Discovery and validation of podocalyxin-like protein as a prognostic biomarker in colorectal cancer

Anna Larsson
Title: Discovery and validation of podocalyxin-like protein as a prognostic biomarker in colorectal cancer

Abstract: Colorectal cancer (CRC) is the third most common cancer worldwide with more than 700,000 deaths every year. Prognosis mostly depends on disease stage at diagnosis, however, outcome may vary considerably even within the same stage. Thus, there is a great need for new prognostic biomarkers to better identify patients with a high risk of developing metastases and select right patients for adjuvant treatment. Podocalyxin-like protein (PODXL) has been associated with an aggressive tumour phenotype and adverse outcome in different types of cancer.

The aim of this thesis was to investigate the prognostic and predictive significance of PODXL in CRC. In addition, the associations between PODXL and other biomarkers in CRC, including EGFR and BRAF, were examined.

Expression of PODXL and EGFR was examined by immunohistochemistry on tissue microarrays containing tumours from three independent CRC patient cohorts (n=557, n=270 and n=320 respectively). BRAF mutational status was assessed by pyrosequencing, and quantiative polymerase chain reaction was used to compare mRNA levels and PODXL protein expression in 62 tumours. Further, concordance between PODXL expression in primary tumours and lymph node metastases, and in tumour samples pre- and postirradiation was examined in a subset of tumours (n=31 and n=16 respectively). Finally, western blot was used to analyse expression of PODXL and EGFR in six CRC cell lines.

High PODXL expression was associated with unfavourable clinicopathological parameters and was found to be an independent factor of poor prognosis in CRC. Furthermore, CRC stage III patients with tumours displaying high PODXL expression had a significant benefit from adjuvant chemotherapy, whereas patients with tumours displaying low PODXL expression did not. We found no correlation between mRNA levels and protein expression of PODXL, and the expression of PODXL did not differ between primary tumours and lymph node metastases, nor was it altered by neoadjuvant radiotherapy. Furthermore, PODXL expression was found to correlate with EGFR expression and BRAF mutation, and in vitro studies showed that PODXL and EGFR were expressed in a uniform way in the cell lines. The highest risk of death within 5 years was observed in patients with high expression of both EGFR and PODXL.

In conclusion, the results from this thesis demonstrate for the first time that PODXL is an independent factor of poor prognosis in CRC, and a potential predictive marker for stratifying patients for adjuvant chemotherapy.

Key words: PODXL, colorectal cancer, prognosis, treatment prediction, EGFR, BRAF mutation

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Discovery and validation of podocalyxin-like protein as a prognostic biomarker in colorectal cancer

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I’ve learned that I still have a lot to learn

Maya Angelou
# Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of papers</td>
<td>9</td>
</tr>
<tr>
<td>Papers included in the thesis</td>
<td>9</td>
</tr>
<tr>
<td>Paper not included in the thesis</td>
<td>9</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>11</td>
</tr>
<tr>
<td>Background</td>
<td>15</td>
</tr>
<tr>
<td>Epidemiology</td>
<td>15</td>
</tr>
<tr>
<td>Etiology</td>
<td>16</td>
</tr>
<tr>
<td>Hereditary colorectal cancer</td>
<td>16</td>
</tr>
<tr>
<td>Colorectal carcinogenesis</td>
<td>17</td>
</tr>
<tr>
<td>Staging</td>
<td>20</td>
</tr>
<tr>
<td>Prognostic and treatment predictive markers</td>
<td>22</td>
</tr>
<tr>
<td>Kirsten rat sarcoma viral oncogene homolog (KRAS)</td>
<td>22</td>
</tr>
<tr>
<td>V-raf murine sarcoma viral oncogene homolog B (BRAF)</td>
<td>23</td>
</tr>
<tr>
<td>Microsatellite instability (MSI)</td>
<td>23</td>
</tr>
<tr>
<td>Treatment</td>
<td>24</td>
</tr>
<tr>
<td>Surgery</td>
<td>24</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>25</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>26</td>
</tr>
<tr>
<td>Targeted therapy</td>
<td>27</td>
</tr>
<tr>
<td>Podocalyxin-like protein</td>
<td>29</td>
</tr>
<tr>
<td>PODXL in cancer</td>
<td>30</td>
</tr>
<tr>
<td>Epidermal growth factor receptor</td>
<td>31</td>
</tr>
<tr>
<td>Aims of the thesis</td>
<td>33</td>
</tr>
<tr>
<td>Patients</td>
<td>35</td>
</tr>
<tr>
<td>Paper I</td>
<td>35</td>
</tr>
<tr>
<td>Paper II</td>
<td>35</td>
</tr>
<tr>
<td>Paper III</td>
<td>36</td>
</tr>
<tr>
<td>Paper IV</td>
<td>36</td>
</tr>
</tbody>
</table>
Methods

- Tissue microarray
- Immunohistochemistry
- Western blot
- Quantitative polymerase chain reaction
- Pyrosequencing

Statistics

Results

- Paper I
- Paper II
- Paper III
- Paper IV

Discussion

- PODXL expression in primary tumours and lymph node metastases
- PODXL as a prognostic factor in colorectal cancer
- PODXL as a treatment predictive factor in colorectal cancer
- PODXL and EGFR

Strengths and limitations

Conclusions

Future perspectives

Populärvetenskaplig sammanfattning

Acknowledgements

References
List of papers

Papers included in the thesis


Paper not included in the thesis

# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU</td>
<td>Fluorouracil</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
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<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>ASCO</td>
<td>American Society of Clinical Oncology</td>
</tr>
<tr>
<td>BRAF</td>
<td>V-raf murine sarcoma viral oncogene homolog B</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CIMP</td>
<td>CpG island methylator phenotype</td>
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<tr>
<td>CIN</td>
<td>Chromosomal instability</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>CRT</td>
<td>Chemoradiotherapy</td>
</tr>
<tr>
<td>CSS</td>
<td>Cancer-specific survival</td>
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<td>CT</td>
<td>Computed tomography</td>
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<tr>
<td>DFS</td>
<td>Disease-free survival</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DSS</td>
<td>Disease-specific survival</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial mesenchymal transition</td>
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<tr>
<td>ESMO</td>
<td>European Society for Medical Oncology</td>
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<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
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<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridisation</td>
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<tr>
<td>FOLFIRI</td>
<td>Fluorouracil/leucovorin + irinotecan</td>
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</table>
FOLFIRINOX Fluorouracil/leucovorin + irinotecan + oxaliplatin
FOLFOX Fluorouracil/leucovorin + oxaliplatin
FOTB Fecal occult blood test
GDP Guanosine diphosphate
GTP Guanosin triphosphate
Gy Gray
HNPCC Hereditary nonpolyposis colorectal cancer
HPA Human Protein Atlas
HRT Hormone replacement therapy
IBD Inflammatory bowel disease
IHC Immunohistochemistry
KRAS Kirsten rat sarcoma viral oncogene homolog
LV Leucovorin
MDCS Malmö Diet and Cancer study
MDT Multidisciplinary team
MLH1 mutL homolog 1
MMR Mismatch repair
MRI Magnetic resonance imaging
MSH2 mutS homolog 2
MSH6 mutS homolog 6
MSI Microsatellite instability
MSS Microsatellite stable
NHERF Na+/H+ exchanger regulatory factor
NRAS Neuroblastoma v-ras oncogene homolog
NSAID Non steroidal anti-inflammatory drugs
NSCLC Non-small cell lung cancer
NSGCT Non-seminomatous germ cell tumour
OS Overall survival
PCR Polymerase chain reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>PFS</td>
<td>Progression-free survival</td>
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<tr>
<td>PMS2</td>
<td>Postmeiotic segregation increased 2</td>
</tr>
<tr>
<td>PODXL</td>
<td>Podocalyxin-like protein</td>
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<tr>
<td>RFS</td>
<td>Recurrence-free survival</td>
</tr>
<tr>
<td>RR</td>
<td>Response rate</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor alpha</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
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<td>TMR</td>
<td>Total mesorectal</td>
</tr>
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<td>TNM</td>
<td>Tumour node metastasis</td>
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<tr>
<td>TP53</td>
<td>Tumour protein p53</td>
</tr>
<tr>
<td>TTR</td>
<td>Time to recurrence</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>wt</td>
<td>Wild-type</td>
</tr>
<tr>
<td>WT1</td>
<td>Wilms tumour suppressor-1</td>
</tr>
<tr>
<td>XELOX</td>
<td>Capecitabine + oxaliplatin</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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</table>
Background

Epidemiology

Colorectal cancer (CRC) is a major cause of morbidity and mortality throughout the world. It is the third most common cancer worldwide with more than 1.3 million new cases and close to 700,000 deaths every year [1]. Approximately 25% of patients with CRC present with metastatic disease at diagnosis [2], and almost half of all patients will develop metastases, explaining the high mortality rates [3, 4]. Incidence varies significantly throughout the world with North America, Australia, New Zealand and Europe having much higher incidence rates than Africa and South-Central Asia, presumably due to environmental and lifestyle differences [1].

Figure 1.
Global variations in CRC incidence [5]. Reproduced with permission from GLOBOCAN.
In Sweden, the incidence of CRC has slowly increased during the last 30 years and 6300 new cases were reported in 2013 [6]. In the United States, however, the introduction of colonoscopy screening programmes has led to a decrease in CRC incidence in the past decade [7]. Mortality rates are also declining due to increased screening and improved surgical and oncological treatment [8].

Etiology

The pathogenesis of CRC is complex and influenced by multiple factors, some of which are related to diet and lifestyle. CRC risk increases with age and approximately 75% of the patients are older than 65 at diagnosis [5]. Epidemiological studies have demonstrated that a diet rich in red and processed meat [9] and low in fibers [10] is associated with CRC. Obesity is a risk factor, mainly for men [11], as is smoking [12] and alcohol consumption [13]. Inflammatory bowel disease (IBD) increases the risk of developing CRC [14, 15], whereas physical activity [16] and long-term therapy with low-dose aspirin [17, 18] and hormone replacement therapy (HRT) in postmenopausal women [19] have shown preventive effects. However, due to the potential side effects of gastrointestinal bleeding from aspirin, and the increased risk of breast cancer from HRT, none of these medications are recommended for CRC prevention in the general population.

Hereditary colorectal cancer

Approximately 20% of patients with CRC have a close relative who has been diagnosed with the same disease. However, only 5% of all cases are caused by a well-defined genetic syndrome [20].

Lynch syndrome or hereditary nonpolyposis colorectal cancer (HNPCC) is the most common form of hereditary CRC, and accounts for approximately 3% of all CRC cases [21]. It is caused by germline mutations in mismatch repair (MMR) genes (MLH1, PMS2, MSH6 and MSH2), and characterised by an increased risk of CRC as well as other malignancies such as endometrial, ovarian and gastric cancer [22]. Inheritance is autosomal dominant. Individuals with Lynch syndrome have a 50-80% lifetime risk of developing CRC, and the median age at diagnosis is 45 [22]. Tumours associated with Lynch syndrome are located in the proximal colon in the majority of cases, and display specific histopathological characteristics, such as poor differentiation, mucinous or signet ring cell histology and lymphocytic infiltration. Synchronous tumours are often found [20]. In addition to prevention by screening,
there is evidence that long-term therapy with aspirin reduces the risk of CRC in this group [23].

Familial adenomatous polyposis (FAP) is an autosomal dominant disorder defined by germline mutation in the adenomatous polyposis coli (APC) gene. It is characterised by hundreds of adenomatous colorectal polyps and a close to 100% lifetime risk of CRC [24]. To reduce the risk of CRC, individuals with FAP undergo systematic endoscopy screening from the age of 10-12, and are offered prophylactic colectomy [25].

Individuals with a strong family history of CRC should be offered genetic testing, as well as CRC patients who are diagnosed at an early age, have multiple primary cancers, or have a history of other cancers related to known genetic syndromes, such as endometrial cancer [26].

**Colorectal carcinogenesis**

For many years, CRC tumourigenesis was explained by the "adenoma-carcinoma sequence", first introduced by Vogelstein in 1988 [27]. In this model, oncogenesis begins with an uncontrolled growth in cryptal cells in the intestine, continues with the formation of an adenoma and eventually after 10-20 years evolves into adenocarcinoma. The transition occurs through a multi-step process, including alterations in oncogenes, loss of tumour suppressor genes and inactivation of genes involved in DNA repair [28].

**Figure 2.**
The adenoma-carcinoma sequence [29]. Reproduced with permission from Massachusetts Medical Society.
Further research has, however, shown that the genetic alterations required for CRC development may not occur in the sequential and uniform way described above, but can be acquired through several different pathways, resulting in different molecular phenotypes with distinct clinical, pathological and genetic characteristics. Three major pathways have been suggested: chromosomal instability (CIN), microsatellite instability (MSI) and the CpG island methylator phenotype (CIMP) [30]. These pathways illustrate the heterogeneous biology of CRC, and support the fact that CRC is not a single disease, but rather an assortment of subtypes of tumours.

**Chromosomal instability phenotype**

CIN is the most common phenotype, accounting for approximately 85% of all sporadic CRC [31]. It involves alterations of tumour suppressor genes and oncogenes such as APC, Kirsten rat sarcoma viral oncogene homolog (KRAS) and tumour protein p53 (TP53) [30].

A key initiating event is the early mutation of APC, involved in both sporadic CIN and, when germline mutated, in all FAP [32, 33]. APC functions as a negative regulator of the Wnt/β-catenin signalling pathway, and mutation of APC results in altered apoptosis and cell-cycle control, thus promoting tumourigenesis [34].

Mutations of KRAS occur in 30-50% of CRC [35-39]. As an upstream mediator of the RAS/RAF/MEK/ERK pathway, mutations in this proto-oncogene leads to an unrestricted activity of signalling, resulting in increased cell proliferation and suppression of apoptosis [40].

TP53 is a tumour suppressor gene which is activated in response to cellular stress and DNA damage [41]. Activated p53 leads to arrest in the G1 phase of the cell cycle where DNA damage is repaired, or initiation of apoptosis if DNA damage proves to be irreparable. Loss of p53 function allows for accumulation of DNA damage and this is the reason why p53 has earned the nickname “guardian of the genome” [41]. Germline mutations of TP53 cause Li-Fraumeni syndrome defined by early onset of a variety of malignancies including breast cancer, sarcoma, brain tumour and adrenal cortical carcinoma [42].

**Microsatellite instability phenotype**

Microsatellites are short, repetitive DNA sequences that are prone to errors during replication. The MMR system acts to recognise and correct these errors. Thus, inactivation of MMR genes (MLH1, MSH2, MSH6 and PMS2) leads to accumulation of errors in DNA, producing a phenotype known as microsatellite instability (MSI) [43].

Approximately 15% of all CRC display MSI and they share distinct pathological features such as location in the proximal colon, poor differentiation, mucinous histology and lymphocytic infiltration [44, 45]. BRAF mutation is also more common
in these tumours [46]. However, despite all these adverse pathological factors, MSI is generally associated with a favourable prognosis [47].

The familial form of MSI is Lynch syndrome. Whilst Lynch syndrome results from germline mutations in MMR genes, the majority of MSI CRCs occur sporadically due to methylation-associated silencing of MLH1 [48]. Hence, these cancers arise through a process that is part of the CIMP pathway described below.

MSI can be detected directly by polymerase chain reaction (PCR) or indirectly by identifying the loss of MMR proteins through immunohistochemistry (IHC). Results from the different techniques have shown to be largely concordant [49]. In PCR-based MSI-testing, five microsatellite sequences are analysed in the tumour DNA. According to the Bethesda guidelines, tumours are classified as MSI-high (MSI-H) if two or more sequences have been mutated, and MSI-low (MSI-L) if only one of the five microsatellite sequences has been mutated. Tumours with no mutation are termed microsatellite stable (MSS). As MSI-L and MSS tumours do not differ clinically or pathologically, they are generally categorised together as MSS [50].

*CpG island methylator phenotype*

Epigenetic silencing of genes, mediated by aberrant DNA methylation, is another mechanism to inactivate tumour suppressor genes in CRC [51].

DNA methylation occurs in CG-rich regions, so called CpG islands, which can be found in the promoter regions in approximately half of all protein-encoding genes in the human genome. Hypermethylation of CpG islands results in repression of gene expression, and hence is an alternative to mutation for switching off tumour suppressor genes. Tumours with extensive CpG island methylation are classified as CpG island methylator phenotype (CIMP) [51].

Abberant DNA methylation can be induced by smoking [52] and increases with age [53]. Consequently, CIMP is more common in older patients, particularly women, and is associated with proximal tumour location, poor differentiation and *BRAF* mutation [54, 55].

Presence of methylation is assessed by PCR, using a panel of CpG island markers. Tumours are categorised as CIMP-high or CIMP-low depending on the extent of methylation.

Unlike mutations, epigenetic alterations of DNA are potentially reversible. Hence, inhibitors of DNA methylation are of interest for the development of new strategies to treat and prevent CRC.
Figure 3.
Schematic model of CRC carcinogenesis. The development from normal mucosa to metastatic cancer is contributed to the interaction of multiple molecular factors. Reproduced with permission from Tidsskrift for Den norske legeforening [56].

Staging

Disease stage is the strongest prognostic factor in CRC and the basis on which treatment decisions are made. Imaging, clinical examination and biomarker assessment are used to determine the stage of the disease. Rectoscopy and colonoscopy with tissue biopsies and computed tomography (CT) scan of the chest and abdomen is usually sufficient for staging of colon cancer. In rectal cancer, investigation is supplemented by magnetic resonance imaging (MRI) of the pelvis. Carcinoembryonic antigen (CEA) is analysed for prognostication, evaluation and follow-up.
Table 1. The TNM staging system according to the American Joint Committee on Cancer (AJCC), 7th edition [57]

<table>
<thead>
<tr>
<th>Primary tumour (T)</th>
<th>Regional lymph nodes (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>NX Regional lymph nodes not assessable</td>
</tr>
<tr>
<td>T0</td>
<td>N0 No regional lymph node metastasis</td>
</tr>
<tr>
<td>Tis</td>
<td>N1 Metastasis in 1-3 regional lymph nodes</td>
</tr>
<tr>
<td>T1</td>
<td>N1a Metastasis in one regional lymph node</td>
</tr>
<tr>
<td>T2</td>
<td>N1b Metastasis in 2-3 regional lymph nodes</td>
</tr>
<tr>
<td>T3</td>
<td>N1c Metastasis without regional lymph node metastasis</td>
</tr>
<tr>
<td>T4a</td>
<td>N2 Metastasis in 4 or more regional lymph nodes</td>
</tr>
<tr>
<td>T4b</td>
<td>N2a Metastasis in 4-6 regional lymph nodes</td>
</tr>
<tr>
<td></td>
<td>N2b Metastasis in 7 or more regional lymph nodes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Distant metastasis (M)</th>
<th></th>
</tr>
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<tbody>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
<tr>
<td>M1a</td>
<td>Metastasis confined to one organ or site</td>
</tr>
<tr>
<td>M1b</td>
<td>Metastases in more than one organ/site or the peritoneum</td>
</tr>
</tbody>
</table>

The TNM staging system by AJCC is based on the size and extent of the tumour (T), involvement of lymph nodes (N) and presence or absence of distant metastasis (M). By combining the T, N and M parameters, tumours can be assigned an overall stage of I-IV, where stage I represents the least advanced CRCs with very good prognosis, and stage IV encompasses advanced CRCs with few patients alive after five years, as shown in Table 2.
Table 2.
Survival according to TNM stage (AJCC cancer staging manual, 7th edition) [58].

<table>
<thead>
<tr>
<th>Stage</th>
<th>TNM</th>
<th>5-year OS (%)</th>
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<tbody>
<tr>
<td>I</td>
<td>T1-T2</td>
<td>N0</td>
</tr>
<tr>
<td>IIA</td>
<td>T3</td>
<td>N0</td>
</tr>
<tr>
<td>IIB</td>
<td>T4a</td>
<td>N0</td>
</tr>
<tr>
<td>IIC</td>
<td>T4b</td>
<td>N0</td>
</tr>
<tr>
<td>IIIA</td>
<td>T1-T2</td>
<td>N1/N1c</td>
</tr>
<tr>
<td>IIB</td>
<td>T1</td>
<td>N2a</td>
</tr>
<tr>
<td>IIB</td>
<td>T3-T4a</td>
<td>N1/N1c</td>
</tr>
<tr>
<td>IIC</td>
<td>T2-T3</td>
<td>N2a</td>
</tr>
<tr>
<td>IIB</td>
<td>T1-T2</td>
<td>N2b</td>
</tr>
<tr>
<td>IIB</td>
<td>T4a</td>
<td>N2a</td>
</tr>
<tr>
<td>IIB</td>
<td>T3-T4a</td>
<td>N2b</td>
</tr>
<tr>
<td>IIB</td>
<td>T4b</td>
<td>N1-N2</td>
</tr>
<tr>
<td>IVA</td>
<td>any T</td>
<td>any N</td>
</tr>
<tr>
<td>IVB</td>
<td>any T</td>
<td>any N</td>
</tr>
</tbody>
</table>

Prognostic and treatment predictive markers

Kirsten rat sarcoma viral oncogene homolog (KRAS)

KRAS is a proto-oncogene in the RAS/RAF/MEK/ERK pathway, responsible for sending extracellular signals to the nucleus of the cell where genes involved in cell proliferation, differentiation and cell survival are transcribed [40]. Through intrinsic enzymatic activity, the KRAS protein cycles between an inactive GDP-bound and an active GTP-bound state [59]. Mutations in KRAS, predominantly in codons 12 and 13, result in an altered protein, insensitive to inactivation [60]. By keeping the protein GTP-bound, mutated KRAS leads to persistent activation of the pathway, even in the absence of growth factor stimulation, thus contributing to cell proliferation and growth. Mutations in KRAS are present in 30-50% of all CRC [35-39, 61], and determination of mutation status is of clinical significance since studies have shown that only patients with KRAS wild-type (wt) tumours benefit from treatment with monoclonal antibodies against the epidermal growth factor receptor (EGFR) [62].
Figure 4.
The EGFR pathway. Inhibition of EGFR leads to reduced KRAS activity and impaired cell survival, proliferation, metastasis and angiogenesis. Reproduced with permission from Current Oncology. [63]

**V-raf murine sarcoma viral oncogene homolog B (BRAF)**

BRAF is a serine-threonine protein kinase downstream of RAS in the RAS/RAF/MEK/ERK pathway. Mutations in the proto-oncogene BRAF are seen in 10-15% of CRCs and lead to sustained activation of downstream signalling through the pathway [64-66]. The most common mutation in BRAF (V600E) occurs early in the progression of CRC and is associated with CIMP. BRAF mutations are more frequent in older patients with poorly differentiated tumours located in the proximal colon [67]. Furthermore, there is an association between BRAF mutation and poor prognosis, especially in MSS tumours [68]. Mutations in BRAF and KRAS are rarely observed together [69].

**Microsatellite instability (MSI)**

As mentioned above, MSI is detected in approximately 15% of all CRC, 3% of which are associated with Lynch syndrome. MSI is associated with a lower rate of recurrence [70] and a favourable prognosis [47, 71-73]. In recent years, MSI testing has been implemented in clinical practice for prognostic stratification and identification of Lynch syndrome patients. In addition, a potential predictive role of MSI has been suggested since several studies have shown that patients with colorectal tumours with
MSI have no benefit from adjuvant chemotherapy [71-75]. However, other studies have demonstrated conflicting results [70, 76], and therefore, according to current guidelines from the European Society of Medical Oncology (ESMO), MSI status should not be used for guidance on treatment decisions other than to identify a small subset of CRC stage II patients who have a very low risk of recurrence, and who will most likely not benefit from chemotherapy [77].

### Treatment

Management of CRC has improved immensely over the past decades and is contributed to several factors. Refined surgery techniques and the use of neoadjuvant radio- and chemotherapy and adjuvant chemotherapy have lead to a reduced mortality and morbidity. The introduction of targeted therapies, including bevacizumab and cetuximab/panitumumab, has further improved outcome for patients with metastatic CRC. Other factors of importance include increased awareness and screening programmes.

As CRC is a preventable disease and curable when diagnosed at an early stage, the use of screening and chemoprevention that can reverse or delay the process before invasive cancer develops, is an appealing approach. In the US, screening using fecal occult blood test (FOTB) and colonoscopy is recommended for average-risk individuals aged 50 to 75 years [78], and has lead to a rapid decline in CRC incidence during the past decade [7]. In Sweden, a general screening programme has not yet been introduced. However, a study has recently been initiated with the aims to study the impact of screening on CRC mortality, and to compare FOTB and colonoscopy as screening methods (Screening of Swedish Colons, SCREESCO).

As of chemoprevention, several potential agents have been investigated, including non-steroidal anti-inflammatory drugs (NSAID) [17, 18], HRT [19], eicosapentaenoic acid [79], calcium [80] and selenium [81]. In the ongoing Swedish ALASCCA study, the addition of low-dose aspirin to standard adjuvant treatment and its effect on recurrence is investigated. However, based on existing literature, there are no sufficient data to support the use of any of these agents in CRC risk reduction practice.

### Surgery

Treatment of CRC is a multidisciplinary teamwork involving pathologists, radiologists, surgeons and oncologists. All cases should be discussed at multidisciplinary team (MDT) meetings.
Surgery is the cornerstone for management of CRC. The goal is to remove the primary tumour with negative margins, resect its vascular supply and lymphatic drainage, and re-establish continuity of the bowel [82]. All surgical procedures begin with a thorough evaluation of the abdominal organs to rule out metastatic disease.

The extent of resection for a colon cancer is based on colonic blood supply [83]. A right hemicolectomy should be performed for tumours of the cecum and ascending colon, and a left hemicolectomy for tumours of the distal transverse or descending colon. Tumours of the sigmoid colon require a sigmoid colectomy [83].

Surgical management of rectal cancer has improved by the introduction of the total mesorectal (TMR) technique which is now the gold standard [84]. This procedure involves removal of the entire rectal mesentery as an intact unit, facilitating nerve preservation and removal of potential tumour deposits in the mesentery [84]. With the TMR technique, and neoadjuvant radiotherapy in selected cases, sphincter preservation can be achieved for most patients [85].

In approximately 20% of all CRC, surgery is performed in an acute setting due to obstruction, perforation or major bleeding [86]. Acute surgery is associated with a higher mortality and a reduced 5-year overall survival (OS) compared to elective surgery, and should be counted as a risk factor when deciding on adjuvant treatment [87-89].

It has become clear in the last years that the number of examined lymph nodes and the ratio of positive to total lymph nodes have an impact on survival for patients with resected CRC [90]. Thus, it is recommended that a minimum of 12 lymph nodes are analysed to reduce the risk of understaging [91].

The presence of distant metastasis in CRC used to imply a palliative treatment approach and exclude patients from curative surgery. However, due to improvements in the oncological and surgical management of CRC, long-term survival or even cure can be achieved in selected stage IV patients with limited disease in the liver, lung or peritoneum by a combination of chemotherapy and surgery.

**Radiotherapy**

For patients with early, resectable rectal tumours, surgery alone is adequate to provide local control. In more advanced tumours though, the risk of local recurrence is substantial, and studies have shown that preoperative radiotherapy can reduce that risk by half [92]. Intermediate tumours are normally treated with short-course radiotherapy 5 Gy x 5 one week before surgery [93]. Patients with locally advanced tumours, who have a high risk of treatment failure both locally and systemically, are recommended chemoradiotherapy (CRT) delivered as 50.4 Gy/28 fractions with the oral fluoropyrimidine capecitabine as radiosensitiser. Surgery is performed 6-8 weeks after end of treatment [93]. In frail patients with poor performance status or
significant comorbidity, short-course radiotherapy with delayed surgery might be an option to CRT [94, 95].

The optimal neoadjuvant treatment for locally advanced rectal tumours is debated, and in the ongoing RAPIDO trial, patients with locally advanced rectal cancer are randomised to standard CRT or short-course radiotherapy followed by 6 cycles of fluorouracil (5-FU)/leucovorin (LV) plus oxaliplatin (FOLFOX) before surgery [96].

Radiotherapy is also an option in the palliative setting where a short treatment of 5 Gy x 5 or 3 Gy x 10 can be effective in reducing symptoms like bleeding, soiling and pain.

**Chemotherapy**

The risk of recurrence in stage III CRC is as high as 40-60% after potentially curative surgery, and adjuvant chemotherapy is used in selected patients in order to eradicate micrometastases and improve the survival rate.

According to current guidelines, adjuvant chemotherapy is recommended for all patients with colon cancer stage III, and includes 6 months treatment with FOLFOX or capecitabine plus oxaliplatin (XELOX) [77]. In patients older than 70 years, the benefit of adding oxaliplatin is small, and single treatment with 5-FU is recommended in this setting [2, 97, 98]. Adjuvant chemotherapy should be initiated within 8 weeks of surgery [99, 100]. Side effects from chemotherapy include neutropenia, nausea, fatigue and mucositis. Sensory neuropathy, which cumulates during treatment and in some cases becomes chronic, is a common side effect from oxaliplatin and normally the limiting factor in the duration of such treatment [101].

While adjuvant chemotherapy is standard of care for colon cancer stage III, there is an ongoing debate whether the same should apply for stage II patients. According to current guidelines from the American Society of Clinical Oncology (ASCO), adjuvant therapy should be discussed with the patient in the event of risk factors including T4 lesion, resection of less than 12 lymph nodes, bowel obstruction or perforation at diagnosis, poorly differentiated histology and perineural or vascular invasion [77].

The evidence for a beneficiary effect of adjuvant chemotherapy in rectal cancer is limited, in particular if neoadjuvant CRT has been administered. In line with the recommendations for colon cancer, adjuvant treatment is recommended for patients with rectal cancer stage III or high-risk stage II disease when no or short neoadjuvant radiation therapy has been given. However, in patients who have received neoadjuvant CRT, adjuvant chemotherapy is generally not recommended [93].

In the palliative setting, 5-FU (or capecitabine) is used alone or in combination with oxaliplatin or irinotecan, sometimes with the addition of targeted drugs. Combination therapy with 5-FU/LV plus irinotecan (FOLFIRI) or FOLFOX is
superior to 5-FU/LV alone in terms of response rates (RR), progression-free survival (PFS) and OS [102, 103], and either combination can be used as first line treatment. Capecitabine is an alternative to 5-FU/LV [104, 105]. Combining all three chemotherapeutic agents (FOLFIRINOX) is also an option in fit patients for a rapid induction of tumour response, or in patients with BRAF mutated tumours who have a poor prognosis [106].

Second line palliative treatment should include the chemotherapy not used in first line. Hence, patients with progression on an oxaliplatin-based regime should be offered FOLFIRI/XELIRI as second-line chemotherapy, and FOLFOX/XELOX should be considered in patients refractory to an irinotecan-based regime.

In the neoadjuvant setting where downsizing is necessary to enable surgery and a major tumour response is wanted, FOLFOX/FOLFIRI combined with anti-EGFR antibodies, or FOLFIRINOX with or without bevacizumab seem to be the most effective combinations [106, 107].

**Targeted therapy**

The introduction of new targeted therapies has improved the outcome in patients with metastatic CRC during recent years with median survival now reaching 30 months in clinical trials [107-109].

Bevacizumab is a monoclonal antibody that binds vascular endothelial growth factor A (VEGF-A), a key player in angiogenesis. It is used in the palliative setting in combination with fluorouracil-based chemotherapy, and side effects include hypertension, proteinuria, gastrointestinal perforation and impaired wound healing. In addition to blocking VEGF-A, fusion protein aflibercept targets other members in the VEGF pathway, including VEGF-B and placental growth factor. Aflibercept has similar side effects as bevacizumab. In addition to these established drugs, new therapies targeting angiogenesis have been developed in recent years, including ramucirumab, which binds to VEGF receptors, and tyrosine kinase inhibitor (TKI) regorafenib, which has shown a modest increase in survival in patients with progression after all established treatment options [110].

EGFR is another target for antitumoural therapy in CRC. Anti-EGFR antibodies cetuximab and panitumumab are used in combination with FOLFOX or FOLFIRI in metastatic disease, and can also be used as a single agent in the palliative setting with a documented increase in RR and survival [109, 111, 112]. Since patients with mutations in the RAS genes do not respond to anti-EGFR treatment, analysis of KRAS and neuroblastoma V-Ras Oncogene Homolog (NRAS) status is a prerequisite for such treatment [109]. Side effects include acneiform rash, hypomagnesia and allergic reactions, the latter being slightly less frequent in human monoclonal antibody panitumumab compared to the chimeric antibody cetuximab.
Podocalyxin-like protein

Podocalyxin-like protein (PODXL) is a transmembrane protein belonging to the CD34 family of sialomucins, that is closely related to the stem cell marker CD34 and endoglycan [113, 114]. It has an extracellular domain which is extensively glycosylated, giving it a high negative charge [115]. The intracellular part is linked to the actin cytoskeleton and contributes to regulation of cell morphology. The PODXL gene is located on chromosome 7q32-q33, and is regulated by the tumour suppressor genes TP53 and Wilms tumour suppressor-1 (WT1). While WT1 acts as a positive regulator, PODXL is repressed by TP53. Hence, inappropriate PODXL expression might be the result of aberrant expression of WT1, as well as of mutation of TP53 or the PODXL gene itself [116].

PODXL was first described in podocytes of the kidney [117], and has since been found to be expressed by vascular endothelial cells [118], hematopoietic progenitors [119] and a subset of neurons [120]. Though often described as an anti-adhesive protein, PODXL has opposite effects on adhesion depending on where it is expressed. In podocytes of the glomeruli, PODXL acts as an anti-adhesive protein essential for formation and maintenance of the filtrative slits between podocyte foot processes [121]. In fact, knockdown of PODXL in mice results in anuria and perinatal death [122]. However, in specialised lymph node endothelial cells, so-called high endothelial venules, PODXL acts as an adhesion molecule binding to L-selectin on circulating lymphocytes [113]. In addition, PODXL can regulate cell morphology by interacting with the actin cytoskeleton through Na+/H+/exchange regulatory factor (NHERF1/2) and ezrin [123, 124].

During development, PODXL is highly expressed in all tissues with hematopoietic activity, including liver, spleen and bone marrow [119]. In adults, PODXL expression in the hematopoietic system is limited to megakaryocytic progenitors and hematopoietic stem cells. In addition, PODXL is rapidly upregulated by erythroid progenitors under conditions of erythropoietic stress, such as hemolytic anemia and erythropoietin treatment [125].
PODXL in cancer

In addition to its important role in normal development, PODXL has been found to be expressed in subsets of tumours in a variety of malignancies. It was first described in testicular cancer as a marker of non-seminomatous germ cell tumours (NSGCT) [126]. Prior to the initiation of this thesis work, PODXL had been found to be upregulated in leukemia, hepatocellular carcinoma and pancreatic ductal adenocarcinoma [127-129]. Moreover, high expression of PODXL had been associated with a more aggressive tumour phenotype and poor prognosis in breast cancer and renal cell carcinoma [130, 131]. In the breast cancer study encompassing 272 patients, Somasiri et al. found that the mean survival time was six years less for patients with tumours displaying high expression of PODXL compared with patients whose tumours had low or no expression of PODXL [130]. Likewise, Hsu et al. demonstrated that overexpression of PODXL was associated with an increased frequency of distant metastasis and a significantly shorter survival in renal cell carcinoma [131]. Furthermore, PODXL mutation, rather than overexpression, had been associated with aggressive prostate cancer [132].

The mechanisms behind the adverse prognostic role of PODXL expression in cancer are not fully understood. However, its ability to disrupt cell adhesion suggests that it might facilitate metastasis. In vitro studies have shown that overexpression of PODXL leads to increased invasive and migratory potential in breast and prostate cancer cell lines through interaction with ezrin [133]. Furthermore, knockdown of PODXL in testicular tumours results in suppression of cancer invasion [134].

PODXL also plays an important role in epithelial mesenchymal transition (EMT), wherein epithelial cells lose their tight cell-cell adhesions and acquire a mesenchymal phenotype [135]. This process is essential for organogenesis and wound repair, but is also believed to contribute to invasive cancer. EMT is characterised by loss of cell adhesion and cytoskeletal alterations, resulting in a mesenchymal phenotype with enhanced migratory capacity, invasiveness and stem cell-like properties, including resistance to apoptosis [136]. Considering the anti-adhesive effects of PODXL and its described role as an undifferentiated embryonic stem cell marker, it is not surprising that PODXL has been demonstrated as an important regulator of TGF-β-induced EMT [135].

Prior to the initiation of this thesis work, PODXL had been described in CRC cell lines [137], but the significance of PODXL in CRC in vivo had not been reported.
Epidermal growth factor receptor

EGFR is a transmembrane receptor tyrosine kinase that plays an important role in CRC initiation and progression. It belongs to the erbB family of receptor tyrosine kinases also including erbB2 (HER2/neu), erbB3 (HER3) and ErbB4 (HER4) [138]. Binding of a specific ligand, e.g. epidermal growth factor (EGF) or transforming growth factor alpha (TGF-α), leads to downstream signal transduction by various pathways. Two main pathways are activated by EGFR: the RAS-RAF-MEK-ERK pathway and the PI3K–PTEN–Akt pathway. These pathways transmit mitogenic signals to the nucleus, and regulate the expression of genes involved in proliferation, differentiation and cell survival [139]. EGFR activation also stimulates VEGF, which is the primary inducer of angiogenesis [140].

Figure 5.
Schematic picture of the EGFR pathways [141]. Reproduced with permission from Nature Publishing Group.
Overexpression of EGFR is reported in 25-75% of CRC [142]. The prognostic significance of EGFR overexpression remains unclear with conflicting results in the literature [143-146]. However, several studies have demonstrated a link between EGFR expression and adverse outcome in CRC [142, 147-150]. Moreover, due to its role in the progression of CRC, EGFR has become an interesting target for antitumoural therapy. The activity of EGFR can be inhibited in two ways; either by preventing binding of growth factors to the extracellular domain of the receptor with the use of monoclonal anti-EGFR antibodies, or through tyrosine kinase inhibitors (TKIs) that target the tyrosine kinase domain of the receptor, thus blocking downstream signalling from the receptor. TKIs are approved for treatment of non-small cell lung cancer (NCSLC), but since studies have shown that TKI efficacy is restricted to tumours with EGFR mutations, there is no support for use of TKIs in CRC wherein EGFR mutations are very rarely detected [151]. However, as mentioned before, monoclonal anti-EGFR antibodies cetuximab and panitumumab are registered for use in metastatic CRC as single agents or in combination with chemotherapy with improved PFS and OS as a result [106, 111, 152, 153].
Aims of the thesis

Prior to this thesis work, PODXL had been found to be associated with a poor prognosis in breast cancer and renal cell carcinoma [130, 131]. Apart from a study on PODXL in CRC cell lines [137], no prior research had been done on PODXL in human CRC in vivo.

The aim of this thesis work was to investigate the potential prognostic and predictive role of PODXL in CRC. Moreover, the concordance between PODXL expression in primary tumours and lymph node metastases was examined, as was the effect of neoadjuvant treatment on PODXL expression. Finally, we wanted to investigate possible correlations between PODXL and other biomarkers in CRC, including EGFR and BRAF.
Patients

Paper I

The Malmö Diet and Cancer Study (MDCS) is a prospective population-based cohort study with the primary aim to examine whether a Western diet rich in fat and low in fruit and vegetables increases the risk of certain forms of cancer. Between 1991 and 1996, a total number of 28098 individuals, 11063 (39.4%) men and 17035 (60.6%) women aged 44-74 years were enrolled (from a background population of 74138). Baseline examinations included questionnaires, anthropometric measures and blood samples. Follow-up is done annually by record linkage to national registries for cancer and cause of death. Until the end of follow-up 31 December 2008, 626 incident cases of CRC had been registered in the study population. Median age at diagnosis was 71.3 years. Histopathological, clinical and treatment data were obtained from pathology and hospital records.

Paper II

The study population in paper II consisted of two independent patient cohorts:

Cohort 1 is a consecutive, retrospective cohort of 270 patients, 133 (49.3%) men and 137 (50.7%) women who underwent surgery for CRC at Skåne University Hospital in Malmö, Sweden between 1 January 1990 and 31 December 1991. Median age at diagnosis was 73.4 years. Histopathological and clinical data were obtained from pathology and hospital records, and information on vital status was obtained from the population register.

Cohort 2 is a prospective cohort of 337 patients, 170 (50.4%) men and 167 (49.6%) women, surgically treated for CRC at the Central District Hospital in Västerås, Sweden between June 2000 and December 2003. Paraffin-embedded tumour tissue was available from 320 cases. Median age at diagnosis was 73.0 years. Histopathological, clinical and treatment data were obtained from pathology and hospital records, and information on recurrence, death and cause of death was gathered from the clinical database for CRC in the Uppsala/Örebro region.
Paper III

The study comprised 73 patients, 37 (50.7%) men and 36 (49.3%) women, from the prospective South-Swedish Colorectal Cancer Biobank who were surgically treated for CRC at Skåne University Hospital in Malmö between 1 January and 30 September 2012. Histopathological, clinical and treatment data were obtained from pathology and hospital records.

Paper IV

The study population in this paper consisted of the three patient cohorts from paper I and II described above.
Methods

Tissue microarray

Tissue microarray (TMA) is a technology for high-throughput evaluation of protein expression that enables screening of hundreds of tissue samples simultaneously, using a fraction of antibody and tissue material compared to analysis of full-face tissue sections. The technique was introduced in 1998 and has become one of the backbones of modern cancer biomarker research [154] TMAs are constructed by taking cylindrical core biopsies, generally 0.6-2.0 mm in diameter, from representative parts of paraffin-embedded tumours and assemble them in a single recipient paraffin block. The new recipient block is cut into sections and mounted on microscope glass slides, allowing detection of protein expression by means of different techniques such as IHC, fluorescence in situ hybridisation (FISH) and mRNA in situ hybridisation [154, 155]. One TMA block can be sectioned up to 300 times, thus conserving valuable tissue material.

Figure 6.
Schematic picture of the TMA technique. Reproduced with permission from Yale University School of Medicine [156].
Concerns have been raised regarding the TMA technique in terms of not being representative for the entire tumour, in particular in heterogeneous cancers. However, studies have shown that two or three cores are sufficient to accomplish a high degree of concordance with large tissue sections [157, 158], and that the TMA technique can provide equal, or even better, prognostic information than conventional whole tissue sections [155, 159].

Immunohistochemistry

IHC, first introduced in the 1940s by dr Albert Coons, is a widely used technique to localise antigens (proteins) in tissue samples by the use of antibodies [160]. Antibodies used are either poly- or monoclonal, the latter being more specific as they bind to only one epitope. To expose the antigens, tissue is pre-treated with heat and an antigen retrieval solution that breaks the cross-links formed by formalin fixation. A primary antibody reacts with the tissue antigen, and the reaction can be visualised by adding a secondary antibody labelled with an enzyme, such as peroxidase, or a fluorescent agent [161].

![Figure 7](image)

**Figure 7.** Immunohistochemical images of PODXL staining in colorectal tumours with membranous PODXL expression in a varying proportion of tumour cells.

In comparison with other quantitative molecular assays, IHC has the advantage of not only providing information on whether a protein is expressed or not, but also enabling simultaneous evaluation of morphology and antigen localisation.
Western blot

Another technique for detection of specific proteins in a tissue sample is western blot. In this technique, proteins are first separated by molecular weight through gel electrophoresis, and then transferred to a membrane where a band for each protein is created [162]. Protein detection is achieved by staining with antibodies specific to the protein of interest, and the thickness of the band on the membrane corresponds to the amount of protein present. Thus, western blotting not only verifies the expression of a protein, but also determines the relative amount of this protein present in a sample.

Figure 8.
Schematic picture of the western blot technique [163]. Reproduced with permission from ASM Press.
Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) is a technique used for quantifying DNA or RNA in a sample [164]. In traditional PCR, a short specific sequence within a DNA template is amplified in cycles by DNA polymerase. In short, the reaction begins with raising the temperature to 95°C in order to melt double-stranded DNA into single strands. Temperature is then lowered to 50°C, allowing small pieces of DNA complimentary to the gene of interest, so called primers, to bind, thereby providing a site for the polymerase to begin copying the DNA strand. Thereafter, temperature is raised again to 72°C which is the optimal temperature for the polymerase. The cycle of changing temperatures is repeated 40-50 times, and for every cycle the number of DNA sequences is doubled, leading to an exponential amplification of DNA. In qPCR, the amplified DNA is fluorescently labelled and the fluorescence intensity is directly proportional to the amount of amplified DNA [164]. Fluorescence is monitored after each cycle, and the intensity is used to calculate the amount of DNA present at the beginning of the reaction.

Pyrosequencing

Pyrosequencing is a method used to determine DNA sequences. In short, nucleotides are sequentially added to a DNA-template. If the nucleotide is complimentary, it binds to the DNA and through a cascade of enzymatic reactions visible light is produced. The light emitted is proportional to the number of incorporated nucleotides, and since the added nucleotide is known, the sequence of the template can be determined [165].
Statistics

Categories of PODXL expression were trichotomised into negative, weak-moderate and strong, or dichotomised into low and high expression based on immunohistochemical staining. EGFR expression was dichotomised into low and high according to the intensity of membranous staining. In paper II, PODXL mRNA levels were dichotomised into low and high according to the mean value.

Associations between PODXL expression and clinicopathological parameters were analysed using Spearman’s rho and Pearson’s chi-square tests. Kaplan-Meier analysis and log rank tests were applied to illustrate differences in survival according to PODXL and EGFR expression. Hazard ratios for death and recurrence were calculated by the Cox’s proportional hazards model, adjusting for age, gender, T-, N-, M-stage, differentiation grade, vascular and neural invasion in multivariable analysis. A backward conditional method was used for variable selection in the adjusted model. The interaction between PODXL expression and adjuvant treatment was analysed using a Cox model including a treatment variable and an interaction variable.

In paper III, Spearman’s rho and Pearson’s chi-square tests were used to illustrate the concordance between PODXL expression in primary tumour and lymph node metastases, and in tumour samples pre- and postirradiation. In paper IV, the same tests were used for comparison of PODXL and EGFR and other established prognostic and predictive markers in CRC.

All statistical analyses were conducted using SPSS version 18-20 (SPSS Inc Chicago, IL, USA). All test were two-sided and a p-value < 0.05 was considered significant.
Results

Paper I

In this paper, immunohistochemical expression of PODXL and its potentially prognostic value was examined in a cohort of 626 incident cases of CRC in the MDCS. TMAs were constructed from 557 tumours and, in addition, 20 full-face sections were examined to assess possible heterogeneity.

PODXL expression could be evaluated in 536 tumours and was denoted as low in 464 (86.6%) cases and high in 72 (13.4%) cases. There was an excellent concordance between analysis of full-face sections and the TMA-based scoring. High expression of PODXL was significantly associated with adverse clinicopathological characteristics including more advanced T-, N-, M-stage, low differentiation grade and vascular invasion. Furthermore, high expression of PODXL correlated with a shorter CSS (HR=1.98, 95% CI 1.38-2.84, p<0.001) and 5-year OS (HR=1.85, 95% CI 1.29-2.64, p=0.001), which remained significant for 5-year OS and borderline significant for CSS in multivariable analysis adjusted for age, gender, TNM status, differentiation grade and vascular invasion.

In addition, the impact of PODXL expression on survival in relation to adjuvant treatment was analysed in 122 curatively resected stage III patients of whom 62 (50.8%) had received adjuvant treatment and 60 (49.2%) had not. The worst prognosis was seen in untreated patients with tumours displaying high PODXL expression. However, patients with tumours displaying high PODXL expression who received adjuvant chemotherapy had a similar CSS and OS as patients with low PODXL-expressing tumours. A significant interaction was found between PODXL expression and adjuvant treatment with regard to both CSS (pinteraction =0.004) and 5-year OS (pinteraction=0.015) in multivariable analysis.

This is the first study to demonstrate the prognostic impact of PODXL in CRC.
Paper II

The aim of this study was to validate the results from paper I in two additional patient cohorts, and to examine the correlation between PODXL mRNA and protein levels in a subset of tumours. To this end, immunohistochemical expression of PODXL was examined in a retrospective cohort of 270 CRC patients (cohort 1) and a prospective cohort of 337 CRC patients (cohort 2). Using real-time qPCR, the expression of PODXL mRNA was determined in 62 tumours from cohort 2.

PODXL expression could be evaluated in 260 tumours in cohort 1 and in 316 tumours in cohort 2. High PODXL expression was denoted in 25 (9.6%) cases in cohort 1 and 25 (7.9%) cases in cohort 2.

The results revealed significant associations between high PODXL expression and unfavourable clinicopathological parameters in both cohorts. Furthermore, high expression of PODXL correlated with a shorter 5-year OS (HR 2.28, 95% CI 1.43-3.63, p=0.001) in cohort 1, and a shorter TTR (HR 2.93, 95% CI 1.26-6.82, p=0.013) and DFS (HR=2.44, 95% CI 1.32-4.54, p=0.005) in cohort 2, all remaining statistically significant in multivariable analysis after adjusting for established prognostic factors.

No significant correlation could be found between mRNA levels and protein expression of PODXL. Moreover, PODXL mRNA was not associated with any clinicopathological parameters, and was not found to be prognostic.

Paper III

In this paper, the expression of PODXL was examined on full-face sections of all primary tumours and 140 corresponding lymph node metastases from 31 cases in a consecutive cohort of 73 CRC patients. In addition, the correlation between PODXL expression pre- and postirradiation was examined in 16 rectal tumour samples.

High expression of PODXL was denoted in 18 (24.7%) primary tumours and the concordance between primary tumours and related lymph node metastases was good. However, while all PODXL negative primary tumours had negative lymph node metastases, a discrepancy between positive primaries and a fraction of their corresponding metastases was noted in some cases.

As for the impact of irradiation, PODXL expression was found to be mainly unaltered in tumours before and after neoadjuvant radiation therapy. However, in two cases, a positive conversion from negative expression in the pre-irradiation biopsy to positive expression in the post-irradiation tumour was observed.
In this study, the potential associations between immunohistochemical expression of PODXL and EGFR were examined in three independent cohorts encompassing a total number of 1233 patients with CRC, previously analysed for PODXL expression and KRAS and BRAF mutations. In addition, levels of PODXL and EGFR were determined by western blot in six different CRC cell lines.

EGFR could be analysed in 533/626 (85.1%) cases in cohort 1, 259/270 (95.9%) cases in cohort 2, and 310/337 (92.0%) cases in cohort 3. High PODXL expression was significantly associated with high EGFR expression (p<0.001) in all three cohorts, and with BRAF mutation (p<0.001) in cohort 1 and 3. There was no correlation between PODXL expression and KRAS mutation.

As for EGFR, high EGFR expression was associated with adverse clinicopathological parameters and BRAF mutation (p<0.001) in cohort 1. Furthermore, high EGFR expression was found to be an independent predictor of a reduced 5-year OS in cohort 1 (HR=1.77, 95% CI 1.27-2.46), cohort 2 (HR=1.58, 95% CI 1.05-2.38) and cohort 3 (HR=1.83, 95% CI 1.19-2.81). The highest risk of death within 5 years was observed in patients with tumours displaying high expression of both PODXL and EGFR in cohort 1 and 3.

In addition, the results from the in vitro studies revealed a uniform expression of PODXL and EGFR in the examined CRC cell lines.
Discussion

PODXL expression in primary tumours and lymph node metastases

PODXL was expressed in the cytoplasm of the tumour cells, with an accentuation towards the membrane in some cases. No expression was seen in the nuclei. Tumours with a distinct membranous expression in any proportion of cells were denoted as having high expression (overexpression) of PODXL.

In all four papers, PODXL was found to be overexpressed in a relatively small proportion of cases. The rate of tumours with high expression of PODXL evaluated on TMAs varied between 7.9% and 13.4% in the studied cohorts. This observation is in line with results from a study by Kaprio et al., in which, using the same antibody, PODXL was found to be overexpressed in 8.1% of the tumours [166]. Furthermore, in previous studies on breast and bladder cancer, overexpression of PODXL has been demonstrated in 6-21% of the tumours [130, 167]. In contrast, in highly aggressive malignancies such as pancreatic cancer and glioblastoma multiforme, the number of PODXL positive cases is higher (49.5% and 64.8% respectively) [168, 169], further corroborating the association between high PODXL expression and a more aggressive tumour phenotype. In line with these results, the observed variations in frequencies of PODXL overexpression between the cohorts in this thesis might be explained by the larger number of patients with stage IV disease in cohort 1 (18.3%) compared to cohort 2 (9.6%) and 3 (7.9%).

In paper III, analysis of full-face sections revealed a larger proportion of PODXL positive tumours (24.7%) compared to TMA-based analysis. Although this cohort was much smaller than the others (n=73), and previous studies have shown an excellent concordance between analyses of TMAs and large sections [159], a possible underestimation of positive cases with the TMA-technique cannot be ruled out. However, while the TMA-technology has huge advantages in terms of screening and characterisation of investigative biomarkers, analysis of full-face sections is the applicable method in the clinical setting.

Interestingly, in the majority of positive cases, PODXL was expressed in a subset of cells at the invasive tumour front, corresponding morphologically with tumour
budding [170]. First described in the 1950s, tumour budding is defined by the presence of small clusters of cancer cells, highly prone to metastasis, at the invasive edge of carcinomas, and associated with an adverse outcome in CRC [171-173]. Tumour budding is closely related to EMT, and as described before, PODXL is a regulator of EMT [135].

The lack of correlation between PODXL mRNA and protein expression levels demonstrated in paper II is likely explained by the way in which the protein is expressed. While PODXL expression in the cytoplasm can be seen in a considerable proportion of CRC tumours, it is the presence of distinct membranous expression (most often in only a fraction of tumour cells) that confers a poor prognosis. In addition, PODXL is expressed in the vascular endothelium, which is present in various amounts in the tumour-associated stroma. Hence, PODXL positive tumours do not necessarily have the highest level of protein in total, and overexpression of PODXL will not be reflected in high mRNA levels.

In paper III, PODXL expression was found to correlate well between primary tumours and corresponding lymph node metastases. However, a discrepancy was observed in a few cases where positive primaries displayed positive as well as negative lymph node metastases. This discordance might be explained by the heterogeneous fashion in which PODXL is expressed in the primary tumour, however, it may also indicate that PODXL is important in initiating cancer progression, but once metastasis has occurred, PODXL expression is reduced and further progress depends on other factors. However, in a study on breast cancer in vivo by Snyder et al., it was demonstrated that treatment with an antibody against PODXL not only prevented primary tumour growth and metastasis, but also had an effect on established distant metastases in a late stage of the disease [174].

Thus, our results indicate that for prognostic purposes, evaluation of PODXL expression in the primary tumour is sufficient. However, the expression of PODXL in lymph node metastases can provide prognostic information when no primary tumour is available for analysis. In addition, our results on PODXL expression in rectal tumour samples before and after irradiation indicate that, unlike other histopathological prognostic factors such as T and N-stage, PODXL overexpression is not affected by neoadjuvant radiation therapy.

**PODXL as a prognostic factor in colorectal cancer**

The role of PODXL as an independent factor of poor prognosis in CRC was described for the first time in paper I, and then confirmed in two additional independent patient cohorts in paper II. In total, these three patient cohorts represent
more than 1200 CRC patients. In addition, another study was published last year by Kaprio et al. showing the same result in a cohort of 840 Finnish patients with CRC [175].

Prior to the initiation of this thesis work, PODXL had been described as a factor of poor prognosis in breast cancer and renal cell carcinoma [130, 131]. Since then, its prognostic value has been investigated in numerous malignancies. In 2012, Cipollone et al. showed that PODXL is highly overexpressed in high-grade serous ovarian carcinoma, and correlates with a significant decrease in DFS [176]. In another study from our research group, the prognostic value of PODXL expression was examined in two independent cohorts of patients with urothelial bladder cancer (n=100 and n=343 respectively) [167]. The results demonstrate that high expression of PODXL is an independent predictor of a reduced 5-year OS and DSS. Furthermore, high expression of PODXL has been correlated with increased glioma grade and decreased OS in glioblastoma multiforme [169], and in vitro studies have shown that PODXL increases invasion and resistance against temozolomide in astrocytoma [177]. Finally, the results from a study by Heby et al., published earlier this year, indicate that PODXL expression is an independent factor of poor prognosis in intestinal-type pancreatic cancer [168]. Of note, this study also demonstrates that patients with tumours displaying high PODXL expression have a beneficial effect of adjuvant chemotherapy, indicating a potential predictive, as well as prognostic, role for PODXL in this setting.

Taken together, the results from these studies provide convincing evidence for PODXL as a predictor of poor prognosis in human cancer. While the underlying mechanisms are not yet altogether elucidated, several reports on the functional role of PODXL in tumour progression have been published in the last few years. In the study by Cipollone et al., forced expression of PODXL in serous ovarian carcinoma cells was found to decrease adhesion of the cells to the mesothelial monolayers, indicating a possible role in the initiation of peritoneal metastasis [176]. Moreover, knockdown of PODXL in an oral squamous cell carcinoma cells led to reduced tumour migration and invasion in a study by Lin et al. [178]. In addition, two recent studies have shown that silencing PODXL in highly aggressive breast cancer cell lines severely impairs both primary tumour growth and the formation of metastasis in vivo [174, 179]. In one of these studies by Snyder et al., treatment with a novel monoclonal antibody against PODXL was found to inhibit primary tumour growth and metastatic progression in xenografted mice [174].
PODXL as a predictive factor in colorectal cancer

As mentioned before, the risk of recurrence in CRC is as high as 40-60% after potentially curative surgery, and adjuvant chemotherapy is used in selected patients since the mid 90’s in order to eradicate micrometastases and improve the survival rate. In paper I, the patients were included in the study during the first part of the 90’s when adjuvant treatment in CRC was not yet standard of care in Sweden. Thus, a unique opportunity to compare the impact of PODXL expression on prognosis in relation to adjuvant chemotherapy was provided in this cohort of patients. The results from paper I demonstrate that stage III patients with tumours displaying high PODXL expression had a significant benefit on survival from adjuvant treatment, whereas patients with PODXL-low tumours did not. The results indicate that, in addition to its prognostic value, PODXL might be a predictive marker for the response to adjuvant treatment in this group of patients. Whether the same is true for patients with CRC stage II could not be determined as the number of patients in this group receiving adjuvant treatment was too low for statistical analysis. Meanwhile, the same results have been demonstrated in a study on pancreatic cancer by Heby et al., wherein patients with intestinal type tumours displaying high PODXL expression had a significant beneficial effect on survival of adjuvant treatment [168].

The results from our study indicate that colorectal tumours displaying high expression of PODXL respond well to standard chemotherapy, i.e. fluorouracil with or without the addition of oxaliplatin. However, based on the study by Snyder et al., targeting PODXL with monoclonal antibodies may also be a future treatment option [174]. Since PODXL is expressed in normal cells in the podocytes of the kidney and vascular endothelium, extensive studies regarding safety will be required before such treatment can be considered. However, in the preclinical mouse experiments, treatment with antibodies against PODXL was well tolerated without adverse effects [174].

PODXL and EGFR

The PODXL gene is located to chromosome 7, which is frequently amplified in CRC. In addition to PODXL, chromosome 7 harbours several other pivotal genes related to CRC, including proto-oncogene MET, EGFR and BRAF [180]. In fact, the PODXL and BRAF genes are located right next to each other at 7q32-33 and 7q34.

In paper IV, high expression of PODXL was found to correlate with high EGFR expression in all three cohorts, and with BRAF mutation in cohort 1 and 3. This is the first study to show an association between PODXL, EGFR and BRAF in CRC.
High EGFR expression was found to be an independent predictor of poor prognosis in all three cohorts. Furthermore, high expression of EGFR was significantly associated with adverse clinicopathological parameters, including more advanced T-, N-, M-stage, low differentiation grade and vascular invasion in cohort 1. Despite diverging reports in the literature regarding the prognostic significance of EGFR, these results are in line with several previous studies linking high EGFR expression to advanced disease stage and adverse outcome in CRC [142, 147-149, 181-183]. For validation purposes, in cohort 1, protein expression of EGFR was examined with two different antibodies, showing high concordance. In addition, EGFR gene amplification was assessed using brightfield double-in situ hybridization (BDISH), demonstrating a significant correlation between EGFR protein expression and EGFR gene copy number alterations. In contrast to EGFR protein expression, however, there was no significant association between EGFR gene copy number alterations and PODXL expression (unpublished data).

Of note, in cohort 1 and 3, the highest risk of death was observed in patients with tumours displaying high expression of both PODXL and EGFR. This could indicate a possible synergistic adverse effect on survival of the two proteins. In cohort 2 with a smaller number of patients, high EGFR expression was an independent predictor of poor prognosis in PODXL low, but not high tumours, indicating that PODXL is a stronger prognostic factor than EGFR. Furthermore, in this cohort, there were no significant associations between EGFR expression and established unfavourable clinicopathological factors.

The association between PODXL and EGFR was further demonstrated in the in vitro studies in paper IV, wherein western blot analysis showed a uniform expression of the two proteins in all six examined cell lines. Of note, in cell lines derived from the same patients, PODXL and EGFR were expressed in the primary tumour cell line (SW480), but not in the metastatic derivative (SW620).

From a clinical perspective, these results are interesting as previous studies on NSCLC and CRC cell lines have demonstrated that tumour cells that have undergone EMT are much less sensitive to anti-EGFR treatment [184, 185]. In a study by Buck et al., using the same cell lines as in paper IV, tumour cells from the primary tumour (SW480) showed epithelial characteristics and sensitivity to EGFR TKI erlotinib, whereas tumour cells from the liver metastasis (SW620) exhibited a mesenchymal phenotype and were not sensitive to erlotinib [185]. Prior research further suggests that EGFR signalling can trigger EMT [186], but once EMT is established, signalling associated with EGFR activation is reduced [187]. Thus EMT, and possibly PODXL, may have a role in resistance to anti-EGFR drugs.

One possible link between PODXL and EGFR may be the NHERF proteins. In vitro studies have demonstrated that expression of PODXL leads to recruitment of NHERF proteins to the apical domain of epithelial cells [188]. NHERF-1 in turn has
been shown to stabilise EGFR at the cell surface to restrict receptor downregulation, thus enhancing EGFR signalling [189]. Furthermore, unpublished data from our group suggests that knockdown of PODXL in CRC cell lines results in downregulation of EGFR, whereas PODXL is not affected by silencing of EGFR.
Strengths and limitations

Regarding the choice of antibody, the same polyclonal anti-PODXL antibody has been used in all four papers. The specificity of this antibody has been validated using western blotting and protein arrays, and PODXL expression has been mapped by IHC in 48 types of normal tissues and 20 common cancers within the Human Protein Atlas (HPA) project [190, 191]. The same antibody has also been used to detect PODXL expression in other studies on testicular, colorectal, bladder and pancreatic cancer [134, 166-168, 192]. Furthermore, as PODXL is a marker of vasculature, blood vessels in the tumour-associated stroma could serve as internal positive controls.

In paper IV, two different antibodies against EGFR were used with concordant results. In addition, BDISH was performed demonstrating a significant correlation between EGFR protein expression and gene copy number alterations (unpublished results).

The pros and cons of using the TMA technique have been touched upon previously. For screening and characterisation of new biomarkers in large patient cohorts, the TMA-technology is indispensable as it allows a large number of tissue samples to be analysed simultaneously, and provides maximal use of valuable tumour tissue. However, this technique has been criticised in that the small cores sampled may not be representative of the whole tumour, particularly in heterogeneous cancers. To reduce the influence of tissue heterogeneity, two tissue cores from the tumour were sampled in all studies included in this thesis. In paper I, IHC expression of PODXL was also analysed in 20 full-face sections, and the results displayed an excellent concordance with the TMA-based analysis. However, based on the results from paper III, a potential underestimation of positive cases with the TMA technique cannot be ruled out.

Predictive biomarkers are ideally evaluated in randomised prospective studies. However, the MDCS cohort with equal proportions of patients receiving and not receiving adjuvant treatment, enabled us to investigate the impact of PODXL on adjuvant chemotherapy in a way that would be impossible to perform in a randomised trial today when adjuvant treatment is standard of care in this setting.

Furthermore, while most studies concerning the concordance between primary tumours and lymph node metastases usually have examined two lymph nodes per patient, we strived to include all corresponding metastatic lymph nodes in paper III, which meant that up to 17 lymph nodes per patient were evaluated.
Conclusions

- High expression of PODXL is associated with adverse clinicopathological factors in CRC, including a more advanced T-, N-, M-stage, low differentiation grade and vascular and neural invasion.
- High expression of PODXL independently predicts a poor prognosis in CRC.
- CRC stage III patients with tumours displaying high PODXL expression benefit from treatment with adjuvant chemotherapy.
- The expression of PODXL is highly concordant between primary tumour and lymph node metastases, and is not affected by neoadjuvant radiotherapy.
- High PODXL expression is significantly associated with high EGFR expression and \textit{BRAF} mutation in CRC.
- High expression of EGFR is associated with advanced disease stage and poor prognosis in CRC.
- The worst prognosis is seen in CRC patients with tumours displaying high expression of both PODXL and EGFR.
Future perspectives

Despite the advances in management of CRC over the past decade, almost half of all patients undergoing curative surgery will develop recurrent disease. Adjuvant chemotherapy reduces the risk of relapse, however, a majority of patients receiving such treatment has no benefit from it. In stage III colon cancer, the RFS increases with 10-20% by adding adjuvant chemotherapy, and in stage II the incremental gain is only around 5%. Furthermore, chemotherapy has side effects, a few of which can be potentially persistent and life-threatening. Similarly, despite the use of predictive markers KRAS, NRAS and BRAF, only approximately 40% of CRC patients benefit from treatment with anti-EGFR antibodies [193]. Thus, it is of great clinical importance to find new prognostic and predictive markers in CRC so as to enable a more personalised treatment.

Although the results from this thesis indicate the potential clinical utility of PODXL as a prognostic and treatment predictive biomarker in CRC, these findings need to be validated in prospective randomised trials. It would, for instance, be of interest to perform a study wherein stage II CRC patients without risk factors would be randomised to receive adjuvant treatment or not. In addition, stage III CRC patients who today are recommended treatment with 5-FU only, could be randomised to 5-FU with or without the addition of oxaliplatin. With this study design, several potential prognostic and treatment predictive markers, including PODXL, could be investigated.

The observed association between PODXL and EGFR should be followed up with further in vitro studies. In addition to silencing and forcing expression of the two proteins, it would be of interest to evaluate how PODXL-positive CRC cells respond to treatment with anti-EGFR antibodies.

Finally, the results from previous preclinical mouse studies suggest that targeting PODXL might be an additional strategy to treat CRC in the future, and this interesting observation merits further investigation.
Populärvetenskaplig sammanfattning

Tjock- och ändtarmscancer är den tredje vanligaste cancerformen i världen och den skördar mer än 700 000 liv per år. Sjukdomen är vanligare i västvärlden, sannolikt beroende på bakomliggande livsstilsfaktorer. Högt intag av fet och fiberfattig kost samt rött kött ökar risken för tjock- och ändtarmscancer, liksom övervikt, rökning, alkohol och låg fysisk aktivitet. Det är en sjukdom som framför allt drabbar äldre, och majoriteten av dem som insjuknar är över 65 år. I Sverige diagnostiseras mer än 6000 nya fall varje år.


Podocalyxin-like protein (PODXL) är ett protein som påverkar hur celler sitter samman och som normalt uttrycks i bl. a. blodkärl och njurar där det spelar en viktig roll för filteringsförmågan. På senare år har man också funnit att PODXL är uttryckt i vissa cancerformer, och mycket tyder på att proteinet är involverat vid metastasering då tumörceller lämnar moder tumören (primärtumören) och ger upphov till dotter tumörer (metastaser). Innan arbetet med denna avhandling påbörjades hade PODXL inte undersömts i tjock- och ändtarmscancer. Studier på bröst- och njurcancer hade dock visat att uttryck av PODXL var förknippat med en mer aggressiv tumörtyp med sämre prognos.

Syftet med denna avhandling har varit att undersöka uttrycket av PODXL i tjock- och ändtarmscancer, och hur detta påverkar prognos och svar på
cytostatikabehandling. För detta ändamål har tumörvävnad undersöks med s.k. immunhistokemisk analys där förekomst av protein visualiseras med hjälp av antikroppsbinding.

I det första delarbetet analyserades uttrycket av PODXL i tumörer från drygt 500 patienter med tjock- och ändtarmscancer. Vi fann att högt uttryck av PODXL, definierat som förekomst av PODXL i tumörcellers membran, var kopplat till en sämre prognos oavsett tumörstadium eller förekomst av andra riskfaktorer. Patienter vars tumörer hade högt uttryck av PODXL levde betydligt kortare efter diagnos än de patienter vars tumörer saknade eller hade lågt uttryck av PODXL. Kortast överlevnad sågs hos patienter som inte hade fått tilläggsbehandling med cytostatika och vars tumörer uppvisade högt uttryck av PODXL. Behandlade patienter med högt tumöruttryck av PODXL levde dock lika länge som patienter vars tumörer uppvisade lågt uttryck av PODXL.

I det andra delarbetet undersökt uttrycket av PODXL i tumörer från yttreligare drygt 500 patienter med tjock- och ändtarmscancer. Dessutom studerade vi sambandet mellan nivåer av PODXL mRNA (budhärar-RNA) och proteinuttryck i drygt 60 tumörer. När en gen aktiveras bildas en kopia av genen i form av en mRNA-molekyl som sedan fungerar som mall för tillverkning av protein. Genom att mäta nivåer av mRNA kan man således få en uppfattning om en gens aktivitet. Resultaten bekräftade fynden från det första delarbetet, d.v.s. patienter vars tumörer uppvisade högt uttryck av PODXL hade en sämre prognos och levde inte lika länge som patienter med tumörer med lågt uttryck av PODXL. Vi fann inget samband mellan nivåer av mRNA och uttryck av PODXL i tumörer, sannolikt beroende på att PODXL också uttrycks i blodkärl i anslutning till tumören och att det är lokalisationen (i cellmembranet) och inte totalnivån av proteinet som avgör prognosen.

I det tredje delarbetet jämfördes uttrycket av PODXL i primärtumörer och lymfkörtelmetastaser från drygt 70 patienter med tjock- och ändtarmscancer. Dessutom analyserades huruvida uttrycket av PODXL påverkas av strålbehandling i tumörprover från 16 patienter med ändtarmscancer som genomgått strålbehandling före operation. Vi fann att uttrycket av PODXL i de flesta fall överensstämde väl mellan primärtumör och lymfkörtelmetastaser. Vi fann också att uttrycket av PODXL var väsentligen oförändrat efter genomgången strålbehandling.

Slutligen, i det fjärde delarbetet jämfördes uttrycket av PODXL med andra markörer vid tjock- och ändtarmscancer såsom epidermal growth factor (EGFR) och mutation i genen BRAF. EGFR är en receptor som sitter på cellytan och tar emot signaler från yttre tillväxtfaktorer vilket resulterar i ökad tillväxt och celldelning. BRAF är ett av flera proteiner som hjälper till att förmedla dessa signaler in till cellkärnan, och
mutationer i BRAF-genen (vilket förekommer i ca 10-15% av all tjock- och ändtarmscancer) leder till kontinuerlig cellsignallering och ohämmad tillväxt.

EGFR-uttryck och förekomst av BRAF-mutation undersöktes i tumörer från drygt 1000 patienter vilka tidigare analyserats avseende PODXL-uttryck i delarbete I och II. Dessutom analyserades uttryck av PODXL och EGFR i sex olika celllinjer av tjock- och ändtarmscancer. Resultaten visade att högt uttryck av PODXL var kopplat till högt uttryck av EGFR och mutation av BRAF. Vi fann vidare att högt uttryck av EGFR var förknippat med dålig prognos, och den sämsta prognosen och kortaste överlevnaden sågs hos patienter vars tumörer hade högt uttryck av både PODXL och EGFR. Sambandet mellan PODXL och EGFR sågs också i cellinjerna där proteinerna uttrycktes tillsammans.

Sammanfattningsvis har denna avhandling visat att högt uttryck av PODXL är kopplat till en sämre prognos vid tjock- och ändtarmscancer, och att patienter med tumörer som uppvisar högt uttryck av PODXL har god nytta av tilläggsbehandling med cytostatika. Resultaten talar för att PODXL skulle kunna användas som markör för att hitta patienter med aggressiv sjukdom där tilläggsbehandling med cytostatika minskar risken för återfall. Fler studier som bekräftar dessa fynd krävs dock innan PODXL kan införas i den kliniska vardagen.
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