Cell adhesion proteins in different invasive patterns of colon carcinoma

En resa på 100 mil startar med ett steg
(Koreanskt ordspråk)

To my father
Professor Per Ki-Jik Hahn M.D

Örebro Studies in Medicine 24

Victoria Hahn-Strömberg

Cell adhesion proteins in different invasive patterns of colon carcinomas

A morphometric and molecular genetic study

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Abstract

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Colorectal carcinoma is the second most common type of cancer in both men and women in Sweden. Cancer of the colon and rectum are often considered together and their ten year survival rate is approximately 50–60 % depending on sex and location. Different histopathological characteristics of such cancers, including the complexity of growth, are of importance for prognosis.

This thesis has compared different morphometric methods in order to achieve a quantitative and objective measurement of the invasive front of colon carcinoma. Since the growth pattern is dependent on the cell adhesiveness of different proteins we studied the distribution and localization of E-cadherin, Beta-catenin, Claudin 1,2,7 and Occludin as well as screened the genes for mutations.

We found a perturbed protein expression of E-cadherin, Beta-catenin, Claudin 1,2,7 and Occludin in tumor sections compared to normal mucosa, but no relation to tumor volume or growth pattern could be seen. The tumor volume was found to be correlated to the growth pattern but not responsible to the perturbed protein expression. In the mutation screening we found a SNP in exon 13 the E-cadherin gene in the tumor, as well as in exon 2 of Claudin 1 and exon 4 of Claudin 7 in both tumor and normal mucosa. No correlation between mutations and growth pattern or tumor volume was found.

In conclusion, this thesis shows that the computer image analysis with estimation of fractal dimension and number of free tumor cell clusters is superior to the semi quantitative visual grading of tumor invasive complexity. The aberrant expression of cell adhesion proteins in the tumor compared to normal mucosa as well as polymorphisms in the cell adhesion genes CLDN1 and CLDN7 in both tumor and normal mucosa. This can suggest that these aberrations are important in the tumorigenesis of colon carcinoma.

Keywords: colon carcinoma, growth pattern, tight junction, Complexity Index, cell adhesion, E-cadherin, Beta-catenin, Occludin, Claudin.

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Sammanfattning

Colorektal cancer är den näst vanligaste cancerformen hos såväl män som kvinnor i Sverige och svarar för ungefär 12 % av all cancer. Insjuknandet i denna cancerform har stadigt ökat sedan 1960-talet och totalt insjuknar ca 5000 individer per år.

Ca 95 % av alla colorektala carcinom är adenocarcinom. Tumörens växtsätt är viktig för prognosen där en tumör med infiltrativt växtsätt och separata tumör cells öar har sämre prognos än en tumör med expansivt växtsätt och en jämn invasionsfront.

I denna avhandling jämförs olika bildanalys metoder för att få ett objektivt kvantitativt mått på graden av tumör komplexitet hos colon cancer och ett Complexity Index har räknats fram. Det visades sig att räkna antalet fria tumörcells öar samt beräkna den fraktala dimensionen var de bästa metoderna för att beräkna komplexiteten i invasionsfronten. Eftersom celladhesion är en central del av växtmönstret i colon carcinom tumörer så undersöktes olika cell adhesionsproteiner, E-cadherin, Beta-catenin, Claudin och Occludin för att se om avvikelser i dessa proteiners uttryck kunde relateras till tumörens växtsätt. Mutationsanalyser utfördes för att se om det fanns mutationer i dessa gener som kunde korreleras till växtsättet hos colon cancer tumörer.

Ett avvikande uttryck av cell adhesionsproteinerna hittades i tumörerna jämfört med den normala omkringliggande vävnaden. Dessutom fann man en homozygot mutation i exon 13 av E-cadherin genen samt homozygota och heterozygota mutationer i exon 2 av Claudin1 och en homozygot mutation i exon 4 av Claudin 7 genen.

Sammanfattningsvis så visar denna avhandling att en morfometriskt analys av invasionfrontens komplexitet hos coloncancer ger en säkrare värdering än en visuell bedömning. Vidare sågs ett avvikande uttryck av olika cell adhesionsproteiner och mutationer i generna för dessa proteiner som kan vara en viktig del av tumör utvecklingen hos colon cancer.

Abbreviations

ANOVA	analysis of variance
APC	Adenomatous polyposis coli
Bp	base pairs
CAMs	Cell adhesion molecules
CLDN	Claudin
ddNTP	dideoxy nucleoside triphosphate
DAB	diaminobenzidine
DCC	Deleted in colorectal carcinoma
DNA	deoxyribonucleic acid
DSH	Dishevelled
EDTA	Ethylene diamine tetra acetate
FAP	Familiar adenomatous polyposis
FZD	Frizzled (G-protein)
HNPCC	Hereditary non-polyposis colorectal carcinomas
IHC	Immunohistochemistry
Lef	Lymfoid enhancer factor
LMD	Laser micro dissection
LRP	Receptor related protein
MSI	Microsatellite instability
NaAc	Sodium acetate
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
SSCP	Single stranded conformation polymorphism
TCF	T-cell receptor
TE	Tris EDTA
WHO	World health organization
ZO	Zonula occludens

Original Papers

Paper I. Franzen LE, **Hahn-Stromberg V**, Edvardsson H, Bodin L. Characterization of colon carcinoma growth pattern by computerized morphometry: Definition of a Complexity Index. Int J Mol Med 2008;22(4):465-72.

Paper II. **Hahn-Stromberg V**, Edvardsson H, Bodin L, Franzen L. Disturbed expression of E-cadherin, Beta-catenin and tight junction proteins in colon carcinoma is unrelated to growth pattern and genetic polymorphisms. APMIS 2008;116(4):253-62.

Paper III. **Hahn-Strömberg V,** Edvardsson H, Bodin L, Franzén L. Tumor volume of colon carcinoma is related to the invasive pattern but not to the expression of cell adhesion proteins. APMIS 2008; In Press.

Paper IV. **Hahn-Strömberg V**, Edvardsson H, Bodin L, Franzén L Claudin 1 and Claudin 7 gene polymorphisms and protein derangement are unrelated to the growth pattern of colon carcinoma. Manuscript.

These papers are referred to in the thesis as I,II,III and IV.

Contents

Introduction

Colorectal carcinoma is the second most common form of cancer in Sweden and the third most common type of cancer in the world. In Sweden about 5 000 new cases are diagnosed each year. The disease is a little more common in men than women and about half of those diagnosed with the disease are over 70 years old. Colorectal carcinoma is more frequent in the southern parts of Sweden and about 50-60% of the patients have a ten-year survival rate. Ninety-five percent of colorectal cancer is adenocarcinom.

Tumor grading and staging the extent of growth are important means to characterize colorectal carcinoma when prognosticating the disease. The tumor can be graded as highly, moderately or poorly differentiated [38]. The TNM classification system is a staging system for describing the clinical behaviour of the tumour where T is the depth of tumor penetration, N describes lymph node involvement and M the presence of distant metastasis [91].

Growth pattern of colorectal carcinoma

A colorectal tumor can grow in two different ways, either with an expansive growth pattern or with an infiltrative growth pattern (Figure1). In the infiltrative growth pattern the tumor splits up into tumor cell clusters of varying sizes giving the tumor front an irregular outline. The expansive growth pattern has a smooth and even border and a better prognosis compared to the infiltrative growth pattern [45, 46]. The concept of tumor budding was introduced and indicates the presence of isolated single cells or small cell clusters scattered in the stroma at the invasive margin [40]. Tumor budding has been defined as the occurrence of tumor cell clusters comprising five cells or less in the invasive border [74] and has been recognized as a prognostic marker in colorectal cancer [88].

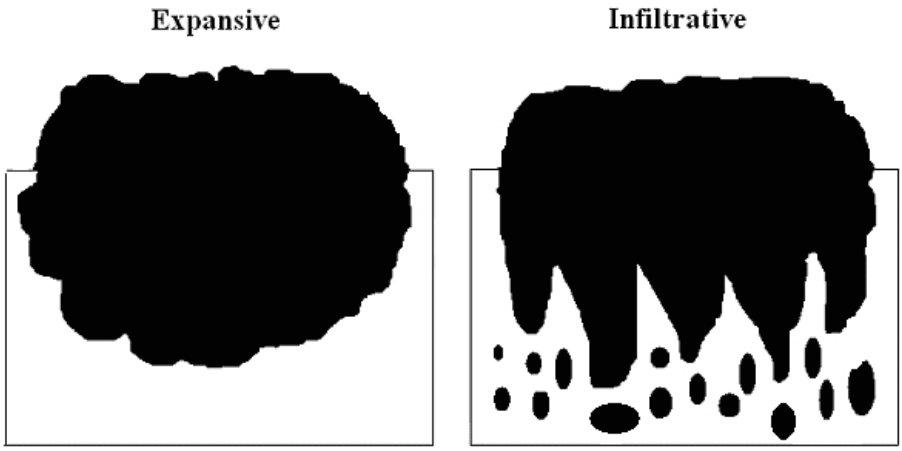

Figure1. Schematic drawing of the invasive front showing an expansive growth pattern with a smooth even border to the left and an infiltrative growth pattern with separate tumor cell clusters to the right.

Tumor development

Colorectal cancer can be divided into sporadic and hereditary forms. The sporadic form is the most common and accounts for 90% of colorectal carcinomas. In sporadic carcinoma it has been proposed that the tumor development starts as an adenoma, which eventually develops into an infiltrative carcinoma. This development is accompanied by a sequence of mutational events in the DNA (Figure 2). The entire process has been designated the adenoma-carcinoma sequence [26, 65].

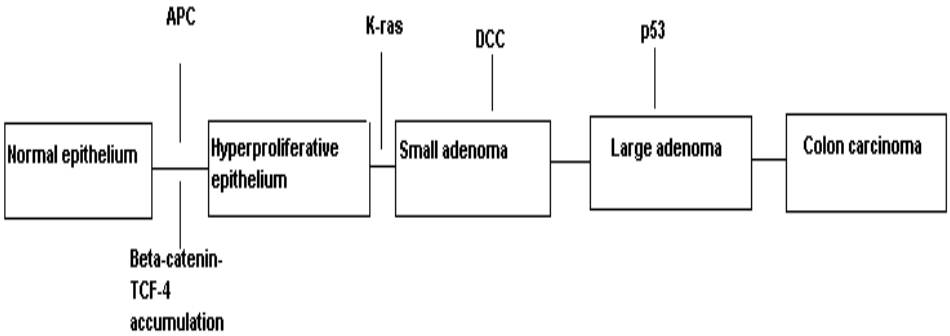

Figure 2. Adenoma-carcinoma sequence, the development of colon carcinoma from normal epithelium due to several mutations.

Loss of the APC gene is thought to be the earliest event in the development of adenomas. APC is involved in the regulation of Beta-catenin by binding to the Beta-catenin-cadherin complex and is inactivated in more than 80% of colorectal carcinomas. About 50% of cancers that do not have the APC mutation have mutations in Beta-catenin. For adenomas to develop into carcinoma several mutations are required such as mutations in K-ras, p53, DCC, p53 etc (Figure 2). The accumulation of mutations per se enhances the risk of developing cancer rather than the order in which they appear [6, 31].

Hereditary colorectal carcinoma include Familial adenomatous polyposis (FAP) and Hereditary non-polyposis colorectal cancer (HNPCC). Familial adenomatous polyposis is an inherited disease caused by mutations in the APC gene located on chromosome 5q21. Patients with FAP often develop numerous colonic adenomas [1]. Hereditary nonpolyposis colon cancer is also known as Lynch syndrome I and II. In Lynch I colon cancer can develop and in Lynch II cancer also occur in other sites of the gastrointestinal or reproductive system. This syndrome arises due to defects in DNA mismatch repair so called microsatellite instability [2, 28].

Microsatellite instability is a condition derived from defects in the DNA repair function. They are sections of repeated nucleotide sequences from 1-6 base pairs long and consist of 10-50 copies, which occur randomly in the human genome between and

within genes. These sections can be lengthened or shortened due to genetic instability. It is not known if microsatellites have a specific function but it has been suggested that they act as promoters, are sites of recombination or binding sites for topoisomerases [37, 89, 95]. Microsatellite instability has been seen in several forms of human cancer such as colon cancer, endometrial, gastric, pancreatic and oesophageal cancer and occurs in 10-15% of sporadic cases and in HNPCC syndrome [25, 39, 60, 80]

Cell adhesion and adhesion proteins

Tight Junction

The tight junction is also called zonula occludens and is mainly made up of two proteins, Occludin and Claudin. The tight junction bind to different membrane proteins like cadherins and catenins on the intracellular side of the plasma membrane, which in turn binds to the cytoskeleton and so the tight junction bind the cytoskeleton of adjacent cells [76]. The tight junction is located at the apical end of the junctional complexes between adjacent epithelial cells where they encircle the lateral surface to interact with each other in order to form a molecular seal that prevents uncontrolled diffusion or leakage of molecules [101, 102]. The tight junction is also assumed to play a major role in controlling cellular adhesion [102] as well as the organization of epithelial cell polarity by separating the plasma membrane into apical and basolateral domains. This results in the polarized localization of ion channels, receptors and enzymes to proper membrane domains in order to form structurally and functionally polarized cells [33, 51, 101]. It has also been suggested that tight junction is involved in the regulation of cell growth and differentiation [7].

Adherens junction

The adherens junction or zonula adherens are protein complexes that occur at cell-cell junctions in epithelial tissues. They are located more basal than the tight junctions and appear as bands encircling the cell or as spots of attachment to the extra cellular matrix. They are composed of three major proteins, E-cadherin, beta and alpha-catenin.

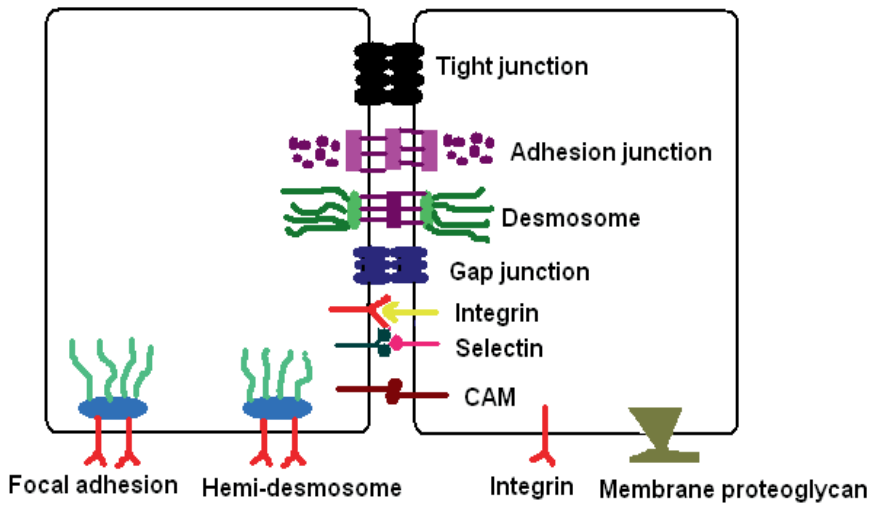

Figure 3. . The location of tight junction in relation to adhesion junction, desmosomes and gap junction.

Proteins located at the adherens junction play an important role in tumorigenesis, tumor progression and metastasis due to changes in adhesion molecule expression and function. These changes can occur with mutations in the cadherin and/or catenin proteins resulting in a separation of the cadherin-catenin complex and disorganized morphological features [68, 70]. Mutagenesis and deletion of the catenin-binding domain of cadherins have shown that this domain is essential for the cadherin binding to the cytoskeleton and for connecting adjacent cells [66].

Gap junction

Gap junctions are intercellular channels that allow different molecules and ions to pass freely between cells. This junction connects the cytoplasm of cells and is composed of two connexons, which are formed from six connexins each, which connect across the intercellular space. It is the connection of connexons from each cell across the gap that results in the formation of the pores which are defined as the gap junction [29]. Gap junctions that are formed from two identical hemi channels or connexons and are called

homotypic and those with differing hemi channels are heterotypic. Loss of gap junctions has been seen in advanced metastatic disease [42, 67].

Desmosomes

Desmosomes are disc shaped junctional complexes that are found in a variety of tissues especially tissues that are subjected to mechanical stress. Like adherens junction, desmosomes contain cadherins that link the two cells. The cadherins of desmosomes are referred to as desmogleins and desmocollins. They are localized as spot adhesions on the later side of the plasma membrane where they help to resist shearing forces [14, 81].

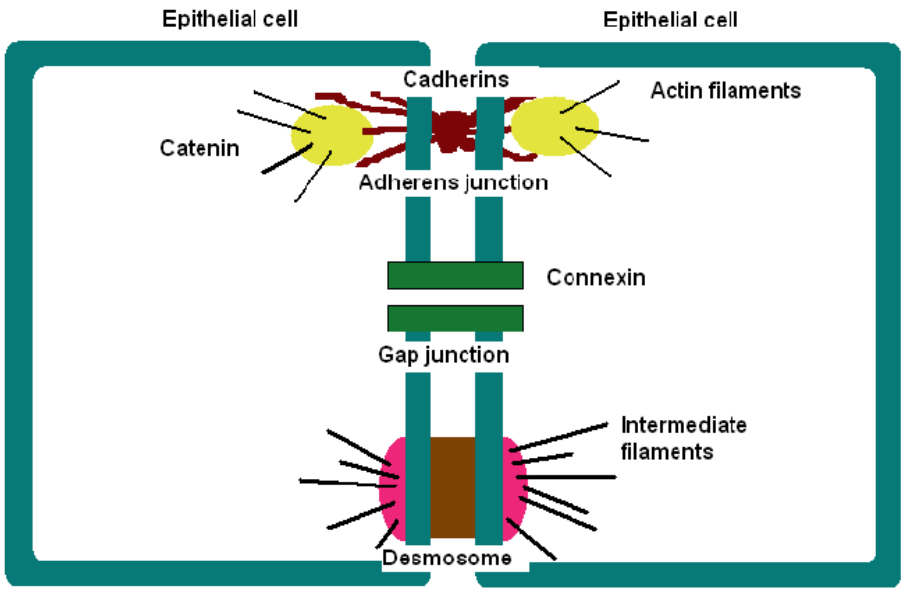

Figure 4. The locations of the different junctions between epithelial cells and their proteins.

Wnt signalling pathway

The Wnts consists of a family of growth factors that are responsible for various developmental processes including cancer development [30]. In cells not exposed to Wnt Beta-catenin forms a complex with APC/GSK3β/Axin which keeps the cytoplasmic levels of Beta-catenin low through phosphorylation by GSK3β. The phosphorylated Beta-catenin becomes ubiquitylated and degraded by the proteosome. When phosphorylated, Beta-catenins binding to E-cadherin decreases. This loss of binding promotes loss of cell adhesion and the accumulation of Beta-catenin within the cytoplasm. As a result the cadherin-related proteins also affect the relative amounts of free Beta-catenin.

In cells exposed to the Wnt proteins, Wnt binds to receptors of the Frizzled and LRP families on the cell surface, which induces phosphorylation of LRP as well as DSH, The APC/GSK3β/Axin complex is then inhibited which causes a block in the phosphorylation of Beta-catenin leading to the accumulation of Beta-catenin in the cytoplasm. The accumulated Beta-catenin then translocates to the nucleus and binds to TCF and works as a transcription factor on activates target genes [9, 30, 86] (Figure *5*).

In colorectal carcinoma APC regulates the degradation of Beta-catenin and is involved in the moving of Beta-catenin from the nucleus to the cytoplasm. The APC is somatically mutated in a majority of sporadic colorectal cancers with most of the mutations found in APCs central region corresponding to the Beta-catenin/Axin binding domain. This results in truncated gene products and a nonfunction of the degradation of Beta-catenin leading to the accumulation and formation of the TCF/Beta-catenin complex and the activation of Wnt target genes [4, 9, 73].

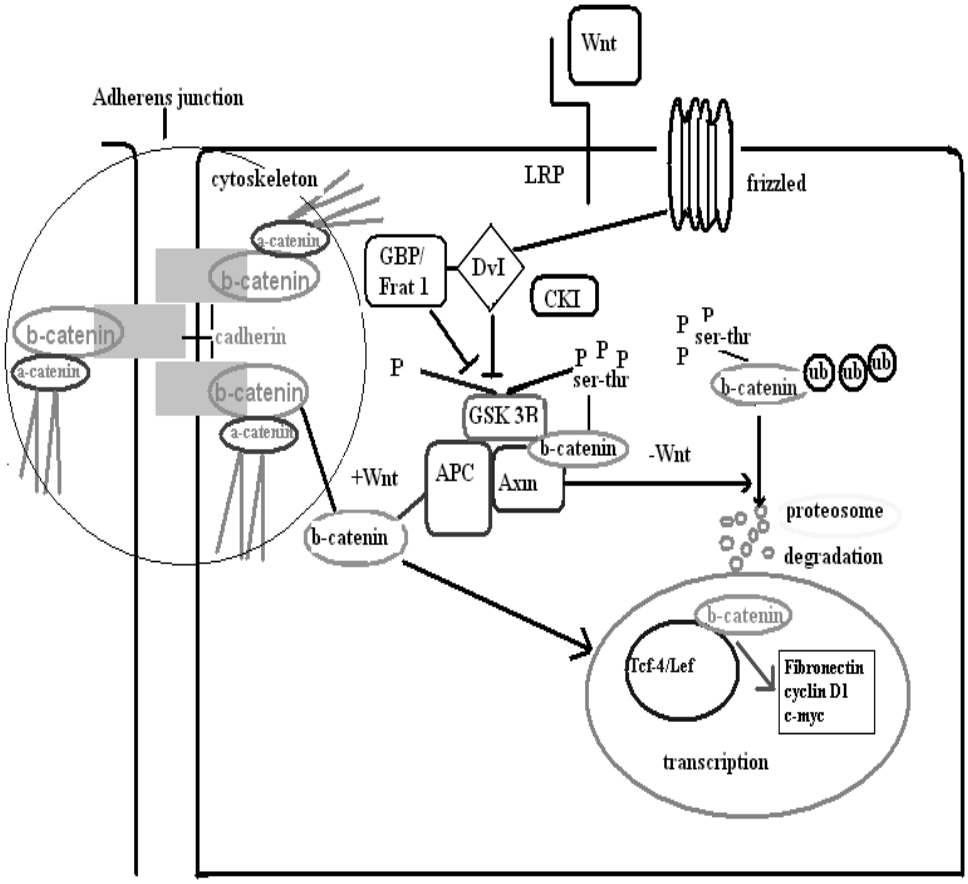

Figure 5. Wnt signalling pathway

Adhesion proteins

Cell adhesion molecules, CAMs

CAMs are proteins that are located in the cell membrane or are stored in the cytoplasm and function as receptors binding to other cells or with the extracellular matrix. They can be divided into four main families, the integrins, the cadherins, the immunoglobin superfamily and the selectins. These proteins can bind to similar or different molecules in other cells, they are composed of three domains, an intercellular domain which reacts with the cytoskeleton, a transmembrane do-

main and an extracellular domain that interacts with other CAMs of the same kind, which results in a homophilic binding or with the extracellular matrix, heterophilic binding [41, 68, 70].

E-cadherin

E-cadherin (epithelial) is a member of the cadherin family, which also consists of N-cadherin (neural) and P-cadherin (placental). The cadherins are a major class of adhesion molecules, which are calcium dependent and involved in homophilic cell-cell adhesion in all solid tissues of the body. The cadherins mediate cell-cell recognition events and together with the actin cytoskeleton are involved in morphological transitions that include tissue formation and the maintaining of tissue architecture. E-cadherin is expressed on the cell surface in most epithelial tissue and plays an important role in epithelial tumorigenesis [3, 105].

The reduced adhesiveness of cancer cells may result from defects in the cadherin-catenin complex. Reduced expression of E-cadherin is regarded as one of the main molecular events involved in dysfunction of the cell-cell adhesion system, triggering disaggregation of cells, cancer invasion and metastasis [10, 22, 50, 70, 85]. Studies have suggested that loss of E-cadherin protein expression in colon cancer may be explained by mutations in the promoter region, abnormalities at the translation or protein level or mutations in other parts of the gene not investigated [56, 87].

The gene structure of E-cadherin is similar to that of other cadherins. The E-cadherin gene (CDH1) is located on chromosome 16 and contains three transcripts with 16 exons spanning approximately 100 kb of genomic DNA http://www.ensembl.org/. Various mutations in the genes for E-cadherin have been found in colorectal carcinoma. Richards et al described a heterozygous polymorphism in exon 13 [79] and Wang et al a polymorphism in exon 9 of E-cadherin [104]. Salahshor et al found two missense mutations in exon 12 (Ala592Thr) of E-cadherin in patients diagnosed with colon cancer. They suggested that this mutation may play an important role in colorectal carcinogenesis as a tumor suppressor [83].

Beta-catenin

Beta-catenin is part of the catenin family, which consists of Alpha, Beta, Gamma and p120 catenin. Beta-catenins can influence tumordevelopment by bindning to Alpha-catenin and E-cadherin and thereby influencing cell adhesion or by acting as a transcription factor in the Wingless/Wnt signal transduction pathway [8, 9].

Decreased Beta-catenin disrupts homotypic cell adhesion and contributes to cellular motility and invasiveness [49]. An aberrant expression of Beta-catenin in colorectal cancers has been found and indicates tumor progression according to Brabletz et al [12].

The Beta-catenin gene (CTNNB1) is located on chromosome 3. It consists of four transcripts with 16 exons and spans 23.2 kb http://www.ensembl.org/. Most of the mutation in the Beta-catenin gene occur in exon 3 at one of the four phosphorylation sites [73]. APC and Beta-catenin are in most cases linked to an increase of nucleus accumulated Beta-catenin. Korinek et al and Morin et al established that the APC gene is a negative regulator of Beta-catenin signaling [53, 63]. Morin et al found that the protein products of mutant APC genes present in colorectal tumors were defective in down regulating transcriptional activation mediated by Beta-catenin and T-cell transcription factor-4 (TCF4) [63].

Furthermore, colorectal tumors with intact APC genes were found to contain activating mutations of Beta-catenin that altered functionally significant phosphorylation sites. These results indicated that regulation of Beta-catenin is critical to the tumor suppressive effect of APC and that this regulation can be caused by mutations in either APC or Beta-catenin [63]. Other studies suggest that activation of Beta-catenin by deletions in exon 3 is an early event in colorectal tumorigenesis even though it is less frequent than APC gene alterations [64]. Nuclear accumulation of Beta-catenin in colorectal cancer has been seen in a many studies and correlated to ulcerative growth, tumor size and tumor progression [11-13].

Claudin

Claudins were first described by Mikio Furuse and Shoichiro Tsukita in 1998 and today there are about 24 members of the Claudin family [102]. The Claudins are the most important components of the tight junction. They are small about 20-27kD, found in many organisms and are very similar in structure. They have four transmembrane do-

mains, with the N-terminus and the C-terminus in the cytoplasm. Claudins span the cellular membrane 4 times with both the N-terminal and C-terminal located in the cytoplasm. One main function is to establish the paracellular barrier that controls the flow of molecules in the intercellular space between the cells of an epithelium.

The localization and expression of Claudins may differ depending on the type of tissue and neoplasm. In malignant tumor, tight junction frequently shows structural and functional abnormalities [72, 94]. For example, in colorectal carcinoma Claudin1, 2 and 7 have shown to be upregulated while an increased expression of Claudins 3 and 4 have been seen in prostate cancer [43, 58, 62]. Loss of Claudin 1 expression has recently been found to be of prognostic significance in colon cancer [78].

According to a study by Wu et al over expression of Claudin 1 is related to abnormal differentiation, invasiveness and metastasis of gastric carcinoma [106]. Dhawan et al reported an increased expression of Claudin 1 in colon cancers where metastatic colon cancer cells expressed the highest levels of Claudin 1 and had the highest rate of nuclear dislocation. In that study an important role in the regulation of cellular transformation, tumor growth and metastasis was suggested for Claudin1, which is in agreement with a study by Wu et al [20, 106]. Claudin 1 (CLDN1) is located on chromosome 3 and contains 4 coding exon regions http://www.ensembl.org/ [36].

Claudin 2 was one of the first Claudins to be discovered together with Claudin 1. It is regularly expressed in the epithelial tissue of many organs including colon, liver, gut, pancreas and kidney as well as in different kinds of carcinoma [93]
[27, 75]. In patients with ulcerative colitis a strong upregulated expression was seen for Claudin 2 [107], a disease that has an increased risk of colon carcinoma. A differential expression of Claudin 2 was seen in crypt and villous cells of the small intestine and undifferentiated crypt cells in the colon [5, 23]. Claudin 2 (CLDN2) is located on chromosome X contains 2 exons and spans 2959 bp [82]http://www.ensembl.org/.

Claudin 7 is normally expressed in different kinds of epithelial tissue. Dysregulation of Claudin 7 protein causes a loss of E-cadherin expression but E-cadherin expression does not regulate Claudin 7 protein expression [57]. Other studies suggest that protein expression of Claudin7 is an early event in gastric tumorigenesis showing over expression in gastric dysplasia, but no correlation was found with tumor localization, stage or grade of established cancer [47]. Furthermore it has been suggested that Claudin 7 has both structural and regulatory functions and may be involved in cell differentiation [109].

Claudin 7 (CLDN7) is mapped to chromosome 17 and contains 4 exons and spans 1,534 bps http://www.ensembl.org/.

A variation of Claudin expression can be seen in different cancers. Claudin 7 was decreased in ductal carcinoma in the breast [52] and Claudin 4 and 5 showed a reduced expression in hepatocellular and renal carcinomas [92] and a loss of Claudin 22 expression has been found in breast and prostate cancer [93]. On the other hand Claudin-3 and Claudin-4 show an increase protein expression in various types of cancer such as pancreatic adenocarcinoma and ovarian cancer [61, 77] and an upregulation of Claudin 1 has been described in colorectal carcinoma, which has been linked to Beta-catenin/Tcf signalling [32]. It has also been shown that reduced expression of Claudin 1 was associated with loss of differentiation and with a worse prognosis in Dukes B colon cancer [78]. No studies seem to have been performed regarding mutations in the Claudin 1,2,7 genes and correlated to growth pattern or other characteristics of colon carcinoma.

Occludin

Occludin is an integral membrane protein, which together with Claudin are the most important parts of the tight junction. It was first described in 1993 by Shoichiro Tsukita who localized this membrane protein in both epithelial and endothelial cells. Disruption of Occludin regulation appears to be an important mechanism in the development of cancer. Kimura et al suggested in their study that Occludin together with Claudin has important functions in the formation of gland like structures and that they are reduced in cancer cells in correlation with loss of differentiation [51]. More recent studies also suggest that Occludin expression can be used as a possible marker for glandular differentiation in rectal carcinoid tumors as well as lung carcinoma [33, 51, 99-101]. Kimura et al (1997) studied the expression of Occludin in cancer of the stomach and colon and observed that its expression was significantly reduced in poorly differentiated carcinomas. Tumor cells, particularly in those cancers that manifest high metastatic potential, often have loss of functional tight junctions [69] and the expression of Occludin has been shown to be decreased during tumor formation and metastasis [48, 98]. Occludin (OCLN) is mapped to chromosome 5 and contains 9 exons http://www.ensembl.org/.

Morphometry

Morphological grading of different characteristics of cells and tissues is usually based on semiquantitative estimations by the viewer. These estimations do not show a good reproducibility and low kappa -values for either intra- or interindividual estimations are often found. For instance, in the colorectum, the invasive patterns of colorectal carcinoma show only fair interindividual (0.37) to moderate intraindividual (0.41) agreement [19]. This is also valid for the grading of dysplasia in advanced colorectal adenomas (= 0.20 and 0.42 for intra- and interindividual grading, respectively) [97]. New and more sophisticated image analysis software for computer based morphometrical analysis has made it possible to quantitatively analyze complex biological structures in a standardized way.

Morphometry is used to calculate or measure different features in images such as area, form and texture. Different morphometrical methods can be used to assess the complexity of structures including the estimation of fractal dimension, lacunarity and number of structures.

A fractal is "a rough or fragmented geometric shape that can be split into parts, each of which is (at least approximately) a reduced-size copy of the whole" [59] which means that the structure shows self-similarity under scale changes. Growth in nature has this quality and thus follows fractal geometry. The fractal dimension is a measure that gives an indication of how completely a fractal appears to fill space and can be calculated in many different ways [15]. Natural objects, in contrast to mathematical fractals, are not the result of a construction by endless iteration and therefore the fractal dimension shows self-similarity only over a limited scale range and if a linear segment is present on the log-log graph, the gradient of this will accurately reflect that dimension [15]. One method to measure the fractal dimension, that is widely used in biology [15, 16] is the box counting technique where boxes of different sizes are put on top of the image and the number of boxes needed to cover the one pixel outline of a structure is then counted [15, 16].

Fractal geometry has been used in molecular biology and bone, vascular and tumour pathology [16]. In tumour pathology, the fractal dimension was able to differentiate between tubular, tubulovillous and villous adenomas of the colon [17]. Fractal geometrical analysis has also been shown to be able to differentiate severe dysplasia and cancer from benign conditions in the epithelial-connective tissue interface in the floor

of the mouth [54] and to quantify the nature of tumour borders in colorectal carcinomas which are often subjectively-divided into 'pushing' or 'infiltrative' types [18].

Objects that look very different may have very similar fractal dimensions since fractals do not uniquely describe irregular binary features of biological objects [90]. Lacunarity is a method to classify fractals and textures, which have the same fractal dimension value but a different visual appearance. Lacunarity is a measure of how the fractal fills space, if the fractal is dense the lacunarity is small, if a fractal has large gaps or holes the lacunarity is high. Different fractals with the same dimension but with different appearance have different lacunarity [71, 90].

Small structures organized in an ordered manner do not show complexity or irregularity. However, at the invasive front of a carcinoma the tumor may split up into cell clusters of different sizes and give the tumor outline an irregular appearance. The number of such tumor differently sized cell islands can be used as a measure of the tumor complexity at the invasive front. Measuring the length of the tumor-stromal interface can also serve as an indirect measure of the complexity of the tumor invasion front, the longer the border, the more irregular or complex it is. This can be accomplished by placing a measuring grid over an image of the structure as is done in stereology [34, 35].

Aims

The aims of this thesis were to:

- Compare human visual assessment of the irregularity of the invasive border of colon carcinoma to computer assisted techniques and define a Complexity Index to grade the growth pattern of the border

- Assess the relationship between expression of the cell adhesion proteins E-cadherin, Beta-catenin, Claudin 1,2,7, Occludin and tumor growth pattern.

- Assess the relationship between tumor volume and protein expression of E-cadherin, Beta-catenin, Claudin 2 and Occludin.

- Analyze if there are any mutations in the genes of E-cadherin, Beta-catenin, Claudin 1,2,7 and Occludin that can account for the variability of the tumor growth pattern or the expression of the cell adhesion proteins.

- Assess the relationship between tumor volume and tumor localization, TNM stage, differentiation and tumor growth pattern.

Materials and Methods

Two sets of colon carcinomas are studied in this thesis. Only carcinomas from the colon were considered since most patients with rectal carcinomas obtain local radiation to the tumor preoperatively. Mucinous carcinomas were not included. All samples were unidentified. Tumor stage was assessed according to the TNM classification [91] and tumor grade as degree of differentiation according to the WHO classification of tumors [38]. The studies were approved by the ethics committee at Örebro University Hospital Sweden.

In paper I, twenty-nine patients diagnosed with colon carcinoma 2002-2003 were studied. The tumors were selected from archived paraffin embedded tissue blocks at the Department of Pathology, Örebro University Hospital. One section from each tumor was selected from regular hematoxyline-eosine stained slides. The selection of tumors and slides was made so that there was a representation of tumors with both infiltrative and expansive growth patterns.

In paper II, we used the same tumors as in paper I but added three more tumors in order to obtain a more even distribution of the growth patterns. Altogether, thirty-two formalin fixed paraffin embedded tissue samples from colorectal carcinomas diagnosed 2002-2004 were used in the study.

In paper III and IV thirty-three whole mount tissue sections from colon carcinomas diagnosed 2001-2002 at Karlstad Regional Hospital and Örebro University Hospital were used. Whole mount sections from the tumors were used for volume assessment. Two samples from each tumor were collected for protein and gene analysis.

Morphometry (I-IV)

In order to measure the complexity of the invasive front of the tumors images from the tumor-stromal interface using a Leica DC200 digital camera mounted on a Leica DMRXE microscope (Leica Microsystems Wetzlar GmbH, Germany) (objective 10X). The images were digitized and stored in uncompressed TIFF-format. No compression of the images was performed during the image processing and handling. The number of images depended on the length of the tumor-stromal border. Areas with artifacts and necroses in the invasive margin were not used. The images were then digitized and

thresholded so that all immunohistocemically stained areas were black. The visual estimation of tumor borderline complexity was then performed in the thresholded black/white images.

The morphometrical calculations were performed using two image analysis software. Adobe Photoshop 7.0 (Adobe Systems Inc. San Jose, California, USA) with the Fovea Pro plug ins for image analysis (Reindeer Graphics, Inc., North Carolina, USA) was used to threshold and delineate the tumor margin and tumor cell clusters. The image analysis software Image J was used to calculate the Fractal Dimension with the box counting method and the Lacunarity.

Immunohistochemistry

Staining was performed using a Dakos Techmate and DAB Envision according to manufacturers protocol (Dako, Denmark). The slides were incubated with the primary antibody for 30 minutes. For paper III, the whole mount sections were stained manually for the cytokeratin marker (Cam5,2) for Complexity Index estimation of the entire tumor borders.

For the paper I,II,III and IV, four microns thick sections were cut onto silano slides for DAKO TechMate Horizon (Dako, Denmark). The sections were deparaffinised in xylene twice for 10 min, rehydrated in a descending series of ethanol (99%, 96%, 70%) followed by washes in distilled water. Antigen retrieval was achieved by heating the samples in TE (Tris EDTA) buffer, pH 9.0±0.2 in a microwave oven at 650W for 30 minutes. The sections were then washed in distilled water.

For paper II and III the primary antibodies used were monoclonal anti-E-cadherin 1:800 (36) BD Biosciences, San José, USA, anti-Beta-catenin (14) BD Biosciences, San José, USA) dilution 1:1000, anti-cytokeratin (Cam 5.2) BD Biosciences, San José, USA, 1:25, anti-Claudin 2 1:200 (ab15100), Abcam, Cambridge, UK, anti-Occludin 1:200 (z-T22) Zymed, San Francisco, USA.

For paper IV the primary antibodies used were anti-Claudin 1(rabbit), Abcam, Cambridge, UK, dilution 1:200, anti-Claudin 7 (5D10F3) Zymed, San Francisco, USA, dilution 1:1000 and dilution 1:25 anti-cytokeratin (Cam 5.2) BD Biosciences, San José, USA. After staining the sections were transferred through ascending ethanol series and xylene before mounting and evaluated under a light microscopy.

Evaluation of staining

Slides were consecutively numbered and anonymous to the observer. All slides were stained simultaneously in a DakoTechmate with a control slide that was exposed only to the secondary antibody. In paper II, the staining of E-cadherin, Beta-catenin, Claudin 2 and Occludin was graded semiquantitatively into four categories 0= absence of staining, 1= reduced staining, 2= moderate staining, 3= strong staining. This was assessed in the nucleus, cytoplasm and membrane respectively, both in tumors and normal mucosa. In normal mucosa the goblet cells compressed the cytoplasm and membrane and they could therefore not be assessed separately as in the tumor cells and were evaluated together. Claudin 2 and Occludin were evaluated in membranes only.

For paper III and IV another set of tumors were analyzed. A different technique to assess the staining pattern was used compared to paper II. Staining of E-cadherin, Beta-catenin, Claudin 1,2,7 and Occludin were graded (0-3) according to the extent of staining in the membrane, cytoplasm and nucleus (0 = 0-10%, 1 = 10-50%, 2 = 50-80% and 3 = 80 - 100%) [84]. Claudin 1,2,7 and Occludin were evaluated in the membranes only.

LMD

In this study a laser capture micro dissection microscope from Leica was used (Leica Microsystems GmbH, Wetzlar, Germany). In laser capture micro dissection an ultraviolet laser micro beam melts a thermoplastic ethyl vinyl acetate membrane that overlays the tissue. The melted membrane sticks to the selected cells, after cutting, the instrument uses an additional pulse of laser energy to catapult the cut region into a microfuge cap.

For laser micro dissection 10-micron sections were mounted on plastic membrane slides The slides were then manually stained with anti-cytokeratin Cam 5.2 as described in the immunohistochemistry part in order to identify he tumor border.

A 1mm^2 piece from the invasive front of the tumor samples was extracted using a laser micro dissection microscope. If the sample contained a small amount of tumor cells, several LMD pieces were pooled together in the same tube. A similar procedure was used to obtain corresponding normal mucosa for controls.

DNA extraction and PCR

DNA was extracted using proteinas K according to manufactures protocol (Qiagen,Sweden) and the amplification was performed in an optimized PCR according to the following protocol á 45-50 cycles on a thermocycler (Eppendorf gradient) at 95°C denaturation for 1 min, 56-60°C annealing, depending on the amplicons for 1 min and 72°C extension for 1 min. PCR was performed using in a volume of 50uL containing 1-10ng of genomic DNA in a buffer containing 1.5-2,0mM $MgCl_2$, 200uM each of deoxyribonucleoside triphosphate, 5pmol of each primer and 0.5 units of Taq Gold polymerase (Applied Biosystems, Foster City, USA).

SSCP

Single strand conformation polymorphism, SSCP, is an electrophoretic separation of single-stranded nucleic acids, which is based on differences in sequence, this results in a different secondary structure and a measurable difference in mobility through a gel. This method was used in paper II.

The mobility of double-stranded DNA in gel electrophoresis is dependent on strand size and length but is relatively independent of the particular nucleotide sequence. The mobility of single strands is noticeably affected by small changes in sequence. Small changes are noticeable because of the relatively unstable nature of single-stranded DNA; in the absence of a complementary strand, the single strand may experience intrastrand base pairing, resulting in loops and folds that give the single strand a unique 3D structure, regardless of its length. A single nucleotide change could affect the strand's mobility through a gel by altering the intrastrand base pairing and its resulting 3D conformation (Melcher, 2000).

SSCP analysis can detect DNA polymorphisms and mutations at multiple places in DNA fragments (Orita et al, 1989). After PCR amplification (II), products were loaded onto 12.5% polyacrylamidgels (Gene Gel 125 Pharmacia Biotech, Uppsala, Sweden) and underwent electrophoresis at 12°C. The single and double strands were visualised by silver staining according to the manufacturers protocol (Amersham Biosciences, NJ, USA).

DNA Sequencing

DNA sequencing is used to determine the order of the nucleotide bases, adenine, guanine, cytosine, and thymine, in a DNA oligonucleotide. It is based on Fredrick Sangers method from 1975 called the dideoxy termination method of the Sanger method.

DNA sequencing reactions are just like the PCR reactions for replicating DNA. However in DNA synthesis the reaction is performed using only one oligonucleotide primer in each tube. Fluorescence labelled dideoxy-dNTP is added to the mixture, these lack the OH group that will be randomly incorporated during the DNA synthesis and will terminate the continuing synthesis of the specific strand. This will generate a mixture of different sizes of DNA sequences where each terminal ddNTP is labelled with different fluorescent colors, R6G, ROX,R110 and TAMRA. A detector reads the color of the fluorescent label and a computer puts together the nucleotide sequence. The DNA sequence products are separated by a capillary electrophoresis ABI 310,3100 and 3130xl (Applied Biosystems).

For paper II the PCR product was purified using Dye Ex spin removal (Qiagen, Solna, Sweden) and sequencing was performed using the ABI Prism Big Dye Terminator cycle sequencing Ready Reaction Kit v.1.1on the ABI 310 and ABI 3100 (Applied Biosystems, Foster City, USA). The DNA sequences were subjected to NCBI Blast; http://www.ncbi.nlm.nih.gov) for verification of the amplified amplicons.

For paper IV the EDTA/NaAc/Ethanol precipitation was used for purification of the amplified products according to manufacturers protocol (Applied Biosystems, Fosters City,CA,USA). DNA sequencing was performed using ABI Prism Big Dye Terminator cycle sequencing Ready Reaction Kit v.1.1on the ABI 3100 and ABI 3130xl (Applied Biosystems, Foster City, USA).

Statistics

In paper I, the Pearson correlation coefficient was used to find correlations between different image analyses parameters obtained from the computer. Discriminant analysis showed the connection between the visual grading and the image analysis data. We performed cluster analysis of the 265 images with Ward's algorithm in order to find homogenous subsets of images with respect to the objective measurements obtained from the image analysis. Cluster solutions from the Ward method were refined with the K-means algorithm using the cluster centers from the Ward method as starting points. Tree diagram analysis or recursive partitioning provided a nonlinear and non-additive approach to classify and predict classifications of images, based on visual gradings and on cluster solutions.

Computations were performed with the statistical packages SPSS and SPLUS. The principles for cluster analysis and tree diagram analysis from Everitt, Landau & Leese and Zhang & Singer were followed [24, 108]].

For paper II,III and IV the tree analysis described above was used to show the Complexity Index factor of the tumor samples. Analysis of variance (ANOVA) was used to analyze differences in volume between groups of objects specified by the different categorizations for tumor complexity. For some of these analyzes we had to recode the variables into fewer categories to avoid cells with very few observations. In those cases where correlations were evaluated we used Spearman's rho due to weaker distributional properties of the analyzed variables. To analyze differences in the distributions of the cell adhesion proteins in tumor samples compared to normal mucosa we used the chi-square test for homogeneity in distribution. The calculation was performed with permutation tests that were especially adopted to handle small samples since the number of cells was too small for the asymptotic chi-square test. P-values < 0.05 were classified as statistical significance. All statistical testing was done with SPSS (version 15, SPSS Inc, IL) or StatXact (Version 8, Cytel Inc, MA).

Decision Tree analysis

Decision trees are useful tools for helping you to choose between several courses of action by using a graph or model of decisions. It is used as a descriptive means for calculating conditional probalbilities. They provide a highly effective structure in which you can see options and study the possible outcomes of choosing those options. Decision tree analysis has been used in the diagnosis of gastrci cancer [96].

Results

Tumour growth pattern (I-IV)

Using computer image analysis we assessed different characteristics in the images.
The irregularity of the tumor border was assessed by either visual estimation of the complexity in a four-graded scale (1-4) or by a computer assisted technique and the two methods were compared.

In order to quickly determine a classification into the four visual gradings, we performed a tree diagram analysis. Only four decision points were necessary to obtain a correct classification as high as 80.3 %.

The complexity of the front was also assessed using four different image analysis techniques, i.e. estimation of Fractal Dimension, Tumor Front Length, number of Tumor Cell Clusters and Lacunarity. Fractal Dimension and Tumor Cell Clusters together gave the best correlation to visual grading using discriminant analysis. A cluster analysis and a tree diagram analysis were then performed and the tree diagram analysis of the data resulted in rules that gave correct classification into the five clusters of 97.0 %. It was thus found that the computer assisted technique was superior to the semiquantitative visual grading.

Celladhesionproteins (II-IV)

In paper II, immunohistochemical staining was performed for E-cadherin, Beta-catenin, Claudin 2 and Occludin. An even distribution of the staining of all adhesion proteins was found in the epithelial cells of mucosa adjacent to the tumors whereas the tumors showed a perturbed staining pattern. A semi quantitative evaluation of the staining pattern and intensity was performed(II). Nuclear staining of E-cadherin was found in one tumor and in none of the normal controls. A large variation was obtained for E-cadherin regarding both cytoplasmic and membrane staining in tumors and differences in staining compared to controls was statistically significant for both. Similar significant results were found regarding the staining for Beta-catenin in both cytoplasm and membranes. Staining of the nucleus for Beta-catenin was seen in 21/32 tumors whereas no nucleus was stained in normal cells.

Claudin 2 and Occludin caused an irregular staining of the cytoplasm of the tumor cells whereas the staining of normal cells was strong and even. The difference in stain-

ing was significantly for Claudin 2 ($p<0.0001$) and near significance for Occludin ($p<0.053$).

In paper III all of the tumors showed a perturbed and reduced expression of E-cadherin compared to normal controls. These differences in staining were significant for E-cadherin in cytoplasm, Beta-catenin in nucleus and cytoplasm (all $p<0.0001$).

Similar results were obtained for Claudin 2 and Occludin as in paper II ($p<0.001$ for both).

In paper IV, Claudin 1 and Claudin 7 showed a strong, even distribution of the staining in epithelial cells in normal mucosa. A significantly reduced expression was seen in tumors for both Claudin 1 and Claudin 7 ($p<0.0001$). In the invasive front a reduced expression was seen compared to the more central areas of the tumor.

Only few tumor clinicopathological characteristics were correlated (rho>0.5) to the expression of adhesion proteins and these included membraneous expression of E-cadherin and degree of differentiation (rho 0.53), cytoplasmic expression of Beta-catenin and tumor localization (rho 0.55) and, expression of Occludin and Complexity Index (rho 0.52)

SSCP analysis was performed on all tumors and the surrounding normal mucosa. No aberrant patterns were found for any of the genes (II).

In paper II, DNA sequencing was performed in about half of the samples with high and low Complexity Index values. One tumor showed a SNP in the Beta-catenin gene exon 3. In the E-cadherin gene no polymorphisms were found in exons 6 and 12. In exon 13 of E-cadherin ten tumor samples showed a single nucleotide polymorphism (Iso650Leu). In the normal mucosa no polymorphisms were found regarding E-cadherin and Beta-catenin. Nor were there any mutations found in the genes for Claudin-2 and Occludin in either the tumors or the normal mucosa.

In paper IV, DNA sequencing was performed on the Claudin 1 and 7 genes. In Claudin 1, 17 out of 26 samples showed a homozygous A/G (Gly123Gly) polymorphism in exon 2 **rs9869263**. Eight of the samples showed a heterozygous AG polymorphism and one sample showed no polymorphisms in Claudin 1. These polymorphisms were found in both tumor and normal mucosa.

In Claudin 7, 15 samples from tumor and normal mucosa showed a polymorphism A/G (Val197Ala) in exon 4 **rs4562**. The remaining eleven showed no polymorphisms in this gene either in the tumor or in the normal mucosa. No significant correlations

were found with, tumor size, localization, pT, pN, Complex Index ,degree of differentiation or protein expression.

Tumor volume (III,IV)

The volume of the tumors were assessed using Cavalier´s principle and ranged from 1.54-94.4 cm^3 with an average of 24.1 cm^3. Statistical significance for differences in tumor volume was obtained for growth pattern ($p=0.05$), tumor stage including depth of invasion (pT) ($p<0.001$) and nodal stage (pN) ($p=0.023$). No significant differences in tumor volume were found regarding differentiation, localization and adhesion protein expression.

DISCUSSION

Tumour growth pattern

The morphological grading of different characteristics of cells and tissues is based on semiquantitative estimations made by a pathologist. It has been shown that such estimations do not give a good reproducibility and low kappa-values for both inter- and intraindividual estimations [15]. By using computer based image analysis it is possible to quantitatively analyze complex biological structures in a reproductive way. In this thesis a methodological study was performed in order to compare different image analysis techniques (Fractal Dimension, Tumor Cell Clusters, Lacunarity and Tumor Border Length) to assess the complexity of the tumor-stroma interface of colon carcinomas in comparison to the visual, semiquantitative estimation done by a pathologist and to construct a Complexity Index based on cluster and tree diagram analyses.

The different clusters indicate various degrees of complexity of the tumor invasive border and the results of the tree analysis can be used to discriminate tumors regarding their irregularity as a Complexity Index. This technique can be used in scientific studies and in clinical contexts when detailed knowledge of the invasive pattern is of importance.

Cell adhesion proteins

Cell adhesion proteins are important for the structure and integrity of the tissue. In tumorigenesis, phenotypic changes occur in the tumor which may result in a disaggregation of the tumor causing an irregular tumor growth. The change in growth pattern has to be the result of mutations in genes that are involved in the expression of adhesion proteins. This means that mutations could occur either in the genes of the adhesion proteins themselves or in the genes of proteins that regulate the expression of adhesion proteins. In these studies (II-IV)

We have chosen to assess the expression of some adhesion proteins and mutations in their genes using SSCP and DNA sequencing and correlated it to the growth pattern of colon carcinoma. Frequent and known polymorphisms(see below) were found in E-cadherin, Claudin 1 and Claudin 7 but these were not correlated to the growth pattern. Nor were other mutations found that could explain the complexity of the tu-

mors. The protein expression and distribution of E-cadherin, Beta-catenin, Claudins and Occludin was assessed by immunohistochemistry. All proteins showed a significantly perturbed distribution in tumors compared to normal mucosa and these results are in agreement with earlier findings [12, 13, 21, 64, 110]. No mutations were found that could explain the aberrant protein expression and no correlations were found between growth pattern and the perturbed protein expression.

This means that mutations responsible for the aberrant expression of the adhesion proteins and the infiltrative growth pattern has to be sought for among other proteins that influences the expression of the adhesion proteins. There is a complex interaction between the different adhesion proteins and other proteins, for instance those involved in the Wnt/Wingless pathway. The APC tumor suppressor gene binds to Beta-catenin but it is not clear whether APC and E-cadherin compete for binding to Beta-catenin, which may result in alterations in the E-cadherin-mediated adhesion [70]. Mutagenesis and deletion of the catenin binding domain of cadherins have shown that this domain is essential for the cadherin binding to the cytoskeleton and for connecting adjacent cells [66]. It has been suggested by Dhawan et al (2005) that the regulation of E-cadherin expression and Beta-catenin/Tcf signalling is a possible mechanism underlying Claudin 1 dependent changes in colon cancer phenotype and behaviour [20].

Different clinicopathological characteristics have been shown to be influenced by the aberrant expression of cell adhesion proteins. Variable Beta-catenin expression has been shown to disrupt homotypic cell adhesion and contribute to cellular motility and invasiveness in colorectal cancer [12]. Loss of E-cadherin expression is associated with perturbed tumor differentiation and has been shown to be correlated with an increased likelihood of distant metastasis [10, 50, 85]. Knock down of Claudin 7 in squamous carcinoma cell lines lead to decreased E-cadherin expression, increased cell growth and enhanced invation [57]. In these studies only few clinicopathological characteristics were noteworthy correlated to the expression of adhesion proteins and these included membraneous expression of E-cadherin and degree of differentiation, cytoplasmic expression of Beta-catenin and tumor localization and, expression of Occludin and Complexity Index. Only around 30 tumors were used in these studies and larger numbers of tumors may be needed to obtain significant correlations.

Various mutations in the genes for E-cadherin and Beta-catenin have been found in colorectal carcinoma. Several groups have described a heterozygous polymorphism in

exon 13 [79] and in exon 9 of E-cadherin [104]. Salashor et al (2001) found a missense mutation in exon 12 of E-cadherin codon 592 in two patients with colon cancer suggesting that this mutation may play a role in colorectal carcinogenesis. The exons 6,12 and 13 were therefore chosen (II). However, no mutations were found in exons 6 and 12. In exon 13 a homozygous mutation (Iso650Leu) was found in 8/15 tumor specimen. No polymorphisms were seen in the corresponding normal mucosa. Mutation in the exon 3 in Beta-catenin has been shown to be an early event of colon carcinogenesis [44] and exon 3 was therefore chosen for mutational analysis. DNA sequencing of CLDN 1 and CLDN 7 was performed (IV) on 26 colon carcinomas and adjacent normal mucosa. In CLDN 1, 17 out of 26 tumors and corresponding normal mucosa showed a homozygous polymorphism in exon 2 and 8 a heterozygous polymorphism. In CLDN7, 15 out of 26 tumors and normal mucosa showed a single nucleotide polymorphism in exon 4. These findings indicate heritable polymorphisms in a high proportion of the individuals. We have not found any information about the frequency of these polymorphisms in the normal population. If this frequency is high, the findings could be unimportant. However, if this frequency is low, there could be a relationship between these polymorphisms and development of colon carcinoma. This remains to be shown in future population studies.

Tumor volume

Tumor volume increases by time and due to tumor progression an increasing number of mutations occur resulting in tumor heterogeneity. This indicates that tumor phenotype, including growth pattern, may change by time due to aberrant protein expression.

We have estimated the volume of 33 colon carcinomas of varying sizes and assessed the growth pattern along the entire invasive border of the tumors (III). It was found that the size of the tumor correlated to growth pattern and tumor stage including depth of invasion (pT) and nodal stage (pN). The volume of a tumor is often related to the depth of invasion and to the number of lymph node metastases, which has been shown for gastric carcinoma [103] and has been shown here for colon carcinoma. During tumor progression mutations accumulate resulting in a change of the phenotype. The degree of differentiation might therefore decrease by time during tumor progression [55]. No statistical significant difference for volume was obtained neither for the degree of

differentiation, nor the tumor localization. Furthermore, the results showed a statistical significance for differences in the volume for the Complexity Index ($p=0.05$). This indicates that when the tumor grows in volume the border becomes more irregular probably due to accumulating mutations influencing the adherence of the tumor cells.

Conclusion

- Computer assisted morphometry with estimation of fractal dimension and number of free tumor cell clusters was significantly superior to human visual assessment of the complexity of the invasive front of colon carcinoma. A Complexity Index was defined that can be used for grading the irregularity of the invasive border.

- A perturbed expression was found in the celladhesionproteins, E-cadherin, Beta-catenin, Claudin 1,2,7 and Occludin compared to normal mucosa. No relationship was found between the tumor growth pattern and expression of the cell adhesion proteins.

- No relationship was found between tumor volume and the expression of cell adhesion proteins.

- No mutations were found in the genes of E-cadherin, Beta-catenin, Claudin 1,2,7 and Occludin that could account for the variability of the tumor growth pattern or the aberrant expression of the cell adhesion proteins.

- A high incidence of polymorphisms was found in the genes of E-cadherin, Claudin 1 and Claudin 7.

- Tumor volume was significantly correlated to tumor growth pattern and tumor stage but not to differentiation or localization.

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