healthy controls have been analyzed, millions of genes are scanned, in order to find differences – risk loci – between the cases and controls. This has generated new hypotheses as to what other genes might be involved in CRC development (34). The benefit of this method is that it gives the possibility to explore thousands of new loci without a prior knowledge of the action of the corresponding gene products, a great method to discover genes previously

unknown that can be further investigated. A problem has been lack of reproducibility (35). Also, the power of GWAS studies are lowered due to low risk estimates for each locus, with most odds ratios (OR) in the range 1.12-1.20. The power is even further lowered because of multiple testing, necessitating a lower p-value to ensure statistical significance. Thus, sample size should ideally be at least 10.000 individuals, and the level of statistical significance must also be more strictly defined to less than 0.00001. (34). Many such low risk alleles are thought to interact in the same individual, in a particular environment, and eventually cause cancer.

High penetrance genes

FAP is a rare disorder caused by a germ line mutation in the *APC* gene (*adenomatous polyposis coli*) on chromosome 5q21-q22. Roughly 8000 different mutations are known in this gene with some variations in phenotype but all associated with CRC (36). Approximately one in 7000 CRC patients has FAP (37). The patients with FAP typically develop adenomas of the colon already in their teens and the disease then progresses with malignant transformation by age 40. They may also develop polyps and cancer in the upper gastrointestinal tract. These individuals should be monitored in endoscopic surveillance programs with annual endoscopies and be offered prophylactic surgery when the amount of polyps is too large for endoscopic removal (30).

HNPCC is a more common cause of CRC than FAP, about one in 300 CRC patients is a carrier of a HNPCC associated mutation (37). This condition is caused by a germ line mutation in any of the mismatch repair genes, *MLH1*, *MSH2*, *MSH6* and *PMS2*. The mismatch repair genes are coding for proteins responsible for correcting errors occurring during DNA replication. A defect mismatch repair system results in defect DNA replication with increased insertion of wrong DNA bases, thus accelerating the amount of genome wide point mutations. If such an event occurs in an important coding area of the DNA, it will affect protein function. This is deleterious especially if it involves a gene with growth- or differentiation regulation functions, and may then promote malignant transformation (38). Among the target genes for the mismatch repair system, many do have important functions like cell signal transduction (*TGFBR2*, *AXIN2*, *IGFR2*) or apoptosis (*BAX*, *PTEN*, *CASP5*) that are frequently mutated in mismatch repair deficient tumors (39). Patients with HNPCC typically also have a family history of CRC. They are affected with CRC at an early age, 40-50 years, and approximately 70% of the tumors are proximal to the left colonic flexure. The adenoma-carcinoma progression time is observed to be shorter in HNPCC associated CRC than in sporadic cases. Patients with HNPCC also have an increased risk of developing other cancers, most commonly cancer of the uterine endometrium, ovary and the upper gastrointestinal tract (40-42). Patients with HNPCC should also be monitored in a surveillance program which includes colonoscopy, gynecological examination, transvaginal sonography and endometrial biopsy (30).

MUTYH (*mutY homologue*) was really the first high penetrant recessive CRC associated gene to be described. The phenotype is usually difficult to distinguish from a mild form of

FAP. The etiology is a biallelic germ line mutation in the *MUTYH* gene, resulting in other mutations, commonly in the *APC* and *KRAS* genes (43).

There are other high penetrance genes causing very rare forms of inherited CRC. These syndromes are grouped together under the name Hamartomatous syndromes and include Peutz-Jegher syndrome, Juvenile Polyposis syndrome, Cowden syndrome and Gorlin syndrome. The genes that are mutated in these disorders are *STK11*, *SMAD4*, *BMPRIA*, *PTEN* and *PTCH* respectively (36).

Many of the high penetrance genes are mutated in the tumor itself in sporadic cancers, i.e where no germ line mutation is present. They are in some cases, like the *APC* gene, crucial steps in the adenoma – carcinoma progression. This will be discussed further in a section below.

Low penetrance genes

The above mentioned high penetrance genes are mutated at a low frequency in the population but frequently cause cancer. The genetic contribution to the majority of CRC cases are by some authors believed to be attributed to interactions between polymorphisms in low penetrance genes and various environmental factors, many studies being conducted according to the hypothesis “common allele-common disease” (44).

The GWAS studies conducted have generated new plausible candidate genes, but also genes already known to be involved in the development of CRC, like for instance the mismatch repair genes or the *APC* gene, have been explored regarding single nucleotide polymorphisms (SNPs) and risk for CRC (45, 46). Other genes of such interest have been *TGFBRIA*, *MUTYH*, *EGFR*, *HER2*, *Src*, *MTHFR*, *NAT1*, *NAT2*, *GSTT1*, *GSTM1* and *TP53* (47-52). In many cases, the first study has been promising but the positive results have not been possible to reproduce (35). A recent review by Dong et al has addressed the problem of false positive report in large candidate gene association studies reviewing 161 studies of a sample size of more than 500 subjects each, encompassing 18 cancer sites and 99 different genes (53). They came to the conclusion that associations with a prior probability of 0.001 and statistical power to detect an OR of 1.5 were less likely to be false positive, which was the case in 13 of the 99 genes. Among these, deletion of the *GSTT1* gene was associated with an increased risk for CRC and *GSTM1* with bladder cancer. These genes are coding for glutathione S-transferases involved in the phase II metabolism of toxic xenobiotics and endobiotics. No other genes associated with CRC were among those 13. The authors also stress the fact that a good biological hypothesis based on previous experimental research or *in silico* searches strengthens a proven association (53).

The problem of identifying low risk genes can be even more complicated. There are data supporting that the transforming growth factor β receptor 1 (*TGFBRI*) polymorphism *6A, claimed to be associated with CRC risk in some studies (47) but not in others (49), is part of a haplotype associated with cancer risk (54). This means that the polymorphism is inherited together with another sequence of the genome that may have a stronger

association to cancer risk, and that haplotypes must be taken into account when identifying risk loci.

There are probably more genes, with common or rare polymorphisms, that could be associated with CRC risk, but larger studies and meta-analyses have to be conducted in order to get reliable results. There may also be epigenetic variability explaining some of the risk. Epigenetic factors refer to molecules and events affecting DNA transcription. Basic cancer epigenetics will be discussed in a section below. An example of epigenetic variability affecting cancer risk is for instance polymorphism in genes coding for micro RNAs (miRNAs) (44, 55).

CRC risk – environmental factors

The lifestyle factors most often discussed in connection with CRC are physical activity, diet, smoking, alcohol and sex steroid hormones. Another issue of increasing interest is that of chemoprevention, especially cyclooxygenase inhibitors, vitamin D and folic acid. In the section below, physical activity and diet will be discussed.

Physical activity

There are investigations, many of them rather old, stating that exercise is protective to CRC and that a sedentary life style is associated with a higher risk of CRC (56-58). There are some weaknesses in the studies, reflecting the difficulties of conducting such investigations, e.g self-reported physical activity as a reliable parameter and the problematic issue of separating the effect of physical activity from that of body-mass index (BMI) and dietary factors. In spite of this, with large sample sizes and the use of adequate epidemiological statistical methods, physical activity seems to have a protective effect in itself and in interaction with other life style related factors. Martinez et al published results from the United States Nurse's Health study, a large prospective study that involved 88751 women age between 34 - 59 years at inclusion. They report a risk reduction of almost 50% in women who were physically active and an increased CRC risk (OR 1.45,[95% CI 1.02-2.07]) in women with BMI > 29(56). A Norwegian study found similar results in men (58). In the study by Slattery et al, the protective effect of a healthy diet was greater in the group with a sedentary life style compared with the group reporting a higher extent of physical activity (57). The postulated mechanisms behind these findings involve a diverse spectrum of pathways: altered immune function, changes in prostaglandin and insulin levels, altered insulin like growth factor 1 (IGF-1) levels and bile acid secretion pattern (59).

Diet

The studies addressing dietary factors and CRC risk are many and to some extent contradictory (60, 61). A good example to this is Willett et al, who also published results from the United States Nurse's Health study. After 10 years follow-up, 150 women had developed CRC, and after adjustment for total energy intake, animal fat consumption was associated with increased risk for CRC (OR 1.89, [95% CI 1.13-3.15]), and daily intake of red meat (beef, pork, lamb) was also associated with CRC risk (OR 2.49, [95% CI 1.24-5.03]) compared with women eating meat only once per month. Supported by these data, the authors suggested that people should be recommended to eat fish and chicken instead of red meat (61). Ten years later, the same author, supported by new larger cohort studies, meta-analyses and longer follow-up time, blames the CRC risk increase seen in Western countries mainly on high total energy intake rather than the consumption of specific nutrients like fat (60).

Consumption of red meat was associated with a doubled risk for CRC in the Nurse's Health study (see above), and this association has been studied in many other investigations during at least the past three decades, with conflicting results. One such study that has been often cited, is a large cohort study of 148,610 patients followed from 1982 and published in JAMA 2005 (62). The persons included in the study answered detailed questionnaires about life style factors and diet, one in 1982 and one ten years later. During follow-up until 2001, 1667 CRCs were diagnosed in the study population. Individuals reporting red meat consumption within the highest tertile both in 1982 and 1992, had a higher risk to develop CRC compared with those in the lowest tertile (RR 1.50, [95% CI 1.04-2.17]) (62) The conclusion was that long term consumption of meat led to higher CRC risk.

The question whether or not consumption of red meat causes cancer cannot be addressed through randomized controlled trials, the golden standard when exploring the association of exposure with a certain outcome. The large bulk of studies published on this issue have varied a lot regarding study design, definitions of various types of meat, definitions of amounts of meat consumed as well as methods to assess it. The range of meat consumption in the study population has also varied, and also the different cultural context. Meat consumption varies immensely between for instance Asian countries, Mediterranean countries and the United States, and yet these different studies are compared with each other. This makes meta-analysis a difficult issue, but still, there are at least five meta-analyses performed in the last ten years trying to analyze whether or not eating red meat causes cancer (58-62). All of them are more cautious in their conclusions, producing lower OR estimates for the CRC risk in consumers of red meat above a certain level than the JAMA study cited above. OR estimates are in the meta-analyses (63) calculated to be in the range between 1.17 – 1.28 depending on study. There are studies claiming that the way of preparing and cooking the meat is more strongly associated with CRC risk than meat consumption *per se* (64, 65).

Carcinogenic compounds in food

There are many mutagenic substances believed to be present in food. Most of them are formed through reactions when the food is conserved or cooked (66). They belong to three main classes of compounds: heterocyclic amines (HCAs), polyaromatic hydrocarbons (PAHs) and N-nitroso compounds (NOCs).

HCAs are generated during high temperature cooking, like grilling, pan-frying and barbecuing, from the reaction between creatinine from the meat and muscular proteins and sugars in the meat. The amount of HCAs in the product is a function of cooking temperature and glucose or glycogen content of the meat, beef and chicken contain more HCAs than pork (67). Boiling and micro-wave cooking do not produce high enough temperatures to create HCAs (68, 69).

PAHs are formed by incomplete combustion of organic material and are ubiquitous contaminants in our environment. All kinds of organic compounds can create PAHs: fossil fuel, vegetable fuel, tobacco and food. They are inert, hydrophobic compounds that can be metabolized in mammal cells into metabolites capable of covalent binding to DNA, ultimately causing mutations and replication errors. In nonsmokers, the main source of human PAH intake is food which stands for 70%, the remainder comes from polluted air. Tobacco smoking adds about the same amounts in micrograms of PAH per day as food does.

Several studies from different countries have investigated what kind of food is the main source of PAHs in an ordinary diet. In all of them, the major contribution was found to come from cereals, about 30%, and vegetable oils, also 30%. Vegetables and fruits and, to a lesser extent meat, contributes to the rest (70). The PAH content in vegetables comes from air pollution. Fish and shellfish contain high levels of PAHs due to sedimentation of the molecules on the sea floor, and in meat, the PAH content is dependent on cooking method (70). There is a great inter individual variability in the sensitivity to PAHs. In a report by Maanen et al, the number of benzo[α]pyrene-DNA adducts was assessed in 21 individuals who, after a period of abstinence from smoked or barbecued food, had to consume 170 g grilled hamburgers daily for 5 days. The PAH content in an index hamburger was measured by high performance liquid chromatography (HPLC). The characteristic DNA adducts were detected in mononuclear blood cells of 8 of the 21 subjects. The experiment was repeated with a lower dose PAH as measured with HPLC in 6 new subjects in the same manner. They saw no DNA adducts in the mononuclear cells in these subjects. This experiment indicates both a dose dependent potentially mutagenic effect of PAH and an inter individual difference in sensitivity (71).

NOCs are also carcinogenic because of their capacity to form DNA adducts. They appear in food mainly because of food preparations like curing and salting in order to prevent growth of bacteria like *Clostridium botulinum* (72). Exposure to NOCs can come from endogenous routes where high intake of red meat leads to elevated NOC levels in humans (73). NOCs can also originate from decarboxylation of amino acids by gut bacteria (72).

Interactions between diet and low penetrance genes

As mentioned above, common polymorphisms in low penetrance genes can modify the risk of CRC from an environmental risk factor.

In a recent review, Andersen et al had the ambition to review all prospective studies analyzing interactions between gene and diet. No such studies were found and they had to review large case-control studies instead (74). The review addressed interactions between genetic polymorphism and various dietary elements like meat, fish, fruit, fiber, vitamins and alcohol. ATP-binding cassette transporters, coded by the genes *ABCB1*, *ABCC2* and *ABCG2*, are proteins responsible for transport of fatty acids, bacterial products and dietary carcinogens in the bowel. Polymorphism in the *ABCB1* gene was associated with CRC and meat intake in a dose dependent manner (75), although the authors cannot find a biological explanation since no difference in affinity for meat constituents was seen in an experimental model, making this epidemiological finding less reliable (74). Polymorphism in the gene coding for the pro inflammatory transcription factor NFκB was interacting with high meat intake and thus increasing risk for CRC. Significance level was borderline but experimental data support this finding. The authors conclude that a low anti-inflammatory response due to reduced synthesis of the p50 subunit of NFκB confers an increased CRC risk if the individual has a high dietary meat intake (74, 76). Other gene-diet interactions were between *GSTM1* and *GSTT1* polymorphisms and fruit and vegetables and between low-activity variant of the anti-inflammatory cytokine interleukin 10 (IL10) and fiber intake, where the increased CRC risk seen in mutation carriers could be moderated by a high intake of dietary fiber (74).

The impact on CRC risk of polymorphisms in genes encoding various cytochrome P450 proteins in relation to diet has also been investigated. This will be discussed in a section further below.

Development of CRC – molecular mechanisms

Genetics

The development from normal colonic epithelium to invasive cancer is a multistep process, often referred to as the adenoma-carcinoma sequence. The normal epithelium is polyclonal, arising from the colonic stem cells residing in the bottom of the epithelial crypts, while studies of the clonality of CRCs have revealed a monoclonal origin (77, 78).

Fearon and Vogelstein (77) proposed a model for cancer development where a number of genetic and epigenetic alterations in one or a few initiating cells provide such growth advantages that it becomes the predominant cell. This process is named clonal expansion. One important initiating event in the adenoma – carcinoma sequence is a silencing

mutation or loss of the *APC* gene (77, 79). This gene has a silencing germ line mutation in FAP (see above), but is somatically mutated in most CRCs (80). Silencing of this gene activates the Wnt signaling pathway which involves stimulation of transcription factors regulating cell proliferation and migration properties (79). Other important events during the adenoma – carcinoma sequence are the activating mutations of *RAS*, which is seen in about 40% of CRCs, and *BRAF*, present in about 15% of CRCs (81-83). This stimulates cell proliferation. At some point in tumor development, a random global demethylation of genes in the tumor cell occurs which in turn leads to transcription of normally silent oncogenes further promoting proliferation, and at the same time increased methylation of tumor suppressing genes result in enhanced cellular growth and proliferation (77). Mismatch repair genes can be silenced by methylation which is seen in patients with a microsatellite instable non-HNPCC associated tumor (79). Losses of chromosomal material and, secondary to this, silencing of the pro apoptotic gene *TP53* is also often seen in CRC (77). Further mutations affect key pathways such as inactivating mutations in the tumor suppressor *TGFBR2*, present in about a third of CRCs (84, 85), and activating mutations in the proliferation stimulating *PI3K* gene, also present in one third of tumors (86). Numerous mutational events can also occur in genes normally maintaining chromosomal stability during DNA replication, resulting in chromosomal instability and aneuploidia (87).

These findings have been detected in tumors in the same frequency regardless of ethnicity of the patients, indicating that the same molecular mechanisms cause CRC notwithstanding varying hereditary factors and environmental exposures (88-91).

Parallel to the above mentioned genetic and epigenetic events, activation of various growth factor pathways is common. One example of this is the up-regulation of cyclooxygenase 2 (COX-2), which induces synthesis of prostaglandin E2 (PGE2). This is believed to be caused by local inflammation or associated with mitogens (92). PGE2 has been associated with CRC, and COX-2 inhibitors, Aspirin and non-steroidal anti-inflammatory drugs have been shown to prevent the development of colorectal adenomas, and even to mediate regression of such adenomas (93, 94). The above described events are summarized in Figure 1.

The number of mutations in each tumor is both large and variable. Whole genome sequencing of CRCs from 11 patients revealed around 80 mutations resulting in altered amino acid sequence (95). Creation of a two-dimensional map of mutated genes visualized about half a dozen peaks – “mountains” – with previously well-known genes frequently mutated, and a large number of smaller “hills” with less frequently mutated genes. The most important mutations for driving malignant progression are by the authors thought to be those representing the “hills”. A large number of mutations, each one of which only confers a slight advantage, is probably what drives tumor progression, and the challenge is to find out what pathways are involved (95-97).

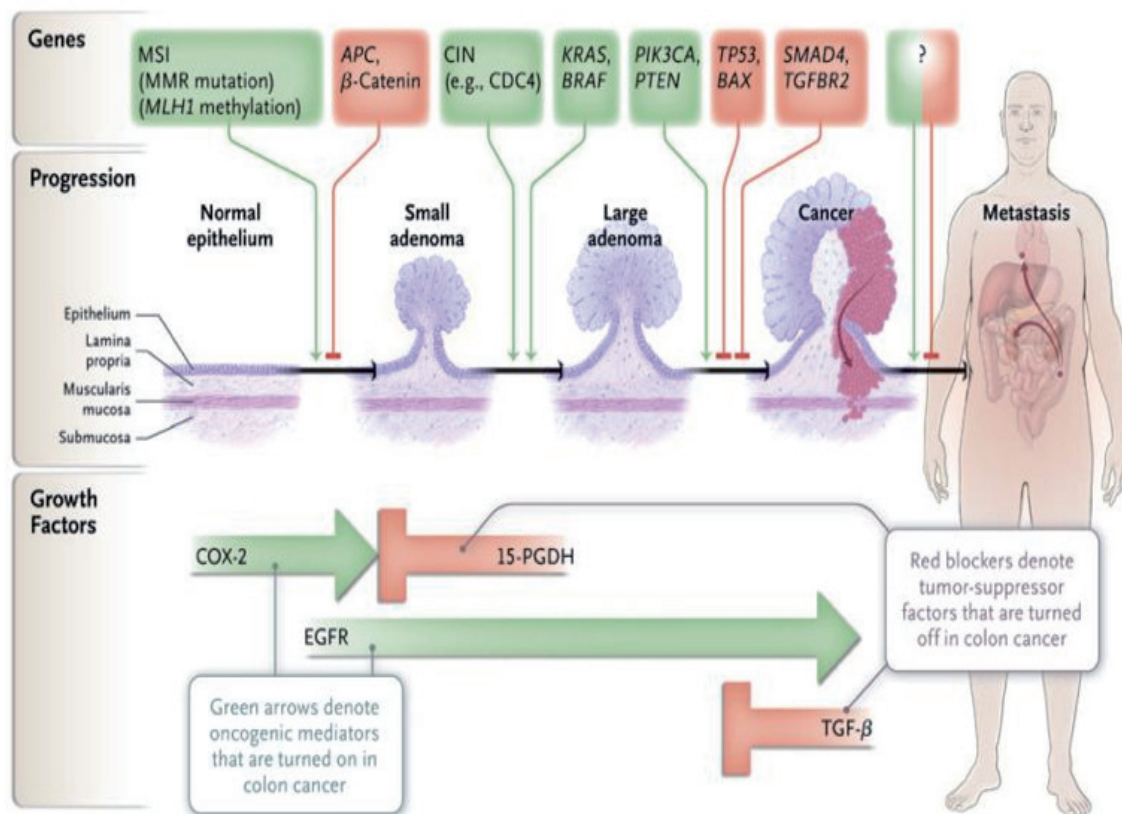


Figure 1. Genes and Growth Factor Pathways That Drive the Progression of Colorectal Cancer.

In the progression of colon cancer, genetic alterations target the genes that are identified at the top of the diagram. The microsatellite instability (MSI) pathway is initiated by mismatch-repair (MMR) gene mutation or by aberrant MLH1 methylation and is further associated with downstream mutations in TGFBR2 and BAX. Aberrant MLH1 methylation and BRAF mutation are each associated with the serrated adenoma pathway. The question mark indicates that genetic or epigenetic changes specific to metastatic progression have not been identified. Key growth factor pathways that are altered during colon neoplasia are shown at the bottom of the diagram. CIN denotes chromosomal instability, EGFR epidermal growth factor receptor, 15-PGDH 15-prostaglandin dehydrogenase, and TGF- β transforming growth factor β .

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Epigenetics

There is some evidence that epigenetic factors also play a role in tumor initiation and progression. Global demethylation can lead to chromosomal instability, and methylation can silence tumor suppressing genes as mentioned above (98). Other epigenetic events include histone modifications and loss of imprinting (LOI). Histone modifications enable the genomic DNA to be either silenced or activated by wrapping or un-wrapping the

chromatin. Imprinting is the normal silencing of one of the two parental copies of a certain gene, predominantly genes regulating cell growth and signaling. This phenomenon is seen in all mammalian cells. Loss of imprinting (LOI) means either activation of a normally silenced allele or silencing of a normally active allele (99). An example of this is the *IGF2* gene, where the activation of the normally inactivated maternal copy of the gene, LOI, is found in several different forms of malignancies like for example Wilm's tumor, lung cancer, breast cancer and CRC (100-104). *IGF2* has been shown in experimental models to have tumor promoting properties, generated by an autocrine self-feeding loop (105, 106). Another epigenetic mechanism is when short non coding RNA fragments can bind selectively to certain promoter regions of the genome and silence that gene. These RNA fragments are called micro RNAs.

There are data supporting the idea of epigenetic alterations in normal tissue increasing the probability of tumor development when a mutation occurs. LOI of the *IGF2* gene was found in 30% of CRC patients both in their tumor and in normal colonic mucosa, but only in 10% of healthy controls in a pilot study by Cui et al (107). Another investigation of LOI in an independent cohort of 172 patients undergoing colonoscopy for various reasons (age, gastrointestinal symptoms, family history of CRC), revealed a strong association between LOI in the colon and a family history of CRC. LOI was also detectable in lymphocytes from peripheral blood in 25 of the subjects and all of them had LOI also in the colon, and there was a concordance of LOI in right versus left colon in all subjects. The results were reproduced by another group and published by Woodson et al (103, 104). These data support the hypothesis of epigenetic alterations both preceding and promoting cancer development, and the issue is thoroughly reviewed by Fienberg et al (99). There are suggestions that epigenetic changes occur primarily in colonic stem cells (99). The biology of colonic stem cells and their hypothesized role in CRC development will be discussed below.

Colonic stem cells and cancer

Stem cells are the progenitors to all different types of cells. There are embryonic stem cells giving rise to all various tissue types in the embryo, but there are also adult stem cells present in specific environments – niches - in specific tissue types in adult humans. These stem cells are organ specific, pluripotent and can differentiate into any of the cell types prevalent in the tissue. For example, the bowel mucosa consists of enterocytes - the mature epithelial cells-, goblet cells producing mucin, entero-endocrine cells producing gastrointestinal peptide hormones, Paneth cells being producers of antibacterial peptides, and less differentiated progenitor cells in the bottom of the colonic crypts giving rise to all the other cell types. There are also myofibroblasts in the stroma, closely interacting with the epithelial cells (108).

The intestinal stem cells can divide symmetrically, giving rise to either two identical new stem cells or two identical daughter cells being a little bit more differentiated. Stem cells can also divide asymmetrically giving rise to one new stem cell and one more differentiated daughter cell that will start propagating up towards the top of the crypt. Symmetrical

division occurs in about 5% of the time, predominantly as a response to injury, while the asymmetric type takes place in about 95% of the time. This leads to monoclonality where one single stem cell will be dominating the crypt during a long period of time, in human colon estimated to be about 8 years, then there is a cyclic substitution of the stem cell dominance (108). The differentiation process and migration towards the top is believed to be made possible by gene expression gradients along the crypt axis. The different epithelial cell types and the gradients are depicted in Figure 2.

The activation of the Wnt signaling pathway is high in the bottom of the crypt and decreasing towards the top while the bone morphogenetic proteins (BMP) signaling pathway is active in the top and decreasing towards the bottom. Just like Wnt signaling, the expression of the EphB receptor and the Ephrine-B trans-membrane ligands is distributed in a gradient where EphB mirrors the Wnt activity and Ephrine-B ligands the opposite (108). Wnt signaling is involved in maintaining stem cells by cell cycle control and inhibition of differentiation. It also controls migration along the crypt axis and terminal differentiation of Paneth cells. As mentioned above, mutations or epigenetic alterations affecting Wnt signaling are important events in carcinogenesis (109, 110). There are also interactions between the epithelium and the stroma where both Wnt signaling, BMP signaling, Hedgehog signaling and transforming growth factor β (TGF β) are involved (111).

The Eph receptors belong to a large tyrosine kinase receptor family and their membrane bound ligands are called ephrins. Their effects include cytoskeleton modulation which affects cellular motility and adhesion, and also cell survival, proliferation and differentiation (112).

If a mutation or an epigenetic event causes activation of the Wnt pathway, EphB receptors are up-regulated in the affected cell. Expansion of these cells makes them bud out from the crypt and into the stroma forming large layers of transformed cells underneath the normal mature colonic epithelium as have been shown in animal experimental models (113, 114). During further tumor progression, the EphB receptor expression is often lost and this event then coincides with the invasion process (115, 116).

Since tumor cells possess self-renewing properties, a typical stem cell feature, it has been postulated that cancers originate from a single mutated or epigenetically modified stem cell (117, 118). This suggestion is further supported by the fact that cancers can reoccur after several years of complete removal and remission. There are experiments where tumor cell lines consisting of a number of cellular phenotypes also contain a low proportion of cells with stem cell like properties. Only the stem cell like cells can give rise to a new tumor clone with all the phenotypic features of the original tumor clone when xenografted into an immunosuppressed mouse while none of the other cells in the clone have this ability (119, 120). In contrast to the single stem cell progenitor model, some studies suggest that during the process of invasion, made possible by transformation of the epithelial cell to acquire more mesenchymal traits, the invading cell becomes more stem cell like by this transformation. This process – epithelial to mesenchymal transition (EMT) will be described in more detail below.

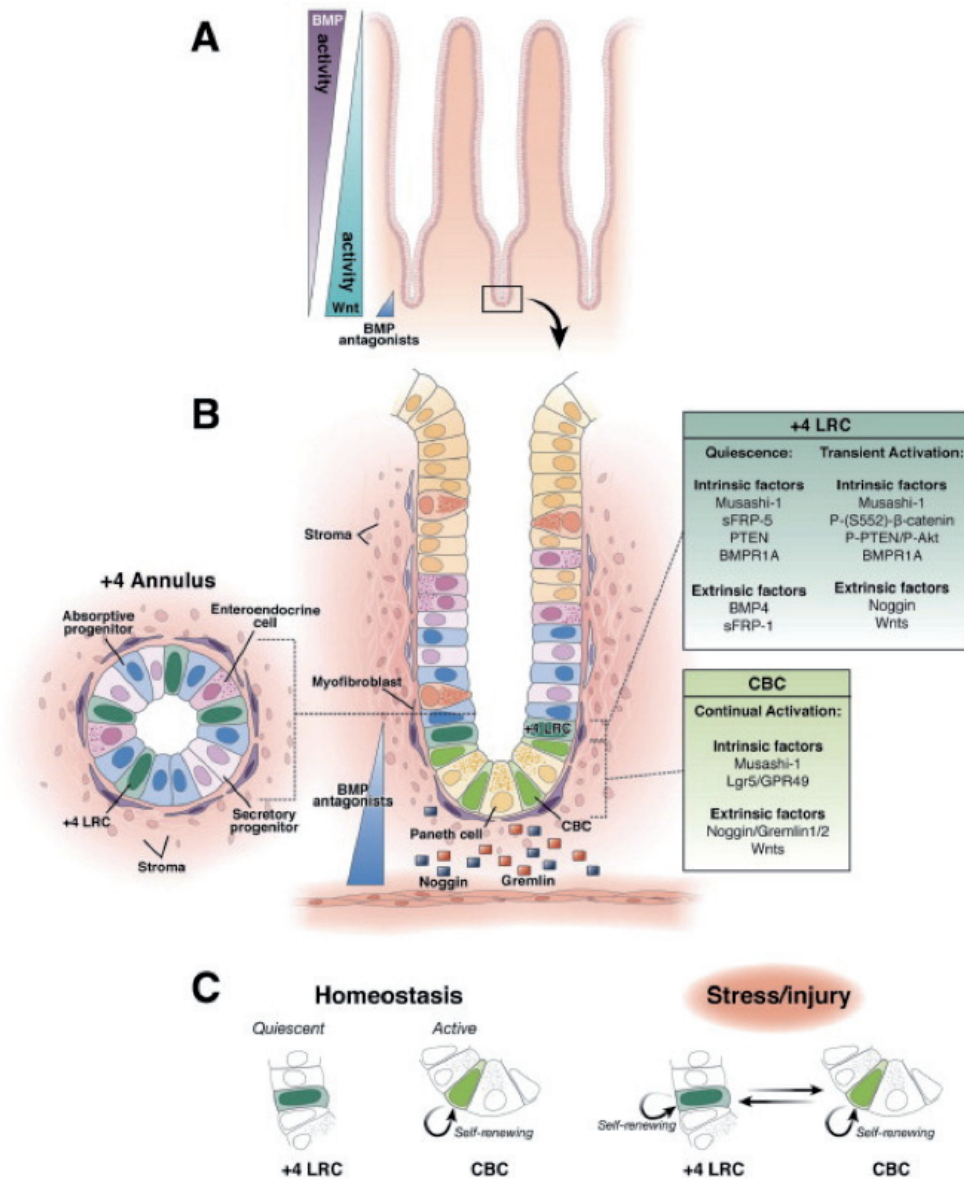


Figure 2: Model for 2 types of intestinal stem cells. (A) Pictorial representation of crypt-villus structure in the small intestine. A gradient of BMP signaling, known to inhibit proliferation, is established along the crypt-villus axis, with relatively high activity throughout the villus and correspondingly less activity within the crypt. An opposite gradient of Wnt signaling, providing an important proliferative stimulus, is highest at the crypt base and decreases toward the crypt-villus junction. In addition a very restricted gradient of BMP antagonists originates from stromal cells near the crypt base and assists Wnt signaling in intestinal stem cells (ISCs). **(B)** An enlarged view of a small intestinal crypt depicting 2 different stem cell regions; a quiescent stem cell zone and an active stem cell zone. Position four label retaining cells (+4 LRCs) are normally maintained in a quiescent state through direct interaction with and signals generated from the niche, such as pericryptal myofibroblasts and adjacent enteroendocrine cells within the +4 annulus. Crypt base columnar cells (CBCs), continually activated by signals generated from stromal cells at the crypt base, such as pericryptal myofibroblasts and smooth muscle cells, are responsible for most of the regenerative capacity of the intestine under homeostatic conditions. **(C)** Under various conditions of stress or injury, +4 LRCs may undergo transient activation to generate progenitors as well as CBCs. In addition, CBCs may be capable of regenerating lost +4 LRCs. Both intrinsic and extrinsic molecules known to associate with +4 LRCs, in both quiescent and transiently active states, as well as CBCs are listed.

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There are problems with the cancer stem cell model as well. One of the most significant problems with CRC stem cells is the lack of specific markers. Many cell surface proteins have been suggested, like CD133, CD44, Lgr5 and EpCAM, but none has been proven robustly expressed in all tumors examined since tumors display very varying phenotypes, probably also reflecting differences in their different stem cell populations. This makes them hard to study in all aspects (108, 121, 122). Other problems include the fact that the true frequency of cancer stem cells within a certain tumor is unclear, and probably also varies between tumors. The fact that many of the studies of cancer stem cells are performed on xenograft models also impose problems on translational relevance regarding their true behavior in patients (122).

Tumor stroma and inflammation

Tumor initiation, growth, invasion and metastasis are complicated events and a result of a complex interplay between genetic, epigenetic and environmental factors. Lots of attention has been paid to the malignant cell itself, but lately more attention also has been drawn towards the surrounding tissue – the stroma – and its role in promoting or repressing tumor growth, progression and invasion (123, 124). The cell types being most studied are tumor associated fibroblasts, immune cells and cells of the tumor vasculature. Tumor associated fibroblasts and immune cells will be discussed further.

Tumor associated fibroblasts are believed to be derived from either myeloid progenitor cells from the bone marrow or from epithelial cells that have undergone transformation into mesenchymal-phenotype cells, a procedure called epithelial-mesenchymal transition (EMT) (124). When recruited from the myeloid precursor cells in the bone marrow, cytokines and chemokines from the tumor are thought to attract these cells to the tumor by similar mechanism as in physiological wound healing. The tumor stroma fibroblasts then can promote malignant behavior of the tumor cells by remodeling of the matrix proteins also present in the stroma, and by cross-talk with tumor cells, immune cells and vascular endothelial cells by secreting cytokines, chemokines and transcription factors. Remodeling of the matrix makes it easier for cancer cells to invade the surrounding tissue and, eventually, to metastasize (125, 126). An important pathway for maintenance of a tumor promoting phenotype of the tumor associated fibroblasts is the activation of NFκB which evokes a pro inflammatory response (127). A key cytokine believed to be an important promoter of tumor supporting inflammation is interleukin 6 (IL-6), a target gene for NFκB, inducing production of the transcription factor signal transducer and activator of transcription 3 (STAT3) which further affect various immune cells present in the stroma (128). Other factors of importance for maintenance and regulation of stem cell properties of cancer stem cells and the properties of the supporting niche are soluble factors like Wnt, Notch, BMPs, hedgehog, epidermal growth factor (EGF), platelet derived growth factor (PDGF) and also hepatocyte growth factor (HGF) (129). There is also evidence for HGF promoting EMT and thus invasion and metastatic properties in the tumor (130, 131).

Immune cells are believed to have a dual role in tumors – they can be both tumor promoting and tumor suppressing depending on context. It is well established that chronic inflammation can lead to cancer, e.g inflammatory bowel disease can lead to CRC (132-134). The tumor microenvironment of patients with no prior history of inflammatory disease is also often infiltrated by immune cells in varying quantities and in a varying state of activation. The inflammatory response is believed to be evoked by the genetic and epigenetic events in the tumor cells (135). The inflammatory cells present in the tumor stroma are mainly tumor associated macrophages and lymphocytes (135).

The human T-lymphocyte response to cancer can be either cytotoxic (Th1 response) leading to tumor suppression, or tolerance inducing (Th2 response) leading to tumor promotion. There are examples of both types of immune response to CRC. The molecular basis for this difference is different expression of cytokines, chemokines and growth factors. The reason for this is largely unknown although believed to be orchestrated by tumor associated macrophages present in the tumor stroma (135). Macrophages, fibroblasts and tumor cells can produce IL-6, IL-1 β , COX-2 and IL-23. These induce expression of STAT3 and STAT5 which promote an immune-tolerant Th2 response that inhibits the cytotoxic T-cells and also promote further expression in the tumor microenvironment of pro-proliferative, pro-angiogenic and anti-apoptotic factors (128). A Th1 response, on the other hand, is evoked by production of IL-12, interferon gamma (IFN- γ), IFN- α and IFN- β from macrophages or other stroma cells, which in turn activate transcription of STAT1 and STAT4, two transcription factors having the opposite effect to those of STAT3.

IL-6 also has systemic effects by stimulating the production of C-reactive protein (CRP) in the liver, creating a systemic inflammatory response which has been linked to a worse prognosis in CRC (136). Lymphocyte infiltration in the tumor is associated with a better outcome and is rarely seen in the IL-6/STAT3 dominated environment but more frequently in presence of a Th1 response (128, 137, 138).

COX-2 is overexpressed in both tumor cells and tumor associated macrophages in about 90% of CRCs (139-141). The induction of COX-2 in CRC is not fully understood although cytokines, growth factors and activated oncogenes probably are important inducers. The tumor promoting effect of COX-2 comes from both PGE2 dependent and PGE2 independent pathways where the PGE2 dependent pathways include pro-proliferative, anti-apoptotic and motility stimulating actions. The PGE2 independent pathways include activation of pro carcinogens, e.g PAHs, and reduction of free arachidonic acid (AA) in the environment. AA has a pro apoptotic action (141).

Biology of the invasion – metastasis cascade

The process of invasion requires certain properties of the cell. A normal epithelial cell has a firm attachment to the neighboring cells, it has an apical-basal polarity and it has a cytoskeleton preserving a constant shape. In order to acquire invasive capacity, the cell must get rid of these features through the process called epithelial-mesenchymal

transition (EMT) described above (142). This process is induced by TGF- β produced by myeloid derived cells in the tumor stroma, or tyrosine kinase receptor ligands, that by up-regulation of transcriptional repressors like Snail, Slug, Zeb1, Zeb 2 and Twist inhibit expression of E-cadherin (143). The reduction of E-cadherin expression causes breakdown of intercellular junctions and induces Wnt signaling which further enhances the process resulting in loss of polarity and gain of motility (144). Tumor cells exhibiting these traits are mainly located in the invasion front of the tumor indicating the dependence of factors from the nearby stroma to obtain this specific phenotype. Cells in the core of the tumor do not as often have these features (145).

TGF- β has a dual role in tumor development – it can be both tumor suppressing and tumor promoting. The tumor suppression is mediated by initiation of a complex against decapentaplegic 2/3/4 (SMAD2/3/4) complex, while the tumor promoting action is mediated by for example extracellular signal-regulated kinase (ERK) or phosphatidylinositol-3 kinase (PI3K). The shift towards a more complete tumor promoting action of TGF- β is probably due to silencing or reduced levels of SMAD4 or to mutations in the *TGFBR2* gene (146-149).

The question why certain tumor cells can spread to and survive in a completely different organ with completely different prerequisites, and why they spread only to some organs but not to others, has interested scientists for centuries. In 1889, Stephan Paget asked the question: “What is it that decides what organs shall suffer in a case of disseminated cancer?” His own observations from autopsies of patients with fatal breast cancer contradicted the prevailing hypothesis that metastasis simply could be explained mechanistically, by the arrest of tumor cells in the capillary bed of an adjacent organ. He formulated the “seed and soil” hypothesis where the “seed” is the initiating cell or metastatic cell, and the “soil” is the microenvironment where the seed, aided by permissive factors in the soil, is able to settle down and sometimes to grow (150, 151). This hypothesis has survived over the centuries supported by many experimental and observational studies. One observation supporting this, is the fact that the drainage of malignant ascites in ovarian cancer into the vena cava does not result in massive metastasizing in spite of the fact that many grams of tumor cells must be shunted into the systemic circulation during that treatment (152). Specific organ preferences to specific tumor cells, regardless of vascular anatomy, have also been demonstrated in animal models (153).

In order to metastasize, the tumor cell – the “seed” - must be proficient in 10 different events. Each event is influenced by the interactions between the malignant cell and the stroma cells, growth factors, cytokines and chemokines in the surrounding tissue. First, the cell must be transformed into a neoplastic cell, and grow. When the tumor mass grows to more than 1-2 mm, angiogenesis is necessary for tumor survival. Local invasion of the host stroma and the capillaries is then imperative, made possible by the EMT process. Later on, the tumor cell aggregates must detach and enter the circulation. Well in the blood stream, a really harsh environment for metastasizing cells, the tumor cell aggregates must not only survive shear forces and lack of nutritional substrates, but also evade recognition and destruction by the host immune defense. The tumor cells must then arrest in the capillary bed of the target organ and successfully extravasate by mechanisms

similar to those involved in invasion. The next step is to survive and start to grow in the new site, the “soil”, establish vasculature, evade host immune response and eventually reinitiate a new metastatic process (154-156). Since most of our diagnostic methods have a detection limit of about 1 cm size, which means that the tumor has actually been growing for approximately 10-15 years, the metastasizing often already has occurred prior to diagnosis. Given the fact that metastatic cells, in many cases, can prevail for years in a state of dormancy in order to adapt to the new and sometimes hostile environment, the metastasis may not be detectable at the time of diagnosis of the primary tumor (154). The metastatic process is astonishingly inefficient – less than one in 10.000 tumor cells entering the blood stream ultimately form a macroscopic metastasis (156).

The seed can be characterized by examining either the circulating tumor cells (CTCs) from the blood stream or tumor cells from the metastatic lesions. A Spanish group has performed whole transcriptome analysis on isolated CTCs from 6 CRC patients revealing 410 genes that characterized the CTC population not being expressed in blood cells from a control group of healthy subjects (157). All these genes were related to cell movement and adhesion, cell proliferation and cell death, and cell signaling and interaction. These features are essential to a tumor cell clone in order to survive the metastatic process (157). The primary tumor displays a high degree of heterogeneity as to cellular phenotype. The phenotype of cells in a metastasis is also heterogeneous although less stable, probably due to increasing genetic instability in this selected, more malignant group of cells (154).

The soil is believed to be prepared for the metastatic lesion by factors emanating from the primary tumor – the tumor cells, the stroma or both – attracting myeloid cells and, later, tumor cells to the metastatic site. This has led to the concept of a premetastatic niche. There is experimental data supporting this hypothesis where hematopoietic VEGFR1-positive cells from the bone marrow can arrive at a distant organ site to further promote homing of circulating tumor cells to that organ (158, 159). Another experimental study found that extracellular matrix from hepatocyte culture stimulated CRC cells more effectively to proliferate than did matrix from fibroblast culture (160).

Prognostic and predictive factors

As probably understood from what has been discussed above, CRC is a heterogeneous, multi-pathway disease where we have few answers to what patients will respond to which treatment and why. In order to understand the reasons for treatment failure and to develop a tool to tailor the right therapy to the right patient, many groups have studied prognostic and predictive factors in CRC. Prognostic factors provide information about the natural course of disease while predictive factors address the question of benefit of treatment or not (161).

The most established prognostic factors are TNM stage and additional histopathological features including venous or lymphovascular invasion, free resection margins and tumor

grade (162-164). Tumor budding and tumor border configuration – pushing versus infiltrative – also seem to be of prognostic value although not yet used regularly in clinical practice. Absence of tumor budding is often associated with intra-tumoral or peritumoral lymphocyte infiltrates which is associated with a better prognosis (165-169). Clinical features such as bowel obstruction, tumor perforation at presentation, somatic performance status and carcinoembryonic antigen levels, all of which probably most reflect the extent of tumor burden at diagnosis, independently affect outcome, although they do not predict response to treatment (163, 170, 171). There is an increasing interest in markers of systemic inflammation as prognostic factors in CRC. The rationale for this is the IL-6 driven vicious circle in the tumor microenvironment causing increased production of CRP in the liver and a systemic inflammatory response. This is believed to be detrimental due to both enhanced tumor growth and to increased drug toxicity (136, 172). Preoperative CRP has not yet been formally included in the treatment planning algorithm.

Many molecular markers have been investigated with, to some extent, conflicting results. A good example for this is the *KRAS* mutation, present in about 30% of CRCs, where some reports have found it to be a prognostic factor while others have not (173-177). The reason why it is useful to know the mutational status of *KRAS* in a tumor, is that it predicts response to treatment with anti-EGFR antibodies, 99% of mutant carriers will not respond to such treatment (178).

There are other common problems when evaluating potential prognostic or predictive markers apart from diverging results and lack of reproducibility. One is that many of the studies are performed on small samples, which often means lack of statistical power – i.e. either the sample is too small to prove a true difference or you find a difference that is due just to chance but is interpreted as a true difference. Another problem is the inconsistent use of a wide array of methods in different studies making the studies hard to compare and impossible to draw any conclusions from performed meta-analyses (164).

The various molecular markers that have been analyzed include loss of the long arm of chromosome 18, *APC* mutation, *TP53* mutation or loss, mismatch repair deficiency, chromosomal instability and differences in genetic polymorphism and gene expression levels in various enzymes taking part in the metabolism of chemotherapeutic agents (161). The enzymes mostly studied are thymidylate synthetase (TS) (179), dehydropyrimidine dehydrogenase (DPYD) (180, 181) and methylenetetrahydrofolate reductase (MTHFR) (182), all three of them involved in the metabolism of 5-fluorouracil (5-FU), a chemotherapeutic agent used in almost all treatment regimens for CRC. Polymorphisms in genes coding for enzymes important for the metabolism of oxaliplatin (e.g. glutathione-S-transferases) and irinotecan (UDP-gucuronosyltransferases) have also been evaluated. None of these have been proven robust enough to be included in the daily clinical practice or decision making due to reasons mentioned above (161).

Table 1. The human CYP families and their main substrates and functions

Family	Number of subfamilies	Number of genes	Substrates/function
CYP1	2	3	Metabolism of eicosanoids* and xenobiotics; in addition, CYP1A2 metabolizes melatonin, oestrogen, uroporphyrin and 24 drugs
CYP2	13	16	Metabolism of eicosanoids*, xenobiotics and many drugs
CYP3	1	4	Metabolism of eicosanoids*, xenobiotics and many drugs
CYP4	6	12	Metabolism of eicosanoids*, xenobiotics and few drugs
CYP5	1	1	Thromboxane A ₂ synthase
CYP7	2	2	Cholesterol, bile acid synthesis
CYP8	2	2	Prostacyclin synthase, bile acid synthesis
CYP11	2	3	Steroidogenesis
CYP17	1	1	Steroid 17-hydroxylase, 17/20-lyase
CYP19	1	1	Oestrogen aromatization
CYP20	1	1	Expressed in gastrula, neural patterning and somitogenesis, organogenesis, fetus and nasopharynx
CYP21	1	1	Steroid 21-hydroxylase
CYP24	1	1	Vitamin D ₃ 24-hydroxylase
CYP26	3	3	Retinoic acid hydroxylation
CYP27	3	3	Bile acid biosynthesis, vitamin D ₃ hydroxylations
CYP39	1	1	24-hydroxycholesterol 7-hydroxylase
CYP46	1	1	Cholesterol 24-hydroxylase in the central nervous system
CYP51	1	1	Lanosterol 14-demethylase

Each gene within each subfamily is numbered in sequence, usually according to the order of discovery (for example, CYP1A1, CYP1A2). If a gene is the only member of that subfamily, the name ends in 'A1' (for example, CYP20A1, CYP27C1).

There are many examples of eicosanoid metabolism by members of the CYP1, CYP2, CYP3 and CYP4 families. In phenotype-genotype association studies that involve cancer as the trait, more than a dozen reasons concerning the difficulties associated with determining an unequivocal phenotype, and about 36 reasons concerning the difficulties associated with determining an unequivocal genotype have previously been detailed. It therefore comes as no surprise that virtually all epidemiological studies of human CYP polymorphisms associated with environmental carcinogenesis remain at least partially ambiguous.

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Cytochromes P450 – a family of enzymes

Cytochrome P450 (CYP) monooxygenases have important and diverse roles in both physiological and toxicological processes in vertebrates. Humans have 57 different active *CYP* genes (183) while for instance rice has 323 and mouse has 102. All *CYP* genes are believed to originate from one common ancestral gene some 2000 million years ago, branching out during evolution to the diversity between and within species that we see today (184).

The human CYP superfamily is divided into 18 families based on similarities in amino acid sequence: sequences being more than 40% identical belong to the same family (e.g CYP1, CYP2). The families are further divided into subfamilies having more than 55% identical amino acid sequences (e.g CYP2A, CYP2B) (185).

The CYPs are monooxygenases important in the phase I metabolism of for example foreign compounds, xenobiotics. Other examples of enzymes in this group are flavin-containing monooxygenases (FMOs), cyclooxygenases (COXs) and monoamine oxygenases (MAO). CYPs account for about 70% of phase I metabolism of xenobiotics in humans. The phase I reaction creates an intermediate metabolite which is further metabolized in a phase II reaction rendering it water soluble and possible to excrete in the urine. Examples of phase II enzymes are UDP-glucuronosyltransferases (UGTs), N-acetyltransferases (NATs) and glutathione S-transferases (GSTs).

The CYPs typically catalyze a reaction where one atom of molecular oxygen is incorporated into the substrate and the other oxygen atom is reduced to water. This reaction requires also a reduction agent like for instance NADPH, and an agent to facilitate electron transfer, most commonly cytochrome P450 reductase. The cellular localization of CYPs in humans is mainly membrane bound in the plasma membrane or in the ER, and to a lesser extent in the mitochondria (186).

CYPs are important enzymes in metabolism of both exogenous and endogenous compounds. Members of the families CYP1-3 are mainly responsible for the metabolism of xenobiotics, CYP family 5-51 members are involved in the biosynthesis of endogenous substances and CYP family 4 members have a mixed function taking part in both metabolism of fatty acids, arachidonic acid and xenobiotics. Some family 1-3 members also have a function in vitamin D and arachidonic acid metabolisms (187). The main functions of the various human CYP families are displayed in Table 1.

Most CYPs are expressed predominantly in the liver, although extrahepatic CYP expression is also seen. We are frequently exposed to various xenobiotics in the environment coming into contact with our skin, respiratory tract and gastrointestinal tract, and thus, these tissues express various CYPs to quickly take care of the first metabolic step.

The human CYP5-51 family members are highly conserved throughout evolution and most of them have orthologs in fugu fish (*Takifugu rubripes*) (188). The CYP2 family is the most diverse with 16 genes and 11 subfamilies in humans and 42 genes and 11

subfamilies in zebrafish. There are a few CYP2 genes/subfamilies that are enough conserved to be considered orthologs, two of these are CYP2U1 and CYP2R1. They are found in all vertebrates examined so far, including birds and lizards. CYP2U1 functions in the hydroxylation of arachidonic acid and CYP2R1 acts as 25-hydroxylase of vitamin D2 and D3(189).

Polymorphism in the *CYP* genes

Many of the *CYP* genes are highly polymorphic. Detailed information is available at the website <http://www.cypalleles.ki.se>. The most polymorphic CYPs are those important to the metabolism of drugs, for example CYP2B6, CYP2C9, CYP2C19 and CYP2D6. This leads to inter-individual variations in the ability to metabolize drugs with ranges from poor metabolizers to ultrarapid metabolizers depending on polymorphism and homo- or hetero-zygosity (190). This in turn leads to large variations in sensitivity to toxic side effects of e.g anti-cancer drugs. CYP3A enzymes are important contributors to the metabolism of about 50% of all drugs on the market. CYP3A5 has a number of common and significant polymorphisms, although the consequences of these are less dramatic since the predominant CYP3A expression in adult life is the much more conserved CYP3A4 (191, 192).

The CYPs mainly metabolizing carcinogens are to a much lesser extent polymorphic with either rare or insignificant polymorphisms. Examples from this group of well conserved CYPs are CYP1A1, CYP1A2, CYP2E1 and the above mentioned CYP3A4, the latter taking part in metabolism of both drugs and pro-carcinogens (193).

CYPs and cancer risk

The carcinogens mentioned previously, PAHs, HCAs and NOCs, can all be bioactivated by CYPs. CYP1A1, CYP1A2 and CYP1B1 are induced by these toxicants via the transcription factor aryl hydrocarbon receptor (AhR). AhR dimerizes with aromatic hydrocarbon receptor nuclear translocator (ARNT), also known as hypoxia inducible factor 1- β (HIF1B), and induce transcription of *CYP* genes (194, 195). No significant polymorphisms in these CYP genes have been found, although polymorphisms are seen in both *AHR* and *ARNT*. After the CYP catalyzed phase I reaction, the substance is further metabolized by phase II enzymes, e.g N-acyltransferases, where also polymorphisms are found (196).

This complex regulation makes it difficult to prove simple correlations between various polymorphisms, diet and other life style factors due to reasons discussed earlier in the text. Briefly, it requires very large samples, strict control of confounding factors and a very stringent and reliable, ideally prospective, reporting of life style factors from the study subjects making such studies very hard to conduct. Numerous attempts have been made in order to evaluate genetic polymorphisms and association with environmental factors in cancer risk, the vast majority suffering from methodological weaknesses. There

are some well performed experimental studies in knockout mice indicating associations between CYPs and cancer risk due to pro-carcinogen activation, but the results from these studies, performed under extremely well controlled conditions, cannot be easily translated into patients (197, 198).

CYPs and prognosis

CYPs may theoretically affect outcome of cancer disease in two ways. One is tumor specific expression of CYPs contributing to either increased bio-activation of carcinogens further enhancing tumor growth or rapid elimination of anti-cancer drug in the tumor cells. Another way is ultra-rapid or slow metabolism of anti-cancer drugs in the liver leading to either reduced effect or toxic side effects. This may be due to inherited factors - polymorphisms – or factors related to the disease like for example systemic inflammation in cancer.

Tumor specific CYP expression is examined in several studies. CYP1B1, a metabolizer of pro-carcinogens, is frequently expressed in tumor tissue (193, 199). There are reports of CYP3A4 expression in breast cancer (200), osteosarcoma (201) and CRC. In one Spanish study analyzing CYP3A4 activity in CRC samples from 17 patients, the enzymatic activity of CYP3A4 towards paclitaxel was about one tenth of that in the liver (202). It should be mentioned, though, that CYP3A4 is also expressed in the normal colonic mucosa. Other CYPs found in CRC are CYP3A5, CYP2U1, CYP2S1, CYP2W1 and CYP51 (203-205).

CYP polymorphisms can also be important determinants of outcome due to variations in individual sensitivity to anti-cancer drugs. One example is CYP2D6, which displays an extensive genetic inter-individual variability resulting in defective or increased enzyme activity. CYP2D6 is involved in the metabolism of tamoxifen which has an impact on the response to tamoxifen therapy in breast cancer (193). Other relevant CYP polymorphisms are those in the CYP2C subfamily where for example CYP2C8 is involved in the metabolism of paclitaxel (193).

CYP2W1

The Human Genome Project (HUGO), started in 1989 and completed in 2003, revealed the existence of a number of previously unknown *CYP* genes, *CYP2R1*, *CYP2S1*, *CYP2U1*, *CYP2W1*, and a number of *CYP4* genes (206).

The *CYP2W1* gene is a typical family 2-gene with 9 exons encoding a 490 amino acid long polypeptide. The human *CYP2W1* gene is located on chromosome 7p22.3, and orthologs are found in other species, for instance birds, lizards, rat and mouse, although the gene is not as well conserved as *CYP2U1* (207). Studies of expression of the gene have been performed showing high levels of CYP2W1 mRNA in samples from various human tumors, predominantly in colon and adrenal tumors, but not in any normal untransformed

tissue in samples from lung, placenta, liver, kidney, bowel, spleen, muscle, heart or brain apart from small trace amounts (207). In rat fetal tissues, the enzyme was expressed at the mRNA level in colon with increasing expression up to just before term. Western blotting of human tumors also showed protein expression in colon and, to a lesser extent, adrenal tumors (207). The function of the enzyme during fetal development is hitherto unknown.

Regulation of the *CYP2W1* transcription

It is unclear what mechanisms are controlling the silencing of the *CYP2W1* gene at term of gestation, and when in the sequential development from normal mucosa to invasive tumor the gene is activated. Gomez and coworkers analyzed methylation status of a CpG island in the exon 1-intron 1 junction. They found that the expression in CRC is associated with the demethylation of this CpG island, but concluded that there are probably other mechanisms influencing gene activation than just this single methylation site. The variable extent of expression between the cell lines examined, in spite of the same methylation status, indicate that other factors are involved as well, specific transcription factors or miRNAs for example. This remains to be elucidated (208). Maybe tumor hypoxia and HIF-1B plays a role for induction of CYP2W1 expression in combination with AhR induced by carcinogens in the diet. These are just speculations since we do not know if CYP2W1 expression is inducible by the AhR pathway.

Polymorphism in the *CYP2W1* gene

Like for other members of the CYP family 2, polymorphisms in the *CYP2W1* gene have been reported (209). Three of these are found in coding regions of the gene but only two of them give rise to changes in amino acid sequence. These two variant alleles are named *CYP2W1**2 (Ala181Thr) and *CYP2W1**6(Pro488Leu). Gervasini and coworkers published a study comprising 150 CRC patients and 263 controls that showed a decreased risk to develop CRC in carriers of the *CYP2W1**2 allele (210). The plausible association between polymorphism in the *CYP2W1* gene and CRC risk is addressed in paper III of this thesis.

Substrates to CYP2W1

Investigations of potential substrates have been performed in various experimental models. Wu and coworkers found catalytic activity of CYP2W1 towards aflatoxin B1 and a number of other pro-carcinogens. They also found CYP2W1 to catalyze the *N*-demethylation of *d*-benzphetamine and, at a much slower rate, oxidation of arachidonic acid (211). Nishida et al found CYP2W1 to effectively activate the new anti-cancer agent AQ4N into a potent topoisomerase inhibitor in a hypoxic environment (212) and Tan et al found CYP2W1 expression to be induced by another novel anti-tumor agent, GW-610, in a breast cancer cell line, and it also seemed as if CYP2W1 further activates the drug (213). Xiao and coworkers have shown that lysophospholipids – phospholipids where one acyl group has

been cleaved off - can be hydroxylated by CYP2W1, giving rise to the hypothesis that an imbalance between phospholipid metabolites may be a contributing factor for cancer development (214). A metabolite of lysophospholipids – lysophosphatidic acid – acts as a lipid mediator that stimulates proliferation and migration of cells.

CYP2W1 has been proven to convert indole to oxindole (215) and in a recently published study to convert various duocarmycin analogues into metabolites, toxic or non-toxic depending on the substance used (216).

Using tumor specific CYP2W1 as a target for pro-drug activation

The pro-drug activation concept has already been tried for other CYPs expressed in tumors, although yet only at an experimental stage, targeting for example CYP1B1 (217, 218). The idea is that a non-toxic substance with selective specificity to the tumor specific CYP-enzyme, is converted into a toxic metabolite killing selectively the tumor cell. Nishida and coworkers and Travica et al have explored this possibility by examining substrates converted by CYP2W1 to treat CRC with so far promising results in experimental models (212, 216). The duocarmycin analogues, indolines, investigated by Travica et al, are small molecules that fit into the active site of CYP2W1. The metabolite forms DNA adducts that cause cell death.

This conversion takes place in the endoplasmic reticulum (ER) of the cell where the largest proportion of CYP2W1 is abundant. About 8-10% is bound to the plasma membrane of the cell and could therefore be targeted by antibody treatment (219).

There are a few requirements for this model to work out. First, we must be sure the enzyme is tumor specific and not expressed in any normal tissue. Second, the substrate must have high affinity to one particular CYP enzyme. Third, the enzyme must be expressed in a fairly high amount of tumors and also in metastases. The enzyme also has to be expressed in an enough large amount of cells within a tumor, since tumors often are heterogeneous as previously discussed. It is unclear how large the minimum amount is. When one tumor cell dies, adjacent cells also die by what is referred to as the bystander effect. The impact of this in relation to CYP2W1 mediated toxicity is not yet fully elucidated although some proof of existence of the bystander effect in this particular reaction is revealed in the study by Travica et al (216). Last but not least, the enzyme must be catalytically active.

If these requirements are fulfilled, targeted therapy using tumor specific CYP2W1 would be a novel complement to standard anti-cancer therapies.

AIMS OF THE THESIS

The overall aim of this thesis is to explore the role of CYP2W1 in CRC.
The more specific aims are:

To evaluate the extent of CYP2W1 expression in primary CRC

To assess the association between CYP2W1 and tumor phenotype

To evaluate the relationship between polymorphism in the *CYP2W1* gene and CRC risk

To evaluate the extent of CYP2W1 expression in colorectal metastases

MATERIALS AND METHODS

Patients

In study I and II, we used paraffin embedded blocks from patients with primary CRC stage II and III. The patients were derived from a randomized clinical Nordic trial aiming to evaluate surgical treatment compared to surgery combined with different adjuvant chemotherapy regimens. The original cohort consisted of 2224 patients under age 75 years, that were treated for stage II and III CRC between 1991 and 1996. They were randomized to either surgery alone or to one of three plausible adjuvant treatment regimens: 5FU/levamisole for 12 months or 5FU/leucovorin for 4-5 months according to a Mayo clinic schedule with or without levamisole or a Nordic schedule with or without levamisole (220-222). From this large clinical trial, we obtained tumor specimen from 162 patients with CRC treated at five different Swedish hospitals for study I and 235 patients with colon cancer treated in 20 different hospitals for study II. Clinical data on patients and tumor characteristics were available, as were survival data. Tumor data were retrieved through the original pathology report while survival data were available through the regional centers of epidemiological oncology. Patient's and tumor characteristics are summarized in table 2.

In study IV, we used paraffin embedded tumor samples from patients having been treated for colorectal liver metastases at our unit for liver surgery between 2004 and 2009. The original population based cohort consisted of 255 patients that had undergone liver resections due to metastases from CRC. From this cohort, we could obtain samples from 96 cases where also the primary tumor including lymph node metastases and all other metastatic manifestations having been resected, were available. The primary tumors were treated at 12 different hospitals between 1999 and 2009, the liver metastases were all operated at Karolinska University Hospital, Huddinge, and the lung metastases were all resected at Karolinska University Hospital, Solna. Patient's and tumor characteristics and follow-up data were retrieved using patient's records and the original pathology reports. These patients are also summarized in table 2 regarding demographics and tumor characteristics.

In study III, DNA preparations were obtained from 1785 patients with sporadic CRC and from 1761 healthy controls. The patients were treated between 2004 and 2006, and recruited from 14 different Swedish hospitals. Data were collected by the Department of Cancer Genetics at the Karolinska University Hospital, Solna, and the aim of the study was to assess risk loci for developing CRC (223). No data regarding tumor characteristics were used since the study aim was cancer risk assessment and not to study tumor phenotype.

The basis for the CRC diagnosis was the finding of invasive CRC in a colonoscopy sample, and patients were excluded if they had a family history of FAP or fulfilled the clinical Amsterdam criteria for HNPCC(223, 224). The control population consisted of 1345 healthy blood donors from the general population, age between 18 and 65, and 416 spouses of the cases, known to be cancer free by health examination, age between 25 and 92. The age span of the cases was 27 – 95 years.

Table 2 Demographic and tumor related factors in the patients included in study I, II and IV

	Study I: Number of patients (%)	Study II: Number of patients (%)	Study IV: Number of patients (%)
Total	162	235	96
Gender: Male	86 (53%)	127 (54%)	59 (61%)
Female	76 (47%)	108 (46%)	37 (39%)
Age: Median	68 years	66 years	63 years
Below median	82 (51%)	114 (49%)	42 (44%)
Stage at primary op: II	73 (45%)	103 (44%)	15 (16%)
III	89 (55%)	132 (56%)	25 (26%)
IV	0	0	56 (58%)
Tumor site: Colon	117 (72%)	235 (100%)	51 (53%)
Rectum	45 (28%)	0	45 (47%)
Tumor grade: Low	40 (25%)	58 (46%)	18 (19%)
Medium	117 (72%)	159 (67%)	74 (77%)
High	3 (2%)	14 (6%)	4 (4%)
Unknown	2 (1%)	3 (2%)	0
Primary tumor treatment:			
Surgery alone	81 (50%)	125 (53%)	Not applicable
Surgery + adjuvant	81 (50%)	110 (47%)	Not applicable
Number of lymph nodes <12	92 (57%)	139 (59%)	<13: 42
≥12	34 (21%)	17 (7%)	≥13: 54
Unknown	36 (22%)	79 (34%)	0
Range	Unknown	Unknown	3-50
Neoadjuvant treatment before primary op: No	145 (89%)	235 (100%)	47 (49%)
Yes	17 (11%)	0	49 (51%)
Chemotherapy before liver op: No	Not applicable	Not applicable	41 (43%)
Yes	Not applicable	Not applicable	55(57%)

Immunofluorescence

In study I, II and IV, a polyclonal antibody towards the c-terminal of the CYP2W1 protein was used. This antibody was developed by Karlgren and coworkers as previously described (207). In western blotting, this antibody has been proven superior to a whole-protein antibody since it gives less background noise and non-specific bands.

In study I, this antibody was validated by immunofluorescence of cells from the human embryonic kidney cell line HEK-293 transfected with either a CYP2W1 containing plasmid vector or just the vector alone. After validation, this antibody was used in immunohistochemistry in study I, II and IV, and western blotting in study III.

Immunohistochemistry

For the analysis of the tumor samples in study I, II and IV, we used immunohistochemistry (IHC). The same methodology was used in all three studies. We used the above described antibody in various dilutions; 1:1250 in study I, 1:1000 in study II and 1:2500 in study IV. The varying dilutions were decided after control staining of positive and negative control samples in order to get equally strong staining.

The interpretation of the immune-staining was performed by two independent investigators with re-reading and consensus in case of disagreement. Disagreement occurred in less than 5% of cases. Since CYP2W1 is a protein to the largest extent abundant in the ER of the cell, and only to about 10% bound to the plasma membrane, staining was seen in the cytoplasm of the tumor cells. The staining intensity was graded from 0 to 3 where 0 represented no staining, 1 weak staining, 2 moderate staining and 3 strong staining. The CYP2W1 staining was quite heterogeneously spread over the tumor slide, which necessitated a cut-off level for how large a proportion had to be stained in order to grade the slide. In study I and II, we stated that the strongest staining grade covering more than 5% of the tumor area was the grade assigned to that slide. In study IV, the limit was raised to 10%. This will be discussed below. Examples of staining grades 0, 1, 2 and 3 in primary tumors are shown in fig 3.

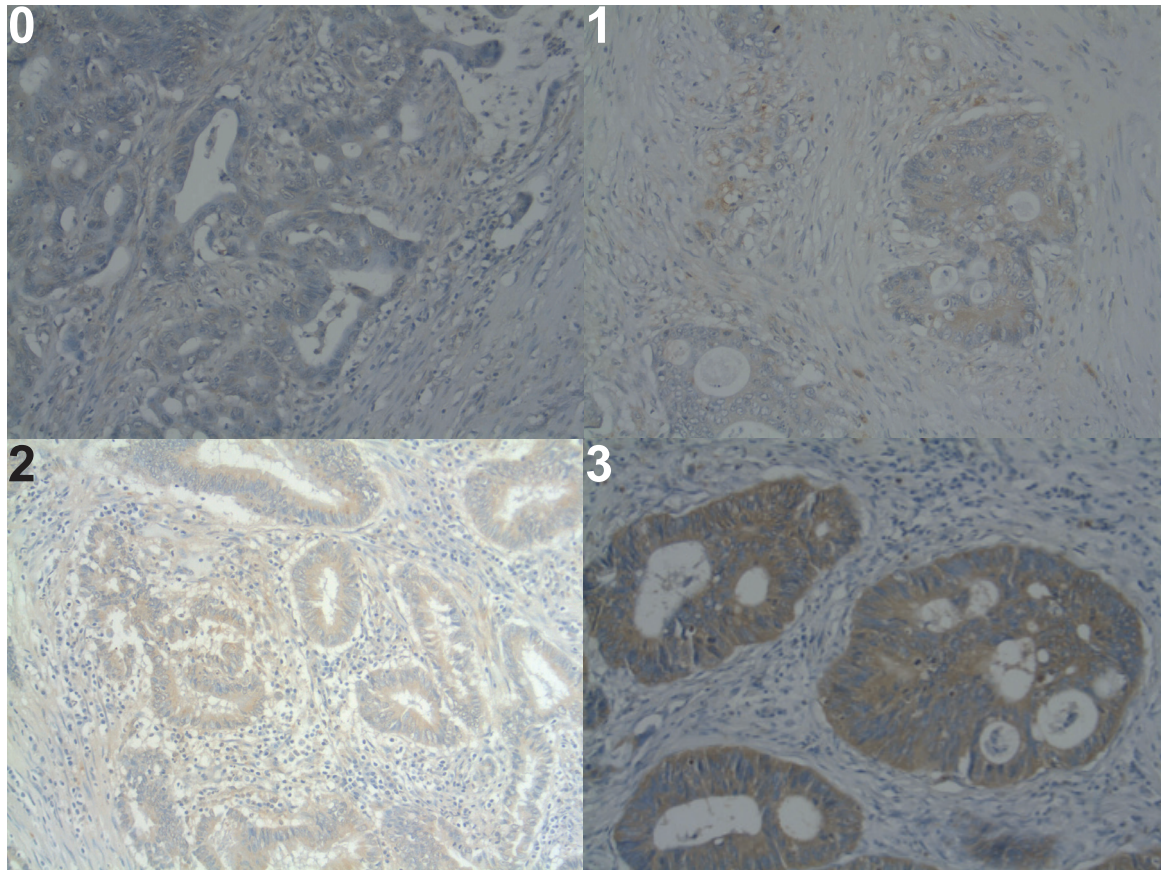


Figure 3. Examples of *CYP2W1* staining intensities in samples of primary colorectal tumors: grade 0, 1, 2 and 3.

Polymerase chain reaction

Polymerase chain reaction (PCR) is a powerful method to amplify DNA. Its advantage is when few genes in many samples are analyzed. If instead many genes in few samples are analyzed, then high throughput methods like Illumina sequencing are preferred. In study III, genetic polymorphism in the *CYP2W1* gene was examined using two different PCR based genotyping methods.

Genomic DNA from the 1785 cases and the 1761 control subjects, was kindly provided by professor Annika Lindblom, Department of Cancer Genetics, Karolinska University Hospital. The single nucleotide polymorphisms (SNPs) g2008G>A (Ala181Thr) (*2) and g5601C>T (Pro448Leu) (*6) were analyzed.

A lot of work had to be done in order to find out which available genotyping method was the most robust and most simple to perform since the number of samples was as large as roughly 3500 samples and two different loci. The *CYP2W1**6 locus is particularly difficult to amplify due to the relative enrichment in C and G nucleotides. This difficulty is caused

by the stronger hydrogen bonding between C and G nucleotides compared to those of T and A. After discarding the two methods High Resolution Melt and pyrosequencing because of too small ratio of interpretable results, the TaqMan® SNP genotyping assay and an allele specific real-time PCR method were proven to fulfill our criteria for a useful method. The Taqman assay was used for the *CYP2W1**2 SNP and the allele specific assay for the *CYP2W1**6 SNP. The Taqman assay, originally described by Heid and coworkers, is an allelic discrimination assay requiring a hybridization probe labeled with two different fluorescent dyes (225). Both alleles can be analyzed in one reaction requiring only one analysis per sample. For the *2 SNP, this method generated readable results in 98% of cases, but used for the *6 SNP, the performance was unacceptably bad, probably due to the limited amount of DNA available in each sample. Instead, we used the above mentioned allele specific PCR method using SYBR Green, a fluorescent dye that binds to double stranded DNA. The dye – DNA complex absorbs blue light ($\lambda=488$ nm) and emits green light ($\lambda=522$ nm) which is then detected. The stringency of the PCR reaction is confirmed by melting curve analysis, an assessment of the dissociation characteristics of the double stranded PCR product during further heating. This melting curve interpretation is based on the fact that the hydrogen bonding between C and G is stronger than that of A and T. For each sample, two analyses have to be made, one for each allele. This was the most robust and reliable method for the *6 SNP generating interpretable results in 94% of cases. For internal validation, we re-ran 100 samples every 1000 samples, and random duplicates were blindly inserted in the first 800 samples. The concordance was good, >95%.

cDNA constructs, transfections and cell viability assay

To evaluate functional activity of the enzymes expressed from the different genotypes, cDNA constructs must be created and, in a second step, transfected into cell lines. To start, the reference allele *CYP2W1*, labeled *1, was cloned into an expression vector generating a construct (pcDNA5/FRT/2W1*1). By a site-directed mutagenesis technique (QuikChange Lightning®, Stratagene, La Jolla, California, USA), the SNPs – G541A (*2) and C1463T (*6) – could be generated in the *1 sequence, resulting in two new constructs (pcDNA5/FRT/2W1*2 and pcDNA5/FRT/2W1*6). The constructs were validated using DNA sequencing.

The above mentioned constructs were transfected into human colon cancer cell line SW480 in order to generate the variant proteins CYP2W1.1, CYP2W1.2 and CYP2W1.6. Control cells – mock transfected cells – were generated by transfecting SW480 cells with empty vector (pcDNA5/FRT). Protein expression in the cells was confirmed using western blot.

Cells were then seeded on plates in medium and incubated with substrate (chloromethylindolines ICT 2706 and ICT 2726) for 60 hours in triplicate. Cell viability was determined using EZ4U assay (Biomedica).

CYP2W1 functional activity assay

Functional activity of the different variants of the CYP2W1 enzymes was assessed after incubation of the CYP2W1 transfected and the mock-transfected SW480 cells with the substrate ICT2726 for 4 hours. This indoline is known to be metabolized by CYP2W1 into non-toxic metabolites. After incubation, the cells were harvested and centrifuged together with the medium in order to separate the cells from the medium. The cell pellet was suspended in acetonitrile and centrifuged once more at a higher speed. The two supernatants were mixed and, after another round of high speed centrifugation, analyzed by HPLC as previously described by Gomez and coworkers and by Travica and coworkers (216, 219). The substrate and metabolite peaks were monitored at 250 nm using Varian UV detector (Varian Inc., Palo Alto, California, USA).

Statistical Methods

Statistical analyses of the results of study I, II and IV were performed using STATISTICA software, release 10 (StatSoft®, Tulsa, Oklahoma, USA). In study III, Graph Pad Prism 5 software package (La Jolla, California, USA) was used for all calculations.

In study I, II and IV, χ^2 test was performed to examine relationships between patient's demographics, tumor characteristics and CYP2W1 expression. The Gehan Wilcoxon univariate test was used to evaluate the relationships between survival and patient's demographics and tumor characteristics. Cox regression multivariate analysis was used in study I and II while in study IV, Cox' proportional Hazard's model was employed for this purpose. Survival analysis was performed using the Kaplan Meier method. In study II, Spearman Rank correlation test was performed for the assessment of correlation of CYP2W1 expression comparing two different slices from the same tumor.

All tests were two-tailed and considered significant at a *p*-value less than 0.05.

In study III, the relationship between genotype and group – case or control – was examined using the χ^2 test. Results from the functional activity assay were compared using two-way ANOVA (Dunnett's or Bonferroni) and data were expressed as means \pm SD. All tests were considered significant at a *p*-value less than 0.05.

RESULTS AND DISCUSSION

CYP2W1 expression in primary colorectal tumors

This aim was addressed in study I, II and IV. In study I, primary CRCs from 162 patients were analyzed by IHC with staining intensity graded 0 - 3 as explained above. 8% had no staining (0) while 18% exhibited grade 1, 38% grade 2 and 36% grade 3. The corresponding numbers from study II, where 235 patients with colonic cancer were evaluated, were 7% grade 0, 26% grade 1, 37% grade 2 and 30% grade 3. In study IV, 96 primary CRCs, the amount of high expressing tumors was slightly lower, 19% had grade 0, 30% grade 1, 25% grade 2 and 26% grade 3. The number of patients in the different staining groups in the three studies is displayed in Table 3.

The IHC methods used in study I, II and IV were similar apart from the dilution of the antibody and the percentage required for a certain staining grade. The difference in percentage cut-off will be discussed in the section about metastases. The staining results in the primary tumors were fairly concordant. We used a method based on a combined scoring of staining intensity and proportion of stained to unstained cells. This method can be criticized for being subjective and with a high inter- and intra-observer variability. We have tried to avoid this as well as possible using the same investigators through the studies.

One problem with IHC described the literature is the lack of consensus regarding staining evaluation protocols. There are also differences in study design and other methodological problems making for example meta-analyses hard to conduct (164). It would be appropriate to validate the IHC findings using another method. We have not done this in our material,

Table 3 CYP2W1 expression assessed by immunohistochemistry in primary colorectal tumors, study I, II and IV.

	Number of patients Study I n=162 (%)	Number of patients Study II n=235 (%)	Number of patients Study IV n=96 (%)
CYP2W1 staining grade 0	13 (8%)	16 (7%)	18 (19%)
CYP2W1 staining grade 1	29 (18%)	61 (26%)	29 (30%)
CYP2W1 staining grade 2	62 (38%)	87 (37%)	24 (25%)
CYP2W1 staining grade 3	58 (36%)	71 (30%)	25 (26%)

simply because we only have access to formalin fixated, paraffin embedded tissue blocks from these particular primary tumors. However, CYP2W1 expression has been found in about 50% of CRC samples from another, although a lot smaller, patient material using western blot (207, 208). In another ongoing study, the amount of patients with strong CYP2W1 expression assessed with western blot is only 10% in the so far analyzed 50 CRC samples (Johansson et al, unpublished data). This illustrates the need for validating studies in a larger cohort of patients.

The advantage of IHC is that the method is fairly easy to use, and also that samples from routine histopathology can be used. Another advantage is that an entire tumor slice can be analyzed in order to assess heterogeneities in expression, which might be missed using methods where only a small piece of the specimen is analyzed, e.g tissue microarray. Disadvantages with this method include the subjectivity in evaluation, the relatively low reproducibility and dependence of how the fixation process was conducted when the specimen originally was prepared for histopathology.

In study II, the question of tumor heterogeneity was also addressed. We could examine CYP2W1 expression in two slices from different parts of the same tumor in 107 patients with a correlation coefficient $r=0.53$, $p<0.001$. Within the same slice, though, the staining pattern is heterogenic with a tendency towards higher staining intensity near the invasion front (data not shown).

CYP2W1 expression in primary tumor and relation to prognosis

Since a more malignant tumor phenotype often means a poor prognosis, we used survival as a proxy for tumor phenotype when addressing the second aim. In study I and II, we evaluated the association between CYP2W1 expression and prognosis. We also analyzed survival in study IV although this was not the primary aim of that study. High expression of CYP2W1, i.e grade 3, did not correlate to gender, age, stage, tumor site or differentiation in any of the studies except for study II where age correlated with high CYP2W1 expression.

In study I (n=162), no difference in survival was seen between patients with grade 0, 1 or 2. Patients with grade 3 expression however, had a significantly worse outcome with both worse 5- and 10-year survival. High expression of CYP2W1 was found to be an independent prognostic factor in all patients in multivariate analysis (OR 1.4, [95% C.I 1.10-1.78], $p=0.007$). When analyzing colonic versus rectal cancer separately, CYP2W1 expression did not fall out as a prognostic factor in rectal cancer (n=45), only in colonic cancer (n=117).

This was the rationale behind the analysis of colonic cancer only, and not CRC, in study II. The sample size in study II (n=235) was twice that of study I counting patients with colonic cancer. Since the result of the first study was highly significant, this sample size was assumed to be enough to ensure statistical power. Since grade 0, 1 and 2 were of no significance in study I, we used the same classification with grade 3 versus the rest, for

the calculations in study II. However, high grade of CYP2W1 expression did not turn out to be of prognostic importance in the entire group of stage II and III colonic cancer patients. It did not reach significant level in univariate analysis although it fell out with borderline significance in multivariate analysis. High expression was of independent prognostic value in multivariate analysis of stage III patients only (n=132), OR 1.4, [95% C.I 1.12-1.75], $p=0.003$. The results of uni- and multivariate analyses of studies I and II are summarized in Table 4.

Table 4 Uni- and multivariate analyses of potential prognostic factors in study I and II

	Univariate analysis p-value Study I	Multivariate analysis p-value Study I	HR for death Study I	95% confidence interval Study I	Univariate analysis p-value Study II	Multivariate analysis p-value Study II	HR for death Study II	95% confidence interval Study II
CYP2W1 expression high vs low	0.01	0.007	1.40	1.10 – 1.78	0.12	0.034	1.22	1.02 – 1.48
Gender	0.4	n.s			0.64	n.s		
Age	0.2	n.s			0.78	n.s		
Stage III vs II	0.003	0.009	1.97	1.19 – 3.27	<0.001	0.003	1.81	1.23 – 2.67
No of analyzed nodes	0.4	n.s			0.09	0.047	0.37	0.14 – 0.99
Site colon vs rectum	0.5	n.s			Colon only			
Grade	0.2	n.s			<0.001	0.008	0.58	0.39 – 0.87
Therapy surgery vs surgery + adjuvant	0.7	n.s			0.12	0.066	0.71	0.50 – 1.02

It is not uncommon when analyzing new proteins as potential prognostic markers, that the initial study is very promising, but attempts at reproducing the results are not as successful due to reasons previously discussed (164). Validation in larger, independent cohorts, preferably using another method, is needed to further elucidate the prognostic value of CYP2W1.

The fact that the study patients were treated for CRC so many years ago is also a problem when trying to assess various prognostic factors. During the early 1990's, the importance of for example extensive lymph node sampling was not widely spread among the surgeons, which means that there is a risk for under-staging of the tumor disease. The total mesorectal/mesocolic excision concept and the cautious techniques discussed in the introduction of this thesis, were not as widely spread among surgeons. Today these methods are the golden standard in surgical treatment of CRC. This can of course also affect survival in the study patients. Another weakness of both study I and II is that the original pathology reports were used and the samples were not re-evaluated by a study pathologist. Some well-known risk factors for tumor spread and recurrence like vascular invasion are not even addressed in the pathology reports. This makes association, or lack of association, between CYP2W1 expression and tumor related factors, like grade, less reliable.

One would assume that high expression of an enzyme normally expressed in fetal life would be associated with some kind of de-differentiation. This has not been evaluated in this study but would of course be of interest to investigate further. CYP2W1 expression might be just a result of random demethylation occurring during the adenoma-carcinoma sequence, and one plausible mechanism by which high expression of CYP2W1 could affect tumor phenotype, is by metabolizing pro-carcinogens into carcinogens, further enhancing tumor progression, as also discussed previously. These issues remain to be further elucidated.

In study IV, CYP2W1 expression was not associated with outcome. Nor were demographic or tumor related factors like for example age, gender, primary tumor location, size and number of liver metastases, neo-adjuvant or adjuvant treatment or synchronous versus metachronous liver metastasis. In the entire population based cohort of 255 liver resected patients, from which our 96 patients were derived, only primary tumor location (rectum better than colon), presence of extra-hepatic metastases, involved microscopic resection margins and disease progression during chemotherapy were independent prognostic factors (Ydsten et al, unpublished data). Survival in the entire cohort was comparable to that of patients in studies from the best international centers, and did not differ from the 5-year survival of our 96 study patients, indicating that our sample is not severely skewed.

Polymorphism in the *CYP2W1* gene and CRC risk

The third aim of this thesis was to elucidate the association between polymorphism in the *CYP2W1* gene and risk to develop CRC. Why would it be of interest to analyze genetic variation in relation to CRC risk, when the gene is known to be expressed only in fetal life and in malignant tumors but not in normal colonic tissue?

CYP2W1 expression is so far only analyzed in tissue panels with various normal human tissues, in fetal rat tissues and in malignant tumors (207, 208). The expression has not yet been evaluated in for example polyps or colorectal adenomas. Hypothetically, expression in an early colorectal polyp of an enzyme that can activate pro-carcinogens may influence the risk of progression to a malignant tumor. This is one answer to that question. Another answer is that the association between *CYP2W1* polymorphism and CRC has already been analyzed by Gervasini and coworkers, who found a protective effect of the *CYP2W1**2 SNP (210).

In study III, we aimed to elucidate this using a case-control approach, with an almost ten times larger group of patients and control subjects, and an experimental approach trying to evaluate the mechanism behind the presence or absence of such an association. The results of both these analyses were congruent – there was no difference in distribution of the various genotypes between cases and controls, and the different genotypes altered neither affinity, nor activity, towards a previously recognized substrate to *CYP2W1* in our experimental model. The most natural interpretation of this is that *CYP2W1* polymorphism is not a risk locus for CRC.

Objections to our results would point at the fact that the age distribution is slightly different between the cases and the controls, the control subjects being younger and less well described as a group. However, this study is designed to address genotype, which is a constant, unaffected by age. The control group is meant to be a representative sample from the background population from which the cases are derived and of a large enough size to rule out the risk of having too many young “potential cases” in the control group. We do not know the exact optimum size of the control group, though, and geneticists and epidemiologists often argue on this issue. The need of a very large sample size decreases if there is a plausible biologic explanation to the findings, as previously discussed. The enzyme activity assay in our study III provides such an explanation.

CYP2W1 expression in colorectal metastases

The fourth aim was to analyze *CYP2W1* expression in colorectal metastases. This was done in study IV where we analyzed *CYP 2W1* expression in all tumor manifestations from 96 patients, i.e all primary tumors, all lymph node metastases, all liver metastases and all lung metastases. Our goal was to obtain tumor samples from all 255 patients in the population based cohort, but the logistical problems were overwhelming, partly since the

primary tumors were operated in 12 different centers and during a long time span. Yet, it is a large material compared with other groups comparing various differences in expression profiles between primary tumor and metastases from the same patient (226-228).

All 96 patients in study IV had undergone surgery for primary CRC and at least one liver resection. 27 had a second metastasis operation: 20 for new liver metastases, 6 for lung metastases and one for local recurrence. At primary surgery, 59 of the 96 patients had lymph node metastases. Calculations were performed on the primary tumor and the first liver metastasis. High expression of CYP2W1 did not correlate to demographic or tumor related factors, neither the expression in the primary tumor, nor in the liver metastasis.

In the primary CRC, 26% had high CYP2W1 expression. Nodal metastases had high expression in 31% and in the first resected liver metastases, high expression was seen in 48%. Of the 20 patients that underwent a second liver operation, 10 had high CYP2W1 expression, 9 had low expression and one had no tumor left in the specimen. In the lung metastases (n=6), 2 had high CYP2W1 expression and 4 had low expression. The amount of liver metastasis with high CYP2W1 expression was significantly higher than the primary tumors (48% versus 26%, $p=0.005$). In figure 4, the dynamics of CYP2W1 expression in primary tumor versus liver metastasis is schematically described.

The IHC method used in study IV did not differ from that in study I and II apart from the dilution of the antibody and that the assessment of grade was slightly stricter. In study I and II, the grade of the slide was defined as the strongest grade that covered at least 5% of the slide, while we in study IV required 10%. This reflects the aim of our investigation of metastases, which is more directed towards a potential treatment pathway rather than just find enzyme expression. Higher amount of high CYP2W1 expressing cells now seemed to be more important, although the arbitrary cut-off at 10% could be discussed. As previously mentioned, we do not know the extent of the bystander effect when it comes to cytotoxicity of the CYP2W1 derived metabolites. The higher cut-off- level of 10% used in study IV could also explain the slightly higher percentages in the weaker staining groups in this study compared with study I and II.

An illustration of slightly heterogeneous staining in a liver metastasis is shown in figure 5.

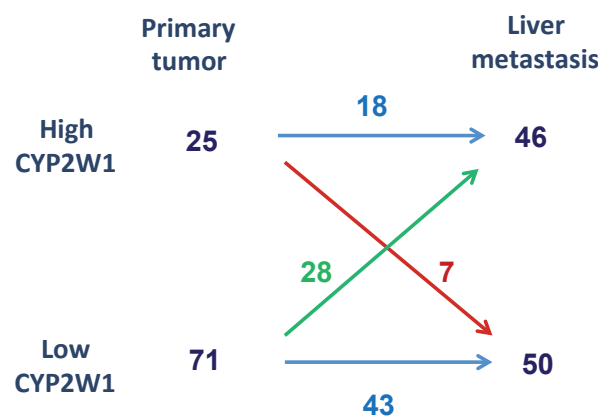


Figure 4. The dynamics of CYP2W1 expression in colorectal primary tumors and liver metastases

As also applied to the investigations of the primary tumors in study I and II, validation of CYP2W1 expression in liver metastases is of great importance.

The reason why CYP2W1 expression seems to increase in the metastases is of course something we just may speculate about. Our study is, although small in sample size, comparatively large when going through the literature. Habermann and coworkers have analyzed transcriptomes and proteomes in tissue samples from 20 primary colorectal tumors and 13 liver metastases, and only in 2 cases the primary tumor and the metastasis came from the same patient (226). The full array comprised some 9000 cDNAs and revealed 158 genes to be differently expressed between primary tumor and metastasis. From the proteome analyses, expression levels of 32 proteins were found to be increased in the metastases compared to the primary tumors. From an omics-study like that, it is hard to draw any conclusions about the expression of a single protein. Conversely, it is also difficult to make any statements on changes in expression patterns in general just studying one single protein. The biology of tumor and metastases is more complicated than that, and depends largely on random events and genomic instability (154, 157). Maybe high expression of CYP2W1 co-varies with other factors that provide survival benefits for the tumor cell in the metastatic process, or maybe it is simply an effect of the random genetic and epigenetic events occurring in the invasion – metastasis cascade.

From the results of our study, it seems as if expression of CYP2W1 is increased in colorectal liver metastases, and if this finding is reproducible, it adds an interesting perspective when searching for novel pathways in the treatment of metastatic CRC.

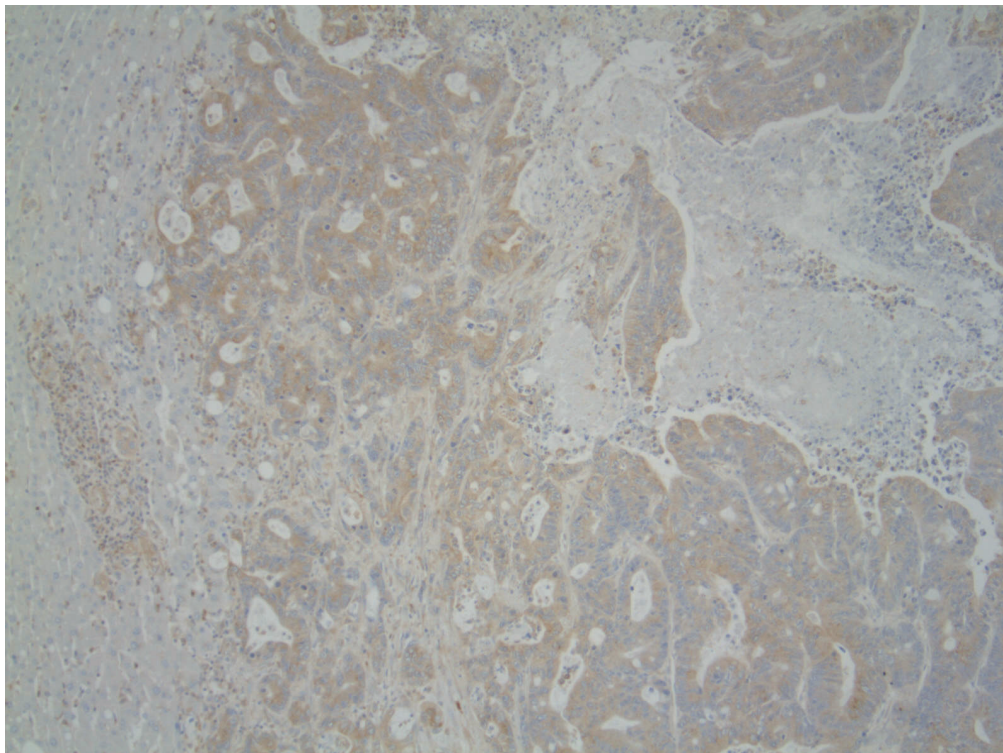


Figure 5. Example of slightly heterogenous staining in a colorectal liver metastasis, overall interpreted as grade 2.

CONCLUSIONS

In summary, the conclusions of this thesis are

CYP2W1 is expressed at high levels in about 30% of primary human colorectal tumors assessed by immunohistochemistry. Expression assessed with another method would be of interest.

High CYP2W1 expression in the primary tumor is associated with poor survival. This needs to be confirmed in a larger, independent patient material.

There is no association between polymorphisms *CYP2W1*2* or *CYP2W1*6* and risk to develop CRC. Neither are there any functional differences between the corresponding gene products.

CYP2W1 protein is expressed in 48% of colorectal liver metastases assessed by immunohistochemistry. The expression in metastases is significantly higher than in the primary tumors. Validation using another method is of importance.

FUTURE PERSPECTIVES

The most interesting aspect of CYP2W1 expression in CRC and metastases is not whether or not it is an independent prognostic factor. What is the most exciting part is the prospect of drug development. As previously mentioned, the need of validation is imperative, especially in this perspective. We hope to be able to start doing this using fresh frozen material from the same liver metastases as already have been investigated by IHC in study IV. The planned method of analysis is liquid chromatography-mass spectrometry (LC-MS).

The prerequisite for pro-drug activation in the patient is of course a functional enzyme expressed in the tumor. CYP2W1 expressed by transfected CRC cell lines is functional but it is of importance also to find out whether the CYP2W1 present in the tumor samples from patients has a good catalytic activity. There is an ongoing prospective study aiming to address this question using fresh material from patients undergoing surgery for primary CRC. In order to assess CYP2W1 expression, these tumors are analyzed with western blot and histopathologic slides from their tumors could be analyzed by IHC also in order to validate the method. Functional activity in the CYP2W1 expressing tumors will be analyzed using the duocarmycin substrates and the assay described in the study by Travica et al (216).

We are also planning to start using fresh material from patients being operated for colorectal liver metastases in order to assess functional activity. This project has some logistic problems that have to be solved before it can start since the liver operations are performed in Huddinge and our laboratory is in Solna.

The question when in the development from normal epithelium to invasive cancer the *CYP2W1* gene is activated remains to be answered. An important study would be to investigate either CYP2W1 expression by IHC or *CYP2W1* gene methylation by bisulfite sequencing in colorectal adenomas of various size and degrees of dysplasia.

The increased CYP2W1 expression in metastases and the fact that the only normal, non-malignant, expression is during fetal life, raises the question whether CYP2W1 is expressed in stem cells or stem cell like cells. This is of course speculative, but an interesting question to find out more about. One problem is, as discussed previously, the lack of reliable stem cell markers for colorectal stem cells. When this area becomes more developed, it would be of interest to find out if CYP2W1 expression in any way is associated with stem-ness of the tumor cells, not least because of the perspective of killing cancer stem cells with a pro-drug.

The biological function of CYP2W1 in fetal life is unknown. A *CYP2W1*^{-/-} knockout mouse model is under development where the aim is to study the phenotype and presence of any malformations.

There are some more thrilling questions also lacking answer. For example how the extremely reactive metabolite to the duocarmycin analogue physically can migrate from the ER to the nucleus to form the DNA adducts? How large is the bystander effect and what are the mechanisms behind it?

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Cancer är den näst vanligaste dödsorsaken i Sverige efter hjärt-kärlsjukdom. Tjock- och ändtarmscancer, kolorektalcancer (CRC) är den näst vanligaste cancerformen hos kvinnor och den tredje vanligaste hos män både i Sverige och globalt. Man vet att det finns rent ärftliga former av CRC. Dessa är emellertid inte så vanliga utan står bara för ungefär 3-4 % av alla CRC fall i Sverige. Hos de andra har ärftliga faktorer också betydelse men inte på ett lika enkelt och tydligt sätt. Man tror att små genetiska skillnader i kombination med faktorer i miljön, t ex kost, har betydelse för vilken risk man har att utveckla cancer och hur cancersjukdomen sedan betar sig när den väl har brutit ut.

Om man drabbas av CRC är det viktigt att man kan operera bort tumören. Ibland behöver man tilläggsbehandling med strålning och cellgifter. Man vet också att prognosen är bättre om sjukdomen upptäcks tidigt. Man kan även operera bort dottertumörer – metastaser – om de finns i begränsad omfattning. Ofta behövs då också cellgiftbehandling som komplement. Om man kan operera bort alla metastaser är prognosen bättre än i de fall där detta inte är möjligt. Det är angeläget att hitta nya sätt att angripa cancer och utveckla nya typer av läkemedel, särskilt när sjukdomen börjat sprida sig, eftersom det är dessa patienter som idag har sämst prognos, även med bästa tillgängliga behandling.

I kroppen finns en familj med enzymer, cytokrom P-450- familjen (CYP), som utgör förutsättningen för en mängd livsviktiga kemiska reaktioner. Ett sådant exempel är t ex CYP21 som är nödvändigt för att kunna tillverka cortisol, ett stresshormon som man inte kan klara sig utan. Ett annat exempel är CYP3A4 som behövs i nedbrytningen av väldigt många av de läkemedel som finns på marknaden. Det finns CYP-enzymmer som bryter ner giftiga substanser i vår miljö som vi utsätts för. I vissa fall blir dessa substanser ännu mera giftiga av den reaktion som CYP bidrar med, men då bryts de ner vidare av andra – fas II-enzymmer – och kan sedan utsöndras från kroppen via urinen. Både växter och djur har CYP-enzymmer, människan har 57 olika CYP, ris har 323.

Av de 57 CYP-enzymerna hos människa, har 13 en mer eller mindre okänd funktion. Ett sådant har fått beteckningen CYP2W1. Det enzymet har visat sig inte finnas i levern, som väldigt många av de andra CYP-enzymerna gör, utan i tjocktarmen under fosterstadiet hos råttan, och i människa finns det inte alls eller bara i mycket små, försumbara mängder i normala vävnader i kroppen. Däremot har man hittat enzymet i olika cancertumörer, främst i CRC. I den här avhandlingen har vi studerat förekomsten av CYP2W1 i dels vävnadsprover från cancertumörer i tjocktarmen och ändtarmen – primärtumörer - och dels från metastaser i lymfkörtlar, lever och lungor. Det är dels med syfte att kartlägga i hur stor andel av CRC som CYP2W1 finns och dels för att se om detta påverkar överlevnaden. Vi har också frågat oss om medfödda olikheter i genen som kodar för just CYP2W1 är förenat med ökad eller minskad risk för att drabbas av CRC.

Vi kom fram till att CYP2W1 uttrycks i ca 25-30% av alla primärtumörer, i 30% av lymfkörtelmetastaser och i nästan hälften, 48%, av alla levermetastaser. Det finns visst fog för påståendet att starkt uttryck av CYP2W1 i primärtumören är associerat med en sämre prognos men man behöver titta mera på det i ytterligare, större studier för att vara säker. Det är ingen ökad eller minskad risk att drabbas av CRC om man har någon av de två genetiska förändringar vi har studerat. Funktionen hos enzymet var dessutom likadan oavsett hur genuppsättningen såg ut när vi testade detta i en experimentell modell.

Kunskaperna vi fått från studierna i denna avhandling är att det förefaller finnas CYP2W1-uttryck i ca en tredjedel av alla primärtumörer och troligen i en högre andel av levermetastaser. Eventuellt är dessa tumörer också lite ilsknare än andra. Man skulle i framtiden kunna utnyttja att CYP2W1 finns i tumörer och metastaser genom att man ger en ofarlig substans till patienten som sedan i själva tumörcellen omvandlas av CYP2W1 till en giftig substans. Eftersom CYP2W1 inte finns i normal vävnad, skulle tumörcellen dödas men inte den friska vävnaden. Djurförsök har visat att detta är möjligt, även om det är en lång väg kvar innan det blir ett färdigt läkemedel som kan ges till patienter.

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