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Polymorphism and expression of NF- κ B in relation to susceptibility and prognosis of colorectal cancer patients

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To my family

Abstract

Normal human cells are strictly controlled in their environment by extrinsic and intrinsic factors. Despite this, some cells begin to develop into cancer cells, and if this process is allowed to continue, it will develop into cancer disease. To become cancerous, a cell must break several biological barriers. Two important barriers are apoptosis and cellular growth control.

Cancer is a multifactorial disease caused by environmental and hereditary factors. The incidence of colorectal cancer varies among different populations around the world. Sweden has a history of a relatively high incidence of colorectal cancer, whereas its incidence in China is relatively low.

Nuclear factor kappa B (NF- κ B) is a transcription factor protein family, regulating genes involved in several aspects of cancer development. In human cells five members have been identified: NFKB1 (p105/p50), NFKB2 (p100/p52), RelA (p65), RelB and c-Rel. They normally form homo- or heterodimers in the cytoplasm of the cells, where they are in an inactive state by binding to inhibitory proteins, I kappaB- α , - β and - ϵ and Bcl-3. Stimulatory signals, both intrinsic and extrinsic, lead the inhibitory proteins to be phosphorylated, which marks them for degradation. On activation, NF- κ B proteins are often posttranslationally modified.

In the first project, we investigated the role of a polymorphism in the promoter region of NFKB1 gene. The polymorphism is a 4-basepair insertion/deletion located 94 basepairs upstream of the gene (-94ins/delATTG). It does not seem to alter the amino acid sequence of the protein and therefore does not alter the function of the protein itself. Instead, it alters the regulation of the protein transcription. The aim of the present study was to investigate whether the polymorphism was related to cancer risk or clinicopathological variables. We found that this polymorphism increased the risk of sporadic colorectal cancer in a Swedish population but not in Swedish populations with a family history of colorectal cancer or in Chinese population.

In the second project we studied an 8-basepair insertion/deletion polymorphism in the promoter region of NFKBIA gene coding for the nuclear factor kappa B inhibitory protein, I κ B α . This polymorphism is located 708 basepairs upstream of the gene (-708ins/del8). The aim of the study was to investigate whether the polymorphism was related to cancer risk or clinicopathological factors. We found that this polymorphism was very rare in a Swedish population of colorectal cancer patients and controls and was totally absent in a Chinese population of patients and controls. Our conclusion is that this polymorphism is too rare to have a major impact on colorectal cancer incidence in the two populations.

In the third project we studied levels of p65 phosphorylated at Serine-536 in colorectal cancers in a Swedish population. After activation and I κ B phosphorylation/degradation, p65 is phosphorylated at Serine-536. This phosphorylation is involved in regulating transcriptional activity, nuclear localisation and protein stability. The aim of the study was to investigate whether the expression of the phosphorylated protein correlated to any clinicopathological

variables, including survival. The expression of p65 phosphorylated at Serine-536 increased from normal mucosa to primary tumour, but no further increase to lymph node metastases was found. We did find, however, that the strong expression in the cytoplasm was correlated to worse survival among the patients, independent of gender, age, tumour location, stage and differentiation.

In the fourth project we continued to study p65 phosphorylated at Serine-536. In this project, however, we studied the expression in a population of rectal cancer patients who participated in a Swedish clinical trial of preoperative radiotherapy. The aim of the study was to investigate whether the expression correlated to response to radiotherapy or to clinicopathological and some biological factors. We found that the expression was increased from normal mucosa to primary tumour, but detected no further increase from primary tumour to lymph node metastases. We found that the expression of p65 protein phosphorylated at Serine-536 was positively related to expression of TEM1, FXYP-3, PRL, p73 and MAC30 in the group of patients who received radiotherapy. Although no such relationship was seen in the group of patients that had not received radiotherapy, we did not find that the expression of p65 protein phosphorylated at Serine-536 was directly related to the clinical response to radiotherapy.

In summary, the -94ins/delATTG polymorphism in the promoter region of NFKB1 gene increases the risk of sporadic colorectal cancer in Swedish but not in Chinese populations. The -708ins/del8 polymorphism in the promoter region of the NFKBIA gene is too rare to have a major impact on colorectal cancer incidence in Swedish and Chinese populations. Strong expression of p65 protein phosphorylated at Serine-536 is independently related to worse survival in Swedish colorectal cancer patients, and the expression is positively correlated to biological factors associated with more malignant features of tumours in rectal cancer patients who received preoperative radiotherapy.

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Populärvetenskaplig sammanfattning

Cancer är en komplex sjukdom. För att en cell ska utvecklas från en normal cell till en cancercell krävs att den erhåller förmågan att bryta de fysiologiska barriärer som cellen och omgivande vävnad besitter för att förhindra utvecklingen av cancer. En cancercell är en cell som delar sig okontrollerat. Den måste även erhålla något sätt att undvika apoptos, programmerad celldöd, vilket är ett program som finns i varje cell och som gör att cellen kan dö utan att skada omgivande vävnad och utan att ge upphov till inflammation. Det finns flera andra fysiologiska barriärer, däribland måste syre och näringsförsörjning erhållas genom nybildning av blodkärl, så kallad angiogenes. Flera av dessa biologiska funktioner regleras delvis utav en proteinfamilj kallad NF- κ B. Denna proteinfamilj har till funktion att reglera tillverkningen av andra proteiner och man har idag funnit att denna proteinfamilj reglerar över 200 olika proteiner med en mängd olika funktioner, däribland celledelning och apoptos.

NF- κ B proteiner binder normalt till varandra i så kallade dimerer. Dessa dimerer binder i sin tur till ett inhiberande protein, så kallade I kappa B, och hålls inaktivt i cellens cytoplasma utanför cellkärnan. När det kommer en aktiverande signal så bryts det inhiberande proteinet ner och NF- κ B-dimeren släpps fri och kan därefter transporteras in i cellkärnan där den reglerar tillverkningen av olika proteiner.

Orsaken till att studier av NF- κ B i cancer är intressant är att många av de proteiner som regleras är involverade i de biologiska mekanismer som skyddar mot eller bidrar till cancerutvecklingen.

Fram till idag har fem olika proteiner i NF- κ B-familjen upptäckts i humana celler. I det första arbetet i denna avhandling studerades den gen, NFKB1, som kodar för p50 proteinet. Det tillsammans med p65 proteinet bildar den vanligaste dimeren, p65/p50, i humana celler. Vi studerade en medfödd förändring, en så kallad polymorfi, i den del av genen som reglerar uttrycket av proteinet, vilket betyder att själva proteinet får i sig själv inte någon förändrad funktion. Däremot kan den biologiska funktionen av proteinet i cellen förändras då regleringen av uttrycket av proteinet kan förändras. Vi ville studera denna polymorfi dels i en svensk population, då kolorektalcancer är förhållandevis vanligt i Sverige, samt även i en kinesisk population, där kolorektalcancer är förhållandevis ovanligt. Vi ville även studera om det var skillnad på om cancer var sporadisk eller om patienten hade nära släktingar som tidigare insjuknat i kolorektalcancer. Vi ville även studera om denna polymorfi var relaterad till någon klinisk eller patologisk variabel hos tumören eller patienten. Det vi fann var att de personer som levde i Sverige och som hade denna polymorfi hade ökad risk att utveckla sporadisk kolorektalcancer. Detta samband fanns inte i den kinesiska populationen eller hos personer som hade insjuknande nära släktingar.

Vidare fortsatte vi studera denna proteinfamilj och dess relation till kolorektalcancer genom att undersöka en polymorfi i genen som kodar för ett av de inhiberande proteinerna till NF- κ B. Denna polymorfi fanns även den i den del av genen som reglerar uttrycket av proteinet. Den gen vi studerade var NFKBIA som kodar för proteinet I κ B α . Även denna gång använde vi

både en population från Sverige och en från Kina. Vi fann att denna polymorfi var mycket sällan förekommande. I den svenska populationen fann vi den hos bara någon procent och vi fann den inte alls i den kinesiska populationen. Den slutsats vi drog av detta var att polymorfin var för sällsynt för att kunna ha någon större inverkan på totala förekomsten av kolorektalcancer i något av länderna.

Regleringen av NF- κ Bs funktion är mycket invecklad. En viktig del i denna reglering är fosforylering av proteinerna på specifika aminosyror, och en av de mest studerade är fosforyleringen av aminosyran Serine-536 i p65 proteinet. Denna har visat sig mycket viktig vid regleringen av NF- κ B. Denna aminosyra fosforyleras omgående vid aktivering via flera av de vanligaste aktiveringssignalerna. I det tredje projektet undersökte uttrycket av det fosforylerade p65 protein i kolorektalvävnad samt närliggande normal vävnad. Vi ville undersöka om uttrycket av p65 proteinet fosforylerat på Serine-536 var sammankopplat med olika kliniska och patologiska variabler däribland patientens överlevnad. Det vi fann var att det i cellernas cytoplasma fanns ett högre uttryck av proteinet i tumörvävnad jämfört med närliggande normal vävnad. Vidare fann vi att ett högt uttryck gav sämre chans för patienten att överleva, detta oberoende av patientens kön, ålder, var i kolon eller rektum tumören hade vuxit, hur långt tumören hade utvecklats eller hur väl differentierad tumören var.

I det fjärde projektet fortsatte vi att undersöka uttrycket av p65 fosforylerat på Serine-536, men denna gång i endast rektalcancer. Vi studerade detta i en grupp patienter som hade randomiserats att få radioterapi innan kirurgi, eller inte. Detta för att kunna studera om uttrycket av proteinet hade någon inverkan på den respons som patienterna fick av radioterapin. Vi kunde här se att uttrycket av p65 fosforylerat på Serine-536 korrelerade med uttrycket av andra proteiner, TEM1, FXD3, PRL, p73, MAC30, i gruppen patienter som erhållit radioterapi men inte i gruppen patienter som inte fått denna extra behandling. Vi kunde dock inte direkt se att uttrycket av p65 proteinet var relaterat till den kliniska responsen av radioterapi. Vi kunde ej heller här hitta något samband med patientens överlevnad.

Vi har i detta avhandlingsarbete börjat försöka klarlägga NF- κ Bs roll i utvecklingen kolorektalcancer. Vi har sett att p50 proteinet kan inverka på risken att utveckla sporadisk kolorektalcancer, detta i Sverige men inte i Kina. Vi har vidare kunnat visa att aktiveringen av p65 proteinet påverkar överlevnaden hos svenska kolorektalcancerpatienter. Att detta protein är uppreglerat i tumörceller jämfört med normala celler. Samt att uttrycket av detta protein var samuttryckt med flera andra proteiner hos patienter som hade erhållit preoperativ radioterapi men att detta samuttryck inte kunde finnas hos patienter som inte hade fått radioterapi.

Abbreviations

APC	Adenomatous polyposis coli
ANK	Ankyrin repeats
CRC	Colorectal cancer
DNA	Deoxyribonucleic acid
FAP	Familial adenomatous polyposis
HCRC	Hereditary colorectal cancer
HNPCC	Hereditary non-polyposis colorectal cancer
IHC	Immunohistochemistry
I κ B	Inhibitor of Nuclear factor kappaB
IKK	I κ B kinase
MAC30	Meningioma associated protein 30
MMP	Matrix metalloproteinase
NEMO	NF- κ B essential modifier
NF- κ B	Nuclear factor kappaB
PCR	Polymerase chain reaction
PRL	Phosphatase of regenerating liver
RFLP	Restriction Fragment Length Polymorphism
SSCP	Single-Stranded Conformation Polymorphism
TAD	Transactivation domain
TCR	Two close relatives
TEM1	Tumour endothelial marker 1
TMA	Tissue Microarray
TNM	Tumour node metastasis

List of publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV):

- I. **Lewander A***, Butchi AK*, Gao J, He LJ, Lindblom A, Arbmán G, Carstensen J, Zhang ZY, Swedish Low-Risk Colorectal Cancer Study Group, Sun XF
Polymorphism in the promoter region of the NFKB1 gene increases the risk of sporadic colorectal cancer in Swedish but not in Chinese populations
Scandinavian Journal of Gastroenterology. 2007. 42:1332-8.
* Authors contributed equally to this work
- II. **Lewander A**, Arbmán G, Sun XF
Polymorphism in the promoter region of the NFKB1A gene is rare in Swedish and Chinese colorectal cancer patients and controls
Molecular Medicine Reports. 2010. 3:69-74
- III. **Lewander A**, Gao J, Carstensen J, Arbmán G, Sun XF
NF- κ B p65 phosphorylated at Serine-536 is an independent prognostic factor in Swedish colorectal cancer patients
Submitted
- IV. **Lewander A**, Gao J, Adell G, Sun X-F
Expression of NF- κ B p65 phosphorylated at Serine-536 in rectal cancer with or without preoperative radiotherapy
Submitted

Introduction

The colon is subdivided into the caecum and the ascending, transverse, descending and sigmoid colon. The sigmoid colon ends in the rectum, and the rectum ends in the anus. The function of the colon is to extract water and electrolytes from solid wastes. The rectum stores solid wastes before they are eliminated from the body through the anus. The colon and rectum are histologically divided into four layers. The first layer facing the lumen of colorectal canal is the mucosa layer. Under this there is a layer of submucosa. Responsible for the peristaltic movement of the colorectal canal is a muscle layer, muscularis propria. Finally an outer layer, the serosa, surrounds the entire colon and rectum.

The histology of normal colorectal mucosa show abundant vertically oriented crypts. The surface of the epithelium is composed of columnar absorptive cells. Although the flow of solid wastes through the lumen of the colorectal canal leads to constant physical stress on the mucosa layer, the regenerative capacity of the intestinal epithelium is remarkable. Cellular proliferation takes place in the crypts, and the newly divided cells migrate upwards in the crypts towards the lumen of the bowel. The turnover of the colonic surface epithelium takes 3 to 8 days. This remarkable capacity for repair also renders the epithelium vulnerable to agents that interfere with cell replication.

Humans have 23 chromosome pairs in each cell nucleus. The entire genome extends over 3000 million basepairs and is replicated each time a cell divides. Although the human replication machinery is very accurate, with proof-reading machinery, errors are unavoidable and mutations occur. When this happens in a germ cell, the mutation passes to the offspring. When a specific mutation accumulates in a population and more than one percent of the people in the population carry the variation, it is known as a polymorphism, which is defined as common, inherited genetic variations in a population. Mutations can affect the function of a cell if they are located within the coding region of a gene, resulting in an altered amino acid sequence of the protein. A mutation may also affect the function of a cell if the mutation is located in such a way that it affects the transcription of the protein and not the protein itself. For instance, this may occur if the mutation is located in the promoter region of a gene. The promoter region is located upstream of the gene and recruits the transcription machinery by expressing a specific DNA sequence that is recognised by transcription factors in the cell. Mutation in the coding sequence of the protein that alters the amino acid sequence of the protein often alters the function of the protein, whereas mutation in the promoter region often does not alter the amino acid sequence, but alters the gene regulation instead.

Colorectal cancer

Colorectal cancer is the third most common form of cancer in Sweden. Only breast and prostate cancers are more common. Around 5,000 new cases of colorectal cancer are diagnosed in Sweden each year. The incidence is rising, but mortality is decreasing. This is due to improved treatment, most likely to improved surgery but also to improved adjuvant chemotherapy in colorectal cancers and preoperative radiotherapy in rectal cancers.

Cancer is a genetic disease. Altered function of proto-oncogenes, tumour suppressor genes and genes involved in DNA repair, often in a sequential pattern, are necessary for a normal cell to develop into a cancer cell. In 1990, Fearon and Vogelstein presented a genetic model for colorectal tumourigenesis that shows a series of gene alterations in a sequential order (Figure 1). However, it is the accumulation of genetic changes, rather than their order, that appears to be the most important factor. Tumours continue to alter once carcinomas have been formed and continue to accumulate alterations in other genes related to tumour development (Fearon and Vogelstein, 1990).



Figure 1. A genetic model for colorectal tumourigenesis (adopted from Fearon and Vogelstein, 1990)

Colorectal cancer is often divided into three different categories: hereditary, familial and sporadic. Hereditary colorectal cancer accounts for approximately 1% to 5% of all cases. The two most common forms are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). FAP is associated with inactivation of the adenomatous polyposis coli (APC) gene, with patients developing a large number of adenomas at a relatively young age. If the patients are left untreated, almost all will develop colorectal cancer by the time they are 40 years old. HNPCC involves mutations in DNA repair genes. A further 10% to 15% of colorectal cancers occur in patients who have a family history of colorectal cancer. They experience an apparently inherited increased risk of colorectal cancer similar to hereditary colorectal cancer, but the risk genes have not yet been identified. These cases are called familial colorectal cancers. Sporadic colorectal cancer occurs in patients without any family history of colorectal cancers (Figure 2).

Cancer is a genetic disease, but only a small proportion of cancers follow a Mendelian pattern of inheritance. There is a considerable difference in incidence worldwide, and when lifestyle and environmental exposure change in a population, the incidence of changes. Migration studies reveal that adaptation to a different cancer incidence takes place within only one or a few generations. It is therefore of interest to investigate patients from populations with different cancer incidence. In the present thesis we studied a population from China, with a relatively low colorectal cancer incidence, and a Swedish population, with a high incidence.

There are several risk factors for colorectal cancer. Incidence increases with age. People with inflammatory bowel diseases also have an increased risk of colorectal cancer. Lifestyle also affects the risk. Diets with a high fat and red meat content have been shown to increase the risk, whereas diets high in fruit and vegetables reduce the risk. Alcohol consumption and smoking have been seen to increase the risk, whereas the regular intake of non-steroidal anti-inflammatory drugs reduces risk.

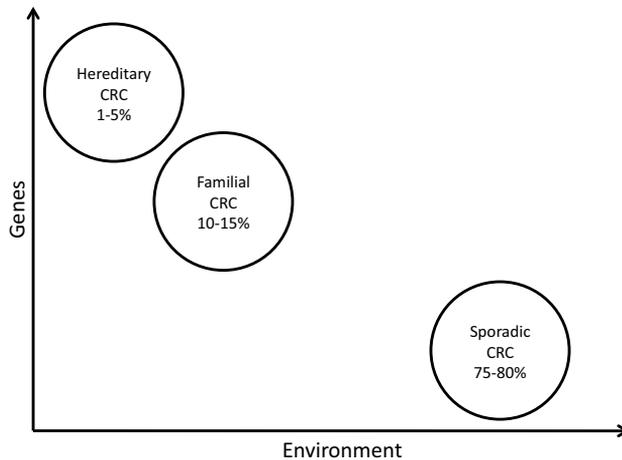


Figure 2. Hereditary colorectal cancer, familial adenomatous polyposis and hereditary non-polyposis colorectal cancer with mutation in high penetrance genes have a strong hereditary component with little environmental influence. The increased risk of colorectal cancer seen in patients with familial colorectal cancer can be caused by germline mutation in low penetrance genes. Somatic mutations contribute to sporadic colorectal cancer.

Radiotherapy

Preoperative radiotherapy has been shown to increase overall survival and reduce the rate of local recurrence in patients with resectable rectal cancer (Swedish Rectal Cancer Trial, 1997). It is now standard practice to give preoperative radiotherapy to patients with rectal cancer, often in high doses distributed over several days. In the above-mentioned study, the patients were given 25 Gray over 5 times 5 Gray. Ionising radiation results in the release of free radicals, which causes damage on macromolecules in the cells. For the cells, the most lethal consequences of this are double-strand breaks of DNA. This often leads to cell death, either through necrosis or apoptosis. There is a significant variation in the response to radiotherapy among patients. One important protein in radio-resistance is p53 (Adell et al. 1999). Survivin and cyclooxygenase-2 have been implicated in the radio-resistance of rectal cancer (Knutsen et al., 2004. Pachkoria et al., 2005). The expression of phosphatase of regenerating liver at the invasive margin can be used to predict radio-resistance in rectal cancer patients (Wallin et al., 2006). It has also been proposed that NF- κ B is involved in radio-resistance in cells.

Clinicopathological variables

In the present thesis we wished to study NF- κ B in colorectal cancer by identifying correlation to clinicopathological variables. We had information about the patients and the tumours. From surgical and pathological records we obtained information about the patients' age at diagnosis, gender, and the location and stage of the tumours (Dukes or TNM). Tumours from the ascending and the transverse colon were regarded as proximal, whereas tumours from the descending or sigmoid colon and from rectum were considered distal. Dukes stage is an older method of grading the tumour stage than TNM, which is the standard method of grading tumour stage today. Tumour growth pattern, grade of differentiation, inflammatory infiltration

and necrosis were scored by a pathologist. The tumour growth pattern was graded as expansive or infiltrative. The grade of differentiation was graded as well, moderately or poorly differentiated or as mucinous/signet-ring cell carcinoma. Inflammatory infiltration was graded as weak, moderate or strong. Necrosis was graded as little, moderate or strong. Tumour size, gross status and invasive depth were graded by a surgeon or by pathologists. Gross status was classified as polypoid or ulcerative. Invasive depth was grouped as intra- or ultra-bowel wall. Apoptosis was measured with TUNEL and other methods. Survival can be either overall survival or disease-free survival. There is a strong correlation between overall survival and disease-free survival among colorectal cancer patients.

Many clinicopathological variables are associated with prognosis in colorectal cancer patients. Tumour stage, Dukes or TNM, is the strongest prognostic factor of colorectal cancer. Advanced stage is correlated to worse prognosis. Grade of differentiation is also an important variable after the stage. Patients with poorly differentiated cancers have a worse prognosis than those with well-differentiated cancers. Moreover, the tumour growth pattern, in some aspects, affects patient survival. Patients who have tumours with an infiltrative growth pattern have a worse prognosis than patients with tumours with an expansive growth pattern. A low level of inflammatory infiltration (weak) is also a sign of poor prognosis compared with tumours with a high level of inflammatory infiltration (strong).

Age and gender can also be prognostic variables in some groups of patients; older patients have lower survival than younger patients, and men have a higher survival rate than women.

Nuclear factor-kappaB

NF- κ B is a transcription factor protein family. It regulates more than 200 different genes. Five members (subunits) of this family have been found in human cells: p65, p105/p50, p100/p52, RelB and c-Rel, which are transcribed from the RelA, NFKB1, NFKB2, RELB and REL genes, respectively (Figure 3). The subunits normally form homo- or heterodimers, the most common dimer in human cells being the p65/p50 heterodimer. NF- κ B dimers are normally kept in an inactive state in the cytoplasm by inhibitory proteins or by the inhibitory c-terminal part of the p100 or p105 subunits. Four I κ B proteins have been identified in human cells: I κ B α , I κ B β , I κ B ϵ and Bcl-3 (Figure 4). All I κ B proteins contain ankyrin repeats (ANK). The c-terminal part of p100 and p105 also contains ankyrin repeats. All subunits in the NF- κ B protein family contain a 300-amino-acid long domain called the Rel homology domain (RHD), which is responsible for DNA binding, dimerisation and interaction with I κ B proteins. The Rel homology domain also contains a nuclear localisation signal. p65, RelB and c-Rel possess a transcription activation domain (TAD) that is necessary for the transcriptional activity. As p50 and p52 do not have transcription activation domain domains, homodimers of these can suppress transcription of NF- κ B-dependent target genes. I κ B α bonds to the p65/p50 heterodimer mask only the nuclear localisation signal on the p65 subunit and not on the p50 subunit. The exposed NLS on the p50 and the exposed nuclear export sequence on the I κ B lead to constant shuttling of I κ B α /NF- κ B complexes between the nucleus and the cytoplasm, but the steady-state localisation is in the cytosol. Degradation of I κ B on activation signal through the canonical pathway leads to nuclear accumulation of NF- κ B proteins.

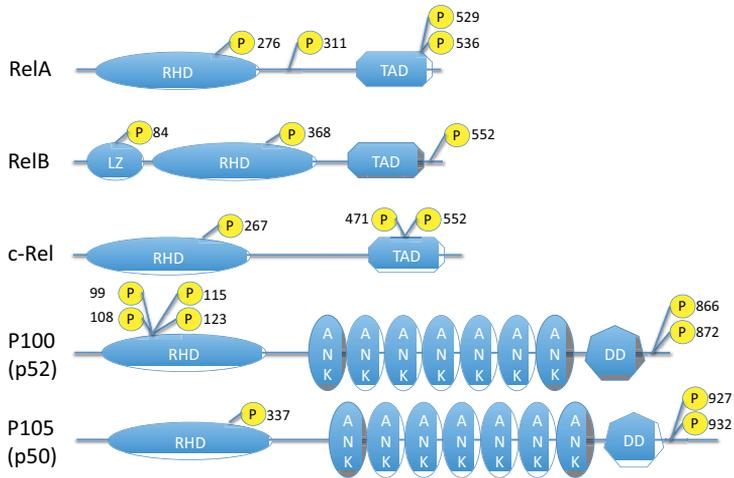


Figure 3. The five members of NF- κ B found in human cells. The proteins contain Rel homology domain (RHD). RelA, RelB and c-Rel have a transactivation domain responsible for recruitment of transcription machinery in the nucleus. p105 and p100 have c-terminal ankyrin repeats that function as inhibitors and keep the dimers in the cytoplasm. The picture also shows common phosphorylation sites on the proteins.

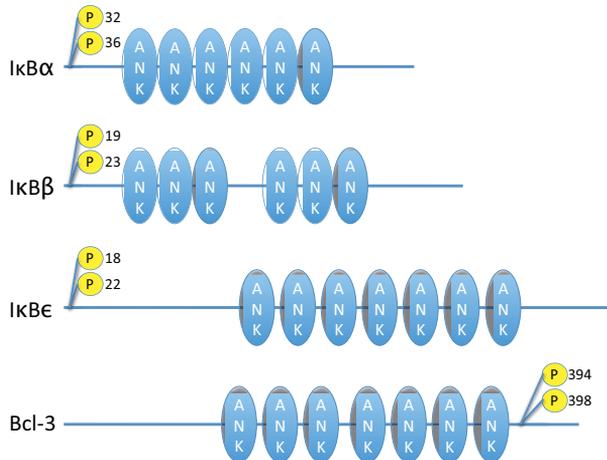


Figure 4. Four members of I kappa B family with common phosphorylation sites. The proteins contain several ankyrin repeats.

NF- κ B signalling is primarily regulated through two major pathways: the canonical and the non-canonical NF- κ B pathways. With the canonical (or classical) NF- κ B pathway, the cytoplasmic I κ B Kinase (IKK) complex is recruited, containing a catalytic α and β subunit and two NF- κ B essential modifiers (NEMO). The IKK β is required for activation of the canonical pathway. The canonical NF- κ B pathway involves dimers with a p50 subunit, either the p65/p50 heterodimer or p50/c-Rel. The non-canonical (or alternative) pathway acts through an IKK complex that contains two IKK α subunits (but no NEMO) and is largely for activation of dimers with a p100/p52 subunit, often the p100/RelA heterodimer. The IKK α complex phosphorylates two Serine residues adjacent to the ankyrin repeat C-terminal I κ B domain of p100, leading to its partial proteolysis and liberation of the p52/RelB complex (Figure 5).

The NF- κ B/I κ B proteins studied in this thesis are the NF- κ B p65 and p50 subunits, and I κ B α . p65 is a protein of 551 amino acids, transcribed from the RelA gene located on chromosome 11q13. NFKB1 is located on chromosome 4q24. The p105 subunit is transcribed from this gene. The 433 amino acid p50 subunit is derived by processing p105, probably through posttranslational degradation of the c-terminal part of the protein or at times perhaps by arrested translation. I κ B α has a mass of 36 kDa and a length of 317 amino acids. It is transcribed from the NFKBIA gene located on chromosome 14q13.

Phosphorylation of NF- κ B/I κ B

Phosphorylation is crucial for the activity of NF- κ B. Two very important phosphorylations occur during activation through the canonical pathway. One is the phosphorylation of I κ B α at two residues, Serine-32 and Serine-36, by IKK β . This marks the I κ B α proteins for proteasome-mediated degradation. This event allows the dimers bound, and thereby inhibited, by I κ B α proteins to be released, and the nuclear localisation signal in the Rel homology domain of p65 is unmasked. NF- κ B dimers then quickly accumulate into the nucleus. At the same time as the I κ B α phosphorylation, NF- κ B p65 is phosphorylated at Serine-536 by IKK β . This phosphorylation affects the transcriptional activity, nuclear localisation and protein stability. Phosphorylation of the NF- κ B protein affects its activity in many ways. Phosphorylation of p100 by IKK α and p105 by IKK β initiates their processing into p52 and p50, respectively. Phosphorylation also affects the normal function of NF- κ B proteins. For example, the phosphorylation of p65 at Serine-536 has been found to decrease the affinity to I κ B α , which enhances/prolongs activation by reducing turn-off through binding to nuclear I κ B α and the subsequent nuclear export. Phosphorylation sites have been found on all NF- κ B and I κ B proteins. Most of these phosphorylations have been shown to fine-tune and optimise NF- κ B activation.

NF- κ B in cancer

NF- κ B is constitutively active in most tumour cell lines. NF- κ B has also been found constitutively active in both solid and haematopoietic tumours. Suppression of constitutively active NF- κ B inhibits proliferation, causes cell cycle arrest and leads to apoptosis (Aggarwal, 2004).

NF- κ B proteins regulate many anti-apoptotic proteins. For example, Bcl-xL, Bcl-2, Survivin, TRAF1, and TRAF2 are all regulators of apoptosis and are regulated by NF- κ B. Inflammatory infiltration is also an important factor in tumour development. NF- κ B regulates many genes involved in the immune response. TNF- α and IL-1 are both regulated by NF- κ B

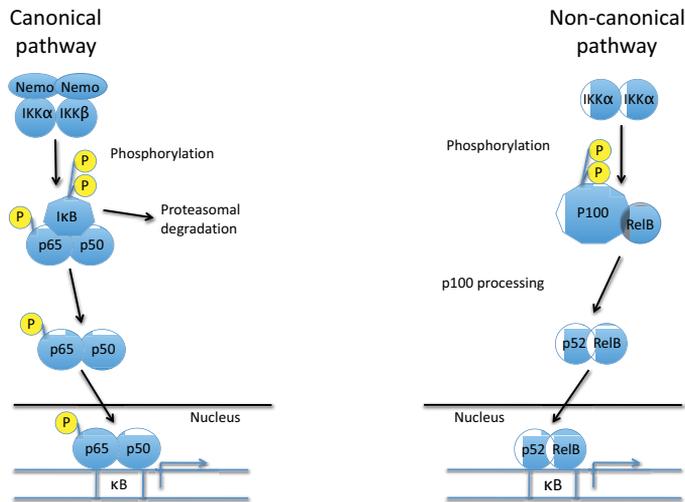


Figure 5. The canonical and the non-canonical pathways. In the canonical pathway IKK β phosphorylate I κ B and p65 resulting in rapid degradation of I κ B and activation of NF- κ B. p65 and p50 heterodimer is shown in the picture but it can also be p50/c-Rel heterodimer. Central in the non-canonical pathway is the p100 NF- κ B subunit. IKK α phosphorylate p100 at c-terminal end causing processing of p100 to p52 NF- κ B subunit. Activated NF- κ B dimers translocate to the nucleus and initiate transcription of target genes.

but are also potent activators of NF- κ B, leading to a positive regulatory loop. Growth pattern and invasive depth are both variable depending on the tumour's ability to remodel surrounding tissue. Metastasis, both to regional lymph nodes and to distant metastasis sites, is also dependent on the ability of the tumour to infiltrate surrounding tissue. Here matrix metalloproteinase (MMP) is important. MMP-2 and MMP-9 are both regulated by NF- κ B. Most of the genes involved in tumourigenesis are regulated by NF- κ B activated through the canonical pathway.

In cell lines, NF- κ B has been seen to be activated after radiation (Ahmed, 2007). Inhibition of NF- κ B in these cell lines has been shown to increase apoptotic response and decrease growth and clonogenic survival after radiation. Irradiation of cells causes single-strand and double-strand breaks in the DNA. The damaged DNA activates nuclear ATM. This protein is then translocated to the cytoplasm where it activates NF- κ B through regulation of IKK activity. NF- κ B regulates many proteins that participate in the regulation of the cell cycle and apoptosis, for example cyclin D1 and B1, Bcl-2 and Bcl-xL. In vitro results show that such inhibition may increase the therapeutic efficiency of radiotherapy.

Aims

The general aim of the present thesis was to begin to investigate the role of Nuclear factor kappaB in colorectal cancer.

Paper I

The aim of this study was to investigate whether the polymorphism (-94ins/delATTG) in the promoter region of the NFKB1 gene has any impact on the risk of colorectal cancer in four different groups of Swedish patients (unselected, HCRC, TCR and sporadic) and in a group of Chinese patients with unselected colorectal cancer, and whether the polymorphism had any clinicopathological significance.

Paper II

The aim of this study was to investigate whether the polymorphism (-708ins/del8) in the promoter region of the NFKBIA gene had any impact on colorectal cancer risk in a Swedish and in a Chinese population. We also wanted to investigate whether this polymorphism had any correlation to clinicopathological variables.

Paper III

The aim of this study was to investigate whether expression of NF-κB p65 phosphorylated at Serine-536 had any impact on prognosis in a population of Swedish colorectal cancer patients, and to investigate whether the expression correlated to clinicopathological variables.

Paper IV

In this study the aim was to investigate the expression of NF-κB p65 phosphorylated at Serine-536 in rectal cancer patients randomised to a Swedish clinical trial of preoperative radiotherapy. We wanted to see whether the NF-κB p65 phosphorylated at Serine-536 was related to response to radiotherapy and whether the NF-κB p65 phosphorylated at Serine-536 was related to clinicopathological variables and expression of other proteins.

Material and Methods

Samples

The patients included in this thesis gave their informed consent for the material to be used in scientific research. The use of the material has been approved by the local Human Research Ethics Committees.

Genomic material (Papers I and II)

Paper I included four groups of Swedish colorectal cancer patients and one group of Chinese colorectal cancer patients. The first group of Swedish patients, diagnosed at the Departments of Pathology at Linköping University Hospital and Vrinnevi Hospital, Norrköping, were unselected patients without identification of sporadic, familial or hereditary colorectal cancer. The second Swedish group included hereditary colorectal cancer (HCRC) patients with three or more affected first-degree relatives. These patients experienced an apparently inherited increased risk of colorectal cancer similar to the known syndromes – familial adenomatous polyposis and hereditary non-polyposis colorectal cancer – but with hitherto unknown high-risk segregating genes. The third Swedish group of patients were patients with two affected first-degree relatives, here called TCR. The fourth Swedish group were sporadic colorectal cancer patients who did not have first- or second-degree relatives with colorectal cancer. The HCRC, TCR and sporadic colorectal cancer patients were unrelated and were selected from a large cohort of consecutive patients who underwent oncogenetic counselling at the Cancer Family Clinic at the Karolinska Hospital, Stockholm, Sweden. The Chinese patients in this study were recruited at Tangshan Gongren Hospital and the Fourth Teaching Hospital of Hebei Medical University. These patients were diagnosed with colorectal cancer between 2001 and 2002, and there were no identification of sporadic, familial or hereditary colorectal cancer.

Paper II included one Swedish and one Chinese group of unselected colorectal cancer patients. In both papers, one Swedish and one Chinese group of controls were used, who were recruited from the same residential areas as their corresponding patients.

Formalin-fixed paraffin-embedded tissue (Papers III and IV)

Material for Paper III was obtained from patients who underwent surgical resection at Linköping Hospital or Vrinnevi Hospital between 1972 and 2004. The patients had primary colorectal adenocarcinoma. The patients' gender, age, tumour location and stage were obtained from surgical and/or pathological records at Linköping or Vrinnevi hospital. The differentiation was graded as better (well and moderate differentiation) and poorer (poorly or mucinous and signet-ring cell). Paper IV included the patients with rectal adenocarcinoma from the Southeast Swedish Health Care region who participated in a Swedish clinical trial of preoperative radiotherapy between 1973 and 1990. The patients were randomised to preoperative radiotherapy, receiving 25 Gray in five fractions before surgery, or surgery alone. Expression analysis with immunohistochemistry on this material for the proteins TEM1, FXYP-3, PRL, p73 and MAC30 has previously been conducted at our laboratory.

Polymorphism genotyping

In the first two projects we investigated two different polymorphisms, both located in the promoter region of the gene of interest, the NFKB1 gene in Paper I and the NFKBIA gene in Paper II. For that we used two different methods: restriction fragment length polymorphism to investigate NFKB1 gene promoter polymorphism; and single-stranded conformation polymorphism to investigate NFKBIA gene promoter polymorphism. For both these methods, we used polymerase chain reaction to amplify the gene section of interest.

DNA extraction

Genomic DNA from Swedish unselected patients and Chinese patients from Tangshan Gongren Hospital was extracted from frozen normal colorectal mucosa. The genomic DNA from HCRC, TCR and sporadic patients used in Paper I was extracted using a standard phenol/chloroform protocol. Genomic DNA was extracted from peripheral blood from the Swedish and Chinese controls, and from the Chinese patients from the Fourth Teaching Hospital of Hebei Medical University.

Polymerase Chain Reaction

This method is used to amplify a chosen segment of the DNA. Within only one to two hours, it is possible to make millions of identical copies of the gene segment of interest. This method uses polymerase to create copies of the DNA. Primers specific to the segment of interest bind to the DNA, enabling the polymerase to extend the primers to make a copy of the DNA. In each cycle of the PCR reaction, new primers are allowed to bind to the DNA or to the copies of the DNA created in the previous cycles. This process almost doubles the number of copies in each cycle. After 30 to 40 cycles, this reaction creates millions of copies.

As the primers cannot bind to double-stranded DNA, the first step in a PCR reaction is an initial denaturing of the double-stranded DNA. This was done by heating the sample to 95°C for five minutes. After the initial denaturation, a PCR cycle is followed and repeated until the desired amount of copies has been gained. The first step of this cycle is a denaturation step with same temperature as the initial denaturation. Then the temperature is lowered, which allows the primers to bind to the denatured DNA. The temperature depends on the primer. In our two projects, the temperature was 58°C in the first and 64°C in the second. The third step was to increase the temperature to optimal working temperature for the polymerase, in our case 72°C, which allowed the polymerase to extend the primers bound to the DNA (or copies of the DNA created in the previous cycles). After extension the temperature was again increased to denature the DNA, and another cycle was initiated. The last step in a PCR reaction is one five-minute extension step at optimal working temperature for the polymerase to allow the extension of all products that had not yet been extended to their full length.

Restriction Fragment Length Polymorphism

Restriction enzymes are proteins that cleave DNA at very specific sites. There are a number of different restriction enzymes, each of which cleave DNA on different sites. The sites at which these enzymes cut the DNA are called restriction sites. The specificity of these enzymes to cut only very specific sites can be used to distinguish normal DNA from DNA that has been altered, for an example by an inherited alteration in the DNA sequence. This technique can be very useful for studying polymorphisms, which are common, inherited changes in the DNA of a population.

In our first project, we used the restriction fragment length polymorphism method to investigate a polymorphism in the promoter region of NFKB1 gene. At the site of this polymorphism, there is a restriction site for the restriction enzyme Pflm I. Close to this site is another restriction site for this enzyme. We used PCR to amplify a segment around this polymorphism that included the two other restriction sites for Pflm I. Then the PCR product with wild-type DNA sequence was cut into fragments by the enzyme. From the original PCR product of 289 basepairs, three smaller fragments were generated, with the respective sizes of 206, 48 and 35 basepairs. The polymorphism of interest altered one of the restriction sites, which could therefore not be recognised by the enzyme. Thus only two fragments were generated, one with 254 and one with 35 basepairs. DNA normally has a slight negative total charge. In an electric field, DNA molecules in solution will migrate towards the positive anode. Agarose gels form a matrix that, with correct concentration, prevent the PCR product from migrating freely but cause small fragments of the DNA to migrate faster through the gel than larger fragments. This is an easy way to separate PCR products of different sizes. The digested PCR products from our samples were separated in an agarose gel, and the 206-basepair segment from a wild-type allele could easily be distinguished from the 254-basepair fragment in a sample with the altered DNA sequence.

Single-Stranded Conformation Polymorphism (SSCP)

In SSCP, a single-stranded PCR product is separated in a matrix gel. Single-stranded PCR products have a three-dimensional structure depending on the sequence of the basepairs of the DNA sequence. PCR products with different three-dimensional structures migrate at different speeds in a matrix gel in an electric field. This can be used to distinguish between DNA sequences with different nucleotide sequences.

In our second project we used this method to identify patients with a polymorphism in the promoter region of NFKBIA gene. The PCR product was first labelled with radioactive [α -33P]dATP instead of normal dATP by re-amplifying the PCR product for 12 cycles. The samples were heat-denatured, rapidly cooled and then loaded into a non-denaturing acrylamide gel. After separation, the product was transferred to a filter paper and exposed to X-ray film.

DNA sequencing

Short DNA fragments produced in a PCR production can be sequenced using labelled modified dNTP, which prevents further elongation of the PCR product together with normal unlabelled dNTP. The dNTP is labelled with different colours. By measuring which nucleotide (label) each PCR product has, it is possible to determine which dNTP has bound and ended the elongation during the PCR run. The PCR product can be separated according to size to an accuracy of only one nucleotide. This makes it possible to determine the sequence of a PCR product. In the second project, we sequenced the PCR product from the samples that gave a shift band on from the SSCP gels. The first step was to remove excess nucleotides that might otherwise disturb the sequencing analysis. The second step was re-amplification of the PCR product with only forward primer to get DNA with a labelled modified dNTP at the end of each string. This was done with a commercially available kit according to the manufacturer's instructions.

Immunohistochemistry

Immunohistochemistry is used to detect expression of a specific protein in intact tissue. Tissue is preserved by snap freezing or by formalin-fixation and paraffin-embedding. Then 5- μ m sections are sectioned from tissue or paraffin-embedded blocks and placed on slides. To be able to detect protein expression in each cell, it is important that the slides are thin enough to have only one layer of cells. To detect a protein in the tissue, a primary antibody specific to epitopes on the protein is used. To enhance the detection level, a secondary antibody specific to the primary antibody is used. This allows multiple secondary antibodies to bind to each primary antibody, causing an increase in staining.

In our study we used formalin-fixed paraffin-embedded tissue. The sections were deparaffinised with xylene and rehydrated in graded ethanol. Antigen retrieval is often necessary before staining, and we used high-pressure cooking in buffer. The labelling method used was a peroxidase reaction of 3,3'-diaminobenzidine tetrahydrochloride, which is colourless before reaction and produces a brown staining after reaction. A higher expression of the protein causes a more intense staining. This can be used to estimate the degree of protein expressed in the tissue. Counterstaining with haematoxylin was also performed to determine the localisation of the expressed protein. One major drawback of this technique is that the staining intensity is graded manually under a light microscope, which to some extent is subjective. We used two methods to mitigate this drawback; first, we used blinded samples, and second, we used two investigators to grade the staining intensity independently. Where the investigators disagreed, the slides were re-examined until consensus was reached.

Tissue microarray

In the fourth project we used immunohistochemistry on tissue microarray slides. The tissue microarrays were made by taking core needle biopsies from paraffin-embedded blocks and re-embedding the samples in new paraffin blocks. The sections were cut from the tissue microarray blocks and placed on slides. We used three biopsies from each sample to avoid misgrading due to intratumoural variation of protein expression. With this method we stained all samples at the same time and thereby avoided inter-batch variation of staining.

Statistical analysis

The chi-square method was used to test genotype distribution in colorectal cancer patients with the controls or clinicopathological variables. The chi-square method was also used to test relationships between protein expression and clinicopathological variables. We tested the differences in allele frequency between the patients and controls using the Armitage Trend Test. For risk calculations, logistic regression was used to calculate odds ratio and 95% confidence intervals. Differences in expression of NF- κ B phosphorylated at Serine-536 between normal mucosa, primary tumours and metastases were tested using Chi-square or McNemar Methods. Cox's Proportional Hazard Model was used to test the relationship with polymorphism or protein expression and survival of the patients. The Kaplan-Meier Method was used to calculate survival curves. Two-sided p values of less than 5% were considered statistically significant.

Results and Discussion

Paper I

In the Swedish population, unselected colorectal cancer patients had an increased risk of colorectal cancer for both heterozygote and homozygote deletion genotypes compared with homozygote wild-type genotype. A similar result was found in the group of Swedish sporadic colorectal cancer patients. Even after age-adjusted analysis, there were still significant differences among the Swedish unselected colorectal cancer patients and among the Swedish sporadic colorectal cancer patients. When the samples were divided into subgroups, <70 and ≥ 70 years or proximal and distal colorectal cancer for unselected colorectal cancer patients, and <70 and ≥ 70 years old for the sporadic colorectal cancer patients, there was still a similar difference between the genotypes. It is possible that the increase in risk seen in unselected colorectal cancer patients is due to the increased risk of sporadic colorectal cancer, but not hereditary/familial colorectal cancers.

There was a significant difference in genotype distribution between the Swedish unselected patients and HCRC, TCR and Chinese patients. This difference in genotype distribution was also seen when comparing Swedish sporadic patients with HCRC, TCR or Chinese unselected patients. There was no significant difference in genotype distribution between Swedish unselected and sporadic patients, or between Chinese patients and HCRC or TCR patients. The frequency of alleles with the deletion was significantly higher among the Swedish unselected patients than among the controls; this was also true for the sporadic cancer patients compared with the controls. There was no significant difference in allele frequency between the cases and the controls in the HCRC, TCR and Chinese patients.

The analysis shows no gender-polymorphism association in either population. Nor did we find any correlation between genotype and survival among the Swedish unselected colorectal cancer patients. In our analysis the polymorphism did not correlate to gender, age, tumour location, Dukes stage, growth pattern, grade of differentiation, inflammatory infiltration or necrosis in the Swedish unselected colorectal cancer patients. In the group of Chinese unselected colorectal cancer patients, there was no correlation between the polymorphism and gender, age, tumour location, size, Dukes stage, invasive depth, grade of differentiation or gross status.

We found that the deletion, either in heterozygote or in homozygote deletion, of the -94ins/delATTG polymorphism in the promoter region of the NFKB1 gene was related to risk of colorectal cancer both in Swedish unselected and sporadic colorectal cancer patients. This increase in risk was not found in the Chinese population of unselected colorectal cancer patients. The genotype frequencies of the Swedish and the Chinese controls were similar – 26%, 58%, 15% versus 24%, 58%, 17% – for the homozygote wild-type, heterozygote and homozygote deletion, respectively. This supports the evidence that individuals with same genotype but exposed to different environmental factors may have different risks of developing cancer. It is possible that the deletion of this polymorphism did not directly promote

carcinogenesis, but instead increased the susceptibility to carcinogenetic exposure that differed between the two populations.

When analysing the other groups of Swedish patients, the genotype did not increase the risk in HCRC or TCR patients, but did so in patients with sporadic colorectal cancer. These patients were recruited from the same residential area, which lowered the impact of the differences in lifestyle and environmental exposure on the results. The differences we find for these groups of patients could result from differences in molecular mechanisms in the development between sporadic and hereditary/familial cancers. Furthermore, sporadic colorectal cancer development may be more dependent on the NF- κ B pathways than the development of hereditary/familial colorectal cancers. There is increasing evidence to show that there are certain differences in epidemiology, clinicopathology and molecular biology between sporadic and hereditary/familial cancers (Craanen et al. 1996. Lagerstedt et al. 2007).

Our results show that the Chinese unselected cancer patients developed cancer at a similar age as that of the HCRC and TCR patients. We also found that the Chinese patients had genotype distribution similar to those of the Swedish HCRC and TCR patients. These findings highlight the importance of further investigation of the similarities between the Chinese unselected patients and the Swedish patients with a family history of colorectal cancer.

The polymorphism did not correlate to the clinicopathological variables included in the Swedish and Chinese unselected patients. These results are in line with those of a study that found that this polymorphism was related to risk of oral squamous cell carcinoma but not to any clinicopathological factors such as tumour stage and lymph node metastasis (Lin, et al. 2006). They also concur with the results of a study that found that the polymorphism may correlate to risk but not to clinicopathological factors including survival in aged patients with gastric cancer. On the other hand, Riemann et al (2006) found an association with both risk and disease progression in B-cell chronic lymphocytic leukaemia. The inability to replicate genetic associations is not uncommon and could be due to a variety of reasons, such as different populations and exposure to environmental factors, the number of, and criteria for, the cases and controls selected, the clinicopathological features of the cases included, and the methods used in these studies.

Paper II

The -708del8 allele occurred with low frequency in the Swedish population; two of 92 patients and eight of 174 controls had at least one allele with the -708del8 polymorphism. The -708del8 allele was absent in the Chinese population; none of the 93 patients or 159 controls had the -708del8 allele.

In the Chinese population, however, there were four other shifts in the SSCP analysis. Two of these could be identified by DNA sequencing. One mutation in the group of patients – a T to A substitution – was found in a patient, and another mutation – a G to T substitution – in a control.

The clinicopathological data for the two Swedish patients with a -708del8 allele shows that both were males, diagnosed at 72 and 87 years of age. They were heterozygous for the deletion of the -94ins/delATTG polymorphism in the promoter region of the NFKB1 gene and heterozygous with a G to A variation in the 3' untranslated region of the NFKBIA gene (Gao et al. 2007).

The three Chinese patients with a small shift on the SSCP gel were all females diagnosed at 52, 57 and 69 years of age, respectively. Two of these patients were homozygous wild-type for the -94ins/delATTG polymorphism in the promoter region of the NFKB1 gene and one was heterozygous. Two patients were homozygous for the A to G variation in the 3' untranslated region of the NFKBIA gene and one had an unknown genotype for this polymorphism. All tumours from the two Swedish and the three Chinese patients were located in the rectum, had moderately differentiated cells, and were in Dukes stage B or C.

The screening of a Swedish and a Chinese population of colorectal cancer patients with the corresponding controls with SSCP revealed that the -708ins/del8 polymorphism in the promoter region of NFKBIA gene is very rare. Only 2.2% (2/92) of the Swedish patients and 4.6% (8/174) of the Swedish controls had the deletion in one or more alleles. The numbers were too low to carry out statistical analysis, so no conclusions can be drawn as to whether the polymorphism increases the risk of colorectal cancer, or whether it is related to any clinicopathological factors in the Swedish population. The only conclusion we can draw is that the polymorphism most likely does not have a potential effect on the incidence of colorectal cancer due to its rare occurrence. In addition, it seems that there were other mutations in the Chinese population only but not in the Swedish. The two populations may have different genetic profiles concerning the polymorphism and mutations examined. In our analysis, the -708ins/del8 polymorphism was totally absent in the Chinese population; it was not discovered in either patients or controls. We cannot exclude that it was totally absent because of the low number of cases analysed. Osborne et al (2005) screened a population of Hodgkin's lymphoma patients and controls from Scotland and northern England and found the 8-basepair deletion in 1.1%, and the 8- basepair insertion in 2.9% of the patients; they found the deletion in 3.6%, and the insertion in 2.1% of the controls. They found no significant differences by case control status, and cases with the deletion or insertion did not show any consistent clinical features.

Paper III

In the present thesis we investigated the expression of NF- κ B p65 phosphorylated at Serine-536 in samples from Swedish colorectal cancer patients. We graded the staining in normal mucosa, primary tumour, invasive margin of the primary tumour and in metastases in the lymph nodes.

We found that the staining intensity of NF- κ B p65 phosphorylated at Serine-536 increased from normal mucosa to primary tumour. Fifty-six samples had weak staining in normal mucosa but strong staining in primary tumour, whereas only five samples had strong staining in normal mucosa but weak staining in primary tumour. In sixty-seven samples there was no difference in staining between normal mucosa and primary tumour. An increase in expression from normal mucosa to primary tumour has also been found in a previous study using electrophoretic mobility shift assay. Yu et al. (2003) used monoclonal antibodies against NF- κ B p65 and found that the expression was significantly increased from normal mucosa to adenoma and to adenocarcinoma. A similar significant difference was found when comparing staining intensity in normal mucosa with staining intensity on the invasive border of the primary tumour. There was a higher frequency of strong staining in the invasive border than in normal mucosa. There was no significant difference in staining intensity of the cytoplasm in primary tumour compared with metastases. The increased expression of NF- κ B p65 phosphorylated at Serine-536 in primary tumour compared with normal mucosa and the fact that the expression did not further increase from primary tumour to metastases indicate that NF- κ B activation may play a role in the earlier development of colorectal cancer.

The staining intensity in the cytoplasm of primary tumour did not correlate to gender, age, tumour location, stage, or differentiation.

We found that the NF κ B p65 phosphorylated at Serine-536 is an independent prognostic factor in colorectal cancer patients. Strong expression in the cytoplasm correlated to worse survival of the patients. The prognostic significance remained even after adjustment for gender, age, tumour location, stage and differentiation.

The staining intensity in the cytoplasm had a strong positive correlation to the staining in the nucleus. There was no significant difference in staining of the nucleus between normal mucosa and primary tumour, or between primary tumour and metastases. The nuclear expression was not related to clinicopathological variables, including survival.

Paper IV

This study included rectal cancer patients from the south-eastern Sweden. The patients had previously been included in the Swedish Rectal Cancer Trial (1997) in which they were randomised into two groups, one that received and one that did not receive preoperative radiotherapy.

When we compared the staining intensity of NF- κ B p65 phosphorylated at Serine-536 expression in the cytoplasm, we found significantly more samples with strong staining in primary tumour than in normal mucosa in both the radiotherapy and non-radiotherapy groups. This difference was found either in the matched cases or in the whole group of patients. We found no significant difference between primary tumour and metastasis in either the radiotherapy or non-radiotherapy groups.

In chi-square analysis of the differences in expression between the biopsies from the primary tumours and the surgical primary tumours we found significantly more strongly stained samples in the surgical primary tumours than in biopsies. However, when we compared paired samples by using the McNemar test, the difference between biopsies and the surgical primary tumours was still present in the non-radiotherapy group, but there was no difference in the radiotherapy group.

There was no difference in NF- κ B p65 phosphorylated at Serine-536 expression before or after preoperative radiotherapy in normal mucosa, primary tumour or in metastases.

Cytoplasmic expression of NF- κ B p65 phosphorylated at Serine-536 in primary tumour was positively related to the expression of TEM1, FXYD-3, PRL, and p73, and was positively related to the expression of MAC30 in the radiotherapy group. In the non-radiotherapy group, there was no such relationship.

The expression of NF- κ B p65 phosphorylated at Serine-536 in the cytoplasm did not correlate to gender, age or survival, or differentiation or stage of the cancer, or local/distant recurrence in either the radiotherapy group or non-radiotherapy group, or in the whole group of patients.

Our previous studies of TEM1 showed that, in the radiotherapy group, the expression significantly increased from Dukes A to Dukes B-D. We have also found that strong expression of FXYD-3 alone or combined with PRL was related to unfavourable prognosis independent of both TNM stage and differentiation. With strong expression of FXYD-3, the tumour showed less necrosis and a trend toward an increased incidence of distant metastases after radiotherapy. None of these effects was seen in the non-radiotherapy group (Loftås, 2009). In the radiotherapy group, strong PRL expression was related to distant recurrence and poor survival independent of both stage and differentiation; this was not found in non-radiotherapy group (Wallin, 2006). Patients with p73-overexpression tumours had a higher local recurrence after radiotherapy than had the non-radiotherapy cases (Pfeifer, 2006). Taken together, after radiotherapy, the expression of NF- κ B p65 phosphorylated at Serine-536 was positively related to biological factors associated with more malignant features of tumours. However, we did not find that the expression was directly related to clinical response to radiotherapy.

Conclusions

Deletion of the polymorphism in the promoter region of the NFKB1 gene is related to increased risk of sporadic colorectal cancer in a Swedish population but not in Swedish patients with a family history of colorectal cancer or in Chinese patients.

An 8-basepair deletion in the promoter region of NFKB1A gene is very rare in a Swedish and a Chinese population and has, because of this rarity, no major impact on colorectal cancer incidence in the two populations.

The expression of a nuclear factor kappaB p65 subunit phosphorylated at Serine-536 increases from normal mucosa to primary tumour but does not further increase from primary tumour to metastases. The nuclear factor kappaB p65 subunit phosphorylated at Serine-536 is an independent prognostic factor in colorectal cancer patients.

After preoperative radiotherapy in rectal cancer, the expression of nuclear factor kappaB p65 subunit phosphorylated at Serine-536 appears to be positively correlated to biological factors associated with more malignant features of the tumours. However, the expression is not directly related to the clinical response to preoperative radiotherapy.

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References

- Improved survival with preoperative radiotherapy in resectable rectal cancer.* Swedish Rectal Cancer Trial. *N Engl J Med.* 1997. 336:980-7.
- Adell G, Sun XF, Stål O, Klintonberg C, Sjødahl R, Nordenskjöld B. *p53 status: an indicator for the effect of preoperative radiotherapy of rectal cancer.* *Radiother Oncol.* 1999. 51:169-74.
- Aggarwal BB. *Nuclear factor-kappaB: the enemy within.* *Cancer Cell.* 2004. 6:203-8.
- Ahmed KM, Li JJ. *NF-kappa B-mediated adaptive resistance to ionizing radiation.* *Free Radic Biol Med.* 2008. 44:1-13.
- Ahn KS, Sethi G, Aggarwal BB. *Nuclear factor-kappa B: from clone to clinic.* *Curr Mol Med.* 2007. 7:619-37.
- Aranha MM, Borralho PM, Ravasco P, Moreira da Silva IB, Correia L, Fernandes A, Camilo ME, Rodrigues CM. *NF-kappaB and apoptosis in colorectal tumourigenesis.* *Eur J Clin Invest.* 2007. 37:416-24.
- Bardelli A, Saha S, Sager JA, Romans KE, Xin B, Markowitz SD, Lengauer C, Velculescu VE, Kinzler KW, Vogelstein B. *PRL-3 expression in metastatic cancers.* *Clin Cancer Res.* 2003. 9:5607-15.
- Beg AA, Sha WC, Bronson RT, Baltimore D. *Constitutive NF-kappa B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice.* *Genes Dev.* 1995. 9:2736-46.
- Beinke S, Ley SC. *Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology.* *Biochem J.* 2004. 382:393-409.
- Bénard J, Douc-Rasy S, Ahomadegbe JC. *TP53 family members and human cancers.* *Hum Mutat.* 2003. 21:182-91.
- Berenson JR, Ma HM, Vescio R. *The role of nuclear factor-kappaB in the biology and treatment of multiple myeloma.* *Semin Oncol.* 2001. 28:626-33.
- Bharti AC, Aggarwal BB. *Nuclear factor-kappa B and cancer: its role in prevention and therapy.* *Biochem Pharmacol.* 2002. 64:883-8.
- Borm ME, van Bodegraven AA, Mulder CJ, Kraal G, Bouma G. *A NFKB1 promoter polymorphism is involved in susceptibility to ulcerative colitis.* *Int J Immunogenet.* 2005. 32:401-5.
- Broström O, Löfberg R, Nordenvall B, Ost A, Hellers G. *The risk of colorectal cancer in ulcerative colitis. An epidemiologic study.* *Scand J Gastroenterol.* 1987. 22:1193-9.
- Buss H, Dörrie A, Schmitz ML, Hoffmann E, Resch K, Kracht M. *Constitutive and interleukin-1-inducible phosphorylation of p65 NF-kappaB at serine 536 is mediated*

- by multiple protein kinases including I{kappa}B kinase (IKK)-{alpha}, IKK{beta}, IKK {epsilon}, TRAF family member-associated (TANK)-binding kinase 1 (TBK1), and an unknown kinase and couples p65 to TATA-binding protein-associated factor II31-mediated interleukin-8 transcription. *J Biol Chem.* 2004. 279:55633-43.
- Butterworth AS, Higgins JP, Pharoah P. *Relative and absolute risk of colorectal cancer for individuals with a family history: a meta-analysis.* *Eur J Cancer.* 2006. 42:216-27.
- Chien C, Morimoto LM, Tom J, Li CI. *Differences in colorectal carcinoma stage and survival by race and ethnicity.* *Cancer.* 2005. 104:629-39.
- Christian S, Winkler R, Helfrich I, Boos AM, Besemfelder E, Schadendorf D, Augustin HG. *Endosialin (Tem1) is a marker of tumor-associated myofibroblasts and tumor vessel-associated mural cells.* *Am J Pathol.* 2008. 172:486-94.
- Craanen ME, Blok P, Offerhaus GJ, Tytgat GN. *Recent developments in hereditary nonpolyposis colorectal cancer.* *Scand J Gastroenterol Suppl.* 1996. 218:92-7.
- Dolcet X, Llobet D, Pallares J, Matias-Guiu X. *NF-kB in development and progression of human cancer.* *Virchows Arch.* 2005. 446:475-82.
- Domingo-Domenech J, Oliva C, Rovira A, Codony-Servat J, Bosch M, Filella X, Montagut C, Tapia M, Campás C, Dang L, Rolfe M, Ross JS, Gascon P, Albanell J, Mellado B. *Interleukin 6, a nuclear factor-kappaB target, predicts resistance to docetaxel in hormone-independent prostate cancer and nuclear factor-kappaB inhibition by PS-1145 enhances docetaxel antitumor activity.* *Clin Cancer Res.* 2006. 12:5578-86.
- Evertsson S, Sun XF. *Protein expression of NF-kappaB in human colorectal adenocarcinoma.* *Int J Mol Med.* 2002. 10:547-50.
- Fearon ER, Vogelstein B. *A genetic model for colorectal tumorigenesis.* *Cell.* 1990. 61:759-67.
- Fuchs CS, Giovannucci EL, Colditz GA, Hunter DJ, Speizer FE, Willett WC. *A prospective study of family history and the risk of colorectal cancer.* *N Engl J Med.* 1994. 331:1669-74.
- Gao J, Pfeifer D, He LJ, Qiao F, Zhang Z, Arbman G, Wang ZL, Jia CR, Carstensen J, Sun XF. *Association of NFKBIA polymorphism with colorectal cancer risk and prognosis in Swedish and Chinese populations.* *Scand J Gastroenterol.* 2007. 42:345-50.
- Geddes M, Balzi D, Buiatti E, Khlata M, Parkin D. *Cancer in Italian migrants.* *Cancer Causes Control.* 1991. 2:133-40.
- Ghosh S, May MJ, Kopp EB. *NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses.* *Annu Rev Immunol.* 1998. 16:225-60.
- Ghosh S, Hayden MS. *New regulators of NF-kappaB in inflammation.* *Nat Rev Immunol.* 2008. 8:837-48.
- Graham B, Gibson SB. *The two faces of NFkappaB in cell survival responses.* *Cell Cycle.* 2005. 4:1342-5.
- Hanahan D, Weinberg RA. *The hallmarks of cancer.* *Cell.* 2000. 100:57-70.
- Hayden MS, Ghosh S. *Signaling to NF-kappaB.* *Genes Dev.* 2004. 18:2195-224.

- Hayden MS, Ghosh S. *Shared principles in NF-kappaB signaling*. Cell. 2008. 132:344-62.
- Hemminki K, Chen B. *Familial risk for colorectal cancers are mainly due to heritable causes*. Cancer Epidemiol Biomarkers Prev. 2004. 13:1253-6.
- Hoffmann A, Levchenko A, Scott ML, Baltimore D. *The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation*. Science. 2002. 298:1241-5.
- Iscovich J, Howe GR. *Cancer incidence patterns (1972-91) among migrants from the Soviet Union to Israel*. Cancer Causes Control. 1998. 9:29-36.
- Jass JR. *Broadsheet number 52: Molecular genetics of colorectal cancer*. Pathology. 1999. 31:354-64.
- Johns LE, Houlston RS. *A systematic review and meta-analysis of familial colorectal cancer risk*. Am J Gastroenterol. 2001. 96:2992-3003.
- Karban AS, Okazaki T, Panhuysen CI, Gallegos T, Potter JJ, Bailey-Wilson JE, Silverberg MS, Duerr RH, Cho JH, Gregersen PK, Wu Y, Achkar JP, Dassopoulos T, Mezey E, Bayless TM, Novuet FJ, Brant SR. *Functional annotation of a novel NFKB1 promoter polymorphism that increases risk for ulcerative colitis*. Hum Mol Genet. 2004. 13:35-45.
- Karin M, Cao Y, Greten FR, Li ZW. *NF-kappaB in cancer: from innocent bystander to major culprit*. Nat Rev Cancer. 2002. 2:301-10.
- Karin M. *NF-kappaB and cancer: mechanisms and targets*. Mol Carcinog. 2006. 45:355-61.
- Karin M. *Nuclear factor-kappaB in cancer development and progression*. Nature. 2006. 441:431-6.
- Kemp Z, Thirlwell C, Sieber O, Silver A, Tomlinson I. *An update on the genetics of colorectal cancer*. Hum Mol Genet. 2004. 13:R177-85.
- Kewenter J, Ahlman H, Hultén L. *Cancer risk in extensive ulcerative colitis*. Ann Surg. 1978. 188:824-8.
- Kayed H, Kleeff J, Ding J, Hammer J, Giese T, Zentgraf H, Büchler MW, Friess H. *Expression analysis of MAC30 in human pancreatic cancer and tumors of the gastrointestinal tract*. Histol Histopathol. 2004. 19:1021-31.
- Kayed H, Kleeff J, Kolb A, Ketterer K, Keleg S, Felix K, Giese T, Penzel R, Zentgraf H, Büchler MW, Korc M, Friess H. *FXYD3 is overexpressed in pancreatic ductal adenocarcinoma and influences pancreatic cancer cell growth*. Int J Cancer. 2006. 118:43-54.
- Kim HJ, Hawke N, Baldwin AS. *NF-kappaB and IKK as therapeutic targets in cancer*. Cell Death Differ. 2006. 13:738-47.
- Klein W, Tromm A, Folwaczny C, Hagedorn M, Duerig N, Epplen JT, Schmiegel WH, Griga T. *A polymorphism of the NFKBIA gene is associated with Crohn's disease patients lacking a predisposing allele of the CARD15 gene*. Int J Colorectal Dis. 2004. 19:153-6.
- Knutsen A, Adell G, Sun XF. *Survivin expression is an independent prognostic factor in rectal cancer patients with and without preoperative radiotherapy*. Int J Radiat Oncol Biol Phys. 2004. 60:149-55.

- Kolonel LN, Altshuler D, Henderson BE. *The multiethnic cohort study: exploring genes, lifestyle and cancer risk*. Nat Rev Cancer. 2004. 4:519-27.
- Lagerstedt Robinson K, Liu T, Vandrovцова J, Halvarsson B, Clendenning M, Frebourg T, Papadopoulos N, Kinzler KW, Vogelstein B, Peltomäki P, Kolodner RD, Nilbert M, Lindblom A. *Lynch syndrome (hereditary nonpolyposis colorectal cancer) diagnostics*. J Natl Cancer Inst. 2007. 99:291-9.
- Lawrence T, Bebie M, Liu GY, Nizet V, Karin M. *IKKalpha limits macrophage NF-kappaB activation and contributes to the resolution of inflammation*. Nature. 2005. 434:1138-43.
- Lin A, Karin M. *NF-kappaB in cancer: a marked target*. Semin Cancer Biol. 2003. 13:107-14.
- Lin SC, Liu CJ, Yeh WI, Lui MT, Chang KW, Chang CS. *Functional polymorphism in NFKB1 promoter is related to the risks of oral squamous cell carcinoma occurring on older male areca (betel) chewers*. Cancer Lett. 2006. 243:47-54.
- Lind DS, Hochwald SN, Malaty J, Rekkas S, Hebig P, Mishra G, Moldawer LL, Copeland EM^{3rd}, Mackay S. *Nuclear factor-kappa B is upregulated in colorectal cancer*. Surgery. 2001. 130:363-9.
- Loftås P, Onnesjö S, Widegren E, Adell G, Kayed H, Kleeff J, Zentgraf H, Sun XF. *Expression of FXD-3 is an independent prognostic factor in rectal cancer patients with preoperative radiotherapy*. Int J Radiat Oncol Biol Phys. 2009. 75:137-42.
- MacFadyen J, Savage K, Wienke D, Isacke CM. *Endosialin is expressed on stromal fibroblasts and CNS pericytes in mouse embryos and is downregulated during development*. Gene Expr Patterns. 2007. 7:363-9.
- Magné N, Toillon RA, Bottero V, Didelot C, Houtte PV, Gérard JP, Peyron JF. *NF-kappaB modulation and ionizing radiation: mechanisms and future directions for cancer treatment*. Cancer Lett. 2006. 231:158-68.
- Malhotra K, Luehrsén KR, Costello LL, Raich TJ, Sim K, Foltz L, Davidson S, Xu H, Chen A, Yamanishi DT, Lindemann GW, Cain CA, Madlansacay MR, Hashima SM, Pham TL, Mahoney W, Schueler PA. *Identification of differentially expressed mRNAs in human fetal liver across gestation*. Nucleic Acids Res. 1999. 27:839-47.
- Maskarinec G, Noh JJ. *The effect of migration on cancer incidence among Japanese in Hawaii*. Ethn Dis. 2004. 14:431-9.
- Mattioli I, Sebald A, Bucher C, Charles RP, Nakano H, Doi T, Kracht M, Schmitz ML. *Transient and selective NF-kappa B p65 serine 536 phosphorylation induced by T cell costimulation is mediated by I kappa B kinase beta and controls the kinetics of p65 nuclear import*. J Immunol. 2004. 172:6336-44.
- Mayo MW, Baldwin AS. *The transcription factor NF-kappaB: control of oncogenesis and cancer therapy resistance*. Biochim Biophys Acta. 2000. 1470:M55-62.
- Mirza MM, Fisher SA, Onnie C, Lewis CM, Mathew CG, Sanderson J, Forbes A. *No association of the NFKB1 promoter polymorphism with ulcerative colitis in a British case control cohort*. Gut. 2005. 54:1205-6.

- Miterski B, Böhringer S, Klein W, Sindern E, Haupts M, Schimrigk S, Epplen JT. *Inhibitors in the NF-kappaB cascade comprise prime candidate genes predisposing to multiple sclerosis, especially in selected combinations*. Genes Immun. 2002. 3:211-9.
- Miyakoshi J, Yagi K. *Inhibition of I kappaB-alpha phosphorylation at serine and tyrosine acts independently on sensitization to DNA damaging agents in human glioma cells*. Br J Cancer. 2000. 82:28-33.
- Miyamoto S, Chiao PJ, Verma IM. *Enhanced I kappa B alpha degradation is responsible for constitutive NF-kappa B activity in mature murine B-cell lines*. Mol Cell Biol. 1994. 14:3276-82.
- Moparthi SB, Arbman G, Wallin A, Kayed H, Kleeff J, Zentgraf H, Sun XF. *Expression of MAC30 protein is related to survival and biological variables in primary and metastatic colorectal cancers*. Int J Oncol. 2007. 30:91-5.
- Morrison BW, Moorman JR, Kowdley GC, Kobayashi YM, Jones LR, Leder P. *Mat-8, a novel phospholemmann-like protein expressed in human breast tumors, induces a chloride conductance in Xenopus oocytes*. J Biol Chem. 1995. 270:2176-82.
- Moynagh PN. *The NF-kappaB pathway*. J Cell Sci. 2005. 118:4589-92.
- Mucci LA, Wedren S, Tamimi RM, Trichopoulos D, Adami HO. *The role of gene-environment interaction in the aetiology of human cancer: examples from cancers of the large bowel, lung and breast*. J Intern Med. 2001. 249:477-93.
- Murphy M, Pykett MJ, Harnish P, Zang KD, George DL. *Identification and characterization of genes differentially expressed in meningiomas*. Cell Growth Differ. 1993. 4:715-22.
- Nakanishi C, Toi M. *Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs*. Nat Rev Cancer. 2005. 5:297-309.
- Neumann M, Naumann M. *Beyond IkappaBs: alternative regulation of NF-kappaB activity*. FASEB J. 2007. 21:2642-54.
- Oliver J, Gómez-García M, Paco L, López-Nevot MA, Piñero A, Correro F, Martín L, Brieva JA, Nieto A, Martín J. *A functional polymorphism of the NFKB1 promoter is not associated with ulcerative colitis in a Spanish population*. Inflamm Bowel Dis. 2005. 11:576-9.
- Pachkoria K, Zhang H, Adell G, Jarlsfelt I, Sun XF. *Significance of Cox-2 expression in rectal cancers with or without preoperative radiotherapy*. Int J Radiat Oncol Biol Phys. 2005. 63:739-44.
- Parkin DM. *International variation*. Oncogene. 2004. 23:6329-40.
- Perkins ND, Gilmore TD. *Good cop, bad cop: the different faces of NF-kappaB*. Cell Death Differ. 2006. 13:759-72.
- Pfeifer D, Gao J, Adell G, Sun XF. *Expression of the p73 protein in rectal cancers with or without preoperative radiotherapy*. Int J Radiat Oncol Biol Phys. 2006. 65:1143-8.
- Reymond MA, Steinert R, Kähne T, Sagynaliev E, Allal AS, Lippert H. *Expression and functional proteomics studies in colorectal cancer*. Pathol Res Pract. 2004. 200:119-27.

References

- Sager JA, Benvenuti S, Bardelli A. *PRL-3: a phosphatase for metastasis?* Cancer Biol Ther. 2004. 3:952-3.
- Sakurai H, Chiba H, Miyoshi H, Sugita T, Toriumi W. *IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain.* J Biol Chem. 1999. 274:30353-6.
- Sasieni PD. *From genotypes to genes: doubling the sample size.* Biometrics. 1997. 53:1253-61.
- Scaife CL, Kuang J, Wills JC, Trowbridge DB, Gray P, Manning BM, Eichwald EJ, Daynes RA, Kuwada SK. *Nuclear factor kappaB inhibitors induce adhesion-dependent colon cancer apoptosis: implications for metastasis.* Cancer Res. 2002. 62:6870-8.
- Scott ML, Fujita T, Liou HC, Nolan GP, Baltimore D. *The p65 subunit of NF-kappa B regulates I kappa B by two distinct mechanisms.* Genes Dev. 1993. 7:1266-76.
- Sizemore N, Lerner N, Dombrowski N, Sakurai H, Stark GR. *Distinct roles of the Ikappa B kinase alpha and beta subunits in liberating nuclear factor kappa B (NF-kappa B) from Ikappa B and in phosphorylating the p65 subunit of NF-kappa B.* J Biol Chem. 2002. 277:3863-9.
- Solomon MJ, Schnitzler M. *Cancer and inflammatory bowel disease: bias, epidemiology, surveillance, and treatment.* World J Surg. 1998. 22:352-8.
- St John DJ, McDermott FT, Hopper JL, Debney EA, Johnson WR, Hughes ES. *Cancer risk in relatives of patients with common colorectal cancer.* Ann Intern Med. 1993. 118:785-90.
- Ten RM, Paya CV, Israël N, Le Bail O, Mattei MG, Virelizier JL, Kourilsky P, Israël A. *The characterization of the promoter of the gene encoding the p50 subunit of NF-kappa B indicates that it participates in its own regulation.* EMBO J. 1992. 11:195-203.
- Sun SC, Ley SC. *New insights into NF-kappaB regulation and function.* Trends Immunol. 2008. 29:469-78.
- Sun XF. *p73 overexpression is a prognostic factor in patients with colorectal adenocarcinoma.* Clin Cancer Res. 2002. 8:165-70.
- Sun XF, Zhang H. *NFKB and NFKBI polymorphisms in relation to susceptibility of tumour and other diseases.* Histol Histopathol. 2007. 22:1387-98.
- Tam WF, Sen R. *IkappaB family members function by different mechanisms.* J Biol Chem. 2001. 276:7701-4.
- Tomkowicz B, Rybinski K, Foley B, Ebel W, Kline B, Routhier E, Sass P, Nicolaidis NC, Grasso L, Zhou Y. *Interaction of endosialin/TEMI with extracellular matrix proteins mediates cell adhesion and migration.* Proc Natl Acad Sci U S A. 2007. 104:17965-70.
- Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S. *Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation.* Genes Dev. 1995. 9:2723-35.
- Viatour P, Merville MP, Bours V, Chariot A. *Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation.* Trends Biochem Sci. 2005. 30:43-52.

- Wallin AR, Svanvik J, Adell G, Sun XF. *Expression of PRL proteins at invasive margin of rectal cancers in relation to preoperative radiotherapy*. Int J Radiat Oncol Biol Phys. 2006. 65:452-8.
- Wang W, Abbruzzese JL, Evans DB, Larry L, Cleary KR, Chiao PJ. *The nuclear factor-kappa B RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells*. Clin Cancer Res. 1999. 5:119-27.
- Wood KM, Roff M, Hay RT. *Defective IkappaBalpha in Hodgkin cell lines with constitutively active NF-kappaB*. Oncogene. 1998. 16:2131-9.
- Yamagishi N, Miyakoshi J, Takebe H. *Enhanced radiosensitivity by inhibition of nuclear factor kappa B activation in human malignant glioma cells*. Int J Radiat Biol. 1997. 72:157-62.
- Yu HG, Yu LL, Yang Y, Luo HS, Yu JP, Meier JJ, Schrader H, Bastian A, Schmidt WE, Schmitz F. *Increased expression of RelA/nuclear factor-kappa B protein correlates with colorectal tumorigenesis*. Oncology. 2003. 65:37-45.
- Yu Y, Wan Y, Huang C. *The biological functions of NF-kappaB1 (p50) and its potential as an anti-cancer target*. Curr Cancer Drug Targets. 2009. 9:566-71.
- Zhang YZ, Sheng JQ, Li SR, Zhang H. *Clinical phenotype and prevalence of hereditary nonpolyposis colorectal cancer syndrome in Chinese population*. World J Gastroenterol. 2005. 11:1481-8.
- Zhang ZY, Zhao ZR, Adell G, Jarlsfelt I, Cui YX, Kayed H, Kleeff J, Wang MW, Sun XF. *Expression of MAC30 in rectal cancers with or without preoperative radiotherapy*. Oncology. 2006. 71:259-65.
- Zhivotovsky B, Joseph B, Orrenius S. *Tumor radiosensitivity and apoptosis*. Exp Cell Res. 1999. 248:10-7.
- Zlobec I, Lugli A. *Prognostic and predictive factors in colorectal cancer*. Postgrad Med J. 2008. 84:403-11.