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Khadija Slik

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To my family

UNIVERSITY OF TURKU
Faculty of Medicine
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ABSTRACT

The risk of recurrence of stage II colorectal cancer is difficult to predict. A part of recurrences take place among patients without any of the known high-risk factors such as lymphovascular invasion or preoperative obstruction. In addition, microsatellite instability status and tumour budding have been included to risk stratification of colorectal cancer patients. The aim of this study is to find new biomarkers and their combinations, which could more efficiently identify high risk stage II colorectal cancer patients. For this purpose, a cohort of 232 stage II colorectal cancer patients treated at Turku University Hospital has been collected and a tissue microarray has been constructed from their paraffin-embedded tumour material.

Ezrin is a cytoskeleton-associated protein that participates in cellular signaling, cell survival, proliferation and migration. High protein expression of ezrin in cancer cells has been linked to poor outcome in many cancer types. The diminished protein expression of transcription factor CDX2 has been associated with inferior outcome in stage II and III colorectal cancer. The association of high tumour budding with poor outcome in colorectal cancer has been well documented, but the molecular biological mechanisms behind this are poorly known. There is evidence that tumour budding is associated with epithelial-mesenchymal transition (EMT) but the exact molecular biological mechanism of this has not been properly studied. In this study, the protein expression of ezrin, CDX2 and EMT markers integrin 4 beta, E-cadherin and ZO-1 have been studied in relation to clinicopathological variables and survival.

The main results of the studies showed high ezrin protein expression and CDX2 loss in patients with microsatellite stable tumours to be independent risk factors of poor disease-specific survival in stage II colorectal cancer. High expression of EMT-marker integrin 4 beta in tumour buds analysed with digital image analysis correlates with visual tumor budding analysis, and it proved to be an independent risk factors of poor disease-specific survival. In conclusion, high ezrin protein expression, CDX2 loss and EMT-marker integrin 4 beta are new promising biomarkers in risk stratification of stage II colorectal cancer patients.

KEYWORDS: colorectal cancer, biomarker, survival, tissue microarray

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
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KHADIJA SLIK: Levinneisyysasteen II paksu- ja peräsuolisyövän biomarkkerit
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TIIVISTELMÄ

Levinneisyysasteen II paksu- ja peräsuolisyövän uusiutumisriskiä on vaikea ennustaa. Osa taudin uusiutumisista tulee potilaille, joilla ei ole yhtään tällä hetkellä tunnetuista korkean uusiutumisriskin tekijöistä, kuten suoni-invaasio tai kasvaimen ennen leikkausta aiheuttama tukkeutuminen. Näihin riskitekijöihin on lisätty myös mikrosatellitti-instabiliteetti ja syövän silmuileva kasvutapa. Tämän tutkimuksen tavoitteena on löytää uusia biomarkkereita ja niiden yhdistelmiä, jotka voisivat aiempaa tehokkaammin tunnistaa tämän levinneisyysasteen korkean uusiutumisriskin potilaita. Tätä tarkoitusta varten on kerätty Turun yliopistollisessa keskussairaalassa hoidettujen 232 levinneisyysasteen II paksu- ja peräsuolisyöpäpotilaiden aineisto, joista on kerätty kliinispatologiset tiedot ja heidän kasvainmateriaalistaan on valmistettu monikudosblokit eli kudosmikrosiru.

Ezrin on solun tukirangan proteiini, joka osallistuu mm. solusignalointiin, jakautumiseen ja liikkumiseen. Korkea ezrin-proteiinin ilmeneminen syöpäsoluissa on liitetty huonoon ennusteeseen useissa syöpätyypeissä. CDX2-transkriptiotekijän vähentynyt proteiinitason ilmeneminen on liitetty huonoon ennusteeseen levinneisyysasteen II ja III paksu- ja peräsuolisyövässä. Syövän silmuilevan kasvutavan on useissa tutkimuksissa todettu liittyvän huonoon ennusteeseen paksu- ja peräsuolisyövässä, mutta tämän taustalla olevia mekanismeja tunnetaan huonosti. Tässä tutkimuksessa ezrin- ja CDX2-proteiinien ja epiteeli-mesenkyymaaliseen transitioon liittyvien integrin 4 beta, E-cadherin ja ZO-1 proteiinien proteiinitason ilmenemistä on tutkittu potilaiden syöpäkudosta sisältävien monikudosblokkien avulla. Näiden proteiinien ilmenemisprofiileita on verrattu potilaiden kliinispatologisiin tietoihin ja eloonjäämiseen.

Työn päätulokset osoittivat, että korkea ezrin-proteiinin ilmeneminen ja heikko tai puuttuva CDX2 proteiinin ilmeneminen ovat huonon ennusteen tekijöitä niillä potilailla, joiden kasvaimet ovat ns. mikrosatelliitti-stabiileja. Edelleen epiteeli-mesenkymaaliseen transitioon assosioituvan integrin 4 beta -proteiinin korkea ilmeneminen syövässä ns. syöpäsilmujen alueella on huonon ennusteen tekijä. Yhteenvetona voidaan todeta, että korkea ezrin-proteiinin ilmeneminen, CDX-2-proteiinin ilmenemisen vaimeneminen ja integrin 4 beta ovat uusia lupaavia levinneisyysasteen II paksu- ja peräsuolisyövän biomarkkereita.

AVAINSANAT: paksu- ja peräsuolisyöpä, biomarkkeri, eloonjäämisennuste, kudosmikrosiru

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Abbreviations

APC	Adenomatous polyposis coli
CI	Confidence interval
CIMP	CGP island methylator phenotype
CIN	Chromosomal instability
CkPan	Pan Cytokeratin
CMS	Consensus molecular subtype
CRC	Colorectal cancer
CRM	Circumferential margin
CT	Computed tomography
CT-Co	Computed tomographic colonoscopy
DAB	Diaminobenzidine
DCBE	Double-contrast barium enema
DFS	Disease-free survival
dMMR	Defective mismatch repair system
DSS	Disease-specific survival
EGFR	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ERM	Ezrin-radixin-moesin
FAP	Familial adenomatous polyposis
GPS	Glasgow prognostic score
H&E	Hematoxylin and eosin
HP	Hyperplastic polyp
HR	Hazard ratio
IAP	Inhibitor of apoptosis protein
IBD	Inflammatory bowel disease
IS	Immunoscore
ITBCC	International TB consensus conference
ITGB4	Integrin beta 4
ITGB4-high bud	Integrin beta 4 high budding
LGR5	Leucine-rich repeat-containing G-protein
LS	Lynch syndrome

MET	Mesenchymal-epithelial transition
MMR	Mismatch repair
mRNA	Messenger RNA
MSI	Microsatellite instability
MSI-H	MSI-high
MSS	Microsatellite stable
mTOR	Mammalian target of rapamycin
Multiplex IHC	Multiplex immunohistochemistry
NRL	Neutrophil-to-lymphocytic ratio
OS	Overall survival
PIK3	Phosphatidylinositol-3 kinase
PKA	Protein kinase
RFS	Relapse-free survival
SP	Serrated polyp
SSL	Sessile serrated lesion
TB	Tumour budding
TGFBRII	Transforming growth factor beta receptor II
TGFß	Transforming growth factor beta
TMA	Tissue microarray
TSA	Traditional serrated adenoma
TTR	Time to recurrence
VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis protein
WT	Wild type

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Slik K, Kurki S, Korpela T, Carpén O, Korkeila E, Sundström J. Ezrin expression combined with MSI status in prognostication of stage II colorectal cancer. PLOS ONE 2017 Sep 27;12(9):e0185436.

II Slik K, Blom S, Turkki R, Välimäki K, Kurki S, Mustonen H, Haglund C, Carpén O, Kallioniemi O, Korkeila E, Sundström J, Pellinen T. Combined epithelial marker analysis of tumour budding in stage II colorectal cancer. J Pathol Clin Res 2019;5(1):63–67.

III Slik K, Turkki R, Carpén O, Kurki S, Korkeila E, Sundström J, Pellinen T. CDX2 loss with microsatellite stable phenotype predicts poor clinical outcome in stage II colorectal carcinoma. Am J Surg Pathol 2019;43(11):1473–1482.

1 Introduction

In both sexes combined, colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide (Ferlay et al. 2018; Kuipers et al. 2015), and it ranks second in terms of mortality (Bray et al. 2018).

CRC is a disease that reflects socio-economic development (Bray et al. 2018). The incidence rates have continued to increase in economically transitioning countries, along with the consumption of high fat diet, physical inactivity and smoking (Mehta et al. 2017). CRC mortality rates have gradually decreased in highly developed countries with improvements in survival due to using modern surgical and oncological treatments and stage assessment (Bray et al. 2018).

The 5-year survival ranges from over 90 % for stage I, 82–87 % for stage II, 60 % for stage III and under 10% for stage IV CRC (White et al. 2018). In stage I–II disease, colonic resection is the optimal treatment, whereas some stage I cases can be treated with local polypectomy. In stage III disease and in high risk stage II disease, adjuvant chemotherapy is recommended after a colonic resection to reduce the risk of recurrence. (van de Velde et al. 2014). The treatment options for stage IV disease have evolved lately along with the possibility for biologic treatments with the anti-epidermal growth factor receptor (EGFR), the anti-vascular endothelial growth factor (VEGF) receptor targeted therapies, and the advanced surgical procedures for liver metastases (Lang et al. 2018). Stage II CRC patients are still a problematic group in selecting the optimal treatment, because the outcome of these patients is very variable (Amri et al. 2016). Relapses may occur in this group of patients despite the absence of any of the currently acknowledged risk factors: T4 disease, grade 3, preoperative obstruction, perforation, lymphovascular or perineural invasion, and less than 12 examined lymph nodes (Dotan & Cohen 2011). Consequently, these currently known risk factors lack sufficient accuracy in defining the recurrence risk in stage II CRC (Tsikitis et al. 2014). There should be more efficient tools in the identification of stage II CRC patients at risk of relapse, and new biomarkers are still needed.

Several studies have demonstrated that patients with microsatellite instability (MSI) in their tumours have better outcome in relation to time to recurrence (TTR), relapse free survival (RFS) and overall survival (OS) as compared to

patients with microsatellite stable (MSS) tumours, especially among stage II CRC patients (Amri et al. 2016; Merok et al. 2013). Tumour budding (TB) is defined as single tumour cells or cell clusters of up to four cells at the invasive margin of CRCs (Lugli et al. 2017). The evaluation of TB has been used as a tool to identify high risk stage II CRC patients, since high TB has been regarded as a marker of poor outcome in this group of CRC patients (Wang et al. 2009). There is evidence that TB is associated with KRAS mutation (G12D), BRAF-mutations, mismatch repair (MMR) proficient disease and lymphovascular invasion (Prall et al. 2007; Zlobec et al. 2012; Jang et al. 2017; Mitrovic et al. 2012). In addition, several studies suggest a connection between TB and epithelial-to-mesenchymal transition (EMT) (De Smedt et al. 2017), which may reveal new tools for patient prognostication. CDX2 is a marker which has been used in clinical pathology for a long time to identify the cancers of intestinal origin (Groisman et al. 2005). For some years ago it was found that CDX2–negative CRCs were associated with a lower rate of disease-free survival (DFS) than those that had preserved CDX2 protein expression (Dalerba et al. 2016). In addition, a strong association between negative CDX2 protein expression has been shown with MSI-high (MSI-H) phenotype as well as with BRAF mutation (Olssen et al. 2016; Zlobec et al. 2010; Landau et al. 2014). However, there are not many studies of the prognostic role of CDX2 loss specifically in stage II CRC.

In the current study, the combination of these recently acknowledged biomarkers have been studied in relation to survival with a cohort of stage II CRC patients treated at Turku University Hospital between the years 2005–2012.

2 Review of the Literature

2.1 Epidemiology of colorectal cancer

Globally, CRC is the third most commonly diagnosed malignancy in men and the second most commonly diagnosed malignancy in women (Ferlay et al. 2010). Its incidence is higher in many countries among men than among women (Torre et al. 2012). Globally, CRC incidence is variable with about 55% of cases occurring in developed countries, the highest incidence rates in Northern America, Europe, New Zealand and Australia. The areas with low incidence rates are in Africa, South – Central Asia and Central America (Favorti et al. 2016).

CRC affects mainly western people, but its incidence has started to rise in many countries with traditionally lower incidence rates as well (Marley & Nan 2016). European Union statistics of the year 2018 showed that the CRC accounts as a whole for the second highest number of cancer deaths, a total number of 177400, with rates of 15.8/100 000 among men and 9.2/100 000 among women. It ranks the second cause of cancer related deaths in men and the third cause of cancer related deaths in women (Ferlay et al. 2018).

It is estimated that CRC continues to increase worldwide because of the constant aging and growth of population as well as the spread of the western lifestyle in economically developing countries (Favoriti et al. 2016). Incidence, mortality, and survival rates vary by race and ethnicity (Jackson-Thomson et al. 2006). There are large differences in CRC survival rates globally according to stage at the time of diagnosis, which may be explained by the differences in the availability of diagnostic methods and the quality of treatments (Boyle & Langman 2000).

2.2 Etiology of colorectal cancer

CRC is a multi-factorial disease with a complicated environmental and genetic etiology (Aran et al. 2016). From its genomic properties, CRC cannot be regarded as a single disease but a variable group of malignant neoplasms of colonic origin (Testa et al. 2018). Most of the cases are sporadic and occur at 50 years of age or older. Some cases are familial or associated with inherited syndromes and present

with younger age (Emre et al. 2018). Altogether, 20–30% of CRCs have a familial trait and 5% of these tumours arise through Mendelian inheritance pattern (Testa et al. 2018).

2.2.1 Adenomatous or serrated polyps – precursors of colorectal cancer

Many CRC patients have a premalignant lesion before the development of carcinoma, most commonly an adenomatous polyp or less frequently a serrated polyp (SP) (Testa et al. 2018). The conventional adenomas and SPs are believed to arise from distinct pathways (He et al. 2018). These include adenoma-carcinoma pathway and the serrated pathway, which have their own genetic characteristics (Testa et al. 2018).

2.2.2 Chromosomal instability (CIN) and microsatellite instability (MSI) pathways

There are two main pathways involved in the development of CRC. These include CIN and MSI pathways with a progression to advanced adenomas and cancers. CIN is typical of adenoma-carcinoma-pathway, where adenomas develop through inactivation of the tumour suppressor gene adenomatous polyposis coli (APC) followed by the progression of the dysplastic adenoma gradually to adenocarcinoma (Shiller & Boostrom 2015). MSI pathway with a deficient MMR gene is typical of serrated pathway. This pathway is proposed to be the origin of hyperplastic polyps (HPs), which are hypothesized to transform to sessile serrated lesion (SSL) or traditional serrated adenoma (TSA) accompanied with progressive dysplastic changes and finally the development of invasive carcinoma (Bettington et al. 2013). In sporadic cancers with MSI, the MMR gene defect is linked with the epigenetic silencing of the MLH1 gene as a result of hypermethylation of the promoter region, which leads to accumulated mutations and inability to repair them leading ultimately to the development of cancer (Yearsley et al. 2006). These cancers typically also have CPG island hypermethylation and BRAF mutation (Bettington et al. 2013). Alternatively, the MMR gene deficiency can originate from germline mutation in one of the following genes: MLH1, PMS2, MSH2 or MSH6, which is the case in Lynch syndrome (Pochapin 2018).

2.2.3 Hereditary colorectal cancer

Also known by the name of hereditary non-polyposis CRC, Lynch syndrome (LS) is a genetic cancer syndrome with an autosomal dominant trait. It is a disease of

familial cancer aggregation associated with deficient MMR and an increased risk of CRC (Buchanan et al. 2010). Patients with LS have a MSI-H carcinoma because of germ line mutation in one of MMR genes: MLH1, PMS2, MSH2 or MSH6. (Yearsley et al. 2006), and the incidence of this syndrome is about 3 % (Win et al. 2017). It occurs at a younger age than sporadic CRC (Sinicrope 2018). LS also predisposes to several other cancers including endometrial adenocarcinoma, urothelial carcinoma, pancreatic cancer and gastric cancer (Latham et al. 2019). LS may show poor differentiation including mucinous features or a medullary growth pattern, right-side predominance, and abundance of lymphocytic infiltration as a reaction to neoantigens developed due to numerous mutations (Sinicrope 2018).

Familial adenomatous polyposis (FAP) is a familial disease associated with inherited mutations of the APC gene. It presents as a rather rare familial polyposis syndrome with hundreds or thousands of adenomas in colorectum. It inevitably leads to development of CRC at the age of 40 years at the latest. Attenuated familial adenomatous polyposis (AFAP), a milder form of FAP is characterized by fewer numbers adenomas and later onset of CRC than in FAP (Kantor et al. 2017). Juvenile polyposis and Peutz-Jeghers syndrome are rare diseases inherited as an autosomal dominant trait and associate with a high risk to develop CRC (Rosenthal et al. 2018). Furthermore, there are syndromes with increased risk of CRC associated with SPs (Buchanan et al. 2010).

2.2.4 Colorectal cancer related with inflammatory bowel disease

Crohn's disease and ulcerative colitis belong to inflammatory bowel diseases (IBDs). They are associated with an increased risk for CRC (Garg & Loftus 2016). This risk is related to the extent and duration of the disease (Huang & Merchea 2017).

Development of cancer in the colon with IBD is attributed to chronic inflammation and immune dysregulation. Chronic inflammation will induce malignant transformation of the colonic mucosa (Garg & Loftus 2016) through the generation of adverse factors causing inactivation of tumour-suppressor genes (e.g., P53 mutation), and activation of oncogenes (e.g., KRAS mutation). In addition, chronic inflammation activates the spread of inflammatory mediators including IL6, IL1b and TNFa. They activate NF-kB, which results in neoplastic transformation of the intestinal epithelium (Meng et al. 2017).

2.2.5 Colorectal cancer and environmental factors

Lifestyle and environmental factors play an important role in CRC. There are several studies which have shown that food with an abundance of meat and animal

fat can function as carcinogenesis promoters in CRC, while a diet rich in fish meat, fiber, vitamin D and calcium can protect from CRC (Marley et al. 2016). Some of these environmental factors have been observed to associate differently with SPs and conventional adenomas (He et al. 2018). A diet high in fat favors the increase of sulfate-reducing bacteria, Desulfovibrio vulgaris among others. These bacteria participate in the transformation of primary bile acids to tumourigenic metabolites, like deoxycolic acid, for instance. (Meng et al. 2018).

There is evidence that both alcohol and smoking are related with increased risk of CRC. Alcohol intake augments the probability of premalignant polyps and CRC in a dose-dependent manner (Fagunwa et al. 2017). Ethanol has been noticed to cause damage to intestinal epithelial cells by inducing DNA breaks. The main degradation product of ethanol, acetaldehyde, can cause degradation of folate, which may predispose to chromosomal damage (Fagunwa et al. 2017). The association between smoking and increased risk of CRC is related to carcinogens in tobacco, including polycyclic aromatic hydrocarbons (Durko et al. 2014). In addition, the cytochrome P450 system may potent the effect of these carcinogens and thus increase the probability of mutations in colonic epithelial cells, and the probability of key mutations for colorectal carcinogenesis, like those in KRAS and BRAF genes (Leufkens et al. 2011). Smoking has been reported to be linked more strongly with serrated pathway carcinogenesis than adenoma – carcinoma pathway (He et al. 2018).

The significance of infectious agents as carcinogenesis promoting factors ranks third after dietary factors and smoking (De Flora & La Maestra 2015). According to one study about in 15 % of newly diagnosed cancers worldwide infectious agents attribute to neoplastic transformation, such as hepatitis B virus infection (Plummer et al. 2016). In addition to directly carcinogenic infectious agents, microbial dysbiosis increases the susceptibility to cancer. This takes place through inducing inflammation and dysregulation of immunity, which can cause genetic instability and interference of pharmacodynamics regarding anticancer agents (Meng et al. 2018).

2.3 Diagnostics of colorectal cancer

Since among some of the patients the diagnosis is delayed which is associated with poor outcome, (Zarcos-Pedrinaci et al. 2018), screening may reduce these cases and offer an opportunity to detect and treat the disease at an early stage. (Chen et al. 2019). Progression of CRC from adenomatous polyps to invasive cancer takes many years (Brenner et al. 2013). Screening for CRC has been proved to be effective in reducing CRC incidence and mortality and is recommended from the age of 50 years and older. Fecal blood tests and endoscopy are mostly used for this

purpose (Sur et al. 2019). Clinical symptoms, especially a change in bowel habits and blood in the stool, should lead to suspicion of CRC (John et al. 2010). After anamnesis and clinical examination including digital rectal palpation, endoscopy should be made, if there is a clear suspicion of CRC. If an endoscopy is not possible, imaging tests should be done (Lopes et al. 2019). Double-contrast barium enema (DCBE), studying the colon using X-rays, was one of the most frequent past techniques, but it is currently rarely used partly because of high radiation burden (Neri et al. 2010). Computed tomographic colonoscopy (CT-Co) is a technique, where the colon is inflated with air, and the distended colon is studied with the aid of computed tomography. It is a safe procedure, which can detect extracolonic pathologic lesions in non- complaining patients (Mazeh et al. 2013). It has 66.8% of sensitivity and 80.3% of specificity diagnostic rates (Hadjipetrou et al. 2017). When the diagnosis of CRC has been confirmed, the whole body computed tomography (CT) is performed to exclude distant metastasis (Labianca et al. 2013). Pelvic magnetic resonance imaging (MRI) helps to determine the type of operation technique in rectal cancer cases (Glynne-Jones et al. 2017). Preoperative serum marker carcinoembryonal antigen (CEA) is determined as a baseline value for the follow-up of CRC patients (Labianca et al. 2013).

2.4 Histological classification and staging

Approximately 95% of CRCs are adenocarcinomas (Bosman et al. 2010). Of the CRCs that are adenocarcinomas, most are conventional adenocarcinomas with a variably amount of glandular formation. There are histological variants of colorectal adenocarcinoma. The most common of them include mucinous adenocarcinoma which is characterized by an abundant extracellular mucin production of > 50 % of tumour volume and signet ring cell carcinoma which is characterized by cancer cells with signet ring cells of > 50 % of tumour volume. Their prognosis is somewhat worse than conventional adenocarcinoma, but tumours of these histologic types with MSI-H behave as low-grade carcinomas (Fleming et al. 2012). Other histologic types include medullary carcinoma and serrated carcinoma. The former is characterized by a favorable prognosis in spite of poorly differentiated histology and often with lymphocytic infiltration (Pyo et al. 2016). The latter is often associated with CPG island methylator phenotype (CIMP) and BRAF mutation with serrated histological features (Bettington et al. 2017). T-stage describes the extent of the tumour on/through the colonic wall (T1-T4), N-stage the absence/presence as well as the number of metastatic lymph nodes (N0-N2b) and the M-stage the presence or absence of distant metastases, as described in Table 1. Staging of CRC utilizes TNM8 classification currently (Table 1).

Table 1. TNM Classification of colorectal cancer, 8th edition.*

T-Primary Tumour		**N-regional Lymph nodes**	
Tx	Primary tumour cannot be assessed	**Nx**	Regional lymph nodes cannot be assessed
T0	No evidence of primary tumour	**N0**	No regional lymph node metastasis
Tis[1]	Carcinoma in situ: intraepithelial or invasion of lamina propria	**N1**	Metastasis in 1-3 regional lymph nodes **N1a** Metastasis in 1 regional lymph node **N1b** Metastasis in 2-3 regional lymph nodes **N1c** Tumour deposit(s), i.e. satellites*, in the subserosa, or in non-peritonealized pericolic or perirectal soft tissue *without* regional lymph node metastasis
		N2	Metastasis in 4 or more regional lymph nodes **N2a** Metastasis in 4-6 regional lymph nodes **N2b** Metastasis in 7 or more regional lymph nodes
T1	Tumour invades submucosa		
T2	Tumour invades muscularis propria		
T3	Tumour invades subserosa or into the non-peritonealized pericolic or perirectal tissues		
T4	Tumour directly invades other organs or structures and/or perforates visceral peritoneum **T4a** Tumour perforates visceral peritoneum **T4b** Tumour directly invades other organs or structures[2,3]		
	Notes: 1. Tis includes cancer cells confined within the glandular basement membrane (intraepithelial) or mucosal lamina propria (intramucosal) with no extension through the muscularis mucosae into the submucosa 2. Direct invasion in T4b includes invasion of other organs or segments of the colorectum by way of the serosa, as confirmed on microscopic examination or for tumours in a retroperitoneal or subperitoneal location, direct invasion of other organs or structure by virtue of extension beyond the muscularis propria. 3. Tumour that is adherent to other organs or structures, macroscopically, is classified cT4b. However, if no tumour is present in the adhesion, microscopically, the classification should be pT1-3, depending on the anatomical depth of wall invasion.		Notes: *Tumour deposits (satellites), i.e. macroscopic or microscopic nodules of cancer in the pericolorectal adipose tissue's lymph drainage area of a primary carcinoma that are discontinuous from the primary and without histological evidence of residual lymph node or identifiable vascular or neural structures. If a vessel wall is identifiable on H&E, elastic or other stains, it should be classified as venous invasion (V1/2) or lymphatic invasion (L1). Similarly, if neural structures are identifiable, the lesion should be classified as perineural invasion (Pn1). The presence of tumour deposits does not change the primary tumour T category, but changes the node status (N) to pN1c if all lymph nodes are negative on pathological examination.
			M – Distant Metastasis **Mx** Distant metastasis cannot be assessed **M0** No distant metastasis **M1** Distant metastasis **M1a** Metastasis confined to one organ or site (eg. liver, lung, ovary, non-regional nodes) without peritoneal metastasis **M1b** Metastases in more than one organ **M1c** Metastasis to the peritoneum with or without other organ involvement.

*According to Brierley JD, Gospodarowicz MK, Wittekind C (eds). (2017). TNM Classification of Malignant Tumours (Eighth Edition), UICC, John Wiley & Sons, Ltd, pp. 73–76. Reprinted with permission from John Wiley & Sons Limited to be used in Khadija M Slik's thesis.

2.5 Treatment of colorectal cancer

High quality surgery is the most crucial step of treatment (Lee et al. 2017). Surgical resection (colectomy) is the main treatment modality for patients with stage I and II CRC. For high risk stage II CRC patients and for those with stage III disease, adjuvant chemotherapy is given after surgery to decrease the risk of recurrence (van de Velde et al. 2014).

Short-course preoperative radiotherapy is considered for rectal cancer patients with a suspicion of lymph node metastasis and long-course chemoradiotherapy for patients with T4 tumours or tumours with a suspicion of involved circumferential margin (CRM) (Glynne-Jones et al. 2017). Therapeutic modalities for stage IV patients include surgery, chemotherapy and radiotherapy. At the time of diagnosis, approximately 25 % of patients are diagnosed with liver metastases. When the recurrent disease is included, approximately 50 % of patients end up having a metastatic disease. (Cook et al. 2005). The combination of chemotherapy, targeted treatments with anti-EGFR and anti-VEGF monoclonal antibodies, surgery, interventional radiology and radiotherapy have even brought a possibility of curative outcome for selected stage IV CRC patients with lung or liver metastases (Van Cutsem et al. 2016). Finally, palliative systemic chemotherapy with or without bevacizumab or anti-EGFR treatment can be given to patients with unresectable disease, in order to improve quality of life and to prolong survival (Van Cutsem et al. 2016).

2.6 Prognosis of colorectal cancer

The 5-year survival rate of CRC in Finland is 63 % (Finnish Cancer Registry 2016). The prognosis depends on the stage of cancer at the time of diagnosis. During the last decades the treatment and diagnostics of CRC have developed considerably with improvement of survival (Heervä et al. 2018). Patients with stage I CRC have excellent prognosis and their 5-year survival is more than 90 %. Recurrence after surgery for 5–10 % of stage I CRC patients means a worse outcome (Patel et al. 2014). For stage II CRC patients, the 5-year survival is 70–80 % (Sato et al. 2011), and that for subgroup of stage III disease only 40–45 % (Walkers et al. 2018). Moreover, the prognosis of metastasized CRC has improved mostly because of active surgery as well as due to new targeted treatments with anti-EGFR and anti-VEGF monoclonal antibodies. Even if the average 5-year survival rates of stage IV is about 10 % (Siegel et al. 2012), the 5-year survival of selected stage IV patients with combination treatments has markedly improved (Chakedis & Schmidt 2018).

2.7 Established and provisional prognostic factors of stage II colorectal cancer

The stage of CRC at the time of diagnosis is the most important prognostic factor. However, the heterogeneity of outcome especially among stage II CRC patients causes challenges in selecting optimal treatment. The presently known high risk factors for stage II CRC include lymphovascular invasion, involved circumferential margin, poor differentiation grade, perineural invasion, preoperative obstruction, perforation and less than 12 examined lymph nodes (Wibe et al, 2002; Dotan & Cohen 2011). Though, these currently known risk factors lack sufficient accuracy for stage II CRC patients at risk of recurrence (Tsikitis et al. 2014). During the last few years new biomarkers for this purpose have established their position. These include especially MSI-status and tumour budding (TB).

2.7.1 MSI

The MSI-H tumours have a defective mismatch repair system (dMMR) because of inactivation of one of the MMR-genes, either because of epigenetic mechanism or germline mutation (Nojadeh et al. 2018). The typical features of MSI-H CRCs include the location in proximal colon, poor differentiation grade, considerable lymphocytic infiltration, mucinous histological type and mutations in transforming growth factor beta receptor II (TGFBR II) and BRAF-genes (Hyde et al. 2010). About 15 % of CRC patients have been found to have MSI-H phenotype, while the majority of CRCs are MSS tumours (Leicher et al. 2018; Puccini et al. 2017). While the prognosis of patients with MSS CRC is stage and grade dependent, it has been observed that especially in early stages MSI-H tumours have more favorable prognosis than MSS tumours of the same stage (Halpern et al. 2017). Still, MSI-H stage II CRC patients have been found not to benefit from adjuvant 5-FU based chemotherapy (Sargent et al. 2010). MSI-testing aids in selecting adjuvant treatments for stage II CRC patients, since patients diagnosed with MSI-H tumours can be excluded from those in potential need of adjuvant chemotherapy (Copija et al. 2017). Stage IV disease rarely presents with MSI-H phenotype (Koopman et al. 2009). However, if this happens, MSI-H stage IV CRC patients can be candidates for treatment with immune check-point inhibitors, and promising results have been seen in preliminary treatment trials (Gourd 2018).

2.7.2 Tumour budding and related markers

In CRC, the invasive tumour margin morphology may give a clue to aggressiveness of cancer, and it may play a role in predicting the risk of relapse (Karamitopoulou et al. 2015). Tumour budding (TB) refers to single cancer cells or

clusters up to four cancer cells apart in stroma usually at the invasive margin (Ueno et al. 2002). There is evidence that TB is linked with EMT, a phenomenon associated with the metastasizing process (Grigore et al. 2016). In several publications, TB has been reported to be associated with a high TNM stage, high tumour grade, and presence of local and distant metastasis (Rogers et al. 2016). Specifically, it is considered to be an independent predictor of survival in stage II CRC, and patients of stage II CRC with high grade TB should be considered to receive adjuvant chemotherapy (van Wyk et al. 2015).

According to International Tumour Budding Consensus Conference (ITBCC) guidelines TB is assessed by selecting the hematoxylin eosin (H&E) slide with highest amount of TB at the invasive front, the tumour buds are counted from one hot spot within 20x objective field, and finally the numerical value is adjusted with a correction coefficient to end up in a field of 0.785 mm2 (Lugli et al. 2017). Even if the analysis is recommended to be done from H&E slides, cytokeratin immunostaining aided method can be used in challenging cases like those with an abundant disturbing inflammatory reaction in the invasive front (Koelzer et al. 2016; Lugli et al. 2017; Mehta et al. 2018).

EMT is a complex process, during which the epithelial cells acquire features that are typical of mesenchymal cells, and EMT is seen in both normal physiological and pathological processes like in cancer (Nakaya et al. 2013). In CRC, it has been associated with increased invasiveness and metastasizing potential, cancer progression, and treatment resistance (Vu and Datta 2017, Bhangu et al. 2012). During EMT the membranous E-cadherin of cancer cells is down-regulated accompanied by loss of intercellular epithelial junctional complexes (Chand et al. 2018). In addition, several other genes with a fundamental role in EMT are known (Figure 1.). There are several reports, which show EMT to be involved in TB (Grigore et al. 2016). Since TB is of clinical significance even in stage II CRC, markers of EMT may have an increasing prognostic role in CRC even in the near future. This concerns especially integrin beta 4 (ITGB4), which we propose to be a potential surrogate marker of TB in cases where tissue tumour material is too scarce for conventional TB analysis (Original publication II).

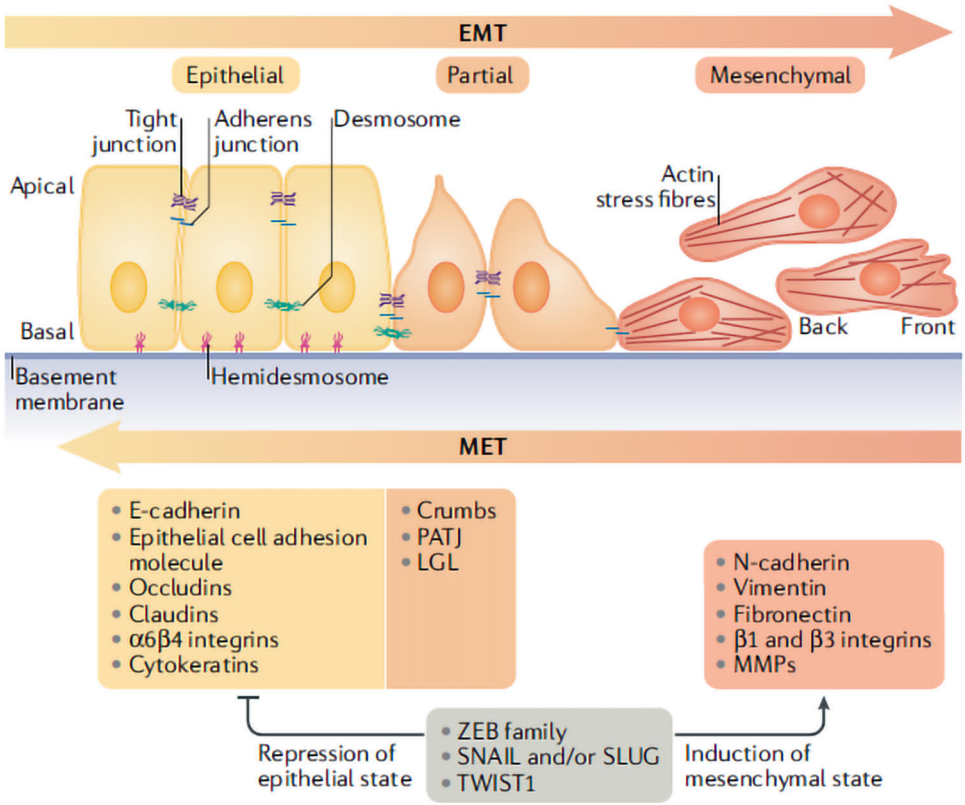

Figure 1. Molecular biological changes in cells during EMT. There is progressive loss of epithelial features and gain of mesenchymal features. The differentiation of cancer cells with mesenchymal features back to epithelial cells, mesenchymal-epithelial transition (MET) is a less known phenomenon. Printed from Dongre A, Weinberg RA. (2019) New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer. Nat Rev Mol Cell Biol 20(2): p. 70. Reprinted with permission of Springer Nature to be used in Khadija M Slik's thesis.

2.7.3 Ezrin

Ezrin belongs to Ezrin, Radixin, and Moesin (ERM) complex, and it combines the actin cytoskeleton to a variety of membrane-bound receptors and adhesion molecules (Neich et al. 2011). It participates to cell migration, proliferation, survival, and signal transduction including mammalian target of rapamycin (mTOR), phosphatidylinositol-3 kinase (PIK3K)/Akt, Src, EGFR, Rho-kinase, and protein kinase C pathways (Brambilla et al. 2009). In malignant cells it has a role in motility, invasion, metastatic potential and survival of malignant cells through the counteracting of inhibitors of apoptosis proteins (IAPs), X-linked inhibitor of apoptosis protein (XIAP) and survivin (Leiphrakpam et al. 2018). High Ezrin protein expression is associated with poor survival in several cancers including

CRC (Li et al. 2015; Elzagheid et al. 2008; Mori et al. 2017; Original publication I). In CRC, ezrin protein expression has been associated with grade, stage, lymph node and distant metastasis, and survival (Liang et al. 2017).

2.7.4 CDX2

Caudal-related homeobox transcription factor 2 (CDX2) encoded by CDX2 gene, is vital for intestinal development and differentiation (Olsen et al. 2014; Verzi et al. 2011). Positive nuclear staining of CDX2 in carcinomas refers to their possible origin from gastrointestinal tract (De Lott et al. 2005). That is why this antibody can be used in clinical pathology marker panels for carcinomas of unknown primary (Varadhachary et al. 2008). There are some rather convincing reports concerning the association of CDX2 loss and poor prognosis in colorectal cancer (Dalerba et al. 2016). However, the prognostic significance of CDX2 staining pattern has not yet been specifically studied in stage II colorectal cancer.

2.7.5 Others

The sidedness of CRC affects its presentation and clinical outcome (Lee et al. 2015). The right-sided colon cancers manifest with less and more obscure symptoms than left-sided colon cancer, which may cause the diagnosis of those with right-sided cancer to be made at a more advanced stage (Nawa et al. 2008). There are significant differences in expression profiles of more than 1000 genes between the right and left-sided colon (Glebov et al. 2003). Of environmental risk factors, high meat consumption increases the risk of left-sided colon cancer more than that of right-sided (Larsson et al. 2005). Right-sided colon cancer has inferior prognosis compared with left-sided colon cancers, which may be related with histopathological factors, including poor differentiation grade, molecular genetic factors, and difficulties to diagnose right-sided cancer at an early stage (Nitsche et al. 2016; Baran et al. 2018). In stage II CRC, the right-sided cancer is associated with favorable prognosis, while after disease recurrence it is a sign of poor prognosis (Kennecke et al. 2018). This may be associated with overrepresentation of MSI-H tumours on the right side, which are known to behave in an indolent way in early stages (Battaglin et al. 2018).

Consensus Molecular Subtypes (CMS) refers to sorting of CRCs to four groups according to their molecular pathological features (Fontana et al. 2019). These four CMS groups include: CMS1 (MSI-immune, 14 %) hypermutated cancers, which are characterized by MSI-H phenotype, CIMP-high and BRAF-mutation; CMS2 (canonical, 37 %) cancers are characterized by high number of somatic gene copy number alterations, activation in WNT and MYC; CMS3 (metabolic, 13 %)

cancers often carry KRAS-mutation and metabolic deregulation; CMS4 (mesenchymal, 23 %) cancers have high number of somatic gene copy alterations, transforming growth factor beta (TGF-β) activation and EMT. In addition, there is an unclassified mixed group (13 %). (Müller et al. 2016). In stage II CRC, CMS4 cancers carry an unfavorable prognosis, since they have a high rate of TB (Trinh et al. 2018).

Some gene panels have been made to identify CRC patients with poor prognosis, especially to recognize high risk stage II CRC patients in need of adjuvant chemotherapy. ColonPrint uses an 18-gene assay (Salazar et al. 2011), and Oncotype DX includes seven cancer-related genes (O'Connell et al. 2010). In one more recent study using an expression profile of 120 genes (Lin et al. 2017), the outcome of stage I-II CRC patients could be predicted with high efficacy. However, only recently the significance of gene panels has gradually been increasing in clinical practice. Along with the increasing importance of precision oncology, especially next-generation-sequencing (NGS) has identified new potential treatment targets of CRC, which could help to improve disease outcome, especially among patients with stage IV disease (Rachiglio et al. 2019). Also, in differential diagnosis of poorly differentiated malignant neoplasms, cancer gene panes may be useful (Ericson-Lindquist et al. 2017).

Cancer stem cells and EMT are both thought to be related with chemo resistance in CRC (Boesch et al. 2018). Cancer stem cells are a minority of tumour cells, which are able to self-renew and differentiate (Clarke et al. 2006). Cancer stem cells have also been observed to be more chemo- and radioresistant than other cells of the tumour (Makena et al. 2018). CRC stem cells can be identified with their cell surface molecules: CD44, CD133, CD166, leucine-rich repeat-containing G-protein (Lgr5) and aldehyde dehydrogenase 1 (ALDH1) (Zhou et al. 2017). Although there are some reports of using these markers in the prognostic assessment of even early CRC (Avoranta et al. 2013), they have not been established yet in clinical use.

MicroRNAs are small non-coding RNA molecules, which have a role in the regulation of gene expression (Bartel 2004). There are several reports showing that dysregulation of microRNAs has a crucial role in neoplastic transformation, cancer progression and invasion including CRC (Chen et al. 2016). A combination of selected microRNAs has shown its ability to detect even early-stage CRC (Guo et al. 2018). However, it is not easy to transfer their use to a clinical practice. For instance, there are several interfering tissue sources for microRNAs, which deteriorate their reliability as a tool for clinical cancer prognosis setting (Chen et al. 2019).

There is evidence that the host immune response plays a role in the outcome of cancer patients (Lu et al. 2006). Specifically, activation of systemic inflammatory

response has been linked with tumour progression, regardless of TNM-class (Roxburg & McMillan 2014). To evaluate this inflammatory response some simple and easily reproducible methods have been developed, such as Glasgow prognostic score (GPS). This score is estimated according to serum CRP and plasma albumin levels (McMillan 2013). According to one study with stage II CRC patient cohort, DSS was worse among patients with GPS 2, as compared to those who had GPS 0 or 1 (Sugimoto et al. 2012). Also, a meta-analysis supports the significance of GPS for survival of CRC (Lu et al. 2018). Also, neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PRL) have been shown to correlate with survival in CRC (Kwon et al. 2012). The host immune response in cancer patients can also be studied using paraffin-embedded tumorous tissue material. Among one of the most applied methods for this is the Immunoscore (IS) system, which utilizes tumour infiltrating lymphocytes (Galon et al. 2006). More specifically, the density of CD3+ and CD8+ tumour infiltrating lymphocytes are evaluated in tumour center and tumour invasive front areas (Galon et al. 2014). There are several studies that support the ability of Immunoscore to predict the outcome in colorectal cancer diagnosed at any stage (Sun et al. 2018), and this result has also been verified in a large international consortium study (Pagès et al. 2018).

3 Aims

The current means to predict the behavior of stage II CRC are insufficient. A significant proportion of relapses are diagnosed in stage II patients without any of the established high risk factors including lymphovascular invasion, poor differentiation grade, emergency operation, obstruction, perforation and less than 12 examined lymph nodes (Dotan & Cohen 2011; Liebig et al. 2009; Labianca et al. 2013; O'Connor et al. 2011). During the last few years some new biomarkers have been introduced, namely evaluation of MSI status and TB (Ribic et al. 2003; Siniscrope et al. 2011; Mitrovic et al. 2012; Lugli et al. 2017). Although they help to identify some additional stage II CRC patients, who could benefit from adjuvant therapy, new biomarkers are still required to further improve patient selection for those, who could get the best advantage from postoperative adjuvant treatments. The focus of this study is to evaluate new promising biomarkers for this purpose. In this study we have evaluated biomarkers related with epithelial integrity (ezrin and CDX2), TB and a phenomenon related to it: epithelial to mesenchymal transition. We have correlated our results in relation to clinicopathological variables including MSI status and BRAF-mutation status.

The specific aims of the study with materials of stage II colorectal cancer patients are:

1) To analyze ezrin protein expression with tissue microarray (TMA) and to correlate the results with MSI and BRAF mutation status and clinicopathological variables
2) To compare the prognostic efficacy of TB analyzed visually from routine stained (HE) whole slides to that of selected EMT markers analyzed from TMA slides with image analysis.
3) To estimate the ability of CDX2 protein expression profile to predict patient outcome.

4 Patients and Methods

4.1 Patients

This retrospective study included histological material from two patient cohorts (Table 2). All studies in this thesis work (I–III) included tissue material from 232 stage II CRC patients treated at Turku University Hospital between the years 2005–2012. One study (II) also included material from 72 consecutive stage II (Dukes B) CRC patients treated at Helsinki University Hospital between the years 1998–2000. The use of paraffin-embedded material from these patients was approved by National Supervisory Authority of Welfare and Health (Valvira Dnro 4423/32/300/02 and 10041/06.01.03.01/2012). For the cohort of patients from Turku permission from Turku University Hospital was approved (T52/2014), and the use of tissue material was also approved by Scientific Steering Group of Auria Biobank (AB15-8108, 25.5.2015). For Helsinki cohort a permission from local ethical committee (Dnro HUS 226/E6/06, extension TMK02 §66 17.4.2013) was approved. To exclude distant metastasis, CT of the abdomen and chest x-ray or whole-body CT were preoperatively performed. The patient files have been carefully checked, including surgery and pathology reports. Patients with lymph node or distant metastases were excluded as well as those who had been operated with palliative-intent surgery and patients with other than adenocarcinoma histology. TNM7 classification of malignant tumours was used for staging, as this staging was in use at that time, Table 3.

Table 2. Clinical characteristics of the patients in the original publications.

	Cohort of Turku University Hospital n = 232 (I–III)		**Cohort of Helsinki University Hospital n = 72 (II)**	
5-year OS		80.1 %		66.70 %
5-year DFS		86.9 %		
5-year DSS		91.1 %		80.60 %
Age				
Median		74		72
Range		34–96		35–94
	≤70	92	≤65	24
	>70	140	>65	48
Gender				
Female		117		29
Male		115		43
Tumour side				
Right		112		20
Left		12		52
pT-status				
T3N0		190	**Duke's classification**	Dukes B
T4aN0		21		
T4bN0		21		
Grade				
G1		21	G1-2	58
G2		154		
G3		51	G3	13
n.d.		1	n.d.	1
Histology				
Conventional		205		68
Mucinous		26		4
n.d.		1		
Preoperative obstruction				
No		196		
Yes		36		
Tumour perforation				
No		212		
Yes		19		
n.d.		1		
Radicality				
R0		214		27
R1		15		6
R2		3		0
n.d.				39
MSI status				
MSS		171		
MSI-H		43		
n.d.		18		
BRAF status				
WT		183		
V600E		28		
n.d.		21		

N.d., not determined. Modified from original publication II, p. 65 and supplementary material of original publication II, p. 14. Creative Commons license, authors are the copyright holders.

Table 3. TNM classification of colon and rectum carcinoma used in the original publications, 7th edition.

T-Primary Tumour		**N-regional Lymph nodes**	
Tx	Primary tumour cannot be assessed	**Nx**	Regional lymph nodes cannot be assessed
T0	No evidence of primary tumour	**N0**	No regional lymph node metastasis
Tis[1]	Carcinoma in situ: intraepithelial or invasion of lamina propria	**N1**	Metastasis in 1–3 regional lymph nodes **N1a** Metastasis in 1 regional lymph node **N1b** Metastasis in 2–3 regional lymph nodes **N1c** Tumour deposit(s), i.e. satellites*, in the subserosa, or in non-peritonealized pericolic or perirectal soft tissue *without* regional lymph node metastasis
		N2	Metastasis in 4 or more regional lymph nodes **N2a** Metastasis in 4–6 regional lymph nodes **N2b** Metastasis in 7 or more regional lymph nodes
T1	Tumour invades submucosa		
T2	Tumour invades muscularis propria		
T3	Tumour invades subserosa or into the non-peritonealized pericolic or perirectal tissues		
T4	Tumour directly invades other organs or structures and/or perforates visceral peritoneum **T4a** Tumour perforates visceral peritoneum **T4b** Tumour directly invades other organs or structures[2,3]		
	Notes:		Notes:
1.	Tis includes cancer cells confined within the glandular basement membrane (intraepithelial) or mucosal lamina propria (intramucosal) with no extension through the muscularis mucosae into the submucosa.		*Tumour deposits (satellites), i.e. macroscopic or microscopic nests or nodules, in the pericolorectal adipose tissue's lymph drainage area of a primary carcinoma without histological evidence of residual lymph node in the nodule, may represent discontinuous spread, venous invasion with extracellular spread /V1/2) or a totally displaced lymph node (N1/2). If such deposits are observed with lesions that would otherwise be classified as T1 or T2, then the classification is not changed, but the nodule(s) is recorded as N1c. If a nodule is considered by the pathologist to be totally replaced lymph node (generally having a smooth contour), it should be recorded as a positive lymph node and not as a satellite, and each nodule should be counted separately as a lymph node in the final pN determination.
2.	Direct invasion in T4b includes invasion of other organs or segments of the colorectum by way of the serosa, as confirmed on microscopic examination or for tumours in a retroperitoneal or subperitoneal location, direct invasion of other organs or structure by virtue of extension beyond the muscularis propria.		
3.	Tumour that is adherent to other organs or structures, macroscopically, is classified cT4b. However, if no tumour is present in the adhesion, microscopically, the classification should be pT1–3, depending on the anatomical depth of wall invasion.		
			M – Distant Metastasis **Mx** Distant metastasis cannot be assessed **M0** No distant metastasis **M1** Distant metastasis **M1a** Metastasis confined to one organ or site (eg. liver, lung, ovary, non-regional nodes) **M1b** Metastases in more than one organ/site or the peritoneum

*According to Sobin L, Gospodarowicz MK, Wittekind C (eds). The TNM Classification of Malignant Tumours, 7th Edition. Singapore: Wiley-Blackwell; 2010, pp. 102–103. Reprinted with permission from John Wiley & Sons Limited to be used in Khadija M Slik's thesis.

4.2 Methods

4.2.1 Constructing of tissue microarray (I–III)

For TMA the next-generation TMA technique was used (Zlobec et al. 2013). Shortly, the suitable paraffin blocks with tumorous tissue were selected from each case and H&E sections of them were cut. The slides were scanned (Pannoramic P250, 3DHistech) and saved into the university digital microscopy server (casecenter.utu.fi). To analyze them, Pannoramic Viewer software (3DHistech) was used. With the 1.2 mm diameter annotation tool, circles of different colours corresponding to center of tumour, front of tumour and normal colonic epithelium were done. The corresponding tissue cores were shifted to the TMA blocks with automated TMA machinery (TMA Grandmaster, 3DHistech). The completed TMA blocks were cut, stained, scanned and moved into the server (casecenter.utu.fi).

4.2.2 Primary antibodies and staining methodology (I–III)

The sections of 3,5 μm thickness were made from TMAs. They were subjected to concerned primary antibodies (Table 4). BenchMark XT (Ventana/Roche) immunoautomate was used with UltraView Universal diaminobenzidine (DAB) Detection kit or OptiView Universal DAB Detection kit. For ezrin staining LabVision immunoautomate (Thermo Fisher Scientific) was used with the Power Vision Plus poly HRP antimouse/rabbit/rat IgG detection kit.

Table 4. Primary antibodies used in the thesis

Study	Antigen target	Clones	Dilution	Provider
I	MLH1	G168-15BD	1:5	Pharmingen
	MSH2	G219-1129	1:200	Pharmingen
	MSH6	EP49	1:200	Epitomoc
	PMS2	EPR3947	Ready to use	Ventana/Roche
	BRAF V600E	VE1	Ready to use	Ventana/Roche
	Ezrin	3C12	0.3mg/mL	Böhling *et al.* 1996
II	Pancytokeratin	C-11, ab7753	1:1500	Thermo Fisher Scientific
		AE1/3, MA5-13156	1:1000	Thermo Fischer Scientific
	ZO-1	D7D12	1:500	CST
	Integrin 4ß	D8P63	1:100	CST
	E-cadherin	36	1:200	BD
III	CDX2	EPR2764Y	Ready to use	Roche Diagnostics

CST, Cell Signaling Technology; BD, BD Bioscience

In thesis work II the primary antibodies of multiplex IHC were detected using tyramide signal amplification (TSA): AlexaFuor488 for Pancytokeratin antibodies, AlexaFluor555 for ZO-1 antibody and AlexaFuor 647 for Integrin ß4 and E-cadherin antibodies (Thermo Fisher Scientific, Invitrogen). The staining methodology has been presented in detail in the original publications (I–III).

4.2.3 Evaluation of immunohistochemical stainings (I–III)

MLH1, MSH2, MSH6, PMS2 and BRAF-mutation stainings were assessed dichotomously as positive or negative. Positive nuclear reaction in all four MSI immunohistochemical stainings refers to MSS phenotype, while the following patterns refer to MSI high phenotype: negative for MLH1/PMS2, negative for MSH2/MSH6, negative for MSH6 and negative for PMS2 (Overbeek et al. 2008). For BRAF V600E mutation staining, a cytoplasmic reaction was recognized. For ezrin staining, bulk and front cores were evaluated separately and the strongest staining was considered. Four staining categories were recognized: negative, weak positive, moderate positive and strong positive, and for statistical purposes also dichotomous categories, negative / weak and moderate / strong, were used. CDX2 was first assessed in four categories from each spot: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). Thereafter, mean values were counted from staining intensities in replicate two spots (front and bulk cores were evaluated separately) as follows: 0–0.5 = 0 (negative), 1–1.5 (weak) and 2–3 = 2 (conventional). For statistical purposes, dichotomous categories of CDX2 loss (negative / weak) and conventional were used. For E-cadherin, ZO-1, ITGB4 and Pancytokeratin: digital image analysis was used (see 4.2.4). The immunohistochemical slides were reviewed by 2 independent evaluators, and consensus formed from discrepant cases.

4.2.4 Visual TB analysis (II)

TB was evaluated according to the instructions of the ITBCC 2016. Shortly, 1–4 buds in hot spot of 20x objective field (0.792 mm2) corresponds to bd1, 5-9 buds to bd2 and 10 or more buds to bd 3. (Lugli et al. 2017). TB was analyzed visually from H&E stained whole sections and TMAs, and also from CkPan stained TMAs.

4.2.5 Digital image analysis (II)

TB was digitally analyzed from TMAs stained with multiplex IHC including EMT markers E-cadherin, ITGB4, ZO-1, and pan-cytokeratin. The multiplex IHC was done as previously described in Blom et al. (2017) using fluorescently labelled

secondary antibodies. Five-channel fluorescent images were generated with the Metafer 5 scanning and imaging system (MetaSystems, Germany), with a ×20 objective (NA 0.8). TIFF images were downscaled to 1:4 from the original resolution for image analysis (final resolution 0.88 μm/pixel), and cell image analysis software was used (CellProfiler version 2.2.0; Carpenter et al. 2006). Its main phases include: (1) spot perception, (2) epithelial cluster and bud perception, (3) determination of channel intensities, and (4) data export (Figure 2). The image analysis protocol has been presented in detail in original publication (II).

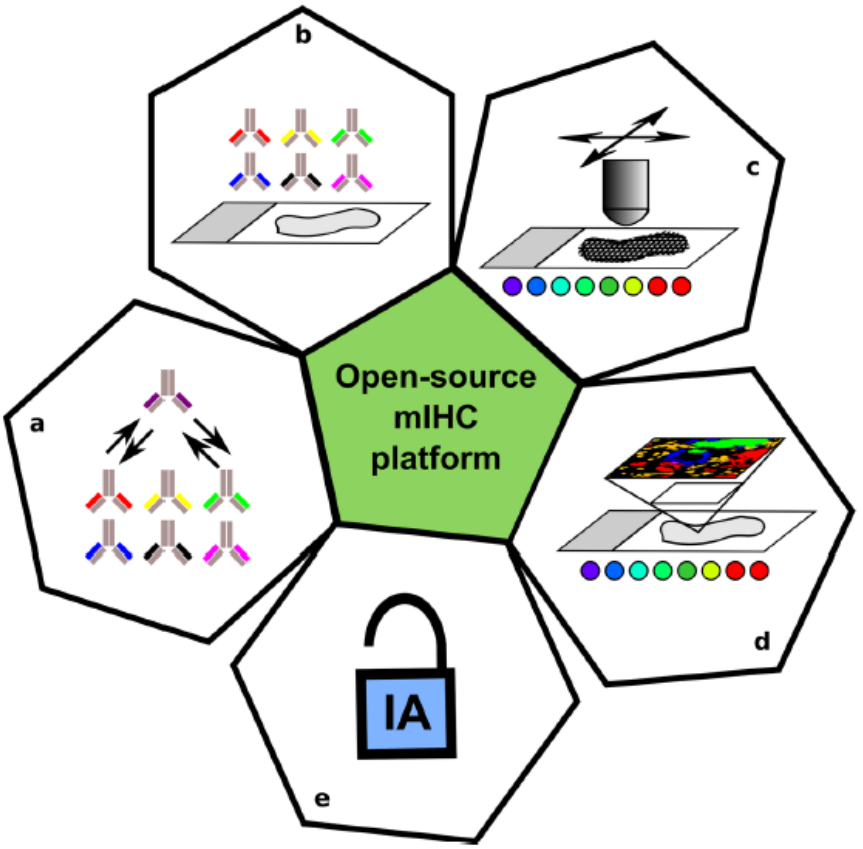

Figure 2. The multiplex IHC platform used in the original publication II includes easy selection of the relevant antibodies (a), multiplex ICH assay, (b), high-resolution whole-slide image scanning, and high-resolution whole-slide image analysis of open source (c-d). Blom S, Paavolainen L, Bychkov D, et al. Systems pathology by multiplexed immunohistochemistry and whole-slide digital image analysis. Sci Rep 2017; 7: 15580, p. 7. Reprinted with Creative Commons license: http://creativecommons.org/licenses/by/4.0.

4.2.6 Statistical analysis (I–III)

Statistical analyses were made with IBM SPSS version 23-24 with standard packages (SPSS Inc., Armonk, NY, USA). Normality of the data was assessed with the Kolmogorov–Smirnov test. The correlations between immunohistochemical staining and clinicopathological variables were analyzed with $\chi 2$ or Fisher's exact test for discrete variables and one-way ANOVA for continuous variables. In the original publication II correlations were evaluated with the non-parametric two-tailed Spearman rho test, and p values for mean and median comparisons were analyzed with either the Student's t test (normal distribution) or the Mann–Whitney U test (non-normal distribution). The Cox proportional hazard regression model and the Kaplan-Meier analysis with log-rank test for survival analysis were made with the aid of R version 3.4.3 (Foundation for Statistical Computing, Vienna, Austria) and RStudio 1.1.383 (RStudio Inc, MA, Boston, USA) with survival package 2.41-3. The methodology of statistical analyses has been presented in detail in original publications I-III.

5 Results

5.1 Ezrin in relation to clinicopathological characteristics and survival in stage II colorectal cancer (I)

High ezrin protein expression was more often seen in tumours with MSI-H phenotype than that with MSS tumours. In addition, high ezrin expression correlated with short disease-specific survival (DSS) (Table 5.). The ability of ezrin expression to identify high risk patients was strengthened by combining it to MSI-status. Specifically, when considering 5-year DSS time, patients with low ezrin expression and MSI-H had better survival when compared to patients with high ezrin expression and MSS (11/18 (61 %) vs. 4/18 (21 %), p = 0.040, Fisher's exact test). In Kaplan-Meyer survival analysis the patients, who had low ezrin expression and MSI-H phenotype had the longest DSS and those with high ezrin expression and MSS phenotype the shortest (p = 0.007, log-rank test, Figure 3).

In multivariate analysis, high ezrin expression with MSS phenotype, as well as tumour perforation were independent factors of poor DSS (Table 6.). Independent factors of poor overall survival included tumours with T4bN0, perforation and BRAF-mutation (Cox model, hazard ratio (HR) 2.86, 95 % confidence interval (CI): 1.06–7.74, p = 0.038; HR 3.8, 95 % CI: 1.57–9.17, p = 0.003; and HR 3.29, 95 % CI: 1.14-9.54, p= 0.028), respectively. Independent factors of poor DFS included tumours with T4bN0, vascular invasion and perforation (Cox model, HR 8.05, 95 % CI: 2.31–28.01, p = 0.001; HR 3.62, 95 % CI: 1.26–10.37, p = 0.017; and HR 4.87, 95 % CI: 1.38–17.23, p = 0.014), respectively. The results have been presented in more detail in the original publication (I).

Table 5. Ezrin expression in relation to MSI- and BRAF mutation status and survival.

Variable	Ezrin low, n (%)	Ezrin high, n (%)	Significance (p)*
Study population (n = 173)			
MSI status			**0.001**
MSS	117 (87)	19 (50)	
MSI	18 (13)	19 (50)	
BRAF status			**0.001**
BRAF WT	121 (91)	25 (66)	
BRAF mutated	12 (9)	13 (34)	
Disease-specific outcome			**0.038**
Alive without CRC	93 (69)	23 (61)	
Alive with CRC	3 (2)	0 (0)	
Dead of CRC	8 (6)	9 (24)	
Dead of other cancer	16 (12)	2 (5)	
Dead of other causes	11 (8)	4 (11)	
Dead cause unspecified	4 (3)	0 (0)	

*Pearson's chi-square test. Modified from original publication I, p. 8. Creative Commons license, authors are the copyright holders.

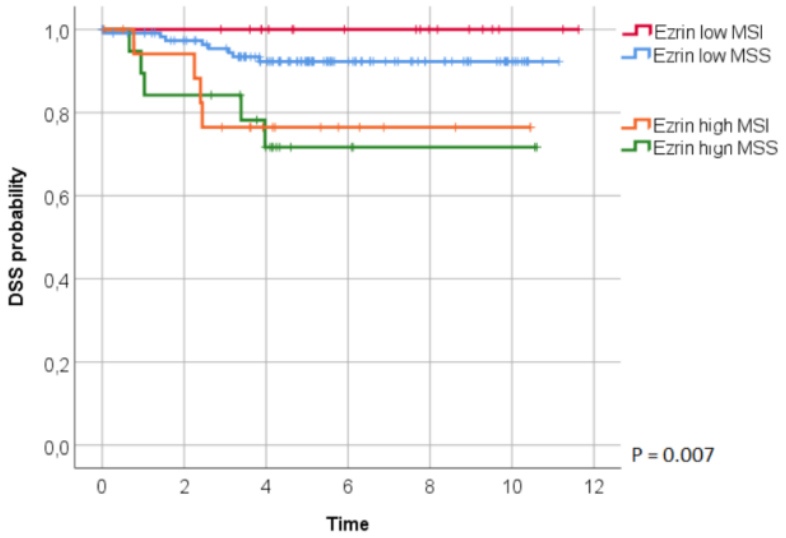

Figure 3. Disease-specific survival of patients with regard to ezrin protein expression and MSI-status. Modified from original publication I, p. 11. Creative Commons license, authors are the copyright holders.

Table 6. Univariate and multivariate Cox regression analysis (DSS) according to selected clinicopathological variables, MSI-status and ezrin protein expression.

Variable	Univariate HR (95 % CI)	p-value	Multivariate HR (95 % CI)	p-value
Stage				
T3N0	1 (ref)		1 (ref)	
T4aN0	2.35 (0.65–8.41)	0.19	3.40 (0.72–15.98)	0.121
T4bN	3.96 (1.1–14.24)	**0.035**	4.58 (0.89–23.62)	0.069
Perforation				
No	1(ref)		1 (ref)	
Yes	6.44 (2.25–18.41)	**0.001**	5.44 (1.3–22.75)	**0.002**
Ezrin & MSI status				
Ezrin low MSS	1 (ref)		1 (ref)	
Ezrin low MSI	0.00 (0.00–0.00)	0.983	0.00 (0.00–0.00)	0.986
Ezrin high MSS	**4.00 (1.31–12.23)**	**0.015**	**5.68 (1.53–21.12)**	**0.01**
Ezrin high MSI	**3.6 (1.08–11.96)**	**0.037**	3.19 (0.61–16.74)	0.17

Ref, reference category; n.d., not determined. Modified from original publication I, page 11 and supplementary Table S1. Creative Commons license, authors are the copyright holders.

5.2 Tumour budding and EMT marker integrin 4 beta in relation to clinicopathological characteristics and survival in stage II colorectal cancer (II)

In three-tiered scoring of TB assessed visually from HE stained whole sections the patients with Bd2 and Bd3 had shorter DSS compared to Bd1 patients. In multivariate analysis Bd2 only but not Bd3 was an independent factor of poor DSS, as well as T4N0 tumours, perforation and vascular invasion (Table 7.). The power of TB as a prognostic factor of poor DSS became even more evident, when 2-tirered scoring was used as seen in univariate analysis (HR 7.55; 95 % CI: 2.64–18.28, $p < 0.001$, Figure 4.), and also in multivariate analysis (HR 6.04; 95 % CI: 2.00–18.20, $p = 0.001$). In multivariate analysis including TB with two-tiered scoring other independent factors of poor DSS included T4N0 stage, and vascular invasion (Table 8.).

The digitally analyzed buds with high integrin beta 4 expression (ITGB4-high bud, higher than median intensity in all epithelial clusters in the full cohort) was prognostic factor of poor DSS in univariate analysis, and it also turned out to be an independent factor of poor DSS in multivariate analysis together with T4N0 stage, perforation and vascular invasion (Table 9.). The same result was obtained when ITGB4-high buds were analyzed from a validation cohort with patients treated in Helsinki University Hospital (HR 3.61; 95 % CI: 1.34–9.74; Helsinki cohort). Strong ITGB4 expression in tumour buds coincided with a localization switch of ITGB4 from basal membrane and cell-cell contacts of intact epithelium to diffusely fill cytoplasm of the budding tumour cells. In addition, small tight junction perimeter (ZO-1 staining) and loss of E-cadherin correlated with strong expression

of ITGB4 in tumour buds. In addition, ITGB4 high-bud count analyzed from TMA material with image analysis correlated better with visually scored TB analysis from H&E stained whole sections than any of the other tested TB scoring methods. These include: visually scored TB analysis from H&E stained TMAs, visually scored TB analysis from pancytokeratin (CkPan) stained TMAs, and TB digital analysis without ITGB4-evaluation. The results of visual and digital TB analyses have been described in more detail in the original publication (II).

Table 7. Univariate and multivariate Cox regression analysis (DSS) for visually assessed TB (three-tier) and for selected clinicopathological variables and biomarkers with statistical significance.

Variable	Univariate HR (95% CI)	p-value	Multivariate HR (95% CI)	p-value
TB H&E				
Bd 1 (n = 192)	1 (ref)		1 (ref)	
Bd2 (n = 24)	**6.95 (2.64–18.78)**	**8.48E-05**	**5.63 81.63–19.39)**	**0.0062**
Bd3 (n = 16)	**5.55 (2.00–15.44)**	**0.0010**	3.13 (0.91–10.70)	0.069
n.d. = 2				
pT-status				
T3N0 (n = 78)	1 (ref)		1 (ref)	
T4abN0 (n = 142)	**3.02 (1.38–6.62)**	**0.006**	**4.38 (1.54–12.46)**	**0.0056**
Perforation				
No (n = 203)	1 (ref)		1(ref)	
Yes (n = 18)	**4.39 (1.76–10.95)**	**0.0015**	**4.04 (1.18–13.82)**	**0.0259**
n.d. = 12				
Radicality				
R0 (n = 203)	1 (ref)		n.d.	
R1 (n = 14)	0.59 (0.08–4.37)	0.606	n.d.	
R2 (n = 3)	**15.71 (3.61–68.35)**	**0.0002**	n.d.	
n.d. = 12				
Vascular invasion				
No (n = 171)	1(ref)		1(ref)	
Yes (n = 37)	2.10 (0.92–4.80)	0.0789	**3.57 (1.33–9.55)**	**0.0114**
n.d. = 12				
Ezrin				
Low (n = 98)	1 (ref)		n.d.	
Intermediate (n = 36)	1.39 (0.42–4.62)	0.5895	n.d.	
High (n = 38)	**3.19 (1.19–8.54)**	**0.0209**	n.d.	
n.d. = 48				

Ref, reference category; n.d., not determined. Modified from original publication II, page 67.

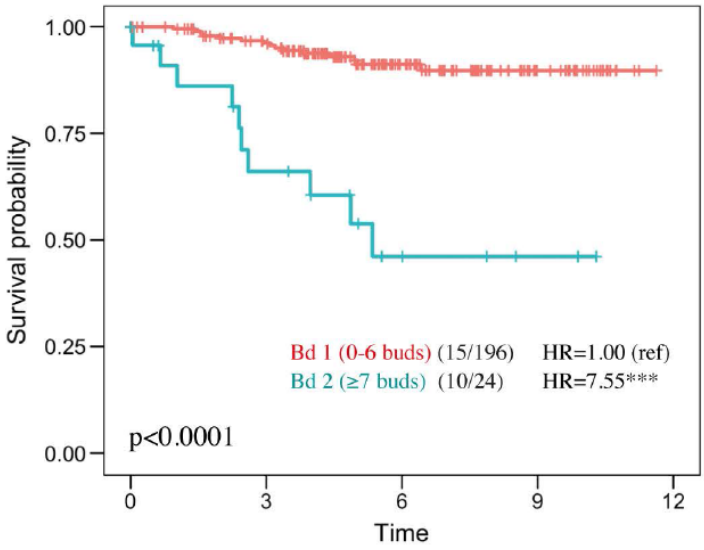

Figure 4. Disease-specific survival of patients with regard to TB has been evaluated visually from HE-stained whole sections (2-tiered scoring). Reprinted from original publication II, page 66. Creative Commons license, authors are the copyright holders.

Table 8. Univariate and multivariate Cox regression analysis (DSS) for visually assessed tumour budding (2-tier) and for selected clinicopathological variables.

Variable	Univariate HR (95% CI)	p-value	Multivariate HR (95% CI	p-value
TumourBudding 2-tier				
Bd 1 (n = 192)	1 (ref)		1(ref)	
Bd2 (n = 24)	**7.55 (2.64–18.28)**	**7.75E-07**	**6.04 (2.00–18.20)**	**0.001**
n.d. = 12				
pT-status				
T3N0 (n = 178)	1 (ref)		1(ref)	
T4abN0 (n = 142)	**3.02 (1.38–6.62)**	**0.006**	**4.12 (1.53–11.14)**	**0.005**
Perforation				
No (n = 203)	1(ref)		1(ref)	
Yes (n = 18)	**4.39 (1.76–10.95)**	**0.0015**	2.93 (0.84–10.26)	0.093
n.d. = 12				
Radicality				
R0 (n = 203)	1(ref)		n.d.	
R1 (n = 14)	0.59 (0.08–4.37)	0.606	n.d.	
R2 (n = 3)	**15.71 (3.61–68.35)**	**0.0002**	n.d.	
n.d. = 12				
Vascular invasion				
No (n = 171)	1(ref)		1(ref)	
Yes (n = 37)	2.10 (0.92–4.80)	0.0789	**3.27 (1.20–8.94)**	**0.021**
n.d. = 12				

Ref, reference category; n.d., not determined. Modified from original publication II, supplementary Table S1. Creative Commons license, authors are the copyright holders.

Table 9. Univariate and multivariate Cox regression analysis (DSS) for ITGB4-high bud count (2-tier) and for selected clinicopathological variable and biomarkers with statistical significance.

Variable	Univariate HR (95% CI)	p-value	Multivariate HR (95% CI	p-value
ITGB4-high buds				
Low (N = 197)	1 (ref)		1 (ref)	
High (n = 19)	**5.09 (2.12–12.22)**	**0.0003**	**4.50 (1.50–13.50)**	**0.007**
n.d. = 16				
Stage				
T3N0 (n = 178)	1 (ref)		1(ref)	
T4abN0 (n = 142)	**3.02 (1.38–6.62)**	**0.006**	**5.61 (2.00–15.71)**	**0.001**
Perforation				
No (n = 203)	1(ref)		1(ref)	
Yes (n = 18)	**4.39 (1.76–10.95)**	**0.0015**	3.95 (1.21–12.90)	**0.0227**
n.d. = 12				
Radicality				
R0 (n = 203)	1(ref)		n.d.	
R1 (n = 14)	0.59 (0.08–4.37)	0.606	n.d.	
R2 (n = 3)	**15.71 (3.61–68.35)**	**0.0002**	n.d.	
n.d. = 12				
Vascular invasion				
No (n = 171)	1(ref)		1(ref)	
Yes (n = 37)	2.10 (0.92–4.80)	0.0789	**3.02 (1.15–7.93)**	**0.0245**
n.d. = 12				
Ezrin				
Low (n = 98)	1 (ref)		n.d.	
Intermediate (n = 36)	1.39 (0.42–4.62)	0.58	n.d.	
High (n = 38)	**3.19 (1.19–8.54)**	**0.0209**	n.d.	
n.d. = 48				

Ref, reference category; n.d., not determined. Modified from original publication II, page 73.

5.3 CDX2 in relation to clinicopathological variables and survival in stage II colorectal cancer (III)

As a categorical variable, CDX2 loss correlated with right-sided tumour, T4N0 stage, tumour perforation, MSI-status, BRAF-mutation status, poor DFS and DSS, high ITGB4 expression in tumour buds, high E-cadherin expression, high tight junction perimeter of tumour cells and high ezrin expression. The results of the associations concerning CDX2 front cores have been presented in Table 10. The results concerning the CDX2 center cores are found in the original publication (III).

When analyzed either from tumour center or front cores, patients with disappearance of CDX2 had poor DSS ($p = 0.012$, log-rank test, Figure 5). The same result was obtained concerning DFS ($p = 0.004$ for tumour center cores, $p = 0.005$ for tumour front cores, log-rank test). In addition, the loss of CDX2 correlated with poor DSS and DFS only in patients with MSS phenotype

($p < 0.001$; $p = 0.019$) but not in patients with MSI-H phenotype ($p = 0.21$; $p = 0.14$). Finally, CDX2 loss remained an independent factor of poor DSS in multivariate analysis concerning both tumour center and tumour front cores (HR 5.96, 95 % CI: 1.55–22.95; HR 3.70, 95 % CI: 1.30 – 10.56). Other risk factors of that Cox model were stage (T3N0 vs. T4N0), number of lymph nodes (>12 vs <12), tumour side (left vs. right), tumour perforation (no vs. yes), and TB (<7 vs >7). The significant results of CDX2 tumour front core multivariate analysis have been presented in Table 11. The results of survival analysis have been presented in more detail in the original publication (III). An example of stage II CRC patient with several poor prognostic features has been presented in Figure 6.

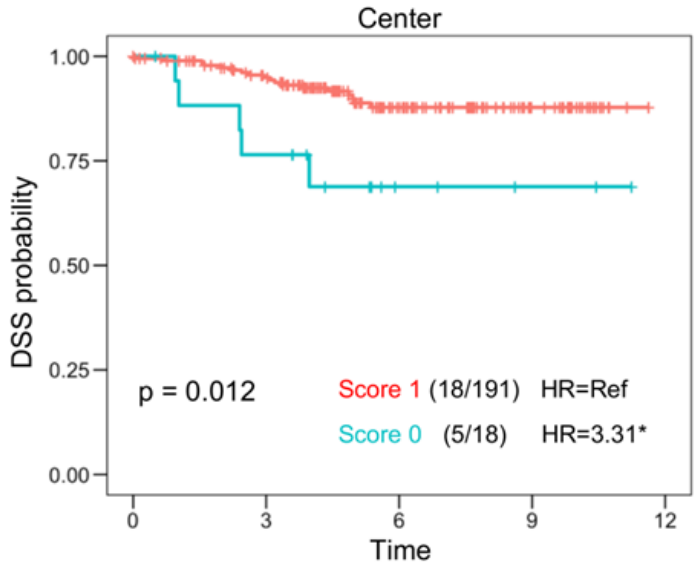

Figure 5. Disease-specific survival of patients in relation to CDX2 loss of center TMA cores. Reprinted from original publication III, page 1479. Creative Commons license, authors are the copyright holders.

Table 10. Association of CDX2 expression (front cores) with selected clinicopathological variables and markers related to epithelial integrity and EMT.

Variable (n, front core)	CDX2 expression Low n(%)	High n (%)	Fisher exact (p)
Tumour side			**0.024**
Right (n, 108)	16 (80)	83 (46)	
Left (n, 112)	4 (20)	96 (54)	
Stage			**0.037**
T3N0 (n, 178)	14 (64)	150 (84)	
T4abN0 (n, 42)	8 (36)	29 (16)	
Perforation			**0.003**
No (n, 203)	16 (73)	168 (94)	
Yes (n, 18)	6 (27)	10 (6)	
MSI status			**4.0E-06**
MSS (n, 170)	8 (36)	150 (84)	
MSI-H (n, 42)	14 (64)	28 (16)	
BRAF status			**0.003**
WT (n, 183)	14 (64)	158 (90)	
V600E (n, 28)	8 (36)	18 (10)	
TB			0.313
Low Bd<7 (n, 196)	18 (82)	157 (89)	
High Bd≥7 (n, 24)	4 (18)	20 (11)	
Disease-free survival			**0.019**
No event (n, 182)	15 (68)	158 (88)	
Event (n, 27)	7 (32)	21 (12)	
Disease-specific survival			**0.033**
No event (n, 186)	16 (73)	161 (90)	
Event (n, 23)	6 (27)	18 (10)	
ITGB4			**0.011**
Low (n, 110)	5 (23)	95 (53)	
High (n, 106)	17 (77)	83 (47)	
E-cadherin			**0.040**
Low (n, 107)	16 (73)	84 (47)	
High (n, 109)	6 (27)	94 (53)	
TJ perimeter			**0.023**
Low (n, 108)	16 (73)	84 (47)	
High (n, 108)	6 (27)	94 (53)	
Ezrin[1]			**2.0E-08**
Low (n, 98)	1 (5)	90 (63)	
Intermediate (n, 36)	5 (24)	30 (21)	
High (n, 38)	15 (71)	23 (16)	

[1]Pearson Chi square test has been used for ezrin instead of Ficsher exact probability test. P-values are 2-sided exact significances. Bolded values mark significance P<0.05.

Table 11. Multivariate Cox regression analysis (DSS) for CDX2 expression, selected clinicopathological variables and TB.

Variable (n front core)	Multivariate HR (95 % CI)	p-value
CDX2		
Conventional (n = 191)	1 (ref)	
Neg/weak (n=18)	**3.70 (1.30–10.56)**	**0.014**
Stage		
T3N0 (n = 173)	1 (ref)	
T4abN0 (n = 36)	**2.87 (1.18–6.97)**	**0.02**
Perforation		
No (n = 193)	1 (ref)	
Yes (n = 15)	**3.14 (1.03–9.56)**	**0.045**
TB		
Low Bd < 7 (183)	1 (ref)	
High Bd > 7 (24)	**3.26 (1.21–8.80)**	**0.02**

Ref, reference category. Modified from original publication III, page 1481. Creative Commons license, authors are the copyright holders.

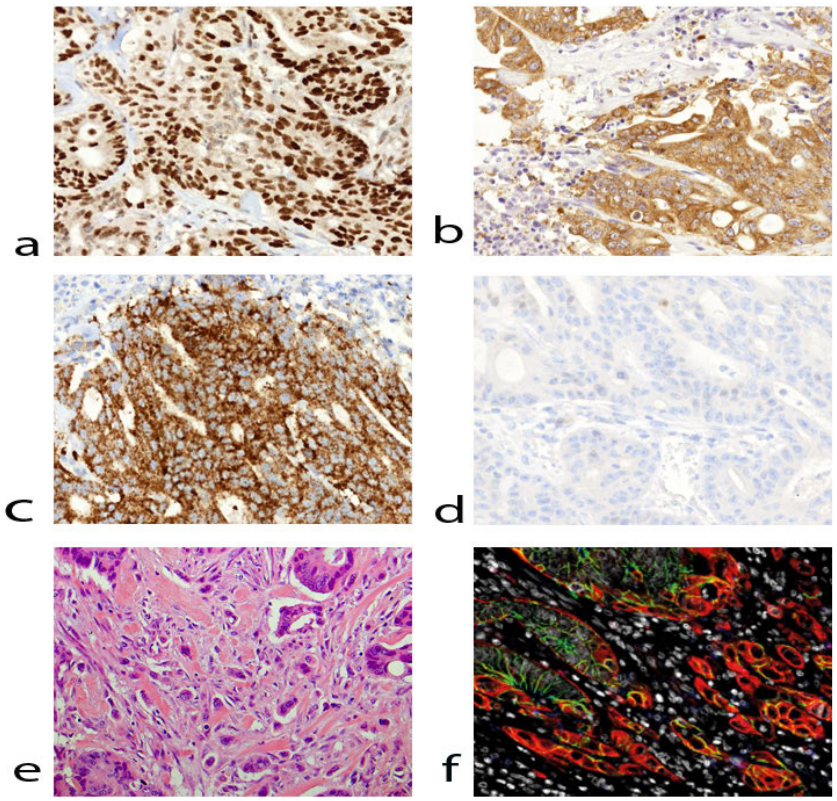

Figure 6. An example of combination of poor prognostic histopathological features in stage II colorectal cancer: 6a. MSS phenotype as demontstrated by MLH1 staining; 6b. high ezrin protein expression; 6c. BRAF-mutation; 6d. CDX2 loss; 6e TB high; 6f. ITGB4 high in tumour buds. Figure 6 f. has been modified from the original publication II. Creative Commons license, authors are the copyright holders.

6 Discussion

Despite the progress in CRC treatment results, stage II CRC still remains a challenge in terms of treatment strategies, and the survival benefit of adjuvant chemotherapy is rather modest (Quasar Collaborative Group et al. 2007). The addition of MSI-testing (Brierley et al. 2017) and TB (Lugli et al. 2017) analysis have helped to some extent in treatment selections, but still new biomarkers are needed to identify patients in risk of relapse.

In this thesis, ezrin and CDX2 protein expression were studied in the material of stage II CRC patients treated at Turku University Hospital by constructing a TMA from paraffin-embedded tumour material of these patients. The results were compared with clinicopathological variables including MSI-status and BRAF-mutation status. In addition, TB was studied as well as TB-associated EMT-markers with multiplex-IHC and image analysis including also a validation cohort from Helsinki University Hospital. The biomarkers tested are easy to introduce in clinical practice of any pathology laboratory with the exception of EMT-markers, which still need additional studies to define their applicability for clinical use.

6.1 Ezrin in relation to prognosis of stage II colorectal cancer (I)

Ezrin belongs to ERM-proteins, which links cell membrane or cell surface molecules to cell cytoskeleton (Arpin et al. 2011). For this reason they have a crucial effect on neoplastic transformation, cell motility, invasion and metastasis (Elliott et al. 2005). In this thesis we have shown, that high ezrin protein expression is related with poor prognosis in stage II CRC with microsatellite stable phenotype. In previous publications it has turned out to be a factor for poor outcome in several malignant neoplasms (Horwitch et al. 2006; Ilmonen et al. 2005; Weng et al. 2005) including CRC and other malignancies of the gastrointestinal tract (Arumugam et al. 2013;Elzagheid et al. 2008; Korkeila et al. 2011; Ling & Chen 2013; Liang et al. 2017). In the study of Ling and Chen 2013 including CRC patients from all stages high ezrin protein expression correlated with large tumour size, serosal invasion, lymph node metastasis, high lymph node ratio, late tumour stage and poor survival. In the current study high ezrin expression correlated with poor DSS

(Fisher's exact test, p = 0.038), and when combined with MSS phenotype, it was an independent factor of poor DSS (Cox model, HR 5.68, 95% CI: 1.53–21.12, p = 0.01). However, it did not correlate with other clinicopathological variables, which may partly be related with the type of patient material including only stage II CRC patients. There is evidence, that ezrin does not predict poor outcome in CRC in protein level only but also in messenger RNA (mRNA) -level (Mori et al. 2017).

Which could thus explain the prognostic power of ezrin in a large variety of different malignant neoplasms? It is envolved in a variety of processes, which favor neoplastic transformation. These include cell signaling pathways including protein kinase C, Rho-kinase, NF-ĸB and Pi3 kinase/Akt (Brambilla and Fais 2009). In addition ezrin is associated with the regulation of several cellular processes like cell-cell adhesion, cell motility and invasion (Chuan et al. 2010). Ezrin belongs to EMT-promoting proteins, since it is involved in EGFR-NF/kB-induced activation of EMT (Li et al. 2017). Downregulation of thrombomodulin has been shown to correlate with poor outcome in CRC, and the mechanism behind this phenomenon has been suggested to result from feed-back upregulation of EMT-proteins including ezrin (Chang et al. 2016). In addition, the activity of the non-receptor tyrosine kinase proto-oncogene src is promoted by ezrin, which contributes to tumorigenesis and metastatic process (Heiska et al. 2011). Still, one of the tumour-promoting mechanisms of ezrin may be its suggested inhibitory effect on apoptosis in CRC cells (Iessi et al. 2015). Furthermore, ezrin regulates the expression of IAPs, XIAP and survivin through protein kinase A (PKA) -activation, further augmenting its tumorigenic potential (Leiphrakpam et al. 2014; Leiphrakpam et al. 2018).

6.2 Tumour budding and EMT in relation to prognosis of stage II colorectal cancer (II)

It is known that the characterstics of tumour border configuration correlate with survival in CRC (Zlobec et al. 2009). More specifically, this concerns the concept of TB. It refers to small clusters of up to four cancer cells in tumor stroma, usually in the invasive margin (Mitrovic et al. 2012). There are several well documented studies indicating the correlation between high grade TB and poor outcome in CRC (Rogers et al. 2016). However, it has been problematic to find a consensus for standardized assessment of TB in CRC. In 2016 there was a consensus conference in Bern of around topic, according to which TB should be assessed primarily from H&E stained sections, and from one hot spot at the invasive front using a three-tier system bd1-3, where bd3 refers to high grade tumor budding (Lugli et al. 2017). It is defined as 10 or more tumor buds counted within one hot spot usually at the invasive tumor front using 20 x objective field and the adjustment of the result to

the area of 0.785 mm^2 (Lugli et al. 2017). The prognostic significance of TB is regarded as most remarkable in stage II CRC, where high grade TB is a risk factor for a short DFS (Lugli et al. 2017; Wang et al. 2009), and in endoscopically resected pT1Nx CRC patients, where high grade TB is a risk factor of lymph node metastasis (Ueno et al. 2004; Bosch et al. 2013). For this reason, analysis of TB has been included as additional prognostic factor of CRC in TNM8 classification of malignant tumours (Brierley et al. 2017). In the original publication II, high grade TB in stage II CRC correlated with poor DSS both in univariate ($p<0.0001$) and multivariate analysis. ($p=0.006$).

The molecular biological mechanisms behind TB are complex, and not yet completely elucidated (Dawson & Lugli 2015). However, there is evidence, that TB and EMT are interrelated (Grigore et al. 2016). During this process epithelial cells lose their characteristic features including polarity and epithelial integrity, and they acquire properties typical of mesenchymal cells allowing them to move and increase their invasive potential (Thiery 2002). Therefore, during EMT CRC cells lose the expression of epithelial markers including E-Cadherin and they acquire the expression of mesenchymal markers like vimentin (Kalluri et al. 2009).

Since TB itself has already been extensively studied in CRC, we wanted to elucidate in more detail the connection between TB and EMT. For this aim we used a multiplex IHC and digital image analysis to study immunohistochemical profile of tumour buds with TMA material of stage II CRC patients. The selected biomarkers included known EMT markers including E-cadherin, ZO-1 and ITGB4. The results of digital image analysis from multiplex IHC stained TMAs were compared with visual image analysis of TB using either H&E stained serial sections from the same TMAs or H&E stained whole sections (cut from corresponding donor paraffin blocks of punch tissue cores). Interestingly, the multiplex IHC analysis with EMT marker ITGB4 predicted patient survival almost as well as visual TB analysis from H&E stained whole sections, where TB was estimated with the ITBCC 2016 consensus conference method (Lugli et al. 2017). Instead, visual TB analysis from H&E stained serial sections of TMAs did not predict survival. This is understandable, since TMAs represent random areas of tumour center and front, not hotspot with highest amount of TB. Yet, why does the multiplex IHC ITGB4 profile from even these randomly selected tumour areas correlate to visually assessed TB? The association of EMT and tumor budding (Gurzu et al. 2016) may explain, that overexpression of EMT-associated genes in randomly chosen tumor areas even without histologically identifiable tumor buds shows correlation with tumor budding evaluated from hot spot area of the same tumor. It is possible, that expression level of EMT-proteins might be used as surrogate markers of histologically confirmed tumor budding, in small biopsies for instance. However, this hypothesis requires further studies.

ITGB4 subunit is a transmembrane protein, which forms a heterodimer with the α6-integrin subunit to act as a receptor for laminin, and in polarized epithelial cells it forms an attachment to basal membrane with the aid of hemidesmosomes (Giancotti 2007). For this reason ITGB4 plays a crucial role in several phenomena associated with cell migration, growth, and survival (Margadant et al. 2008). Specifically, ITGB4 has been reported to have an important role in cancer invasion and EMT (Masugi et al. 2015), possessing also features of cancer stem cell phenotype (Bieriea et al. 2017).

Surgical pathology has traditionally been based on visual analysis of routine histological slides as well as immunohistochemical stainings. Along with increasing demands of standardization and to avoid interobserver variation, digital image analysis has received increasing interest. For several years automated image analysis has been possible in the evaluation of Ki-67-index as well as for estrogen and progesterone receptor stainings from cancer specimens (Tuominen et al. 2010). In addition, tumour-infiltrating lymphocytes and their subclasses have been analyzed for years with digital image analysis (Klauschen et al. 2018). Multiplex immunohistochemistry (multiplex IHC) makes it possible to study several biomarkers from one tissue section simultaneously, but the interpretation of these slides visually may be challenging and not provide reproducible results. For this purpose, whole-slide digital image analysis has shown promising accomplishments (Blom et al. 2017), and this method has been used in the original publication II for analyzing of TB associated EMT-markers. It is remarkable, that digitally analyzed ITGB4 -high bud count correlated better with survival outcome than visually analyzed tumour bud count from either H&E or CkPan stained TMA samples (Original publication II). However, the use of more complicated image analysis techniques may require standardization of equipment, software and configuration. For this reason it may take for some years, before more complicated image analysis techniques including deep learning based tissue analysis (Caie et al. 2016; Bychkov et al. 2018) can be applied more broadly in clinical pathology settings (Kratz et al. 2019).

6.3 CDX2 in relation to prognosis of stage II colorectal cancer (III)

The caudal type homeobox 2 transcription factor (CDX2) is important for proliferation and differentiation of intestinal epithelial cells (Walters et al. 1997). During embryogenesis homeobox genes have a vital role for the development of embryo (Rawat et al. 2012). CDX2 has been observed to possess tumour suppressor function (Bakaris et al. 2008), and it affects Wnt/β-catenin signaling among others (Coskun et al. 2014). In addition, CDX2 expression has effect on

EMT by regulating the expression of claudin-1, which is an essential constituent of tight junctions (Bhat et al. 2012). Furthermore, CDX2 regulates the expression of EMT-associated proteins E-cadherin, vimentin and sialyl Lewis glycans a and x (Zhang et al. 2015; Sakuma et al. 2012). For this reason, it is no wonder, that inactivation of CDX2 contributes to colorectal tumorigenesis (Hryniuk et al. 2014). Decreased expression of CDX2 protein may be associated with gene promoter hypermethylation of this gene (Kameoka et al. 2015). Loss of CDX2-expression is associated with advanced tumour stage, poor differentiation, BRAF-mutation and MSI-H phenotype (Olsen et al. 2014, Sakamoto et al. 2017), and poor outcome (Baba et al. 2009; Dalerba et al. 2016; Lugli et al. 2008; Zhang et al. 2017). MSI-H and BRAF mutation correlate with CDX2 loss, which may suggest the association with serrated pathway in these tumours (Neumann et al. 2018). There are no previous reports concerning our observation of association between CDX2 loss and high ezrin protein expression. Both of these genes have an important role during embryonic development (Liu et al. 2018). Although both CDX2 loss and high ezrin protein expression correlate with poor prognosis in stage II colorectal cancer (Original publications I, III), further studies are needed concerning their possible combinatory synergistic effect. There are hardly any studies, which asses the prognostic significance of CDX2 specifically in stage II CRC only. In the current study we could show that loss of CDX2 to be an independent factor of poor outcome in stage II CRC, but only in patients with microsatellite stable phenotype. Also the study of Ryan et al. (2019) showed that poor prognosis associated with loss of CDX2 is limited to MSS patients, yet that study included CRC patients from several stages. The assessment of CDX2 protein expression in addition to MSI-status may identify high risk patients in need of adjuvant chemotherapy to exclude recurrent disease. However, the results must be confirmed in a prospective setting with a larger patient series.

7 Summary/Conclusions

Treatment of stage II CRC is in dire need of new clinical biomarkers to identify high-risk patients better than before. The methods for this should be easy to apply them in all pathology laboratories. In this study, ezrin, CDX2 and TB-associated EMT markers have been tested for this purpose, and the following conclusions can be drawn:

1) High ezrin protein expression together with microsatellite stable phenotype in tumours is an independent risk factor of poor DSS in stage II CRC patients.
2) High ITGB4 budding count analyzed from TMAs with digital image analysis correlates with TB evaluated visually from H&E stained whole sections, and provides prognostic efficacy comparable to latter (HR = 5.09 vs 7.55, Univariate Cox regression analysis for DSS), and it independently predicted poor DSS in two independent stage II CRC patient cohorts. In patients with high ITGB4 expression in their tumour buds, the localization of staining switched from the basal membrane to the cytoplasm.
3) Loss of CDX2 protein expression together with microsatellite stable phenotype in tumours is an independent risk factor of poor DSS in stage II CRC patients.
4) CDX2 loss correlates with other observed poor prognostic factors including high ezrin expression, EMT markers including low E-cadherin and tight junction disruption, suggesting both some functional relationship and possibility for some joint effects on the prognosis.

Taken together, the results of these studies suggest, that especially stage II colorectal cancer patients with high ezrin expression and CDX2 loss combined with MSS phenotype in their tumours carry a risk of poor outcome. In addition, the biomarkers in the focus of the present study may have a potential to further improve the risk stratification of stage II colorectal cancer patients. However, further studies are needed to validate these findings. (Figure 7.).

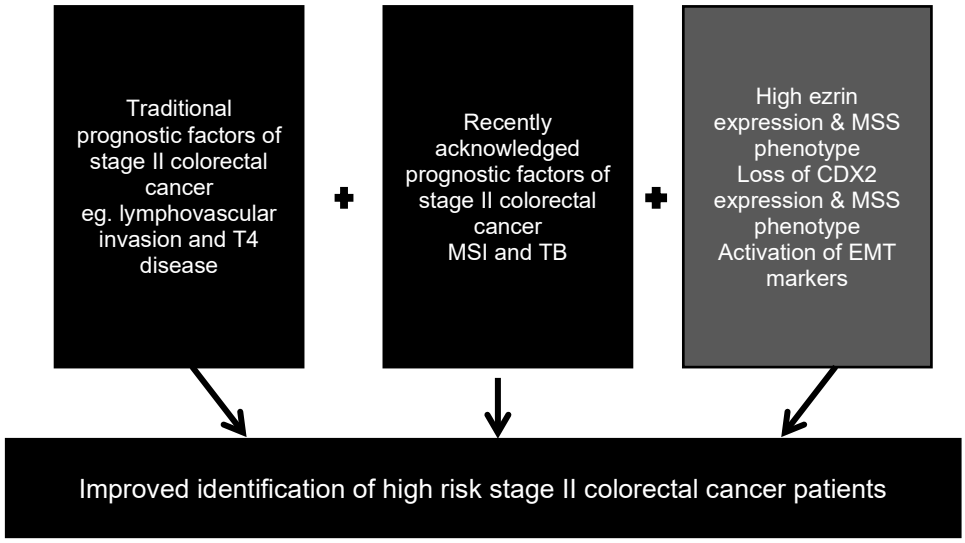

Figure 7. A suggested algorithm to identify high-risk stage II CRC patients. Grey box lists biomarkers identified in this study.

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