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# **Molecular and Biological Characteristics of Stroma and Tumor Cells in Colorectal Cancer**

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*To my beloved Guoliang Shuangji and parents*



# CONTENTS

<b>ABSTRACT</b>	<b>7</b>
<b>ABBREVIATIONS</b>	<b>9</b>
<b>LIST OF PAPERS</b>	<b>11</b>
<b>INTRODUCTION</b>	<b>13</b>
<b>1. The molecular basis of cancer</b>	<b>13</b>
1.1. Oncogenes	13
1.2. Tumor suppressor genes	14
1.3. DNA repair genes	16
1.3.1. Base excision repair	17
1.3.2. Nucleotide excision repair	17
1.3.3. Mismatch repair	17
1.3.4. Double strand break repair	20
1.3.4.1. Homologous recombination	20
1.3.4.2. Non-homologous end joining	21
1.3.4.3. RAD50/MRE11/NBS1 complex	22
1.4. Microenvironment	24
1.4.1. Particularly interesting new cysteine-histidine rich protein	25
1.4.2. Inflammatory infiltration	28
1.4.3. Angiogenesis and lymphangiogenesis	30
<b>2. Colorectal cancer</b>	<b>34</b>
2.1. Epidemiology	34
2.2. Etiology and risk factors	34
2.3. Pathology	36
2.4. Molecular tumorigenesis	37
2.4.1. Chromosomal instability pathway	38
2.4.2. Microsatellite instability pathway	39
<b>AIMS OF THE STUDY</b>	<b>41</b>
<b>MATERIALS AND METHODS</b>	<b>43</b>
<b>1. Materials</b>	<b>43</b>
1.1. Patients	43
1.2. Cell lines	43

<b>2. Methods</b>	<b>45</b>
2.1. DNA extraction	45
2.2. Polymerase chain reaction	45
2.3. Single-strand conformational polymorphism	46
2.4. DNA sequencing	46
2.5. Immunohistochemistry	46
2.6. Immunofluorescence	47
2.7. Western blotting	47
2.8. Statistical analysis	48
<b>RESULTS AND DISCUSSIONS</b>	<b>49</b>
<b>1. Clinicopathological and biological significance of stromal variables in colorectal cancer (Studies I-III)</b>	<b>49</b>
1.1. PINCH and inflammatory infiltration (Studies I-III)	49
1.2. Angiogenesis and lymphangiogenesis (Studies II & III)	51
<b>2. Clinicopathological and biological significance of hRAD50/hMRE11/hNBS1 in colorectal cancer (studies IV &amp; V)</b>	<b>53</b>
<b>CONCLUSIONS</b>	<b>57</b>
<b>ACKNOWLEDGEMENTS</b>	<b>59</b>
<b>REFERENCES</b>	<b>61</b>

## ABSTRACT

Carcinogenesis is a progressive process involving multiple genetic alterations in tumor cells and complex interactions in the tumor-host microenvironment. To better understand the contribution of molecular alterations in tumor cells and stromal variables to the development of colorectal cancer (CRC) and identify prognostic factors, in this study we examined the clinicopathological and biological significance of stromal variables, including particularly interesting new cysteine-histidine rich protein (PINCH), inflammatory infiltration, angiogenesis and lymphangiogenesis, as well as hRAD50/hMRE11/hNBS1 proteins and *hRAD50* mutation in tumor cell in CRC.

PINCH protein expression in the stroma was increased from normal mucosa to primary tumors and further to lymph node metastases. In particular, PINCH expression was most intense at the tumor invasive margin, which was related to low inflammatory infiltration and independently related to an unfavorable prognosis. Low inflammatory infiltration at the tumor invasive margin was related to advanced tumor stage, worse differentiation and microsatellite instability (MSI). Further, it was independently related to an unfavorable prognosis. Increased blood and lymphatic vessel density was observed in the primary tumors compared with the corresponding normal mucosa. However, neither angiogenesis nor lymphangiogenesis was associated with tumor stage and patients' survival. Moreover, PINCH was present in a proportion of endothelial cells of the tumor vasculature, and PINCH expression in tumor-associated stroma was positively related to blood vessel density.

In primary tumor cells of CRC, strong expression of hRAD50, hMRE11 or hNBS1 was related to microsatellite stability (MSS). A high percentage of hMRE11 expression was associated with less local recurrence and high apoptotic activity. Further, we observed that the expression of hRAD50, hMRE11 or hNBS1 among normal mucosa, primary tumors and metastases in MSS CRC differed from that in MSI CRC. In MSS CRC, the expression intensity of hRAD50, hMRE11 and hNBS1 was consistently increased with respect to normal mucosa, but there was no difference between the primary tumors and metastases. In the primary MSS tumors, the expression of individual or combination of hRAD50/hMRE11/hNBS1 was associated with a favorable prognosis in the same series of the CRCs. Moreover, strong/high hRAD50 in MSS primary tumors was related to earlier tumor stage, better differentiation and high inflammatory infiltration, whereas strong hNBS1 expression tended to be independently related to a favorable prognosis in MSS CRC with earlier tumor stage. However, in MSI CRC, there were neither differences in the expression of hRAD50/hMRE11/hNBS1 among normal mucosa, primary tumors and metastases, nor any association of the protein expressions with clinicopathological variables. On the other hand, frameshift mutations of (A)<sub>9</sub> at coding region of *hRAD50* were only found in MSI CRC.

Our study indicates that 1) PINCH is likely a regulator of angiogenesis, and PINCH expression at the tumor invasive margin is an independent prognostic indicator in CRC. 2) Inflammatory infiltration at the tumor invasive margin is also an independent prognostic indicator in CRC. The lack of association between high inflammatory infiltration and MSI may help to explain the non-association of MSI with survival in CRC patients. 3) Angiogenesis and lymphangiogenesis occur in the early stage of CRC development, but do not associate with CRC progression and patients' prognosis. 4) hRAD50/hMRE11/hNBS1 may act dependently and independently, playing different roles in MSS and MSI CRC development. In MSS CRC, the strong expression of the three proteins, associated with a favorable prognosis, may present the cellular response against tumor progression. Expression of hNBS1 may be a prognostic indicator for MSS CRC patients in the earlier tumor stage. In MSI CRC, the frameshift mutations at the coding region of *hRAD50* may contribute to tumor development.



## ABBREVIATIONS

ABC	ATP-binding cassette
<i>APC</i>	Adenomatous polyposis coli
AT	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutated
BER	Base excision repair
BRCT	Breast cancer C-terminus
CH-ILKBP	Calponin homology-containing ILK-binding protein
CRC	Colorectal cancer
DC	Dendritic cell
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
dNTP	Deoxynucleotide triphosphate
DSB	Double strand break
ECM	Extracellular matrix
EGF	Epidermal growth factor
FHA	Forkhead-associated
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GSK-3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphate hydrolase
HNPCC	Hereditary nonpolyposis colorectal cancer
HR	Homologous recombination
IGFIIR	Insulin-like growth factor type II receptor
ILK	Integrin-linked kinase
IRS-1	Insulin receptor substrate-1
ITL	Intratumoral lymphatic
LOH	Loss of heterozygosity
LVD	Lymphatic vessel density
MAPK	Mitogen-activated protein kinase
MMR	Mismatch repair

MSI	Microsatellite instability
MSS	Microsatellite stability
MVD	Microvessel density
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NK	Natural killer
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PH	Pleckstrin homology
PI3K	phosphoinositide 3-kinase
PIGF	Placental growth factors
PINCH	Particularly interesting new cysteine-histidine rich protein
PKB	Protein kinase B
PTL	Peritumoral lymphatic
RMN	RAD50/MRE11/NBS1
TGF	Transforming growth factor
TGF- $\beta$ RII	Transforming growth factor beta receptor type II
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

## LIST OF PAPERS

This thesis is based on the following papers:

- I. **Gao J**, Arbman G, Rearden A, Sun XF. Stromal staining for PINCH is an independent prognostic indicator in colorectal cancer. *Neoplasia*. 2004;6:796-801.
- II. **Gao J**, Arbman G, Wadhra TI, Zhang H, Sun XF. Relationships of tumor inflammatory infiltration and necrosis with microsatellite instability in colorectal cancers. *World J Gastroenterol*. 2005;11:2179-83.
- III. **Gao J**, Knutsen A, Arbman G, John C, Sun XF. Clinicopathological and biological significance of angiogenesis and lymphangiogenesis in colorectal cancer. Submitted.
- IV. **Gao J**, Zhang H, Arbman G, Sun XF. The different roles of hRAD50 in microsatellite stable and unstable colorectal cancers. *Dis Markers*. In press.
- V. **Gao J**, Zhang H, Arbman G, Sun XF. hRAD50/hMRE11/hNBS1 proteins in relation to tumor development and prognosis in patients with microsatellite stable colorectal cancer. Submitted.

Other related papers by the author:

- I. Murthy RV, Arbman G, **Gao J**, Roodman GD, Sun XF. Legumain expression in relation to clinicopathological and biological variables in colorectal cancer. *Clin Cancer Res*. 2005;11:2293-9.
- II. 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.
- III. **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.
- IV. **Gao J**, Arbman G, He L, Qiao F, Zhang Z, Zhao Z, Rosell J, Sun XF. MANBA polymorphism was related to increased risk of colorectal cancer in Swedish but not in Chinese populations. *Acta Oncol*. 2007;:1-7 [Epub ahead of print].

- V. Lewander A, Butchi AK, **Gao J**, He LJ, Lindblom A, Arbman G, Carstensen J, Zhang ZY, Group TS, 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. *Scand J Gastroenterol.* 2007;42:1-7.

# INTRODUCTION

## 1. The molecular basis of cancer

Cancer is a general term for a large group of diseases characterized by self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan & Weinberg, 2000). It can arise in many sites and behave differently depending on its organ of origin. It has been long accepted that cancer develops through sequential morphological steps, with the accumulation of multiple genetic and epigenetic alterations in oncogenes, tumor suppressor genes and DNA repair genes in tumor cells (Vogelstein & Kinzler, 2004). However, it is becoming increasingly evident that those discrete genetic alterations in tumor cells alone cannot explain multistep carcinogenesis. Emerging evidence suggests that cancer is a state that emerges from a tumor-host microenvironment in which the microenvironment of the local host tissue actively participates in tumor initiation and progression (Liotta & Kohn, 2001).

### 1. 1. Oncogenes

The first evidence that there are genes capable of causing cancer (oncogenes) comes from studies carried out with transplantable tumors in chickens, mice and rats (Rous, 1911). The causative agent for such tumors was found to be an RNA virus, and the first oncogene identified was a viral gene (*v-src*) responsible for the sarcoma-producing properties of the Rous sarcoma virus. Subsequently, oncogenes were identified as altered forms of normal cellular genes called proto-oncogenes, with a gain of oncogenic or transforming potential in a dominant fashion (Bishop, 1991; Park, 1998).

Proto-oncogenes are highly conserved in evolution and regulate the cascade of the appropriate events throughout the cell cycle, cell division, and differentiation under normal conditions. Based on their normal function within cells, proto-oncogenes can be classified into growth factors, growth factor receptors, proteins with guanosine triphosphate hydrolase (GTPase) activity, GTPase exchange factors, cytoplasmic serine-threonine protein kinases and nuclear protein family (Bishop, 1991; Park, 1998). The process of activation of proto-oncogenes to oncogenes includes point mutations, insertion mutations, gene amplification, chromosomal translocation, methylation, protein-protein interactions, retroviral transduction or retroviral integration (Park, 1998). Furthermore, it has been identified that, in humans,

proto-oncogenes are frequently located at, or adjacent to, chromosomal translocation breakpoints, making them susceptible to mutation (Rowley, 1983; Yunis et al., 1987).

On activation, a proto-oncogene becomes an oncogene, leading to changes in structure (qualitative change) or level of protein expression (quantitative change), resulting in a continuous or abnormal signal for cell proliferation (Park, 1998).

The first oncogene identified in humans was *ras* (Barbacid, 1987). The *ras* gene family consists of three functional genes: *H-ras*, *N-ras* and *K-ras*. The proto-oncogene *ras* genes encode 21-kD plasma membrane proteins that regulate cellular signal transduction by acting as a one-way switch for the transmission of extracellular growth signals to the nucleus. *Ras* genes are expressed in all tissues. In a normal cell, most of the *ras* proteins are present in an inactive guanosine diphosphate (GDP)-bound conformation. Upon mitogenic stimulation (e.g. CDC25, SOS), *ras* can be activated by the release of GDP and subsequent binding to guanosine triphosphate (GTP). It is inactivated by GTPase-activating proteins (GAPs) through catalyzing the hydrolysis of GTP to GDP. Activation of *ras* leads to the activation of a number of pathways, including the Raf/mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt, and Mekk/JNK pathways. The most important one seems to be the MAPK pathway, which transmits signals downstream to other protein kinases and gene regulatory proteins (Lodish et al., 2000).

The *ras* oncogene is commonly mutated in tumors such as pancreatic and colon cancers. Mutations of *ras* have been detected in up to 90% of pancreatic cancers and 50% of sporadic colorectal cancers (CRCs). Mutations in the *ras* genes prevent GTP hydrolysis and result in constitutively active *ras* (Reuter et al., 2000), thus leading to a continuous growth stimulus, the basis of carcinogenesis.

## **1. 2. Tumor suppressor genes**

The concept that there was gene product that could inhibit or suppress the proliferation of cells was first put forth by Boveri (1914). It was supported many years later by experiments carried out using somatic cell genetics (Harris et al., 1969), and an epidemiological study carried out by Knudson with retinoblastoma (Knudson, 1971), as well as a study of chromosome losses in tumor cells using cytogenetic and molecular genetic techniques (Francke, 1976). Tumor suppressor genes normally restrain cell growth either by inhibiting the cell cycle progression or by promoting programmed cell death. Their function loss results in uncontrolled cell growth. They function in a recessive fashion at the cellular level for

carcinogenesis, meaning that inactivation of one allele of the susceptibility gene is insufficient for tumor formation, whereas alterations of both alleles are necessary for cancer development (Fearon, 1998; Sherr, 2004). The first mutation could be either a germline or somatic mutation, whereas the second mutation is always somatic. Tumor suppressor genes can be inactivated by point mutations, rearrangements and deletions, including small deletion, deletion of entire chromosomal arm or even deletion of whole chromosome, as well as phosphorylation or binding to other proteins.

The first tumor suppressor gene discovered in humans was the *Rb* gene through its association with a familial (inherited) form of retinoblastoma, but base substitution mutations and gene deletions of *Rb* have been found in a proportion of common cancers such as lung, prostate and breast cancers. The Rb protein is a key regulator of the cell cycle, and loss of function can lead to increased cell proliferation and a failure in terminal differentiation (Liu et al., 2004).

The *p53* gene is the most frequently altered gene in human cancers (Levine et al., 1991). It was first described in 1979. The *p53* gene is located on chromosome 17p13.1, and encodes a 53 kDa protein (Levine, 1993; Levine, 1997). The *p53* protein is a sequence-specific DNA-binding protein, capable of transcriptionally activating the expression of genes containing *p53* binding sites in either upstream regulatory regions or introns (Kern et al., 1991; Farmer et al., 1992). It is well conserved in vertebrate species and exists as a tetramer consisting of four functional domains: one for activating transcription factors, one for recognizing specific DNA sequences (core domain), one that is responsible for the tetramerization of the protein, and one for recognizing damaged DNA such as misaligned base pairs or single-stranded DNA.

Wild-type *p53* is a labile protein located in the nucleus (Bell et al., 2002). In normal cells, *p53* is usually inactive and expressed at negligible levels, bound to the protein MDM-2, which prevents its action and promotes its degradation. Active *p53* is induced by various cancer-causing agents such as UV radiation, oncogenes and some DNA-damaging drugs. Once activated, *p53* can induce a variety of growth-limiting responses including cell-cycle arrest, apoptosis, cell senescence, differentiation and antiangiogenesis through different mechanisms (Vogelstein et al., 2000).

The *p53* gene can be inactivated by mutations, resulting in a single amino acid change in the protein. Mutational inactivation of the *p53* gene has been detected in more than 50-60% of sporadic human tumors analyzed, including tumors of the brain, lung, breast, kidney, bladder, bone, esophagus, liver, colon, and anus. All the tumor-related *p53* mutations are in the DNA-binding domain and most of them are missense mutations. In addition, the normal *p53* protein

can be rendered non-functional by binding to other proteins, leading to accumulation of p53 protein within cells. Inactivation of p53 by cytoplasmic sequestration has been reported in breast cancer and neuroblastoma (Levine, 1997).

### **1. 3. DNA repair genes**

The cellular genome is constantly subjected to the threat of DNA damage agents derived from environmental sources, such as UV light, ionizing radiation and certain chemotherapeutic drugs, and cellular metabolisms, such as endogenously generated reactive oxygen species. In response to numerous DNA damage insults, all eukaryotic cells have evolved elaborate DNA repair mechanisms to monitor and repair DNA damage lesions to maintain genomic integrity, through complex signal transduction, activation of cell-cycle checkpoints, repair pathway or when the damage is irreparable, initiation of apoptosis (Hoeijmakers, 2001; Hasty, 2005).

The original insights into DNA repair and the genes responsible (DNA repair genes) were largely derived from studies in bacteria and yeast. Up to now, over 150 genes directly involved in DNA repair have been identified in humans (Wood et al., 2001; Wood et al., 2005). They include DNA base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and DNA double strand break (DSB) repair genes. They do not control cell birth and death directly, but have a general role in ensuring the integrity of the genetic information by controlling the rate of mutation of other genes. Disregulation of DNA repair genes can cripple the repair process and cause a cascade of unrepaired mutations in the genome, which are associated with significant, detrimental health effects, including an increased prevalence of birth defects, an enhancement of cancer risk, and an accelerated rate of aging (Hoeijmakers, 2001; Dixon & Kopras, 2004; Hasty, 2005).

The implication of DNA repair genes in human cancer has been observed in people with rare inherited disease, such as Bloom's syndrome, ataxia telangiectasia (AT), xeroderma pigmentosum, and hereditary nonpolyposis colorectal cancer (HNPCC), which all have the common feature of deficient DNA repair and an increased risk of developing certain cancers. It is believed that defects in DNA repair genes facilitate malignant transformation by failing to produce proteins that correct DNA damage, leading to an accumulation of mutations of other genes that ordinarily have key functions in the cells. Thus, decreased efficiency of repair is now considered as being an important event in the succession of changes required for cancer formation (Hoeijmakers, 2001; Dixon & Kopras, 2004; Hasty, 2005).

### **1.3.1. Base excision repair (BER)**

BER is the main pathway for repairing endogenous DNA damage resulting from reactive oxygen species or methylation in human cells. The BER proteins excise damaged DNA bases by DNA glycosylases, followed by cleavage of the sugar phosphate chain, and then the gap is filled by DNA polymerase I and DNA ligase. It is expected that different tumor types could derive from BER defects. However, no human disorders caused by inherited BER deficiencies have been identified. Interestingly, several DNA variations in BER genes have been linked to lung cancer susceptibility (Divine et al., 2001; Frosina, 2004). Further, defects in BER of oxidative damage have been described in some forms of intestinal cancers (Frosina, 2004).

### **1.3.2. Nucleotide excision repair (NER)**

NER is the most versatile repair system in terms of lesion recognition. It mainly repairs a variety of bulky, helix-distorting lesions in DNA caused by environmental agents. NER consists of a multistep process in which the DNA lesion is recognized and demarcated, followed by strand incision at both sides of the DNA lesion. Then, an approximately 28 bp DNA damage-containing oligonucleotide is excised, and the gap is filled by a newly synthesized oligonucleotide using the undamaged complementary DNA strand as a template (Wood et al., 2001; Leibel et al., 2006). In *E. coli*, the three polypeptides, UvrA, UvrB and UvrC, can locate a lesion and incise on either side of it to remove a segment of nucleotides containing the damage (Wood et al., 2001). In humans, all seven XP genes (*XPA-XPG*) are involved in this process. The XPC protein initiates the NER process, followed by XPA binding to DNA damage. XPB and XPD possess DNA helicase activity for formation of an unwound pre-incision intermediate. XPG endonucleases and ERCC1-XPF function to incise the damage-containing DNA strand. Defects in NER genes are associated with xeroderma pigmentosum, Cockayne's syndrome and trichothiodystrophy, all characterized by exquisite sun sensitivity. Xeroderma pigmentosum exhibits a dramatically increased sunlight-induced cancer risk (Leibel et al., 2006).

### **1.3.3. Mismatch repair (MMR)**

MMR corrects the single-base mismatches and insertion/deletion loops that arise during DNA replication. The mechanism of MMR was first thoroughly studied in *E. coli*, in which MutS, MutL, and MutH proteins mediate the repair process. At least seven human homologs of *E. coli* MutS (*hMSH2*, *hMSH3* and *hMSH6*) or MutL genes (*hMLH1*, *hMLH3*, *hPMS1* and *hPMS2*) have been identified and function as multiple repair complexes by protein binding

(Kolodner & Marsischky, 1999; Jacob & Praz, 2002; Sharova, 2005). Mismatched bases in DNA are first recognized by a heterodimeric complex of MutS-related proteins (hMSH2/hMSH6 or hMSH2/hMSH3 heterodimer). hMSH2/hMSH6 plays a major role in the repair of base:base mispairs and single-base insertion/deletion mispairs, whereas hMSH2/hMSH3 is mainly responsible for correcting large insertion/deletion mispairs (Kolodner & Marsischky, 1999). Then, a heterodimeric complex of MutL-related proteins (hMLH1/hPMS2) interacts with the mismatch recognition complex that has already bound to mismatch bases and other proteins necessary for MMR, including exonuclease 1, proliferating cell nuclear antigen (PCNA) and DNA polymerase  $\delta$ . This leads to activation of exonucleases. Subsequently, the mismatches are removed by the exonucleases followed by gap filling with DNA polymerases and DNA ligases (Jacob & Praz, 2002; Sharova, 2005).

Mismatched DNA repair genes were linked to HNPCC a decade ago (Aaltonen & Peltomäki, 1994; Hemminki et al., 1994). HNPCC is an autosomal dominant inherited disease characterized by an increased risk of CRC and cancers in the extra-colonic organs, including the endometrium, ovary, urinary tract, stomach and biliary system (Lynch & Smyrk, 1996; Lynch et al., 1996). Germline mutations in one of *hMSH2*, *hMLH1*, *hPMS1*, *hPMS2* and *hMSH6* have been detected in up to 70-80% of HNPCCs (Liu et al., 1996; Luce et al., 1996). In addition, acquired defects in MMR may account for 15-25% of sporadic cancers of different organs in the “HNPCC spectrum,” including the colon and rectum, stomach, endometrium, and ovaries (Peltomaki, 2003). In contrast to HNPCC, the cause of these sporadic cancers is often biallelic or hemiallelic methylation of cytosine residues of the cytosine and guanine (CpG)-rich promoter sequences of *MLH1* (Herman et al., 1998; Leung et al., 1999; Simpkins et al., 1999; Wheeler et al., 2000; Baek et al., 2001; Miyakura et al., 2004).

Microsatellites are repetitive DNA sequences with a 2-6 bp repetitive size that occurs between 15-30 times, and most of them are noncoding sequences (Koreth et al., 1996). They are found in great number spread out over the whole DNA sequence and are prone to insert/deletion mutations during replication due to the favorable strand slippage nature of the repetitive sequences. Normally, the DNA MMR system works as a “spell checker” that identifies and corrects the mismatched base pairs in the DNA. However, in the absence of efficient MMR function, these insertion/deletion loops may become permanent, resulting in two alleles with different sizes but a constant number of repeated units. When a microsatellite shows heritable and stable differences from person to person in the number of repeats it involves, it is said to be polymorphic. When a germline microsatellite allele has undergone a

somatic change in length (gained or lost repeat units), compared with matched normal DNA, it is referred to as microsatellite instability (MSI) (de la Chapelle, 2003).

There are no definite consensus criteria for defining the MSI phenotype. A panel of five markers including two mononucleotide markers (BAT25 and BAT26), and three dinucleotide markers (D5S346, D2S123, and D17S250), so-called Bethesda markers, has been recommended by a National Cancer Institute Workshop for MSI analysis (Boland et al., 1998). Using this reference panel, tumors with instability in two or more of the five markers are defined as MSI-high; tumors with instability in only one of the five markers are defined as MSI-low; and tumors without instability in any of the five markers are defined as microsatellite stable (MSS). However, some authors consider that MSI at a single locus, the BAT26 mononucleotide marker, is enough to detect almost all MSI tumors (Zhou et al., 1998, Cravo et al., 1999; Loukola et al., 2001). BAT26 is a repetitive sequence of 26 adenines within an intron of the *hMSH2* gene, and germline polymorphisms are rare in the Caucasian population (Samowitz et al., 1999). Using BAT26, it is possible to identify the MSI status of tumors from different origins with a certainty of 86-99.5% (Zhou et al., 1998, Cravo et al., 1999) and detect MSI-H tumors with 97% sensitivity (Loukola et al., 2001).

Although most MSI resides in untranslated intergenic or intronic sequences, the instability of coding microsatellites results in frameshift mutations of the corresponding genes, inevitably leading to truncated protein. Thus, genes with coding microsatellites might present important mutation targets in human tumorigenesis. A number of genes containing coding microsatellites affected by MSI have been identified, encoding proteins involved in signal transduction, such as the transforming growth factor beta receptor type II (*TGF- $\beta$ RII*) gene (Markowitz et al., 1995; Parsons et al., 1995;), the insulin-like growth factor type II receptor (*IGFIIIR*) (Souza et al., 1996) and *PTEN* (Guanti et al., 2000), apoptosis (*BAX*) (Rampino et al., 1997), DNA repair (*hMLH3*, *hMSH6*) (Malkhosyan et al., 1996), transcriptional regulation (*TCF-4*) (Duval et al., 1999) or immune surveillance ( *$\beta$ 2M*) (Bicknell et al., 1996). The *TGF- $\beta$ RII* gene, harboring an (A)<sub>10</sub>, is a tumor suppressor of prime importance in CRCs. Frameshift mutations of *TGF- $\beta$ RII* have been found in 90% of CRCs showing MSI (Parsons et al., 1995). Studies on *TGF- $\beta$ RII* mutations at various stages of MSI-positive adenoma showed the mutations correlate with the progression of colorectal adenoma to carcinoma (Grady et al., 1998). *BAX* frameshift mutations are found in 35% of all tumors with MSI. Altered *BAX* expression is believed to contribute to carcinogenesis by disrupting the apoptosis pathway (Mandal et al., 1998; Sturm et al., 1999; Giatromanolaki et al., 2001; Jansson & Sun, 2002;

Katsumata et al., 2003; Trojan et al., 2004;). Frameshift mutations of *BAX* may replace the role of the mutation in *p53* in the carcinogenesis of MSI colon cancers (Grady, 2004).

#### **1.3.4. Double strand break (DSB) repair**

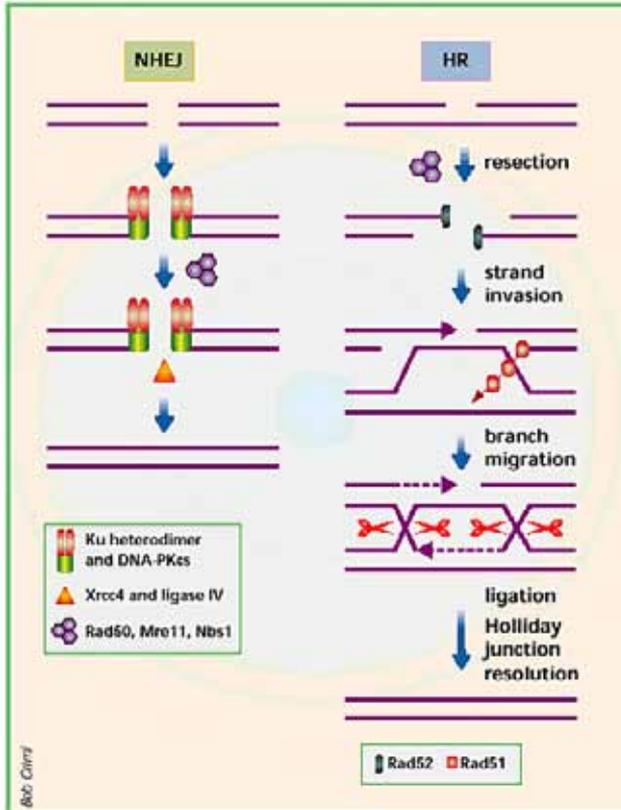
Of the many types of DNA damage that exist within the cell, DSB is probably the most dangerous type and is considered to be particularly biologically important because its repair is intrinsically more difficult than that of other types of DNA damage (Khanna & Jackson, 2001). The presence of DSBs is recognized by a sensor, which transmits the signal to a series of downstream effector molecules through a transduction cascade, to activate signaling mechanisms for cell-cycle arrest, the induction of DSB repair, or apoptosis if the damage is irreparable (Khanna & Jackson, 2001, Jackson, 2002). Ataxia telangiectasia mutated (ATM) protein kinase is the major player in the DNA DSB signaling pathway (Khanna et al., 2001). Activated ATM signals the presence of DNA damage by phosphorylating various downstream substrates, including *p53*, the checkpoint kinase *CHK2*, *BRCA1*, and *NBS1*, leading to a variety of effects on cell cycle progression, DNA repair and apoptosis. ATM deficiency leads to the neurodegenerative syndrome AT, characterized by progressive cerebellar ataxia and telangiectasia, immunodeficiency, genomic instability, predisposition to cancer and sensitivity to ionizing radiation (Chun & Gatti, 2004).

There are two major pathways for DNA DSB repair: homologous recombination (HR) and non-homologous end joining (NHEJ) (Figure 1). These pathways are largely distinct from one another and function in complementary ways for DSB repair. When an intact DNA copy is available, HR is preferred. Otherwise, cells utilize NHEJ. The malfunction of these mechanisms can lead to gross chromosomal rearrangements, loss of chromosome arms, aneuploidy and, ultimately, tumorigenesis in humans (Khanna & Jackson, 2001, Jackson, 2002).

##### **1.3.4.1. Homologous recombination (HR)**

HR rejoins DSBs in an error-free manner using an undamaged and homologous DNA as a template. The majority of HR takes place in late S- and G2-phases of the cell cycle. The *RAD52* epistasis group of proteins, including *RAD50*, *RAD51*, *RAD52*, *RAD54*, and *MRE11* mediate this process. The broken DNA ends are sensed by *RAD52* and processed by *RAD50/MRE11/NBS1* (RMN) to produce 3' single-strand trails. The newly generated 3' single-strand DNA trails are bound by *RAD51* to form a nucleoprotein filament. The *RAD51* then searches the undamaged DNA on the sister chromatid for a homologous repair template.

Once the homologous DNA has been identified, the damaged DNA strand invades the undamaged DNA duplex as DNA exchange. A DNA polymerase then extends the 3' end of the invading strand and subsequent ligation by DNA ligase I yields a heteroduplexed DNA structure (Figure 1) (Khanna & Jackson, 2001, Jackson, 2002).



**Figure 1.** Homologous recombination (HR) and non-homologous end joining (NHEJ) DNA DSB repair pathways. HR joins DSBs using undamaged and homologous DNA as a template. RAD52, a DNA-binding protein, initiates the process and recruits RAD51 to facilitate strand exchange. The resected 3' end invades an undamaged homologous DNA duplex and is extended by DNA polymerase. NHEJ joins the two broken ends directly. The Ku heterodimer binds two free DNA ends and recruits DNA-PKcs, followed by recruitment of Xrcc4 and ligase IV. The RMN complex may be involved in the end processing in the two pathways (adopted from Khanna & Jackson, *Nat Genet.* 2001;27:247-54).

#### 1.3.4.2. Non-homologous end joining (NHEJ)

NHEJ does not require a homologous template for DSB repair and is most relevant in the G1 phase of the cell cycle. It rejoins the two broken ends directly and usually results in the correction of the break in an error-prone manner, such as small deletions of DNA sequence. A

key component of the NHEJ apparatus is the DNA-dependent protein kinase (DNA-PK), consisted of a heterodimeric DNA targeting subunit (Ku70/Ku80) and an approximately 465 kD DNA-dependent protein kinase catalytic subunit (DNA-PKcs) whose catalytic domain is homologous to that of phosphatidylinositol (PtdIns) 3-kinase-like. The Ku heterodimer initiates NHEJ by binding to the free DNA ends, recruiting and activating DNA-PKcs. Then DNA ligase IV along with Xrcc4 are recruited directly or indirectly by the DNA-PK to the site of injury, and activated by the DNA-PK-mediated phosphorylation. The ends of most DSBs are damaged and unable to be directly ligated without processing. The RMN complex, FEN-1 and the Artemis protein, may be responsible for this processing. Subsequently, two broken ends of DNA are ligated by ligase IV in a complex combined with Xrcc4 (Figure 1) (Khanna & Jackson, 2001, Jackson, 2002).

#### ***1.3.4.3. RAD50/MRE11/NBS1 complex***

The RMN complex, consisting of the large coiled-coil ATP-binding cassette (ABC) ATPase RAD50, the nuclease MRE11 and the checkpoint protein NBS1. RAD50 and MRE11 are highly conserved in yeast, mouse and human, whereas NBS1, a homolog of yeast XRS2, is much less sequence conserved and only found in eukaryotes. RAD50 is a ~150 kDa protein that contains two ABC ATPase domains at N-terminus and C-terminus separated by two coiled-coil regions required for intramolecular interactions (D'Amours & Jackson, 2002; Hopfner et al., 2002; Assenmacher & Hopfner, 2004). The ABC folds into an antiparallel coiled-coil domain. At the apex of these coiled-coil domains are intriguing hook structures, which can form interlocked hook/zinc/hook bridges and join two RAD50 coiled-coils. Adjacent to the ABC segments are MRE11 binding sites. MRE11, a ~ 80 kDa protein, is the core of the RMN complex. It has amino-terminal phosphoesterase motifs with nuclease, strand-dissociation and strand-annealing activities, and two DNA binding motifs. MRE11 interacts independently with both RAD50 and NBS1, and its nuclease activity can be modulated by RAD50, NBS1 and ATP (D'Amours & Jackson, 2002; Assenmacher & Hopfner, 2004). NBS1 is a ~ 95 kDa protein composed of three functional regions at the N-terminus, central region, and the C-terminus. The N-terminus contains a forkhead-associated (FHA) domain and a breast cancer C-terminus (BRCT) domain. The C-terminal region of NBS1 contains an MRE11 binding site. The exact biochemical functions of NBS1 remain to be determined. However, the FHA and BRCT domains at its N-terminus bind to the histone  $\gamma$ H2AX, the phosphorylated form of H2AX as a result of the presence of DSBs, leading to the

recruitment of the other members of the RMN complex to the proximity of DSB site (Kobayashi et al., 2002).

Studies of the function of the RMN in the DSB repair process, along with observation of the architecture, indicate that the RMN complex is involved in a number of events of the cellular response to DNA DSB, including DSB detection, DNA damage checkpoint activation, HR, NHEJ, and telomere maintenance (de Jager et al., 2001; Hopfner et al., 2002; van den Bosch et al., 2003). It serves as a multipurpose DNA tether to bind and bridge DNA ends (de Jager et al., 2001; Hopfner et al., 2002; van den Bosch et al., 2003), acts as a primary sensor to DSB and recruits ATM to broken DNA molecules, and is a mediator for the activation of ATM (Petrini & Stracker, 2003; Lavin, 2004; Lee & Paull, 2005). In turn, the activated ATM will phosphorylate MRE11 and NBS1/XRS1 protein involved in cell-cycle checkpoint control (D'Amours & Jackson, 2002; Lavin, 2004). The essential role of the RMN complex in DNA DSB repair has been observed in yeast (Schiestl et al., 1994; Moore & Haber, 1996; Bressan et al., 1999) and in vertebrate cells (Tauchi et al., 2002; Koh et al., 2005). Mutations in components of the complex result in defective HR or NHEJ activity. In addition, RMN contributes to telomere-length maintenance by producing ssDNA at telomeres, or recombination-mediated telomere-elongation (Assenmacher & Hopfner, 2004).

Genetic studies have suggested that the RMN complex is required for genomic stability. Experimental models demonstrated that null mutations in *MRE11*, *RAD50* or *NBS1* lead to embryonic lethality (Xiao & Weaver, 1997; Luo et al., 1999; Zhu et al., 2001), whereas a hypomorphic mutation in *RAD50* results in partial embryonic lethality and cancer susceptibility in mice (Bender et al., 2002). In humans, *MRE11* and *NBS1* have been implicated in genome-instability syndromes. Hypomorphic mutations in *MRE11* and *NBS1* cause ataxia telangiectasia-like disease (Stewart et al., 1999) and Nijmegen breakage syndrome (Varon et al., 1998), respectively. Both disorders are phenotypically similar to AT. Recently, a frameshift mutation at mononucleotide repeats (A)<sub>9</sub> between codon 719 and 722 in *hRAD50* (Duval et al., 2001; Ikenoue et al., 2001; Kim et al., 2001) and a mutation of the poly(T)<sub>11</sub> repeat within *hMRE11* intron 4 (Giannini et al., 2004) have been found in human cancers with MSI, including CRCs, but not MSS cases. These mutations have been associated with reduced mRNA and protein expression of all three members of the RMN complex, impaired S-phase checkpoint and defective NHEJ activity *in vitro* (Giannini et al., 2002; Giannini et al., 2004; Koh et al., 2005). These findings suggest that the genes encoding the component of the complex are novel and major targets for inactivation by MMR defects.

## 1. 4. Microenvironment

The tumor microenvironment is composed of an insoluble extracellular matrix (ECM), fibroblasts, immune cells, vasculature, pericytes, and adipocytes and a milieu of cytokines and growth factors. Its importance in tumor progression has been recognized since Paget's "seed and soil" theory (Paget, 1989). It is not only supportive, in providing growth factors and blood supply (angiogenesis), and responsive, such as remodeling ECM and immune response to tumors, but also active in tumorigenesis (Liotta & Kohn, 2001; Tlsty TD, 2001). During tumor development and progression, tumor cells interact with the microenvironment exchanging growth factors and cytokines such as transforming growth factor (TGF)  $\beta$  and platelet derived growth factor (PDGF), or by directly interacting with stromal cells and ECM by cell surface proteins, e.g., cadherins, integrins and others, which activate fibroblasts, modify the local ECM, recruit inflammatory cells, and stimulate the endothelium (Liotta & Kohn, 2001; Zigrino et al., 2005). In turn, the altered tissue surrounding tumor cells, such as modified fibroblasts (myofibroblasts), ECM remodeling, increased inflammatory cells and angiogenesis may suppress or induce tumor progression by stimulating tumor growth or providing nutrients, oxygen or an environment favorable local tumor growth, invasion and metastatic spreading.

Myofibroblasts present the majority of stromal cells within various types of human primary and metastatic carcinomas. They are morphologically characterized by large spindle-shaped cells with indented nuclei. In tumors, myofibroblasts are normally defined by the concurrent express  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA, smooth muscle marker), and vimentin (mesenchymal marker) (Micke & Ostman, 2004). Myofibroblasts can develop in many ways. They may originate from existing fibroblasts in the surrounding tissue stroma (partial smooth muscle differentiation of fibroblasts), vascular bed (smooth muscle cells and pericytes), circulating mesenchymal precursors derived from the bone marrow or local epithelial-mesenchymal transition (Desmouliere et al., 2004; Micke & Ostman, 2004). Experimental models of tumor-stroma interaction *in vivo* have shown that myofibroblasts are able to promote tumor initiation, growth, local invasion and metastasis by secretion of tumor-promoting factors, angiogenic factor, ECM proteins and proteases (Tlsty, 2001; Desmouliere et al., 2004; Micke & Ostman, 2004).

Cell-ECM adhesion, a fundamental process that controls cell shape change, migration, proliferation, differentiation and survival, is considered critical for tumor invasion and metastasis. Cell-ECM interaction is mediated primarily by integrin at the cell-ECM contact

sites, e.g., focal adhesions (Hynes, 1992). Focal adhesions are integrin-rich sites where a selective group of membrane and cytoplasmic proteins, such as focal adhesion kinase, integrin-linked kinase (ILK), talin and paxillin, are recruited and through which the ECM is linked to the actin cytoskeleton and signals are transduced bidirectionally between the intracellular signaling network and the ECM (Burridge & Chrzanowska-Wodnicka, 1996).

ILK is a 59 kDa protein containing a pleckstrin homology (PH)-like domain flanked by an N-terminal ankyrin repeat domain and a C-terminal serine/threonine protein kinase domain (Persad & Dedhar, 2003). The PH-like domain, through an interaction with phosphatidylinositol 3,4,5-triphosphate [PtdIns (3,4,5) P3] in response to either cell adhesion to ECM or growth factor stimulation, participates in the regulation of ILK kinase activity in a PI3K-dependent manner. ILK can phosphorylate protein kinase B (PKB/Akt) and glycogen synthase kinase 3 (GSK-3) (Delcommenne et al., 1998), leading the activation of PKB/Akt and inhibition of GSK-3 $\beta$ . This results in suppression of apoptosis and promotion of cell survival. ILK interacts with the cytoplasmic domains of integrin beta1 and beta3 subunits (Hannigan et al., 1996). Genetic and biochemical evidence has established the essential role of ILK in connecting integrins to the actin cytoskeleton. Apart from integrins, ILK interacts with several components of focal adhesion proteins including the particularly interesting new cysteine-histidine rich protein (PINCH), (Tu et al., 2001; Zhang et al., 2002), calponin homology-containing ILK-binding protein (CH-ILKBP) ( $\alpha$ -parvin, actopaxin) (Nikolopoulos & Turner, 2000; Olski et al., 2001),  $\beta$ -parvin (affixin) (Yamaji et al., 2001) and paxillin (Nikolopoulos & Turner, 2001), resulting in its activation and localization to focal adhesion plaques. Gain and loss of function strategies have shown that overexpression and/or constitutive activation of ILK results in oncogenic transformation and progression to invasive and metastatic phenotypes (Persad & Dedhar, 2003).

#### **1.4.1. Particularly interesting new cysteine-histidine rich protein**

PINCH was originally identified by Rearden (1994) as a widely expressed, evolutionarily conserved protein that contains an autoepitope homologous to “senescent cell antigen”. The *PINCH* gene is located on chromosome 2q12.2 and encodes a 38 kDa protein. PINCH is an adaptor protein with five LIM domains that are cysteine-rich motifs implicated in mediating protein-protein interactions (Michelsen et al., 1993; Schmeichel & Beckerle, 1994). It is widely expressed in different types of human organs and tissues such as the heart, lung, kidney, liver, thymus, spleen, stomach, small intestine, colon, pancreas, prostate, ovary, skeletal muscle and peripheral blood leucocytes (Rearden, 1994).

Although PINCH does not contain a catalytic domain, it participates in a key convergence point of integrin and growth factor signaling pathways by intermolecular interactions (Figure 2). PINCH interacts directly with ILK through its N-terminal LIM1 domain binding to the N-terminal ankyrin repeat domain of ILK, co-localized in focal adhesions (Tu et al., 1999; Wu, 1999; Zhang et al., 2002). Through the binding of ILK with CH-ILKBP ( $\alpha$ -parvin, actopaxin), PINCH forms a ternary complex with ILK-CHILKBP (Nikolopoulos & Turner, 2000; Tu et al., 2001; Olski et al., 2001; Zhang et al., 2002). The formation of PINCH-ILK-CHILKBP increases protein stability, facilitates their localization to cell-ECM adhesions and is essential for cell shape modulation, motility and survival (Wu, 1999; Guo et al., 2002; Zhang et al., 2002; Fukuda et al., 2003). Inhibition of PINCH-ILK interaction retards cell spreading, cell motility, cell proliferation and fibronectin matrix deposition (Guo et al., 2002).

PINCH also interacts with Nck-2 through its N-terminal LIM 4 domain with the third SH3 region of Nck-2 (Tu et al., 1998) (Figure 2). Nck-2 is a novel Src homology2/3-containing adaptor protein implicated in growth factor receptor signaling pathways including epidermal growth factor (EGF) receptor, PDGF receptors and insulin receptor substrate-1 (IRS-1) (Tu et al., 1998; Chen et al., 2000). It can modulate actin dynamics by interacting with the Wiskott-Aldrich syndrome protein and DOCK 180 (Tu et al., 2001), respectively. Therefore, PINCH, by mediating the formation of complexes between ILK and Nck-2, participates in the fundamental cellular process such as cell-ECM interaction and intercellular signal transduction pathways, regulating cellular proliferation, differentiation, migration and survival.

A new member of the PINCH family has been identified, called PINCH-2 (Zhang et al., 2002; Braun et al., 2003). Therefore, PINCH has been renamed to PINCH-1. The gene of PINCH-2 is mapped to chromosome 2q14.3 and encodes a 39 kDa protein. PINCH-2 protein is also highly conserved in vertebrates and has five LIM domains (Zhang et al., 2002; Braun et al., 2003). In humans, PINCH-2 is 82% identical to PINCH-1 at the amino acid sequence level and co-expressed with PINCH-1 in a variety of human cells (Zhang et al., 2002).



revealed that PINCH protein expression was markedly upregulated in the tumor-associated stroma of many common human cancers, including breast, prostate, lung, skin and colon cancers, and especially abundant in stromal cells at the invasive margin in these tumors (Wang-Rodriguez et al., 2002). Since tumor-stroma interactions are important for cancer progression, it is possible that increased PINCH in tumor-associated stroma may have a role in promoting tumor progression.

#### **1.4.2. Inflammatory infiltration**

The role of the immune system during cancer development is complex, involving extensive reciprocal interactions between genetically altered cells, adaptive and innate immune cells, their soluble mediators and structural components present in the neoplastic microenvironment. Today, an overwhelming amount of data from animal models—together with compelling data from human patients—indicate that the immune system not only protects the host against tumor development through immunosurveillance but also sculpts tumor immunogenicity (Dunn et al., 2004). Evidence of the increased susceptibility of immunodeficient mice to carcinogen-induced tumors, and that the transfer of immune T lymphocytes protects mice from tumor challenge, suggest the role of adaptive immunity in the control of tumor development. In contrast, it is evident that tumor-associated innate immune cells, especially macrophages and mast cells, have powerful effects on tumor development (Coussens & Werb, 2002).

Solid tumors are commonly infiltrated by immune cells, including T cells, B cells, natural killer (NK) cells, macrophages, dendritic cells (DCs), neutrophils, eosinophiles, basophiles and mast cells. T lymphocytes are the key players of adaptive cellular immune responses. T lymphocytes arise in the bone marrow and migrate to the thymus for maturation. During this process, T cells somatically rearrange gene segments, eventually leading to the expression of a unique antigen-binding molecule, the T-cell receptor. This receptor allows them to monitor all cells of the body, ready to destroy any cell posing a threat to the host, directly through the Fas or perforin pathway and/or indirectly by the release of cytokines. The important role of T cell response, in particular, antigen-specific CD8<sup>+</sup> T cells with cytotoxic activity, in tumor surveillance has been recognized for many years by studies in both animal models and humans (de Visser et al., 2005; Zimmermann et al., 2005). Data from previous studies suggest that antitumor T cell immune responses may influence patients' prognosis (Clemente et al., 1996; Zhang et al., 2003). Most recently, a combination of genotypic and phenotypic analyses have showed that T cell migration, activation and differentiation are increased in CRC

without sign of early metastatic invasion, suggesting that an adaptive immune reaction of the host might influence the tumor dissemination from the early steps of the metastatic process to the established metastasis in lymph nodes and distant organs (Galon et al., 2007). Further, immunohistochemical analyses of adaptive immune markers provided evidence of the correlation between adaptive immune reaction and clinical outcome regardless of the local extent and spread of the tumors (Galon et al., 2007).

B-lymphocytes proliferating in the draining lymph node migrate into the tumor, where they undergo further rounds of antigen-driven stimulation and proliferation, resulting in antibody secretion. The antibodies bind to tumors, causing tumor destruction via phagocytes in the presence of complement. However, experiments on animal models demonstrated that activation of B cells and humoral immune responses increase tumor growth and invasion, as well as human tumor-cell xenografts through the recruitment and activation of granulocytes and macrophages. Furthermore, anti-tumor antibodies are frequently detected in the serum of cancer patients, and an increase in the titer correlates with an unfavorable prognosis. It is now unknown whether this correlation indicates that the individuals with tumors have a high antigen load, triggering greater antibody production, or whether the presence of antibodies is essential for promoting tumor growth (de Visser et al., 2006).

NK cells represent a highly specialized innate immune population with a potent cytolytic activity against virus-infected or tumor cells. Their function is regulated by a series of inhibiting or activating signals. NK cells can recognize tumor cells expressing abnormal or downregulation of MHC-class I molecules. They kill tumor cells through the granule exocytosis pathway by perforin, a membrane-disruption protein, by granzymes, a family of structurally related serine proteinases, and by the death receptor pathway mediated by the members of the tumor necrosis factor (TNF) superfamily, e.g., Fas ligand, TNF- $\alpha$ . NK cells lack B-cell and T-cell receptors, but can modulate the subsequent adaptive immune response by releasing cytokines and chemokines (Moretta et al., 2005; Wallace & Smyth, 2005). The importance of NK cells in anti-tumor immunity has been established in a number of experimental models in mice (Wallace & Smyth, 2005). Moreover, it has been shown that extensive infiltrates of NK cells in gastric cancer or CRC are associated with a favorable prognosis (Coca et al., 1997; Ishigami et al., 2000).

Macrophages are a major component of innate immune cells. They are derived from blood monocytes and differentiate into different resident tissue macrophages, including alveolar macrophages in the lungs, microglial cells in the brain, and Kupffer cells in the liver. Despite the diverse names and locations, many of these macrophages share common functions,

including their ability to bind and engulf particulate materials and antigens, to regulate normal cell turnover and tissue remodeling, and to help repair sites of injury. Macrophages are a significant component of inflammatory infiltration in neoplastic tissues and have dual roles in neoplasm. They inhibit tumor growth by secreting lytic enzymes such as lysosomal enzymes and TNF- $\alpha$  into cancer cells, after being activated by IFN- $\gamma$  and macrophage activation factor (Benjamini et al., 2000; Brigate et al., 2002). However, recent studies have shown that tumor-associated macrophages are actually a distinct M2 polarized population. They promote tumor cell proliferation and angiogenesis, tumor invasion and metastasis by producing a wide variety of growth factors, cytokines, chemokines and proteases that stimulate tumor growth directly or can act on stromal cells to stimulate angiogenesis, break down and/or remodel ECM, or suppress adaptive immunity (Lewis & Pollard, 2006). The correlation of a high density of these tumor-associated macrophages with a poor prognosis has been found in over 80% of studies published (Condeelis & Pollard, 2006).

DCs are key players in the interface between innate and adaptive immunity. The principal function of DCs is as “professional antigen presenting cells” in directing and regulating the activation of adaptive immune response. The immature DCs are differentiated from monocytes in the presence of granulocyte-macrophage colony-stimulating factor and interleukin-4. After taking antigens and maturing in inflamed peripheral tissue, DCs migrate to lymph nodes to stimulate T-lymphocytes activation. Interestingly, DCs found in neoplastic infiltration are frequently immature and defective in a T cell stimulatory capacity (Sharma & Browning, 2005). Immature DCs might maintain tolerance to tumor antigens, and tumor-associated DCs, analogous with tumor-associated macrophages, might, in some tumors, promote tumor progression and dissemination. Several studies have documented the presence of tumor-associated DCs in tumors and noted that an increased frequency of tumor-associated DCs in tumors is associated with a poor prognosis for cancer patients (Sharma & Browning, 2005).

#### **1.4.3. Angiogenesis and lymphangiogenesis**

Angiogenesis, the formation of new blood vessels from existing blood vessels, is an important process occurring in the body, both in physiological condition, such as embryonic development and the reproductive cycle, and in pathological condition, such as wound healing, diabetic retinopathy, muscular degeneration and cancer. The occurrence of angiogenesis around tumors was observed 100 years ago (Goldman, 1907; Ide et al., 1939; Algire & Chalkley, 1945). In 1971, Folkman (1971) proposed that tumor growth and

metastasis were angiogenesis-dependent. Today, various preclinical *in vivo* experiments have proven that angiogenesis is essential for solid tumor growth, invasion and metastasis by providing nutrients and oxygen, and allowing tumor cells to intravasate into the circulation and metastasize to distant sites (Folkman, 1992; Folkman, 2002). By contrast, in the absence of blood vessels, tumors cannot grow beyond the size of a few mm<sup>3</sup> or metastasize to distant organs, and inhibition of blood vessel formation suppresses tumor growth. In humans, the importance of angiogenesis in cancers has been confirmed by analyzing angiogenic growth factor expression in tumors and estimating angiogenesis, most commonly by microscope quantification of microvessel density (MVD) on tissue immunostained with a variety of endothelial markers. Most previous studies have shown upregulation of angiogenic growth factors in tumors and a positive correlation of the upregulated angiogenic growth factors with increased MVD. MVD is a powerful prognostic indicator of tumor progression and of the risk of future metastases for a variety of cancers (Folkman, 2002). However, the clinicopathological significance of angiogenesis in CRCs is controversial, probably due to methodological differences among these studies including the variety of endothelial markers with differences in sensitivity (factor VIII, CD31 and CD34), different cut off values and microvessel counting techniques as well as the difference in patient selection (such as tumor size, stage). Therefore, future studies on assessing the prognostic significance of angiogenesis should be carried out in a large series of patients with stratification of tumor stage, using specific antibody (CD31 or CD34), standard microvessel counting techniques, and multivariate regression survival analysis (Des Guetz et al., 2006).

The lymphatic system collects extravasated fluid, macromolecules, and returns them to the blood circulation. It plays an important role in the immune response by directing lymphocytes and antigen-presenting cells to lymph nodes. It also serves as one of the most important routes of tumor dissemination (Pepper & Skobe, 2003). Lymphatic capillaries are thin-walled, relatively large vessels, composed of a single layer of endothelium. They have little or no basement membrane and lack associated pericytes (Pepper & Skobe, 2003). It has been proposed that the entry of tumor cells into the lymphatic system may be easier due to the nature of lymphatic capillaries. Tumor cells can escape from a primary site by actively entering existing or new vessels (Ruoslahti, 1996). However, it remains unclear whether lymphatic vessels actually exist in solid tumors, whether lymphangiogenesis occurs in tumors, or what is the role of lymphatic vessels in tumor progression and metastasis. This lack of clarity is mainly due to the lack of specific markers for lymphatic vessels.

Recent identification of molecular markers to discriminate lymphatic endothelium from blood vessel endothelium has enabled the study of the characterization of tumor lymphatics and the assessment of the role of lymphatic vessels during tumor progression. These include vascular endothelial growth factor receptor (VEGFR)-3, Prox-1, LYVE-1, podoplanin and D2-40. Enlarged, dilated lymphatic vessels have been found frequently in peritumoral areas of many types of tumors. Several experimental models have demonstrated that intratumoral lymphatics (ITLs) are absent, suggesting that ITLs are more easily destroyed, compressed or collapsed due to proliferating cells and high intratumoral pressure, proposed as non-functional. Structurally, ITLs, if they exist, could provide more direct routes and extensive interfaces for lymphatic invasion than peritumoral lymphatics (PTLs). Indeed, intratumoral lymphangiogenesis, and increased lymphatic vessel density (LVD) in tumors with respect to normal tissue, have been observed. In tumors, high LVD appears as an increased risk factor for lymph node metastasis development and correlates with poor survival in various human cancers (Swartz & Skobe, 2001; Stacker et al., 2002; Pepper et al., 2003; Ji, 2006). However, the relative importance of peritumoral versus intratumoral lymphatics and preexisting versus newly formed lymphatics in promoting tumor cell dissemination in humans remains to be determined.

Angiogenesis is precisely regulated by a balance between multiple stimulators and inhibitors produced by various cell types, such as tumor cells, fibroblasts or inflammatory cells, and the functional outcome might be dependent on the combined effect of these factors. Overexpression of angiogenic stimulators and downregulation of angiogenic inhibitors may switch on angiogenesis (Folkman, 1992). Based on what we learnt from angiogenesis, tumor lymphangiogenesis appears to be in an analogous fashion to angiogenesis (Cao, 2005).

Among the pro-angiogenic factors known today, the vascular endothelial growth factor (VEGF) family and their corresponding tyrosine kinase receptors are recognized as the most important mediators of angiogenesis and lymphangiogenesis. The VEGF family includes VEGF-A, VEGFR-B, VEGF-C, VEGF-D and placental growth factors (PIGF). The angiogenic and lymphangiogenic activities of the members of the VEGF- family are mediated through activation of tyrosine kinase receptors predominantly expressed on vascular endothelial cells (VEGFR-1 and VEGFR-2), or lymphatic endothelial cells (VEGFR-3), respectively (Otrock et al., 2007). VEGF-A is a highly specific mitogen for vascular endothelial cells. Several VEGF-A isoforms are generated as a result of alternative splicing from a single *VEGF* gene (Neufeld G, et al., 1999). It was originally described as vascular permeability factor (Bates & Harper, 2002). It increases vascular permeability of water and large molecular weight

proteins, and stimulates proliferation, migration, as well as sprout and tube formation of vascular endothelial cells by binding to VEGFR-1 and VEGFR-2 (Otrock et al., 2007). The expression of VEGF-A is potentiated in response to hypoxia (Neufeld et al., 1999). PlGF and VEGF-B only bind to VEGFR-1, and their biological functions remain largely unknown although they may modulate the function of VEGF-A. VEGF-C and VEGF-D are secreted as preproteins that undergo extensive proteolytic processing to alter their receptor binding affinities for VEGFR-2 and VEGFR-3. They have the dual capacity to induce angiogenesis and lymphangiogenesis by binding to both VEGFR-2 and VEGFR-3. The angiogenic versus lymphangiogenic responses to VEGF-C might depend on the degree of proteolytic processing of its precursor, and on the expression of its receptors on blood versus lymphatic endothelial cells of target tissue. VEGFC has a mature form that consists of the VEGF homology domain, which contains receptor-binding sites and is 30% identical in amino-acid sequence to VEGF-A. Like VEGF-A, VEGFC stimulates the migration of endothelial cells and induces vascular permeability and endothelial-cell proliferation. Unlike VEGF, the expression of VEGF-C does not seem to be regulated by hypoxia, but increases in response to proinflammatory cytokines (Stacker et al., 2002). VEGF-C is an essential chemotactic and survival factor during embryonic lymphangiogenesis (Karkkainen et al., 2004).

Tumor necrosis is a common feature of CRC. It begins with swelling of the cell body and mitochondrial contents, followed by vacuolization of the cytoplasm, irregular breakdown of nuclear DNA, rupture of cell membrane and cell lysis (Xiao et al., 2002). It is assumed to be due to the vascular blood supply in the tumor not being able to multiply fast enough to nourish the rapid growth of tumors. This leads to ischemic injury, infarction and subsequent necrosis. It can also be secondary to infarction. Infarction may be a relatively common occurrence in the tumor environment due to the brittle and relatively inconsistent nature of tumor microvasculature (Swinson et al., 2002). Other possible pathway leading to necrosis formation may involve increased tumor cell secretion of TNF (Raza et al., 2002). The degree of tumor necrosis reflects the level of intra-tumor hypoxia. Indeed, *in vitro*, exposure of cells to hypoxia can induce necrosis (Swinson et al., 2002). Necrosis usually indicates that the tumor is aggressive and is linked to a poor prognosis in this disease (Leek et al., 1999; Swinson et al., 2002).

## **2. Colorectal cancer**

### **2. 1. Epidemiology**

CRC is one of the most common cancers and a leading cause of cancer-related deaths worldwide. Based on the age standardized rates estimated globally, CRC is the third most frequent malignancy in both sexes, next to lung and prostate cancer in males, and breast and cervix/uterine cancer in females. Of the 1,023,152 new cases of CRC diagnosed worldwide in 2002, 528,978 led to deaths (Ferlay et al., 2002). The incidence of CRC varies approximately 20-fold around the world, with the high rates seen in industrialized countries such as North America, Australia and New Zealand, and the lowest in India (Ferlay et al., 2002). In Sweden, it is the third most common cancer after prostate cancer and breast cancer, with approximately 5,000 new cases diagnosed each year (Cancer incidence in Sweden 2005, Statistics, Health and Diseases 2007:3). The lifetime risk for developing CRC is about 5% in the Swedish population, and the risk increases with age, beginning around the age of 50. The incidence of CRC in Sweden has increased slightly over the last 20 years (1986-2005), with approximately 42.7 in the colon and 26.8 in the rectum in males, and 38.9 in the colon and 18.5 per 100,000 in the rectum in females in 2005. Meanwhile, the mortality rate has been decreasing markedly, with the five-year relative survival rate for colon cancer improved from 39.6% in 1960-1964 to 57.2% in 1995-1999, and for rectal cancer from 31.2% to 57.6%, respectively (Birgisson et al., 2005). The survival improvement is probably because of earlier detection and more efficient treatment of cancers, such as enhanced screening, improved surgical techniques, preoperative radiotherapy and adjuvant chemotherapy.

### **2. 2. Etiology and risk factors**

The etiology of CRC remains unclear. However, many factors such as heredity, age, inflammatory bowel disease, environment as well as lifestyle have been found to be associated with CRC (Boyle & Leon, 2002; Weitz et al., 2005). Heredity and genetic predisposition are major identifiers. Approximately 5-10% of all CRCs develop in the setting of defined hereditary cancer syndromes, mainly familial adenomatous polyposis syndrome and HNPCC. A family history of CRC in a first-degree relative (parents, sibling, or offspring), especially where the person developed cancer before the age of 45, also presents a high risk factor. However, the majority of CRCs (85%) are sporadic and not associated with a known inherited syndrome or genetic predisposition (Weitz et al., 2005). It has been well

accepted that age is an unmodifiable risk factor for CRC, and all people aged 50 years and over are considered to be at elevated risk. In addition, a personal history of medical diseases, such as ulcerative colitis or Crohn's disease, is associated with an increased likelihood of developing CRC.

The striking geographic difference in CRC incidence among ethnically different populations and the increased incidence of the offspring of immigrants migrating from a low (unindustrialized country) to a high incidence area (industrialized country with Western life style) suggest that environmental components, especially diet and lifestyle, might be important risk factors for CRC. Much experimental and epidemiological evidence suggests that diets high in fat, particularly animal fat and red meat, increase the risk of CRC, whereas the consumption of vegetables, fruit and fiber reduces the risk of CRC (Potter, 1999; Boyle & Leon, 2002). A high fat intake increases the levels of cholesterol and bile acids, produced by the liver, which in turn can be converted to carcinogens by intestinal bacteria (Kumar et al., 2003). High fiber consumption is thought to have a positive effect. A low fiber content decreases the stool bulk, increases fecal retention and can also alter the intestinal bacterial flora (Kumar et al., 2003).

Most case-control studies have identified that cigarette smoking and alcohol consumption are risk factors for several cancers, including CRC. The risk of developing any of the smoking/alcohol-related cancers is dose-related; that is, the more cigarettes/alcohol consumed daily, the younger the age at which one initiates smoking/alcohol consumption, and the more years one smokes/drinks, the greater the risk. Carcinogens from tobacco could reach colorectal mucosa through either the alimentary tract or the circulatory system and then damage or alter the expression of cancer-related genes. Alcohol can induce or suppress the liver's microsomal monooxygenase system (metabolizing system). Furthermore, acetaldehyde, which is the first metabolite or breakdown product of alcohol, has been classified as a carcinogen in animals by the International Agency for Research on Cancer. Moreover, it has been showed that alcohol consumption and folate deficiency have a synergistic action in the promotion of CRC (Giovannucci & Willet, 1994). In addition, physical activity has been shown to protect against adenoma and CRC, and is postulated as being due to the effect of exercise on bowel transit time, the immune system or serum cholesterol and bile acid metabolism (Boyle & Leon, 2002).

### **2. 3. Pathology**

CRC comprises cancer in the cecum, ascending, transverse, descending and sigmoid colon, and rectum. Approximately 60-70% of CRCs are located distal to the splenic flexure, particularly in the rectum and sigmoid colon (Ponz de Leon et al., 2001). Several studies have shown a tendency towards a more frequent occurrence of CRC in the proximal colon over the last 20-40 years (Sariego et al., 1992; Cady et al., 1993; Cheng et al., 2001; Ponz de Leon M et al., 2004). It remains unclear whether this is a true biological event or simply the consequence of a wider use of colonoscopy.

Most CRCs, at least two-thirds and perhaps as much as 95%, are adenocarcinomas, arising from the columnar surface epithelium, and approximately 20% of adenocarcinomas show a mucinous component within tumor cells or in the glandular lumen. Few CRCs are signet-ring cell carcinoma (1%), squamous carcinoma, undifferentiated carcinoma and medullary-type adenocarcinoma (solid carcinoma with minimal or no glandular differentiation and slight cellular pleomorphism). Squamous carcinomas usually develop from the squamous epithelium of the anal canal. In addition, carcinoid tumors can be found rather frequently in the appendix (Ponz de Leon et al., 2001).

Colorectal adenocarcinomas can be histologically graded as well, moderately or poorly differentiated lesions. Well-differentiated adenocarcinomas have a well-defined glandular pattern and regularly shaped tumor cells. Moderately differentiated carcinomas have a more irregular glandular pattern than the well-differentiated carcinomas and less regularly shaped tumor cells. Poorly differentiated carcinomas have little or no glandular formation and irregular tumor cells (Ponz de Leon et al., 2001). The histological grade has been demonstrated as a prognostic indicator in CRC patients where patients with well-differentiated adenocarcinomas usually result in a good prognosis. However, there was no consistent evidence that grading may be of help in evaluating the prognosis or deciding the management of CRC patients.

The adenoma-carcinoma sequence is the basis for the development of CRC. It initially arises in a stem cell in the normal mucosa with a disorder of cell replication and renewal such as enhanced cellular proliferation, inhibited apoptosis, and prevented senescence by an inherited or induced DNA-damage, leading to the formation of adenoma. These adenomas are true neoplastic lesions and can be precursors of carcinomas (Kumar et al., 2003). Adenomas can be small tumors, most often with stalks, or sessile, rising above the surface of surrounding normal mucosa without any stalk (Kumar et al., 2003). The potential for an adenoma to

become malignant is correlated with its size, villous architecture and grade of dysplasia. Adenomas with a size larger than one centimeter in diameter, a villous appearance or high-grade dysplasia are most likely to progress to cancer (Kumar et al., 2003). However, the most important risk factor for the patients is the number of adenomas.

CRC grows within the tissue of origin by expansion and infiltrating the normal structure. Slowly expanding cancer may push along a broad front into adjacent normal structure. However, in general, cancer cells penetrate the margin and infiltrate the adjacent normal structure, displaying a crablike pattern of growth (Walker & Quirke, 2001). Based on patterns of growth and invasiveness, CRCs are divided into expansive and infiltrative types as recommended by Ming (1977) for gastric carcinoma. The growing margin of expansive type is sharply defined and circumscribed, while in infiltrative tumour, there is no recognizable margin of the growth, with single tumor cells or clusters spread through the bowel wall and a host response is often lacking. Cancer growth often leads to the breakdown of basement membrane and cancer invasion outside the tissue of origin. After invasion, the cancer cells may detach from the primary tumor, survive in the blood stream and then lodge themselves in the other organs, preferably the liver and the lungs (Walker & Quirke, 2001).

Based on the invasion depth of the primary CRC and the absence or presence of nodal and distant metastasis at the time of resection, various pathological staging systems have been described to assist clinicians in planning treatment and assessing prognosis. Dukes' stage classification, originally described in 1932 and modified over the years, is still commonly used as the most powerful prognostic indicator. The stages and correlation of five-year survival with Dukes' stage are presented in the following Table 1 (Crissman et al., 1989).

**Table 1.** Dukes' stage and 5-year survival rate in colorectal cancer patients (Crissman et al., 1989)

Dukes' stage	Description	5-year survival
A	Penetration through submucosa	80-95%
B	Penetration through muscularis propria	60-80%
C	Regional lymph node metastasis	30-55%
D	Distant metastasis	< 3%

## 2. 4. Molecular tumorigenesis

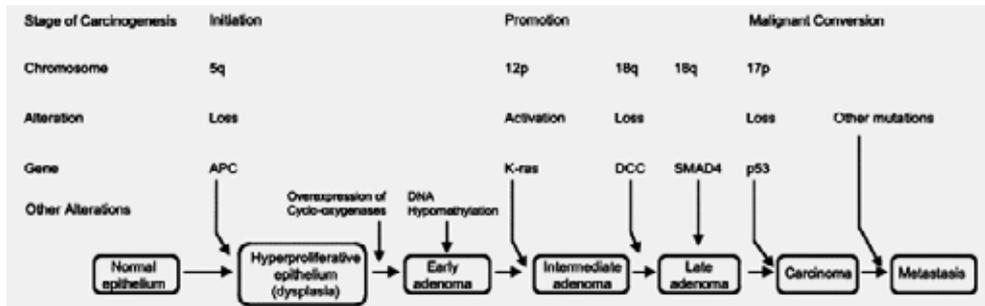
It is now generally accepted that CRC results from the progressive accumulation of genetic and epigenetic alterations that lead to the transformation of normal epithelium to adenocarcinoma. Traditionally, CRC arises through at least two distinct genetic pathways:

chromosomal instability and MSI pathways. Recent progression in molecular biology has shown that colorectal carcinogenesis is rather more complicated than these two pathways. CRCs associated with inflammatory bowel disease do not develop from adenoma, suggesting a different genetic pathway for sporadic cancers. In addition, the serrated and epigenetic pathways have been reported (Fukushima & Takenoshita, 2001; Takayama et al., 2006).

#### **2.4.1. Chromosomal instability pathway**

Chromosomal instability refers to numerical and structure chromosomal alterations, namely gain and/or loss of whole chromosomes or chromosomal segments at a higher rate in a population of cells, such as cancer cells, compared with normal cells (Lengauer et al., 1998). The chromosomal instability pathway proposed by Fearon and Vogelstein (1990) described that cumulative genetic alterations in oncogenes and tumor suppressor genes, occurring in a specific order and each conferring a selective growth advantage, lead to the stepwise progression from normal to dysplastic epithelium to carcinoma (Figure 3). This pathway, accounting for about 85% of CRCs, is characterized by allelic imbalance (mostly involving chromosomal arms, 5q, 17p and 18q), chromosomal amplification and translocation. One of the key steps in this pathway is mutation of the tumor suppressor gene, adenomatous polyposis coli (*APC*). The human *APC* gene is located on chromosome 5q21-22, encoding a 312 kDa intracellular protein presumably involved in epithelial cell adhesion, cellular transcription, differentiation, and intracellular transmission of extracellular signals (Kinzler et al., 1991). *APC* protein normally binds to  $\beta$ -catenin to form a complex with axin and GSK-3 $\beta$ , which is degraded through ubiquitylation. Loss or mutation of *APC* leads to an accumulation of unphosphorylated  $\beta$ -catenin in the cytoplasm, translocation of  $\beta$ -catenin to the nucleus and subsequent activation of APC- $\beta$ -catenin-T-cell factor (Tcf)/Lef. This stimulates transcription of target genes known to be involved in tumorigenesis (e.g. c-myc, cyclin D1, and c-jun) (Su et al., 1993; Rubinfeld et al., 1993; Behrens et al., 1998; Sparks et al., 1998). Germline mutations of the *APC* gene cause familial adenomatous polyposis, an autosomal dominant disorder characterized by the development of hundreds to thousands of colorectal adenoma appearing in adolescence or early adulthood. *APC* mutation or allelic losses of 5q are observed in 40-80% of sporadic CRCs and are found at a similar frequency in adenomas. Furthermore, mutated *APC* has been detected in the earliest adenoma, suggesting that the mutation is an initiating event for sporadic colorectal tumorigenesis (Powell, et al., 1992). Other somatic alterations involved in this pathway

include mutations in *K-ras*, subsequently in *p53*, and deletion on chromosome 18 (*DCC/SMAD4*) (Takayama et al., 2006). *K-ras* mutations are thought to be correlated with the relatively early event of progression from early adenoma to late adenoma. The loss of *DCC* and *p53* occurs in the late stage and is believed to be what drives the adenoma to carcinoma (Buda & Pignatelli, 2002; Takayama et al., 2006).



**Figure 3.** Chromosomal instability pathway associated with the progression of adenoma–carcinoma. Tumorigenesis proceeds through a series of genetic alterations involving oncogenes (*ras*) and tumor suppressor genes (particularly those on chromosome 5q, 17p and 18q). *APC* mutations occur at a relatively early stage of the tumorigenesis. *K-ras* activation contributes to the progression from early adenoma to late adenoma. Allelic deletion of chromosome 17p and 18q usually occurs at a late stage of the tumorigenesis (Adopted from Borst et al., *Clinics in Colon and Rectal Surgery*. 2002,15:97-104).

#### 2.4.2. Microsatellite instability pathway

MSI is the hallmark of HNPCC and can also be found in approximately 15% of sporadic CRCs (Soreide et al., 2006). MSI in HNPCC is associated with defective DNA MMR proteins caused by germline mutation in one of the DNA MMR genes, mainly *hMLH1*, *hMSH2* or *hMSH6* (Soreide et al., 2006). In the about 15% of sporadic CRCs that display MSI, around 80% have hypermethylation of a CpG island within the promoter region of the *hMLH1* gene, whereas a minority, approximately 20%, have somatic mutation, sometimes within the same MMR genes causing HNPCC (Cunningham et al., 1998). *MLH1* promoter hypermethylation is also present in non-neoplastic colorectal mucosa and colorectal adenoma from CRC patients with MSI, indicating that it is an early event in the MSI pathway (Nakagawa et al., 2001).

MSI CRCs, including HNPCCs and sporadic CRCs, have some unique biological and clinicopathological features compared with MSS CRCs. They are more likely to be diploid, displaying infrequent mutation in *K-ras*, *APC* and *p53*, and less frequent loss of heterozygosity (LOH) on chromosomes 5q, 17p and 18q, but frequent mutations in specific

target genes, such as *TGF- $\beta$ RII*, *IGFIIR* and *Bax*. They are observed more frequently in women and in a right-sided location, and exhibit poor histological grade, mucinous or signet-ring cell type. They are less likely to metastasize to either regional lymph nodes or distant organs, and are accompanied by intense peri- and intra-tumoral inflammatory reaction, and are associated with improved survival (Gryfe & Gallinger, 2000; Pawlik et al., 2004; Søreide et al., 2006). An increased rate of mutation in other genes might lead to aberrantly expressed proteins in membranes, which may trigger antitumor immune response typified by lymphocytic infiltrates surrounding MSI tumors (Dolcetti et al., 1999; Phillips et al., 2004; Prall et al., 2004). The enhanced host-immune response in MSI tumors provides a possible mechanism that explains the improved survival of patients with MSI CRCs. Furthermore, MSI tumors are more likely resistant to the cytotoxicity induced by certain chemotherapeutic agents, thus not gaining a survival benefit from chemotherapy (Ribic et al., 2003; Benatti et al., 2005). However, conflicting data exist about the relevance of predicting the prognosis and benefit of chemotherapy in patients with MSI CRCs (Curran et al., 2000; Evertson et al., 2003; Wang et al., 2003; Carethers et al., 2004; Emterling et al., 2004; Popat et al., 2005; Lamberti et al., 2006). The prognostic significance and response of MSI CRCs to adjuvant chemotherapy remain to be demonstrated.

## **AIMS OF THE STUDY**

1. To investigate the clinicopathological and biological significance of stromal variables, including PINCH, inflammatory infiltration, angiogenesis and lymphangiogenesis in CRC.
2. To study the clinicopathological and biological significance of hRAD50/hMRE11/hNBS1 proteins and hRAD50 mutation in tumor cells in CRC.



## MATERIALS AND METHODS

### 1. Materials

#### 1. 1. Patients

Samples, including formalin-fixed paraffin-embedded tissue sections involved in the five studies and 87 genomic DNA samples extracted from frozen primary CRCs included in study IV for mutation analysis, were consecutively collected from Swedish CRC patients diagnosed 1972-2001 at Linköping Hospital, Linköping, or Vrinnevi Hospital, Norrköping, Sweden. The patients' gender, age, tumor location and Dukes' stage were taken from surgical and/or pathological records. Tumor growth pattern and differentiation were scored by two pathologists. The tumor growth pattern was divided into expansive or infiltrative type based on the pattern of growth and invasiveness. The differentiation was graded as well, moderately, or poorly differentiated, or mucinous/signet-ring cell carcinoma. The follow-up data on the patients were obtained from surgical records, oncological records, the Swedish Cancer Register and the Regional CRC Register in Östergötland, Sweden. Patients who died of CRC were graded as uncensored. The clinicopathological characteristics of the patients are summarized in Table 2. The various numbers of the patients among the different studies were due to the available sections from the cases. The data of microsatellite status determined by the microsatellite marker BAT26, DNA ploidy and S-phase fraction by flow cytometry, apoptotic activity by terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (Tunel), as well as expression of p53, hMLH1 and PCNA by immunohistochemistry, were taken from previous studies carried out at our laboratory.

Seven frozen colon cancer specimens, obtained from the University of California, San Diego Medical Center, USA, were included in study I for Western blotting and immunofluorescence.

#### 1. 2. Cell lines

Three colon cancer cell lines, KM12C, KM12SM and KM12L4a, kindly provided by Prof. I.J. Fidler (M.D. Anderson Cancer Center, Houston, TX), were included in study IV and V, for Western blotting.

**Table 2.** Summarized clinicopathological characteristics of the patients from the five studies.

Characteristics	Studies				
	I	II	III	IV	V
Patients (No.)	174	301	210	268	208
Distant normal mucosa (No.)	39		27	69	41
Adjacent normal mucosa (No.)	96		105	138	130
Lymph node metastasis (No.)	26			44	26
Diagnosis (Year)	1972-2000	1975-2001	1974-2001	1977-2001	1982-2001
Gender (Males/Females)	98/76	158/143	114/97	143/125	114/94
Age (mean, range)	71 (34-94)	71 (34-94)	71 (42-89)	71 (34-94)	71 (42-95)
Tumor location					
Right colon	64	103	68	96	74
Left colon	29	54	41	48	38
Rectum	78	137	98	119	93
Unknown	3	7	3	5	3
Dukes' stage					
A	21	47	32	33	25
B	50	102	80	91	74
C	60	94	60	81	61
D	38	50	33	51	40
Unknown	5	8	5	12	8
Growth pattern					
Expansive	81	128	110	130	110
Infiltrative	85	152	91	122	86
Unknown	8	21	9	16	12
Differentiation					
Well	8	23	11	17	12
Moderately	113	196	136	166	132
Poorly	26	44	27	40	31
Mucinous/signet-ring	25	37	36	44	33
Unknown	2	1		1	
Follow-up end time					
Uncensored	2001.10	2001.10	2006.4	2006.4	2006.4
	81	125	73	108	76

## 2. Methods

### 2. 1. DNA extraction

DNA was extracted from frozen primary CRCs by means of the Wizard<sup>®</sup> SV Genomic DNA Purification System according to the manufacturer's instructions (Promega, Madison, WI). Briefly, approximately 20 mg tissue was cut into small pieces and digested overnight at 55 °C in a 275 µl Digestion Solution Master Mix containing nuclei lysis solution 200 µl, 0.5 M EDTA (pH 8.0) 50 µl, RNase A Solution 5 µl and 20 mg/ml proteinase K 20 µl. DNA was precipitated by using a spin protocol with a microcentrifuge, collected with Wizard<sup>®</sup> SV minicolumn and eluted with Nuclease-Free Water.

### 2. 2. Polymerase chain reaction

Polymerase chain reaction (PCR) is a powerful technique that allows the amplification of a specific DNA region by using oligonucleotide primers (short, single-stranded DNA molecules complementary to the ends of the template), and the thermo stable enzyme Taq polymerase, under the presence of deoxynucleotide triphosphates (dNTPs). It was invented by Dr, Kary Mullis in 1983, for which he received the Nobel Prize in chemistry 10 years later. It is a cycling process that usually consists of a series of 20-35 cycles. Each cycle contains the following three steps: 1) Denaturation at 94-96°C: During the denaturation, high temperature is used to separate the double helix DNA molecule into single strands and stop all enzymatic reactions (for example: the extension from a previous cycle); 2) Annealing at 50-65°C: During the annealing, the primers hybridize (by ways of hydrogen bonds) to their complementary sequence on each side of the target sequence; 3) Extension at 72°C: This is the ideal working temperature for the polymerase. The primers are extended on single-stranded denatured DNA (template) by a DNA polymerase in the presence of dNTPs, resulting in synthesis of new DNA strands complementary to the template strands. Through the repetition of the three steps, each newly synthesized strand becomes a template for further cycles of amplification and the number of copies of the original target region is amplified exponentially, theoretically described as  $2^{\text{number of cycles}}$ . The PCR products can be separated and determined by size using gel electrophoresis.

### **2. 3. Single-strand conformational polymorphism**

Single-strand conformational polymorphism is a simple and sensitive method for detecting mutations in the genomic DNA by analyzing their unique electrophoretic mobility that results from small alterations in nucleotide sequences. It is widely used as an effective screening tool to find variants in a large number of samples. In brief, 1) The target nucleotide sequence is first amplified by PCR; 2) The PCR product is labeled in a secondary PCR with isotopic or non-isotopic markers, incorporated either in the primers or in the nucleotides; 3) The secondary PCR product is mixed with formamide dye and a loading buffer, denatured to create single-stranded DNA, and then separated by electrophoresis in a non-denaturing polyacrylamide gel. Under non-denaturing conditions, single-stranded DNA has a folded structure that is determined by intramolecular interactions and, therefore, by its sequence. Any variants, even a single base mutation, may lead to conformational changes of the single-stranded DNA and, further, to a change of mobility in polyacrylamide gel electrophoresis, which can be detected by the appearance of new bands when compared with wild type sequence in the resulting autoradiograph.

### **2. 4. DNA sequencing**

DNA sequencing is a technique for determining the precise sequence of nucleotides in a sample of DNA. In this thesis, capillary array electrophoresis DNA sequencing with the MegaBACE instrument (Amersham Biosciences, Buckinghamshire, England) is employed. When PCR amplification is completed, the PCR products are cleaned up with ExoSAP-IT containing Exonuclease I, Shrimp Alkaline Phosphatase, and then subjected to a sequencing reaction. Subsequently, sequencing reaction mixtures are purified by applying them to the center of the column resin bed in the wells of an AutoSeq96 plate and centrifuging for 5 minutes at 910x g. Finally, this is detected by capillary electrophoresis with the MegaBACE 1000 sequencing instrument and analyzed using the MegaBACE 1000 sequencing profiler.

### **2. 5. Immunohistochemistry**

Immunohistochemistry is a method for detecting the presence of specific proteins in cells or tissues by using antibodies as specific reagents through antigen-antibody interactions. It can be visualized at both light and electron microscopy by a marker such as an enzyme, fluorescent dye or colloidal gold, or visualized by autoradiography using a radioactive element. There are several methods for immunoperoxidase staining. In this thesis, the chain

polymer-conjugated technology is used (DAKO EnVision Systems, Carpinteria, CA). Briefly, it consists of the following steps: 1) Primary antibody binds to a specific antigen; 2) The antibody-antigen complex is bound by a secondary antibody conjugated to an enzyme-labeled inert “spine” molecule of dextran; 3) In the presence of substrate and chromogen, the enzyme-substrate reaction converts colorless chromogens into colored end products at the antibody-antigen binding sites.

## **2. 6. Immunofluorescence**

Immunofluorescence is a laboratory technique for detecting the location and relative abundance of any protein by using a fluorescent dye that is covalently attached to the antibody. It can be performed on cells fixed on slides and tissue sections. There are two major types of immunofluorescence staining methods: 1) Direct immunofluorescence staining in which the primary antibody is labeled with fluorescence dye, and 2) Indirect immunofluorescence staining in which a primary antibody binds to its specific protein and a secondary antibody conjugated with fluorochrome is used to recognize the primary antibody with specificity to the primary antibody’s species of origin. Immunofluorescence stained samples are examined under a fluorescence microscope or confocal microscope. When a light illuminates the fluorescence dye, it absorbs the light and emits a different color light that is visible to the investigator and can be photographed. Using immunofluorescence, investigators can see when, where and how much of their specific protein is expressed in any cell or tissue.

## **2. 7. Western blotting**

Western blotting (immunoblotting) is a method of determining, with a specific primary antibody, the relative amount of a particular protein presented in different samples after protein separation by gel electrophoresis, and after that the proteins are transferred to a membrane. The name was given to the technique by W. Neal Burnette due to its similarity to Southern blotting, a technique for DNA detection developed earlier by and named after its inventor Edwin Mellor Southern. Briefly, 1) Samples are prepared from tissues or cells that are homogenized in a buffer that protects the protein of interest from degradation; 2) Proteins are separated by gel electrophoresis, usually SDS-polyacrylamide, according to molecular weight, where the smaller proteins migrate through the gel faster than larger proteins; 3) Proteins are transferred to a membrane for detection; 4) The membrane is incubated with a generic protein (such as bovine serum albumin, non-fat dry milk) to prevent non-specific

protein interactions between the membrane and the antibody used to detect the target protein; 5) A primary antibody is then added to the solution which is able to bind to its specific protein; 6) A secondary antibody-enzyme conjugate that recognizes the primary antibody is added to find locations where the primary antibody is present; 7) An appropriate substrate is added that will produce a detectable product upon reaction with the conjugate, which can be detected as a visible band, where the primary antibody bound to the protein. There are several detection methods, including colorimetric detection, chemiluminescence and fluorescent detection. Most commonly, a horseradish peroxidase-conjugated secondary antibody is used in conjunction with a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein.

## **2. 8. Statistical analysis**

Chi-square, Fisher exact test or McNemar's method was used to test the difference in expression of PINCH and hRAD50/hMRE11/hNBS1 proteins between normal mucosa tissues, primary tumors and metastases as well as correlations between these protein expressions, inflammatory infiltration or tumor necrosis and clinicopathological or biological variables. Student t-test and Mann-Whitney U test were used to compare the difference in MVD or LVD between normal mucosa and primary tumors and analyze the association of MVD or LVD with clinicopathological and biological variables. Spearman rank correlation test was used to assess the correlation between MVD and LVD. Cox's Proportional Hazard Model was used to estimate the relationship between PINCH expression, inflammatory infiltration, tumor necrosis, MVD, LVD, or hRAD50/hMRE11/hNBS1 expression and patients' survival in univariate and multivariate analyses. The Kaplan-Meier method was used to calculate survival curves. Two-sided p-values of less than 5% were considered statistically significant.

## RESULTS AND DISCUSSIONS

### **1. Clinicopathological and biological significance of stromal variables in colorectal cancer (Studies I-III)**

Malignancy is a state that emerges from a tumor-host microenvironment in which the host actively participates in the induction, selection and expansion of the neoplastic cells (Liotta & Kohn, 2001). In order to better understand the contribution of stroma to CRC development and progression, and identify prognostic factors in the stroma, we investigated the clinicopathological and biological significance of stromal variables, including PINCH, inflammatory infiltration, angiogenesis and lymphangiogenesis in CRC.

#### **1. 1. PINCH and inflammatory infiltration (Studies I-III)**

PINCH is a family of cell-ECM adhesion proteins involved in integrin-mediated intracellular and growth factor signaling pathways regulating cellular proliferation, differentiation, migration and survival. An earlier study has shown that PINCH protein, by immunohistochemistry using a polyclonal anti-PINCH antibody, was upregulated in tumor-associated stroma in several types of cancers, including five colon cancers, compared with the normal tissue. The most intense expression was observed at the invasive margin of breast cancer (Wang-Rodriguez et al., 2003). We further analyzed PINCH expression in a large series of CRCs (n = 174), along with their corresponding normal mucosa (n = 39) and lymph node metastases (n = 26) by immunohistochemistry using the same antibody as above. The expression of PINCH increased from distant/adjacent normal mucosa (no staining difference between distant and adjacent normal mucosa) to primary tumor, and further to metastasis. In the primary tumors, PINCH expression was stronger at the invasive margin than it was in the inner tumor area. Further, strong expression of PINCH at the tumor invasive margin was related to an unfavorable prognosis, independent of gender, age, tumor location, Dukes' stage, growth pattern, differentiation and inflammatory infiltration. These results suggest that PINCH protein in the stroma may be involved in tumor development and progression, and PINCH expression at the tumor invasive margin is an independent prognostic indicator in CRC.

It is well accepted that tumor invasion or metastasis needs to escape from the immune system. Inflammatory infiltration in or around developing neoplasia represents a tumor-associated immune response and is generally considered as cytotoxic for the tumor cells. Our

results, along with previous findings, showed that a high inflammatory infiltration was related to a favorable prognosis in CRC (Adachi et al., 1989; Ropponen et al., 1997; Funada et al., 2003). By immunofluorescence, we also found that PINCH was present in stromal myofibroblasts in CRC and that PINCH expression in tumor-associated stroma was reversely related to inflammatory infiltration. Myofibroblasts have been considered to be associated with desmoplastic stromal responses to neoplasia, promoting tumor growth and invasion. It has been proposed that they form a barrier against the migration of immunocompetent cells toward the tumor and hence reduce immune surveillance. Indeed, a reverse correlation between myofibroblast and inflammatory infiltration has been demonstrated in the stroma of colon cancer (Lieubeau et al., 1999). Thus, the overexpression of PINCH in tumor-associated stroma may be the tumor-activated stromal reaction against the immune response, which may lead to an escape from immune surveillance and result in tumor development and progression.

Cell-ECM interactions are also required for the differentiation of many types of cells. Several studies *in vitro* have shown that the cellular binding of CRC to ECM is an essential step in the induction of morphological differentiation, and that the differentiation is regulated by factors produced by stromal cells (Del Buono et al., 1991; De Bruine et al., 1993). We found that PINCH expression was related to tumor differentiation. This suggests that PINCH may play a role in the induction or maintenance of tumor differentiation in CRC through the interaction of the stroma with tumor cells. Furthermore, it is particularly notable that strong PINCH staining at the invasive margin was associated with both better differentiation and an unfavorable prognosis. Although better differentiation is usually considered a sign of a favorable prognosis, the prognostic value of the histological grade is still controversial. To our knowledge, there is currently no way of discriminating among patients with well-differentiated CRC to select those who will have an unfavorable prognosis. Our results indicate that PINCH staining at the tumor invasive margin may be an especially valuable clinical marker in a subpopulation of CRC patients in which well-differentiated tumors are associated with an unfavorable prognosis.

Angiogenesis, the process of neovascularization from pre-existing vessels, is regulated by multiple pro- and anti-angiogenic molecules produced by various cell types, such as tumor, endothelial, and other stromal cells. Our results showed that the PINCH protein was expressed in a proportion of endothelial cells of the tumor vasculature stained by CD31, and strong expression of PINCH in tumor-associated stroma was associated with high MVD. Taken together with the finding that PINCH gene expression is upregulated in microvascular endothelial cells (Glienke et al., 2000), these results indicate that PINCH is a likely regulator

of angiogenesis, directly on endothelial cells or indirectly through the tumor-activated stroma reaction.

The relationship between MSI and patients' survival remains a matter of debate. Whereas many have reported better survival in CRC with MSI, even independent of tumor stage or radiotherapy and chemotherapy (Gafa et al., 2000; Gryfe et al., 2000; Gryfe et al., 2001; Samowitz et al., 2001; Colombino et al., 2002), others were unable to confirm these findings (Curran et al., 2000; Evertson et al., 2003; Wang et al., 2003; Carethers et al., 2004; Emterling et al., 2004; Lamberti et al., 2007). Consistent with the latter studies, our results showed that there was no association between MSI and patients' survival. It has been suggested that MSI-associated improved survival is attributable to enhanced host lymphocytic infiltration in MSI tumors, which is predictive of a favorable prognosis in these patients (Gryfe et al., 2000; Gryfe et al., 2001). Our results showed that increased inflammatory infiltration at the tumor invasive margin did relate to a favorable prognosis in CRC. However, MSI tumors had low inflammatory infiltration, either at the invasive margin or in the entire tumor, compared with MSS tumors. It is possible that the non-association of MSI with patients' survival in CRC is due to the lack of high inflammatory infiltration in the MSI tumors.

## **1. 2. Angiogenesis and lymphangiogenesis (Studies II and III)**

The significance of angiogenesis in CRC, estimated by microscope quantification of MVD, in predicting metastasis or prognosis remains controversial, probably due to methodological problems, such as the different endothelial markers used and the different techniques for estimating angiogenesis. In our study, we assessed MVD following the first international consensus report on the methodology and criteria for the evaluation of angiogenesis quantification in solid tumors (Vermeulen et al., 1996). We found a significant increase of MVD in CRCs compared with their corresponding normal mucosa, suggesting the occurring of angiogenesis in CRC. However, MVD was not related to tumor stage or patient survival either in the entire group of the patients or in the subgroups stratified by tumor stage, which is in agreement with other reports in which MVD was also quantified using the CD31 marker (Bossi et al., 1995, Cianchi et al., 2002; Staton et al., 2007). It is generally thought that malignant tumor growth is angiogenesis-dependent. However, recent histomorphological studies indicate that some tumors may be vascularized without significant angiogenesis, probably by using existing vessels, a process later described as vascular co-option, or even by forming vascular channels on their own through a non-endothelial cell process called

“vascular mimicry” (Ribatti et al., 2003). It has been suggested that human cancers consist of a heterogeneous population of tumor cells with diverse biochemical properties, in which abnormal growth is derived by a minority of pathological cancer stem cells. A more recent study has provided direct evidence that glioblastoma stem-like cancer cells could mediate tumor growth by co-opting the host vasculature without any sign of angiogenesis, and that invasion and angiogenesis were two independent strategies for tumor progression (Sakariassen et al., 2006). Thus, the significance of angiogenesis in cancer progression and patients’ survival needs to be re-adjusted in the future.

Tumor necrosis, a common feature of solid tumors caused by chronic ischemia, has been linked to a poor prognosis in several cancers. It is suggested that the hypoxic environment that results in tumor necrosis may stimulate angiogenesis by releasing angiogenic growth factors from infiltrating macrophages (Leek et al., 1999; Lewis et al., 2000; Edwards et al., 2003). We aimed to study the tumor-related angiogenesis rather than the inflammatory reaction related vascularization in CRC. Thus, we did not count microvessels in the area with intense inflammatory infiltration and necrosis, and did not analyze the correlation between tumor necrosis and angiogenesis. Regarding the clinicopathological significance of tumor necrosis, we found that tumor necrosis was related to advanced tumor stage and predicted an unfavorable prognosis in univariate analysis. However, tumor necrosis was not an independent indicator in CRC after adjusting for Dukes’ stage and differentiation. Tumor necrosis also appeared more frequently in moderately/poorly differentiated tumors, but was the lowest in mucinous/signet-ring cell carcinomas. This is probably because mucinous carcinomas grow slowly, require a smaller blood supply, and lack glands.

Lymphatic vessels are a major route for tumor cell dissemination. In CRC, ITLs have been detected, and increased LVD in tumors, compared with normal mucosa, have been observed (Ji, 2006). However, the relative importance of LVD in intratumoral areas versus peritumoral areas and pre-existing versus newly formed lymphatics, to lymphogenous metastasis and prognosis remains to be determined. Our study, using monoclonal antibody D2-40, a specific marker for lymphatic endothelium, recently recommended by the first international consensus on the methodology of lymphangiogenesis quantification in solid human tumors (de Auwera et al., 2006), also showed an increase in LVD in CRCs with respect to their corresponding normal mucosa. However, LVD, either in the intratumoral areas, peritumoral areas or in the entire specimen, was not related to tumor stage or patients’ survival. This suggests that lymphangiogenesis mainly occurs in the earlier, not later, stage of CRC development. With cancer progression, cancer cells may cause lymphatic destruction, or

the mechanical forces generated by the growing tumor cells may cause lymphatic vessels collapse, leaving them non-functional (Padera et al., 2002). Thus, we would speculate that the impact of LVD on patient outcome could not be adjusted. On the other hand, although we examined the clinicopathological significance of LVD in intratumoral and peritumoral areas, our study could not distinguish newly proliferating lymphatic vessels from pre-existing lymphatic vessels. A further study is needed to clarify the clinicopathological significance of proliferating versus pre-existing lymphatic vessels in intratumoral versus peritumoral areas, by double immunostaining with D2-40 and Ki-67 antibodies.

## **2. Clinicopathological and biological significance of hRAD50/hMRE11/hNBS1 in colorectal cancer (Studies IV and V)**

It is now well known that the malignant phenotype of solid tumor depends on the acquisition of multiple genetic changes in tumor cells. DNA repair mechanisms serve to suppress mutagenesis by correcting DNA damage. The RMN complex has been involved in many aspects of the cellular response to DNA DSB, maintaining genetic stability. In order to understand the importance of hRAD50/hMRE11/hNBS1 proteins in CRC, we studied the clinicopathological and biological significance of hRAD50/hMRE11/hNBS1 proteins and *hRAD50* mutation in tumor cells in microsatellite stable and unstable CRC.

Our results, in agreement with previous studies (Duval et al., 2001; Ikenoue et al., 2001; Kim et al., 2001), showed the frameshift mutation of (A)<sub>9</sub> at the coding region of *hRAD50* in MSI, but not in MSS CRC. We also found that strong expression of hRAD50, hMRE11 or hNBS1 was related to MSS. Further, we observed that the expression of hRAD50, hMRE11 or hNBS1 among normal mucosa, primary tumors and metastases in MSS CRC differed from that in MSI CRC. In MSS CRC, the expression of hRAD50, hMRE11 or hNBS1 was increased in the primary tumors, compared with the corresponding normal mucosa, but there was no difference between the primary tumors and metastases. Furthermore, in MSS primary tumors, the expression of hRAD50, hMRE11 and hNBS1 was positively correlated, and the strong expression of individual or combination of hRAD50/hMRE11/hNBS1 was associated with a favorable prognosis in the same series of the CRCs. However, in MSI CRC, there was neither a difference in the expression of the hRAD50/hMRE11/hNBS1 among normal mucosa, primary tumors and metastases, nor any association of the expressions of the proteins with clinicopathological variables. These results supported our hypothesis that hRAD50/hMRE11/hNBS1 proteins may interact with each other, and play different roles in

the development of MSS and MSI CRC. In MSS CRC, the strong expression of the three proteins in the primary tumors, associated with a favorable prognosis, may present the cellular response against tumor progression. In MSI CRC, frameshift mutation at the coding region of *hRAD50* may contribute to tumor development.

Consistent with our study, expression of the hRAD50/hMRE11/hNBS1 proteins was also upregulated in gastric cancer. Further, the upregulation of the complex was associated with increased levels of telomere repeat binding factors 1 and 2, which are negative regulators of telomere length, suggesting the importance of the complex in regulating telomere length (Matsutani et al., 2001). In contrast, expression of the three proteins was reduced in invasive ductal breast cancer, and was associated with a low level of ATM protein kinase and a high level of p53 (Angele et al., 2003). Taken together, the different patterns of the hRAD50/hMRE11/hNBS1 expression among various cancers indicate that the complex is important in cancer development and progression with cancer cell type specificity, although the mechanism behind it is unclear.

Our results also showed a positive correlation between the individual or combined expression of hRAD50/hMRE11/hNBS1 and PCNA expression. PCNA functions as a DNA sliding clamp for replicative DNA polymerases (Maga & Hubscher, 2003). Emerging evidence has shown that, upon DNA damage, PCNA interacts with multiple proteins involved in DNA replication, repair, and cell cycle control, leading to cell-cycle arrest and DNA damage repair. It is still not understood, however, how PCNA exactly interacts with the hRAD50/hMRE11/hNBS1 complex in the DNA DSB repair pathway, although our results suggest that PCNA is required for upregulation of the complex in the early stage of primary CRC.

It is believed that defects in the genes encoding the components of the hRAD50/hMRE11/hNBS1 complex could contribute to tumorigenesis by failing to produce proteins concerning DNA DSB repair, thereby leading to genetic instability. The susceptibility of hypomorphic mutation in *RAD50* to cancer has been observed in mice (Bender et al., 2002). Also, reduced expression of hRAD50 mRNA and protein induced by frameshift mutations at mononucleotide repeats (A)<sub>9</sub> between codon 719 and 722 in the *hRAD50* gene was detected in human colon cancer cell lines (Koh et al., 2005). However, our results did not observe a clear relationship between the mutation and protein expression. Of eight cases with the mutation, two cases displayed negative, three weak, two moderate and one strong expression. Among 16 cases without the mutation, three cases were negative, five weak, five moderate and three strong expression. One possible reason may be the limited

cases examined for both mutation and protein expression analysis. Another reason may be attributable to the limitations in the assay sensitivity of immunohistochemistry for detecting small difference of protein expression. As demonstrated by Koh et al. (2005), the heterozygous mutation of *hRAD50* leads to the impairment, but not the abolishment, of wild-type hRAD50 expression. A subtle difference in expression of hRAD50 protein induced by mutation may not be detected. It was noted that the altered expression of hRAD50 in primary MSI CRC with mutation, compared with the corresponding normal mucosa, differed from that without mutation. In six mutated MSI cases, none of the primary tumors showed increased expression of hRAD50 (three decreased and three unchanged). However, in eight non-mutated MSI cases, four (50%) of the primary tumors showed increased expression, two (25%) decreased expression, and two (25%) were unchanged, compared with their normal mucosa. It seemed that the frameshift mutation in *hRAD50* might have an impact on its protein expression. The decreased hRAD50 expression in two non-mutated MSI tumors with respect to their normal mucosa could be attributable to other mutation within/close to the *hRAD50* gene or other genes regulating the hRAD50 expression, such as *hMRE11* or *hNBS1*. We need a larger number of cases to further investigate this issue.

hRAD50, hMRE11 and hNBS1 are the components of the hRAD50/hMRE11/hNBS1 complex. However, it has been demonstrated *in vitro* that the complex and its individual components are involved in different cellular functions responsible for repair DNA damage induced by ionizing radiation and radiomimetic chemicals (Taylor et al., 2004). Despite the concordant association of the strong expression of individual and combination of hRAD50/hMRE11/hNBS1 with MSS, and further related to a favorable survival in the same series of MSS CRC, we found that each individual protein had additional clinicopathological significance. Strong or high hRAD50 expression was correlated with earlier Dukes' stage, better differentiation and high inflammatory infiltration in MSS CRC, whereas high hMRE11 expression was related to less local recurrence and high apoptotic activity in the whole series of CRCs, but not in MSS or MSI cases. Moreover, only hNBS1 tended to be independently related to prognosis in MSS patients with earlier Dukes' stage. These findings suggest that the components of hRAD50/hMRE11/hNBS1 may have additional roles despite being part of the complex.



## CONCLUSIONS

1. PINCH is likely a regulator of angiogenesis, and PINCH expression at the tumor invasive margin is an independent prognostic indicator in CRC.
2. Inflammatory infiltration at the tumor invasive margin is also an independent prognostic indicator in CRC. The lack of high inflammatory infiltration in MSI tumors may help to explain the non-association of MSI with prognosis in CRC patients.
3. Angiogenesis and lymphangiogenesis occur in the early stage of CRC development, but do not associate with CRC progression and patients' prognosis.
4. hRAD50/hMRE11/hNBS1 may act dependently and independently, playing different roles in MSS and MSI CRC development. In MSS CRC, the strong expression of the three proteins in primary tumors, associated with a favorable prognosis, may present the cellular response against tumor progression. Expression of hNBS1 may be a prognostic indicator for MSS patients in the earlier tumor stage. In MSI CRC, the frameshift mutations at the coding sequence of *hRAD50* may contribute to CRC development.



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