CLINICAL IMPLICATIONS AND REGULATION OF INTESTINAL STEM CELL MARKERS IN COLON CANCER

PhD Dissertation by
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Clinical Implications and Regulation of Intestinal Stem Cell Markers in Colon Cancer
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The present PhD dissertation is based on experimental work conducted at the Molecular Unit, Department of Pathology at Herlev University Hospital and the laboratory of Professor Jesper Troelsen at Department of Science and Environment at Roskilde University from January 2013 to March 2016.

The dissertation is divided into five sections including an introduction to the research field and aims of the studies, followed by a summary of the materials and methods and a summary of the results obtained. Hereafter perspectives and discussions of the findings and a conclusion of the studies are presented. The four papers of which the PhD dissertation is based upon are enclosed at the end of the dissertation. All abbreviations, including abbreviations in tables and figures, are listed in the beginning of the dissertation.
ACKNOWLEDGEMENTS

During my time as a PhD student I have met and worked with many inspiring people whom I owe my sincere thanks.

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grateful for all the help I received during my studies. Special thanks to Sylvester Larsen for providing technical knowledge and a good humorous atmosphere. Also thanks to the members of Louise Torp Dalgaard’s laboratory for many fruitful scientific discussions and help whenever needed.

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Most importantly, I would like to emphasize my deep gratitude to my friends and family. For providing the graphic expertise necessary for designing the figures and setup of the dissertation, I owe Katja my sincere thanks. Jannie, I would like to thank you for being such a supportive and wonderful friend who I deeply value. I wish to thank my parents and brothers for the great support and their fantastic humor (apparently I do not have it from strangers). My younger brother, Peter Filip, deserves a special thank for always being optimistic, enthusiastic, and pacing his sometimes frustrated sister to get the most out of life.

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ABSTRACT

Background
The primary treatment of stage II colon cancer patients is surgery. At present the high risk stage II colon cancer patients are stratified for adjuvant therapy according to certain histopathological features of the primary tumor. Yet, it remains a challenge to identify the 20% of the patients with lower stages (stage I and II) who relapse. Currently, no optimal biomarkers can identify the remaining high-risk patients. The objectives of the present PhD was to investigate whether the intestinal stem cell markers Sex-determining region y-box 9 (SOX9) and B-cell-specific Moloney murine leukemia virus integration site 1 (BMI1) could predict relapse of stage II colon cancer patients and whether tumor necrosis factor alpha (TNF-α) regulate SOX9 through the Nuclear factor kappa B (NF-κB) signaling pathway in human colon carcinoma cell lines.

Material and methods
Formalin fixed paraffin-embedded primary tumors from 144 patients diagnosed with stage II colon cancer from January 2005 to August 2008 were consecutively included. The patient samples were investigated for mismatch repair deficiency (dMMR) using immunohistochemistry (IHC) and promoter hyper-methylation assay. SOX9 and BMI1 protein expression was investigated by IHC both at the invasive front and the luminal surface of the primary tumor. The colon carcinoma cell lines DLD1, SW480, HT29, and Caco2 were stimulated with TNF-α to access the effect of TNF-α on the endogenous SOX9 mRNA expression. The effect of transient exogenous overexpression of NF-κB subunits (p50, p52, and p65) on the SOX9 promoter was investigated by luciferase promoter reporter assay in Caco-2 cells.

Results
A total of 37 (25.7 %) of the patients had relapse of their colon can-
dMMR was present in 33 (22.9%) of the tumors. The majority of the dMMR tumors were hyper-methylated in the MLH1 pro-
moter. Univariate Cox proportional hazards analysis revealed that there was no association between BMI1 expression in the primary tumors and risk of relapse. High SOX9 expression at the invasive front of the tumors was associated with lower risk of relapse when including the SOX9 expression as a continuous variable (from low to high expression) in univariate (HR: 0.73; 95% CI: 0.56-0.94; p=0.01) and multivariate (HR: 0.75; 95% CI: 0.58-0.96; p=0.02) Cox proportional hazards analysis adjusting for dMMR and histopathological risk factors. Low SOX9 expression at the invasive front of the tumors was associated with high risk of relapse in uni-
variate (HR: 2.32; 95% CI: 1.14-4.69; p=0.02) and multivariate Cox proportional hazards analysis (HR: 2.32; 95% CI: 1.14-4.69; p=0.02) adjusted for dMMR and histopathological risk factors. While the endogenous mRNA expression of SOX9 was unaffected in DLD1 and SW480 cells upon TNF-α stimulation, the endoge-
nous mRNA expression of SOX9 significantly increased upon TNF-α stimulation of HT29 and Caco-2 cells. The SOX9 promoter activity was significantly up-regulated in Caco-2 cells when p50 or p52 was transiently overexpressed. Transient overexpression of p65 together with overexpression of p50 or p52 significantly in-
creased SOX9 promoter activity.

**Conclusion**

While BMI1 had limited prognostic value in the investigated co-
hort, SOX9 expression at the invasive was demonstrated to be an independent predictor of relapse of stage II colon cancer patients. Moreover, the studies indicated that endogenous SOX9 mRNA expression is affected by TNF-α and this might be mediated by the NF-κB signaling pathway. Additional studies are needed to con-
firm our indications.
Baggrund
Colorektalcancer er en af de hyppigste cancer former i den vestlige verden med omkring 1.2 millioner nye tilfælde årligt. Den primære behandling af patienter med stadium II coloncancer er operation af det tumorbærende tarmsegment. Dernæst inddeles patienterne i høj-risiko og lav-risiko grupper baseret på histopatologiske karakteristika i primærtumoren. Alligevel får ca. 20 % af stadium II coloncancerpatienterne tilbagefald. Der forefindes stadig ingen biomarkerer til optimal identificering af disse høj-risiko patienter.

Formålet med denne Ph.d.-afhandling var at undersøge om proteinekspressionen af Sex-determining region y-box 9 (SOX9) og B-celle-specific Moloney murine leukemia virus integration site 1 (BMI1) i primærtumor fra stadium II coloncancerpatienter kan anvendes til at identificere patienter med høj risiko for tilbagefald. På baggrund af disse studier blev det ydermere undersøgt, om tumor necrosis factor alpha (TNF-α) regulerede SOX9 gennem nuclear factor kappa B (NF-κB) signalvejen i humane coloncancer-celleliner.

Materiale og Metoder
Formalininfikserede og paraffinindstøbte primærtumor fra 144 patienter diagnosticeret med stadium II coloncancer mellem januar 2005 og august 2008 blev konsekutivt inkluderet i studiet. Primærtumorerne blev undersøgt for defekt mismatch repair system (dMMR) ved brug af immunhistokemi (IHC) og promoter hyper-methyleringsanalyse. For at undersøge TNF-α’s effect på det endogene SOX9 mRNA ekspressionsniveau blev coloncancer-celleline DLD1, SW480, HT29 og Caco-2 stimuleret med TNF-α og mRNA-niveauet undersøgt ved kvantitativ RT-PCR. Effekten af transient overekspRESSION af NF-κB signalmolekyleerne p50, p52 og p65 på SOX9 promoteren blev undersøgt ved luciferase promoter reporteranalyse i Caco-2 celler.

Resultater
I alt havde 37 (25,7 %) af de 144 inkluderede patienter tilbagefald
af deres stadium II coloncancer. dMMR forekom i 33 (22,9 %) af primærtumorerne. Størstedelen af tumorerne med dMMR havde hyper-methyleret MLH1-promoter. Ved univariat Cox proportional hazards analyse fandtes ingen association mellem BMI1-ekspressionen i primærtumorerne og risikoen for tilbagefald. Derimod sås en association mellem høj SOX9-ekspression ved den invasive front i tumorerne og lav risiko for tilbagefald både i en univariat (HR: 0.73; p=0.01) og multivariat (HR: 0.75; p=0.02) Cox proportional hazards analyse med SOX9-ekspressionen som kontinuer variabel og med justering for dMMR samt de kendte histopatologiske risikofaktorer. Desuden sås en association mellem lav SOX9-expression i den invasive front af tumorerne og høj risiko for tilbagefald i en univariat (HR: 2.32; p=0.02) og multivariat (HR: 2.32; p=0.02) Cox proportional hazards analyse med SOX9-ekspressionen som dikromatisk variabel (høj/lav), og med justering for dMMR samt histopatologiske risikofaktorer. Der kunne ikke vises signifikant ændring af det endogene SOX9 mRNA-ekspressionsniveau ved TNF-α-stimulering af DLD1 og SW480 celler. Derimod steg det endogene SOX9 mRNA-ekspressionsniveau signifikant ved TNF-α-stimulering af Caco-2 og HT29. Herudover steg SOX9 promoter aktiviteten signifikant, når p50 eller p52 blev transient overudtrykt i Caco-2 celler. Dette var også tilfældet, når p65 blev transient overudtrykt sammen med enten p50 eller p52.

Konklusion

LISTS OF PAPERS

This PhD dissertation is based on the following four papers which are referred to by Roman numerals (I-IV) in the text.

Paper I
*Espersen ML*, Olsen J, Linnemann D, Høgdall E, and Troelsen JT.
Clinical Implications of Intestinal Stem Cell Markers in Colorectal Cancer.

Paper II
*Espersen MLM*, Linnemann D, Alamili M, Troelsen JT, and Høgdall E.
The prognostic value of polycomb group protein B-cell- specific moloney murine leukemia virus insertion site 1 in stage II colon cancer patients.

Paper III
*Espersen MLM*, Linnemann D, Alamili M, Troelsen JT and Høgdall E.
SOX9 Expression Predicts Relapse of Stage II Colon Cancer Patients.
Human Pathology (2016) 52: 38–46

Paper IV
*Espersen MLM*, Coskun M, Linnemann D, Høgdall E, and Troelsen JT.
Tumor Necrosis Factor-α Induces the Expression of SOX9 through NF-κB Activation in Caco-2 Cells. Manuscript in preparation.
Additional published papers during the PhD study period


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Cancer Committee</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>AXIN</td>
<td>Axis inhibition protein</td>
</tr>
<tr>
<td>BCL-3</td>
<td>B-cell CLL/lymphoma 3</td>
</tr>
<tr>
<td>BMI1</td>
<td>B-cell-specific Moloney murine leukemia virus integration site 1</td>
</tr>
<tr>
<td>BRAF</td>
<td>B-Raf proto-oncogene, serine/threonine kinase</td>
</tr>
<tr>
<td>CC</td>
<td>Colon cancer</td>
</tr>
<tr>
<td>CDX2</td>
<td>Caudal type homeobox 2</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CK1</td>
<td>Casein kinase 1</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>DIM</td>
<td>Self-dimerization domain</td>
</tr>
<tr>
<td>dMMR</td>
<td>Mismatch repair deficiency</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-fixated paraffin embedded</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HMG</td>
<td>High-mobility binding group</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of NF-κB kinase</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of NF-κB</td>
</tr>
<tr>
<td>KLF4</td>
<td>Kruppel-like factor 4</td>
</tr>
<tr>
<td>KRAS</td>
<td>Kirsten rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>LGR5</td>
<td>Leucine-rich repeat-containing G-protein coupled receptor 5</td>
</tr>
<tr>
<td>LPR5/6</td>
<td>LDL receptor-related protein 5 or 6</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MLH1</td>
<td>MutL homolog 1</td>
</tr>
<tr>
<td>MSH2</td>
<td>MutS protein homolog 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MSH6</td>
<td>MutS homolog 6</td>
</tr>
<tr>
<td>MSI</td>
<td>Microsatellite instability</td>
</tr>
<tr>
<td>MSI-H</td>
<td>High microsatellite instability</td>
</tr>
<tr>
<td>MSI-L</td>
<td>Low microsatellite instability</td>
</tr>
<tr>
<td>MSS</td>
<td>Microsatellite stable</td>
</tr>
<tr>
<td>MUC2</td>
<td>Mucin 2</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NRAS</td>
<td>Neuroblastoma RAS viral (v-ras) oncogene homolog</td>
</tr>
<tr>
<td>p100</td>
<td>Unprocessed precursor of p52</td>
</tr>
<tr>
<td>p105</td>
<td>Unprocessed precursor of p50</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase</td>
</tr>
<tr>
<td>pMMR</td>
<td>Mismatch repair proficient</td>
</tr>
<tr>
<td>PMS2</td>
<td>Postmeiotic Segregation Increased 2</td>
</tr>
<tr>
<td>PQA</td>
<td>Proline, glutamine, and alanine rich transactivation domain</td>
</tr>
<tr>
<td>PQS</td>
<td>Proline, glutamine, and serine rich transactivation domain</td>
</tr>
<tr>
<td>PRC1</td>
<td>Polycomb repressive complex 1</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription-PCR</td>
</tr>
<tr>
<td>RPLP0</td>
<td>Ribosomal Protein Large P0</td>
</tr>
<tr>
<td>SOX9</td>
<td>Sex-determining region y-box 9</td>
</tr>
<tr>
<td>TA cells</td>
<td>Transit-amplifying cells</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>TNFR1</td>
<td>Tumor necrosis factor receptor type 1</td>
</tr>
<tr>
<td>TNFR2</td>
<td>Tumor necrosis factor receptor type 2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>UICC</td>
<td>Union of International Cancer Control</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
</tbody>
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1. INTRODUCTION

The present section provides an overview of the current knowledge within the research area of the dissertation. This includes the current clinical aspects of stage II colon cancer (CC), the underlying molecular development of CC as well as the cancer stem cells hypothesis and stem cells of the intestines with emphasis on the two intestinal stem cell markers investigated in the enclosed papers.
1.1 CLINICAL ASPECTS OF COLON CANCER

Colorectal cancer (CRC) is one of the most frequent cancers worldwide with more than 1.2 million new cases per year[1]. It is the second and third most common cancer amongst women and men, respectively[1]. Furthermore, CRC accounts for one of the highest cancer related death rates with estimate of approximately 600,000 deaths annually[1]. The majority of CRC patients are 50 years or older when diagnosed[2]. Along with a population that lives much longer than previously combined with an increasing population worldwide, the international community is facing an ongoing burden of CRC. Therefore, the incentive for proper stratification of the patients for clinical treatments and improving treatments is immense. Today many patients have advanced stages of CRC when they are diagnosed with CRC. National screening programs have been introduced to diagnose the patients at an earlier stage with the intention to improve the prognosis of the patients. Thus, the implementation of screening programs is expected to increase the number of patients diagnosed with earlier stages of disease (stage I and II) and decrease the number of patients diagnosed with advanced stages (stage III and IV).

Currently, the primary treatment of stage I and II CC patients is surgical removal of all tumor tissue, followed by an estimate of the risk of relapse based on certain histopathological features. Patients with high risk of relapse are offered adjuvant therapy. However, approximately 20% of the stage II CC patients have relapse of their cancer[3]. Therefore, the need for novel biomarkers to improve identification of these high-risk patients is imperative.

Within the last decade much attention has been directed towards stem cells and stem cell-like cells, due to their potential role in carcinogenesis. Several intestinal stem cells markers have been proposed to have a predictive or prognostic value in CRC patients[4].

The present PhD dissertation investigates the protein expression of the two proposed intestinal stem cell markers (a) B-cell-specific Moloney murine leukemia virus integration site 1 (BMI1) and (b) Sex-determining region y-box 9 (SOX9) in primary tumors from a comprehensive cohort of 144 patients with stage II CC with the primary objective of identifying patients with high risk of relapse. Interestingly, we found that SOX9 was a robust independent predictor of relapse. Thus, we further investigated the regulation of SOX9 to provide insight into the molecular mechanisms behind the SOX9 expression in CC.
Stage II Colon Cancer
Primary treatment of CC is surgical removal of the tumor. Some patients with large tumors receive neoadjuvant therapy prior to surgery. The excised tumor is staged according to the Union for International Cancer Control (UICC)/American Joint Cancer Committee (AJCC) TNM classification (Table 1)[5].

Table 1 Staging of colon tumors according to UICC/AJCC TNM classification.

<table>
<thead>
<tr>
<th>Primary Tumor Invasion (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1    Tumor invades submucosa</td>
</tr>
<tr>
<td>T2    Tumor infiltrates muscularis propria</td>
</tr>
<tr>
<td>T3    Tumor invades through muscularis propria</td>
</tr>
<tr>
<td>T4    Tumor invades through peritoneum and/or into adjacent organs or structures</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Regional lymph nodes (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0    No metastasis to regional lymph nodes</td>
</tr>
<tr>
<td>N1    Metastasis in one to three regional lymph nodes</td>
</tr>
<tr>
<td>N2    Metastasis in four or more regional lymph nodes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Distant metastasis (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0    No distant metastasis</td>
</tr>
<tr>
<td>M1    Distant metastasis</td>
</tr>
</tbody>
</table>

Based on the TNM classification the patients are further divided into four UICC disease stages[5]. The decision of whether the patient should receive adjuvant therapy, such as chemotherapy or biological regiments, depends largely on the disease stage at the initial diagnosis and the health state of the patient (performance score).

Table 2 UICC disease stage based on the TNM classification system.

<table>
<thead>
<tr>
<th>Disease stage</th>
<th>T status</th>
<th>N status</th>
<th>M status</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>T1-T2</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>II</td>
<td>T3-T4</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>III</td>
<td>Any T</td>
<td>N1-N2</td>
<td>M0</td>
</tr>
<tr>
<td>IV</td>
<td>Any T</td>
<td>Any N</td>
<td>M1</td>
</tr>
</tbody>
</table>

T (Tumor invasion); N (Lymph node involvement); M (Metastasis)
Although it is evident that stage III and stage IV CC patients benefit from adjuvant therapy, the benefits of providing adjuvant therapy to patients with stage II CC are limited\[6–10\]. Up to 80% of the stage II CC patients are cured by surgery alone\[3,7\]. Thus, it remains controversial whether stage II CC patients benefit from adjuvant therapy. Furthermore, this raises the question of whether adjuvant therapy for this patient group can be justified when considering the toxicity, costs, and inconvenience for the patients\[8\]. Presently, only a minority of stage II CC patients receive adjuvant therapy and are considered high-risk patients which is primarily based on histopathological features, including T4 stage, vein invasion, perineural invasion, margin involvement, number of sampled lymph nodes, perforation, and low differentiated histology (if the tumor is not mismatch repair deficient (dMMR))\[6,10–14\]. Importantly, these risk factors are not predictive markers of adjuvant therapy but prognostic markers.

Predictive markers can predict whether the individual patient is likely to respond to the provided treatment or not. An example of predictive markers are the RAS (\textit{Kirsten rat sarcoma viral oncogene homolog} (KRAS) and \textit{Neuroblastoma RAS viral (v-ras) oncogene homolog} (NRAS)) mutations which predicts response to the two anti-epidermal growth factor receptor (EGFR) monoclonal antibody treatments cetuximab and panitumumab in metastatic CC\[15,16\]. In contrast, prognostic markers provide information of the likely course of the disease and can be helpful to select patients for further personalized treatment. As an example CRC patients with \textit{B-Raf proto-oncogene, serine/threonine kinase} (\textit{BRAF}) V600E mutated tumors have a poorer prognosis\[17,18\].

Despite proper surgical intervention and the current stratification of patients, approximately 20% of patients with stage II CC experience relapse at local or distant sites\[3\]. The lack of identification of these high risk patients is becoming an increasing issue since the number of patients diagnosed with earlier stages of CC is expected to increase due to the implementation of the screening initiatives for CC. Thus, an improved stratification of the stage II CC patients is highly needed to select patients who will benefit from adjuvant treatment.

**1.2 MISMATCH REPAIR DEFICIENCY**

One of the hallmarks of cancer is genomic instability\[19\]. Functional defects of the mismatch repair (MMR) system causes replication errors frequently observed in the genomic microsatellites which are small repetitive mononucleotide or dinucleotide sequences present throughout the genome. When a group of tumor cells display one or two alleles with a different number of repeats than the wildtype, the cells are said to be microsatellite instable (MSI). MSI is classified as MSI-high (MSI-H) and MSI-low (MSI-L) dependent on the frequency of MSI\[20\]. If there is no detectable instability of the microsatellites the tumor is referred to as microsatellite stable (MSS).

The MSI phenomenon is a classic phenotype of patients with the hereditary Lynch syn-
drome which is caused by germline mutations in critical MMR genes, predominantly in *MutL homolog 1* (MLH1) but also in *MutS protein homolog 2* (MSH2), *MutS homolog 6* (MSH6), and *Postmeiotic Segregation Increased 2* (PMS2)[21]. dMMR is not only restricted to the hereditary Lynch syndrome but is also found in 15% of all sporadic CRC cases[22].

dMMR observed in sporadic CRC is primarily a consequence of hypermethylation of the MLH1 promoter rather than mutations[22]. However, a recent meta-analysis indicated that some MLH1 mutation carriers do also exhibit MLH1 promoter methylations[22]. To exclude Lynch syndrome, a MLH1 promoter methylation assay can be supplemented by a mutational test for the *BRAF* V600E which is often found in MSI-H and dMMR tumors but rarely in Lynch syndrome[22,23]. In contrast to the poor prognosis of Lynch syndrome patients, patients with sporadic CRC and dMMR tumors have an improved prognosis and dMMR has also been reported as a potential predictive marker of 5-flourouracil (5-FU) therapy[24–26].

1.3 COLORECTAL CANCER AETHIOLOGY

CRC arises as a consequence of dysregulation of essential signaling pathways, such as p53, Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), EGFR, transforming growth factor beta (TGF-β), and the canonical Wnt/β-catenin signaling pathway within the epithelial cells. Consequently, the cells are driven towards immortality, autonomous proliferation and invasion occurs, providing a malignant potential. Mutations in critical oncogenes and tumor suppressor genes, such as *RAS*, *TP53*, and *Adenomatous Polyposis Coli (APC)* are common in this multistep process[27]. The latter, APC, plays a central role in the canonical Wnt/β-catenin signaling pathway, which is an established key driver pathway of colorectal carcinogenesis. The crucial role of APC in CRC is underlined by familial adenomatous polyposis patients who mainly present germline mutations in the APC gene. The disease typically manifests itself by up to thousands of gastrointestinal adenomatous polyps, an early onset, and CRC penetrance of nearly 100%[28].

Wnt Signaling

The Wnt signaling pathway is divided into the non-canonical and the canonical Wnt signaling. The canonical Wnt/β-catenin pathway maintains the self-renewal capacity of intestinal epithelial stem cells and pluripotency[29,30] and is characterized by activation of β-catenin. The non-canonical pathway is independent of β-catenin. Less is known about this pathway which amongst other things has been found to be involved in cell polarity and calcium signaling[31]. The Wnt signaling pathway can be activated by the binding of Wnt ligands to a heterodimer receptor complex consisting of Frizzled and LDL receptor-related protein 5 or 6 (LRP5 /6) located in the plasma membrane[29]. The specific Frizzled receptor, Wnt ligand, and the local concentrations of these deter-
mines whether the canonical or non-canonical Wnt signaling pathway is activated[31]. Due to the importance of especially the canonical Wnt/β-catenin pathway in intestinal stem cells and colorectal carcinogenesis, the focus from hereon will only be on the canonical Wnt/β-catenin pathway.

In the inactive canonical Wnt/β-catenin signaling state cytoplasmic β-catenin is bound by a destruction complex consisting of Axis inhibition protein (AXIN), APC, casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3) (Figure 1). The complex constitutively phosphorylates β-catenin, targeting it for proteosomal degradation[29,30], thus inhibiting transcription of Wnt target genes. The pathway is activated upon binding of Wnt ligands to the receptor complex and the protein Dishevelled is recruited to the receptor complex after which AXIN is translocated to the membrane, inactivating the destruction complex. This stabilizes the cytoplasmic β-catenin which translocates to the nucleus where it associates with TCF/LEF transcription factors, thereby activating transcription of Wnt target genes, such as CMYC, CCND1 (Cyclin D1), Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5), and SOX9[32–34] (Figure 1).

Figure 1: Illustration of the active and inactive canonical Wnt/β-catenin signaling pathway.
Tumor Necrosis Factor Alpha (TNF-α) and Nuclear Factor Kappa B (NF-κB) Signaling

Tumor promoting inflammation is one of the hallmarks of cancer[19]. Evidently, patients suffering from chronic inflammation, such as inflammatory bowel diseases, are predisposed to CRC[35]. Tumor necrosis factor alpha (TNF-α) is a cytokine released from several immune cells (monocytes, macrophages, lymphocytes, neutrophils) and epithelial cells[36]. The pro-inflammatory cytokine activate signaling pathways responsible for cell proliferation, survival, transcription of pro-inflammatory genes, and cell death[37]. The downstream signaling of TNF-α is initiated upon binding of TNF-α to TNF receptor type 1 (TNFR1) or TNF receptor type 2 (TNFR2)[38]. This causes a conformational change in the receptor providing a binding platform for proteins intracellularly, initiating downstream signaling. It is mainly the canonical nuclear factor kappa B (NF-κB) signaling pathway or the Mitogen-activated protein kinases (MAPK) signaling pathways that is activated upon TNF-α stimulation[39].

The mammalian family of NF-κB transcription factors consists of five members (p50, p52, p65(ReA), cRel, and RelB) which form homodimers or heterodimers to bind to their target genes[40]. p100 and p105 are preliminary forms of p52 and p50, respectively. p52 and p50 becomes active transcription factors upon proteolytic processing of p100 and p105[39]. RelB, cRel, and p65 all have transactivation domains facilitating the transcription of target genes through DNA binding, while p50 and p52 exert DNA binding specificity by forming heterodimers with the other NF-κB subunits. The main NF-κB dimer activating transcription is the heterodimer of p65 in association with either p50 or p52[41]. However, homodimers of p50/p50 or p52/p52 can also activate or repress NF-κB target genes by interacting with other activators or repressors[40,42,43].

Two main NF-κB signaling pathways exist, the canonical and the non-canonical NF-κB signaling pathway. The non-canonical NF-κB signaling pathway is activated by TNF receptor family members such as CD40 and lymphotoxin-β receptor, which mainly plays a role in the secondary lymphoid organ development, B-cell survival and maturation[44]. The activation mainly leads to p52/RelB dimers[45].

The canonical NF-κB signaling pathway is activated by stimuli such as TNF-α and Interleukin-1. Following binding to TNFR1 a number of proteins are activated which results in activation of the Inhibitors of NF-κB kinase (IKK) complex (Figure 2). The IKK complex phosphorylates Inhibitors of NF-κB (IxBs) targeting them for degradation (Figure 2). Thereby NF-κB transcription factors are released and translocated into the nucleus where they activate or repress genes involved in inflammation, differentiation, survival, proliferation, angiogenesis, and metastasis (Figure 2)[39].
Cancer Stem Cells

The traditional stochastic tumorigenesis model argues that all cells within a tumor have the potential to initiate and drive tumorigenesis[46]. However, within the last decade much attention has been directed towards stem cells and their potential as tumor initiating cells leading to the cancer stem cell model of tumorigenesis. The cancer stem cell theory proposes that tumors are comprised of a hierarchy of cells that are biologically distinct and cells with stem cell properties have a central role in initiation, progression, reoccurrence of cancer, and treatment response[46,47]. The fact that the adult stem cells already have attributes favorable for tumor development, such as
self-renewal and longevity, has resulted in the hypothesis that they might be the cells of origin. These characteristics make stem cells more potent for malignant transformation, whereas more differentiated cells have to acquire additional mutational events to gain tumorigenic capabilities[48].

Similarly to normal stem cells, cancer stem cells are also dependent on the microenvironment/niche they reside in[49]. Therefore, it is reasonable to propose that the immune infiltrate present in the stroma potentially affect the stem cells and cancer stem cells. Recently, dedifferentiating of non-stem intestinal epithelial cells has been described in a genetic mouse model[50]. The dedifferentiation was dependent on active NF-κB signaling in the epithelial cells which resulted in enhanced β-catenin signaling, leading to dedifferentiation of non-stem cells into cells with stem cell and tumor-initiating properties[50]. Thus, cancer stem cells do not necessarily originate from stem cells but might arise from a differentiated cell becoming dedifferentiated and gaining stem cell properties[50]. Furthermore, a recent paper by Rozhok et al. argue that phenotypes of oncogenic mutations are environment-dependent and thus the effects on somatic cells are different depending on the environment that these cells reside[51]. Accordingly, they extend the evolutionary model of carcinogenesis adding that cancer consists of several clones outcompeting each other depending on the environment they live in and the environment they are subjected to[51]. This provides a much more complicated picture of cancer development and progression than previously thought regardless of the traditional stochastic model or cancer stem cell hypothesis.

To understand the role of intestinal stem cells in CC a brief introduction to the intestinal stem cells is provided in the following section.

1.4 INTESTINAL STEM CELLS

Due to the high turnover of cells, the intestinal epithelium is constantly self-renewing to maintain epithelial homeostasis[52]. Early studies indicated that stem cells residing at the bottom of the crypt are responsible for maintaining cellular homeostasis of the crypts in mice[53,54] (Figure 3). The intestinal stem cells do not immediately turn into differentiated cells. As the cells migrate towards the lumen they become an intermediate progenitor pool of cells referred to as transit-amplifying cells (TA cells). These cells divide rapidly, generally amplifying the population of cells in the crypt. Next the cells migrate towards the lumen, stop dividing, and differentiate into the absorptive or secretory lineage[54]. Finally, the cells are discarded into the luminal space as they reach the top and become senescent.
Figure 3 A simplified model of the location of intestinal stem cells, progenitors, and differentiated cells in the small intestinal and colon crypt together with an indication of the Wnt signaling gradient along the crypts.

Since Barker and colleagues in 2007 identified the Lgr5+ stem cell population by sophisticated in vivo lineage tracing in mice[55], the field of intestinal stem cells and the number of publications within the subject has exploded. The Lgr5+ marks a subpopulation of intestinal stem cells referred to as crypt base columnar cells due to their appearance. These cells are interspersed between Paneth cells in the small intestine (Figure 3). The study by Barker and colleagues showed that Lgr5 expressing cells had a long life-span and were multipotent by harboring the ability to give rise to all the cell types of the colon and small intestine[55]. Shortly after another paper was published reporting another subpopulation of intestinal referred to as the +4 stem cell population (+4 referring to the location of the cells approximately four cells from the bottom of the small intestinal crypt, just above the Paneth cells (Figure 3)[56]. The researchers found that this population was characterized by a distinct expression of Bmi1[56]. Since then several other potential stem cell markers has been described as briefly listed in Paper I. Sox9 has been proposed as a more general marker marking both of the intestinal stem cell populations[57].
The two different subpopulations of intestinal stem cells have been suggested to have diverse roles. The Lgr5+ population drive the intestinal epithelial homeostasis under physiological conditions being constitutively proliferative active[55], whereas the +4 stem cell population is a more quiescent “reserve” stem cell population that becomes active during tissue repair and regeneration in response to injury[58,59]. A reserve stem cell population is also seen in the colon but is not restricted to a certain position[60]. Whether two different adult stem cell populations exist is still controversial.

Intestinal Stem Cell Markers in Colorectal Cancer

A large proportion of the intestinal stem cell markers have been reported to be elevated in CRC. The expression of some of these markers has been correlated to clinicopathological features and may have a prognostic significance as reviewed in Paper I. In the following sections the function and clinical implications of BMI1 and SOX9 in CRC is further elaborated. Even though the field of intestinal stem cell markers has expanded immensely during the last decade, technical detection of the markers in a clinical setting remains an issue. To date most of these markers can still only be detected using RNA in situ hybridization due to insufficient antibodies for protein detection. With the premise of clearly defined cut-off values for the biomarker, immunohistochemistry (IHC) would be preferred as a first line semi-quantitative method in a clinical setting due to the low cost, simplicity of the analysis, the relatively short processing time, high throughput, and many years of experience with the technique. Thus, even though LGR5 is one of the most investigated markers, there is still no commercial optimal antibody for the protein detection in human tissue. Based on availability of commercially available antibodies for protein detection of the intestinal stem cell markers, SOX9 and BMI1 were investigated as potential biomarkers in the present project.

1.5 BMI1

BMI1 encodes for the 37kDa polycomb protein BMI1 and was initially identified as an oncogene playing a role in the initiation of B-cell lymphomas in mice[61]. BMI1 is part of the polycomb repressive complex 1 (PRC1) which plays an important role in gene silencing through chromatin remodeling and modification of histones in various cell types, including embryonic and adult stem cells[62,63]. Besides BMI1 the PRC1 complex consists of several other subunits and the composition of the complex differ among cell types[64]. The composition of the PRC1 complex has an impact on the silencing of specific subsets of genes, however to what extent is still unknown.

As previously mentioned, Bmi1 has been proposed to mark +4 intestinal stem cells[58]. It should be noted that most of the functional studies of Bmi1 have been conducted in the small intestine and not the colon, where the function of BMI1 and the expression might differ slightly. BMI1 has been reported to be completely absent or expressed at low levels in the nucleus of human colon epithelial cells at the crypt bottom with a distinct decreasing expression towards the lumen and with the surface epithelium being mostly negative[65–67].
BMI1 has been reported to play a role in numerous pathways targeting critical genes[68]. One of the main targets of BMI1 is the Ink4a/Arf loci which encodes the critical tumor suppressors p16 and p14 involved in the retinoblastoma protein and p53 signaling pathways[69,70]. Silencing of the locus causes increased self-renewal and proliferation along with decreased senescence and apoptosis[69]. Silencing of this crucial locus has also linked BMI1 to its oncogenic activity in various cancers. Indeed, downregulation of BMI1 has been reported to reduce growth of CRC cells and impair colorectal tumor initiation in vitro and in vivo[71]. Another study demonstrated that BMI1 expression is associated with nuclear β-catenin and is required for CC proliferation in vitro and in vivo[72].

Clinical Implications of BMI1 in Colorectal Cancer

As reviewed in Paper I, several studies have investigated both mRNA and protein BMI1 expression in human CRC specimens[4]. Presently, BMI1 has not been tested as a biomarker for relapse of stage II CC patients. The majority of the studies report that BMI1 is upregulated in CRC[65–67,73–77]. Studies on correlations of BMI1 expression level in colorectal tumors and clinicopathological features are conflicting. Several studies have correlated BMI1 expression to cancer stage, indicating that BMI1 might be associated with cancer progression[73–75]. However, there is also one study reporting that BMI1 expression is not correlated to TNM stage[76].

The prognostic significance of BMI1 is debatable as presented in Paper I. A recent meta-analysis paper indicated that the prognostic value of the BMI1 expression might be different depending on whether the patient cohort is of Asian or Caucasian origin[78]. The study included many different cancers, however with regards to CRC only three Asian studies were included[79]. An univariate analysis showed that CRC patients with BMI1 positive tumors both had a significant lower 5-year disease free survival and 5-year overall survival[75]. In the same study the multivariate analysis showed that BMI1 expression, cancer stage, and tumor differentiation was independent prognostic factors of disease free survival[75]. This was also the case when having 5-year overall survival as an endpoint[75]. In contrast, another study performed by Benard and colleagues showed CC patients with high BMI1 expression in their primary tumor had a tendency to a longer disease free survival than patients with low BMI1 expression[76]. Since the polycomb proteins in general act together in complexes the authors also investigated if a combination of several polycomb proteins as one variable would provide a better stratification of the patients. By combining four polycomb proteins, including BMI1, the longest overall survival and longest recurrence free period was observed in patients who had a high expression of all four proteins in the primary tumor compared to patients with low levels of the markers[76]. These studies indicate that BMI1 may have a prognostic significance as a singular biomarker or in combination with other polycomb proteins. However, further studies are needed to confirm the significance.
1.6 SOX9

SOX9 was initially discovered in patients with campomelic dysplasia syndrome caused by heterozygous SOX9 mutations[80]. The syndrome is characterized amongst other defects by severe skeletal malformations and male-to-female sex reversal. Since then SOX9 has been reported to play a crucial role in several embryonic developmental processes as well as functioning as a tight regulator of adult tissue architecture and homeostasis in, amongst others organs, the intestinal tissue[81,82].

The SOX9 Gene and Protein

SOX9 is part of the SOX family comprising transcription factors containing a highly similar 79 amino acid high-mobility binding group (HMG) box domain to the gene SRY. The family is subdivided into groups from A through to H. Together with SOX8 and SOX10, SOX9 belong to the SOXE group. The self-dimerization region (DIM) preceding the HMG domain is unique to the SOXE proteins and provides the proteins with the ability to homodimerize or heterodimerize SOXE proteins in-between [83]. In addition, SOX9 have two transactivation domains located in the C-terminus. One of the domains is rich in proline, glutamine, and alanine (PQA) and probably potentiate the transactivation properties of the downstream transactivation domain which is rich in proline, glutamine, and serine (PQS)[84,85] (Figure 4). The transcription factor consists of 509 amino acids with a total molecular weight of 65kDa. The protein is encoded by the SOX9 gene located on the long arm of chromosome 17q24.3 (position 70,117,161-70,122,560, GRCh37/hg19 Assembly) stretching over 5,400bp and comprising of three exons and two introns (Figure 4)[80].

Figure 4 Illustration of SOX9. (A) The SOX9 gene and (B) the SOX9 protein (the numbers refers to amino acids).
Implications of SOX9 in the Intestines and in Colorectal Cancer

The exact function of the SOXE proteins are highly context specific and the proteins interact and binds differently in different cellular environments. The SOX proteins generally regulate gene transcription by bending DNA through interaction of the HMG-domain to the minor groove of the DNA helix[83].

SOX9 expression is restricted to the nucleus of cells located in the lower proliferative compartment of the crypts of both the small intestine and the colon of humans and mice[32,86–90]. It has been proposed that SOX9 is expressed throughout the intestinal epithelia in the early embryonic stages and becomes restricted to the crypt cells as the development proceeds[81]. SOX9 expressing cells have been shown by lineage tracings to repopulate the entire small intestinal and colonic tissue in mice, indicating self-renewal and multipotent properties[81]. Furthermore, a recent study indicated that SOX9 is required for radiation resistance and subsequent epithelial regeneration[60], suggesting that the transcription factors is important for the reserve intestinal stem cell population. Colonic epithelial cells with high expression levels of Sox9 is associated with a gene expression profile consistent with a less differentiated population, whereas colonic epithelial cells expressing low levels of Sox9 had a gene expression profile consistent with more differentiated cells[91]. This suggests that the expression of Sox9 itself might not be a stem cell marker but rather the exact expression level of Sox9 may determine its cellular function.

The expression of the transcription factor is confined to a heterogeneous population of proliferative but less differentiated cells with the exception of the postmitotic Paneth cells and enteroendocrine cells of the intestines[32,81,92]. In agreement, SOX9 has been found to indirectly repress Caudal type homeobox 2 (CDX2) and directly repress Kruppel-like factor 4 (KLF4) in vitro which are expressed in more differentiated cells[32,93]. Furthermore, Sox9-deficient mice has decreased levels of goblet cells in the colon[92]. This was also observed in the small intestine, however with the addition of a decrease, and in some cases complete depletion, of the Paneth cells[92]. These studies suggest that SOX9 plays a central role in differentiation of the goblet and Paneth cell lineages of the intestines in vivo[92]. Somewhat contradicting Blache and colleagues showed that Mucin 2 (MUC2) was indirectly repressed by SOX9 in vitro[32] and Mori-Akiyama et al. showed that only the Paneth cell lineage was affected in the small intestine with the remaining lineages intact of Sox9-deficient mice (Figure 5)[94].
In addition to being a regulator of differentiation, Sox9 has also been found to be important for intestinal tissue architecture and homeostasis[60,82,92]. Inactivation of Sox9 has been reported to induce severe alterations of the colonic and small intestinal morphology in mice[92,94]. Hyperplasia and dysplasia was observed throughout the intestines including both the small intestine and the colon of the Sox9-deficient mice[92,94]. However, inactivation of Sox9 itself did not induce malignancy[92], indicating that SOX9 itself cannot trigger malignant transformation but tightly regulates proliferation of the intestinal epithelial cells. The exact role of SOX9 in cancer development and progression is unclear since studies both indicate tumor suppressive and oncogenic properties of the transcription factor[32,82,89,90,92,95,96].

SOX9 regulates a plethora of genes dependent on transcriptional binding partners, the specific environment, and potentially also the level of SOX9 protein available. In addition to potentially regulating itself[97], p65 of the NF-κB signaling pathway has also been shown to bind to the Sox9 promoter (Figure 5)[50]. Furthermore, SOX9 is a downstream target of the active β-catenin/TCF complex in CC cells (Figure 5)[32].

Figure 5 Illustration of the function and regulation of SOX9.
Conversely, the transcription factor has also been reported to inhibit the β-catenin/TCF4 complex in CC cell lines (Figure 5)[92], suggesting a negative feedback loop and a potential tumor suppressive role of SOX9. In contrast, Matheu et al. showed that SOX9 promote tumorigenesis in vivo and potentially regulates Bmi1 in mice and human CC cell lines[95].

Clinical Implications of SOX9 in Colorectal Cancer

The conflicting studies on the clinical implications of SOX9 in CRC might be due to existence of variants of the protein. Interestingly, Abdel-Samad and colleagues showed that a truncated version of SOX9 (referred to as MiniSOX9) was present in human CC tissue and cell lines due to a premature stop codon in intron 2[89]. The variant was nearly undetectable in normal colon[89]. The variant lacked the C-terminal transactivating domain but contained the HMG domain. MiniSOX9 was shown to up-regulate the canonical Wnt signaling pathways whereas full length SOX9 inhibited the pathway in vitro[89]. This study is in accordance with an extensive genomic characterization of human CRC, where SOX9 was reported to be mutated in 9% of the CRC cases[27]. The mutations were exclusively frameshift and nonsense mutations which could indicate a tumor suppressor function of SOX9 in CRC[27].

A low copy number gain of chromosome 17, where SOX9 is located, has been reported[95]. This might be a contributing cause to the SOX9 overexpression observed in some tumors from CRC patients. As reviewed in Paper I, several studies have reported an increase of SOX9 expression at both protein and mRNA level in human adenomas and CRC tissue compared to normal mucosa[4,87,88,95,98–102]. One study described a decrease in SOX9 expression in CRC compared to paired normal colon, however the study included relatively few patients (n=10)[103]. No significant difference in SOX9 expression levels has been reported between colorectal adenomas and carcinomas[87]. SOX9 has been found to be expressed in a heterogeneous manner throughout the tumors[87,91] and the expression has been correlated to several clinicopathological features as reviewed in Paper I.

Univariate analysis and multivariate analysis adjusted for TNM-stage and tumor budding showed that high SOX9 expression was associated with a lower 5-year overall survival[87]. However, this could not be confirmed in a study by Panza et al. who found no association between SOX9 expression and overall survival of CRC patients[101]. Similarly, a Norwegian study neither found any association between SOX9 expressions of primary tumors and 5-year overall survival (n=761 CRC patients) or time to recurrence (n=583 CRC patients)[104]. Interestingly, a study reported that stage III CRC patients having high SOX9 expression in their primary tumors had a significantly poorer survival than patients with low SOX9 expression[88]. This was most likely due to treatment with 5-FU, indicating that SOX9 could be relevant as a predictive marker for this treatment. No association between survival and stage II CRC patients was observed.
In untreated CRC patients there was a tendency to low expression was associated with reduced survival, however this was not significant[88].

Conclusively, SOX9 may have a prognostic and predictive significance, however further studies are needed to validate the findings.

1.7 AIM OF INVESTIGATIONS

The overall objective of this PhD project was to investigate whether intestinal stem cell markers could predict relapse of stage II CC patients and furthermore explore the regulation of SOX9.

The specific aims and hypothesis of the present studies were:

I) **Aim**: Provide an overview of the clinical implications of intestinal stem cell markers in CRC. (Review).

II) **Aim**: Investigate if BMI1 protein expression in primary tumors of stage II CC patients could predict risk of relapse.

   **Hypothesis**: Stage II CC patients with high expression of BMI1 in their primary tumors have high risk of relapse.

III) **Aim**: Explore whether SOX9 protein expression in primary tumors of stage II CC patients could predict risk of relapse.

   **Hypothesis**: Stage II CC patients with high expression of SOX9 in their primary tumors have high risk of relapse.

IV) **Aim**: Investigate whether TNF-α regulate SOX9 through NF-κB signaling in human CC cell lines.

   **Hypothesis**: TNF-α regulate SOX9 through the NF-κB signaling pathway in CC.
2. MATERIALS AND METHODS

In the present section materials and methods used in the different papers are summarized. More detailed descriptions are presented in Paper II-IV.
2.1 PATIENT COHORT AND TUMOR TISSUE (PAPER II AND III)

The cohort was collected retrospectively. A total of 282 stage II CC patients diagnosed and treated at Herlev University Hospital, Gentofte University Hospital, or Glostrup University Hospital were consecutively included from January 2005 to August 2008. The enrollment period was based on a desire of minimum 5 years follow-up. The follow-up was terminated the 14th of April 2014. Registered data, inclusion and exclusion criteria are presented in detail in Paper III. The final patient cohort consisted of 144 stage II CC patients who all had undergone complete surgical resection as primary treatment.

A flow chart over the analysis conducted after patient selection is presented in Figure 6. Two formalin-fixed paraffin embedded (FFPE) tissue blocks of the primary tumor from each of the stage II CC patients were obtained for the further analysis. The FFPE tumor tissue had been processed as part of the diagnostic routine following surgery and is described in more detail in Paper III. A tissue block containing both the invasive front and the luminal surface was used for IHC analysis of SOX9 and BMI1, whereas a secondary tumor tissue block was used for construction of tissue microarrays (TMA). The TMA was constructed as presented in Paper III.

Figure 6 Flow chart of the laboratory analyses conducted in Paper II and III.

![Flow chart of the laboratory analyses conducted in Paper II and III.](image-url)
2.2 SOX9 AND BMI1 IMMUNOHISTOCHEMISTRY ANALYSIS (PAPER II AND III)

All of the analyses were blinded to patient outcome. The IHC analysis was conducted following the diagnostic routine at the Department of Pathology at Herlev University Hospital using the EnVision™ FLEX (Dako, Glostrup, Denmark) system and the automated Autostainer Link 48 (Dako). Following antibody staining, the tissue slides were counterstained with Mayers hematoxylin using the automated slide stainer Tissue-Tek® Prisma® /Film® (Sakura, Alphen aan den Rijn, Netherlands). The slides were scanned using the Nanozoomer 2.0-HT (Hamamatsu, Herrsching, Germany) and partially digitally scored using the image analysis software Visiopharm Integrator System (version 4.5.6.516, Visiopharm, Hoersholm, Denmark).

Antibody Optimization

The antibodies were optimized using a control tissue slide. Tissues from normal ventricle, small intestine, colon, breast, and testis was selected as control tissues for BMI1 and SOX9 based on the clinical studies presented in Paper I and further literature review[105–108]. Likewise, the antibodies applied in Paper II and Paper III was selected based on literature review and manufacturer’s information. Specifications of the antibodies are presented in Paper II and Supplementary Table 1 in Paper III.

Three antibodies, anti-SOX9 (AB5535, Merck Millipore, Darmstadt, Germany) and two anti-BMI1 (clone F6, cat. no. 05-637, Merck Millipore, Darmstadt, Germany; HT-99, cat no. sc-10745, Santa Cruz Biotechnology, Heidelberg, Germany) were tested by different antibody dilutions with and without the amplifier EnVision Flex+ Linker (Dako). Antigen retrieval was tested by EnVision™ FLEX, High pH antigen retrieval buffer (Dako) and EnVision™ FLEX, Low pH antigen retrieval buffer (Dako) to mimic the routine diagnostic settings as close as possible. The antigen retrieval method that provided the most specific staining with the antibody was selected for further use. EnVision™ FLEX, High pH antigen retrieval buffer (Dako) was selected for both the BMI1 antibody (clone F6, Merck Millipore) and the SOX9 antibody (AB5535, Merck Millipore). The antigen stability was tested by obtaining normal colonic tissue from the same individual and dissecting it into four pieces. Next the four tissue pieces were subjected to 3, 27, 51, and 123 hours of 10% neutral buffered formalin. The time points were set such that it fitted with the diagnostic routine work.

Evaluation of Immunohistochemistry

Due to the heterogeneous expression of both SOX9 and BMI1 and the lack of national or international guidelines for the scoring of these, the protein expression was evaluated both at the invasive front and at the luminal surface of full slides. The luminal surface was defined as the luminal surface of the neoplastic glands, whereas the invasive was considered where the tumor periphery invaded deepest into the tissue. The luminal surface was not available in 2 and 3 cases of SOX9 and BMI1 analysis, respec-
tively, as the tissue was unable to stick to the glass slide. A detailed description of the evaluation of BMI1 and SOX9 is presented in Paper II and Paper III.

2.3 TESTING FOR MISMATCH REPAIR DEFICIENCY (PAPER II AND II)

Immunohistochemistry

IHC was performed as part of the diagnostic routine using the constructed TMAs. If one or more of the four MMR proteins (MLH1, PMS2, MSH2, and MSH6) was undetected in the tissue; the tumor was defined as dMMR. If all the MMR proteins were present, the tumor was defined as MMR proficient (pMMR). Specifications of the antibodies and an elaborate description of the IHC are presented in Paper III.

DNA Extraction and Promoter Methylation Assay

Tumors that were dMMR were further analyzed by a promoter hyper-methylation assay. Using the same tissue block as for the TMA, an area with high tumor cell content (≥50%) was selected and steriley punched out. DNA was extracted as previously described[109]. Promoter hyper-methylations of the MMR genes were conducted as part of the diagnostic routine and therefore investigated using the SALSA MS-MLPA ME011 Mismatch Repair Genes kit (MRC Holland, Netherlands). The assay was performed according to manufacturer’s instructions with a 50ng DNA input. Each run included a positive, a negative, and a no template control. All un-methylated cases were rerun to confirm the status.

2.4 FUNCTIONAL STUDIES (PAPER IV)

Cell Culture and Treatment

TNF-α-stimulation experiments were carried out by a collaborative group at the Department of Gastroenterology (Medical Section), Herlev University Hospital and conducted as described in Paper IV. The human colon carcinoma cell lines HT29, DLD1, and SW480 were stimulated for 24 hours with 10nM TNF-α (R&D Systems, Minneapolis, MN, USA) while Caco-2 cells were stimulated for 24 hours with increasing concentrations of TNF-α (0.1, 1, or 10nM)[110].

RNA Extraction and Quantitative Reverse Transcription (qRT)-PCR

RNA extraction and quantitative reverse transcription-PCR (qRT-PCR) was conducted by our collaborative group at Department of Gastroenterology (Medical Section) at Herlev University Hospital. Briefly, total RNA from Caco-2, HT29, DLD1, or SW480 cells was extracted and converted to cDNA as presented in Paper IV. The qRT-PCR reactions were performed using Maxima SYBR Green qPCR Master Mix (Fisher Scientific, Pittsburgh, PA, USA) on a LightCycler 480 (Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer’s instructions. The SOX9 transcript was amplified using the primers: forward-5’GATTACAAGTACCAGCCCGCG-3’ and reverse- 5’GGATTGC-
CCCGAGTGCTC-3' (Eurofins, Ebersberg, Germany). The SOX9 gene expression was normalized to the expression of human Ribosomal Protein Large P0 (RPLP0) which was used as a reference gene.

**Construction of SOX9 Promoter Reporter Plasmid**

The SOX9 promoter reporter plasmid was constructed by amplifying a 703bp region upstream of the SOX9 gene (from position chr17:70,116,683-70,117,385, the GRCh37/hg19 Assembly), including the untranslated region of exon 1. The fragment was cloned into the firefly luciferase reporter vector pGL4.10 using the *HindIII* restriction site and the In-Fusion® HD Cloning Kit (Clontech Laboratories, Mountain view, CA, USA) according to manufacturer’s instructions.

**Transient Transfections and Luciferase Assay**

Caco-2 cells were grown as described in Paper IV. When the cells were 70% confluent they were split and seeded in a 24 well plate in a density of 5×10⁴ cells/ml medium overnight. The promoter reporter construct (pGL4-SOX9) was co-transfected with pCMV4-p50 (plasmid #21965[111], Addgene, Cambridge, MA, USA), pCMV4-p52 (plasmid #23289[112], Addgene), pCMV4-p65 (plasmid #21966[111], Addgene), or a combination of these overexpression plasmids together with the empty pcDNA3.1+ and the pCMV-LacZ plasmid. The pBluescript SK+ plasmid was added to normalize the DNA mix to a total amount of 0.3µg DNA. The DNA mix was mixed with the transfection agent 2µM polyethylenimine (PEI25, Alfa Aesar, Karlsruhe, Germany) dissolved in 150mM NaCl. The mixture was added to the cells. All transfections were carried out in four replicates. Four hours later the media was changed and the cells were harvested after two days. The luciferase levels were measured using the Dual-Light® reporter gene assay system (Termo Fisher Scientific). The luciferase levels were normalized to the level of luciferase exhibited by the pGL4-SOX9 promoter reporter construct because the overexpression plasmids affected the β-galactocidase expression. All of the transfections were repeated three times.

**2.5 STATISTICS (PAPER II-IV)**

The clinical data was explored by non-parametric tests. Associations in Paper IV were investigated by the two-tailed Student’s t-test and the data is presented as mean ± standard deviation.

The primary endpoint of both Paper II and Paper III was risk of relapse. The secondary end point was overall survival of the patients. Time to relapse was defined as the time from surgical excision of the primary tumor to local relapse or metastasis occurring within the follow-up period. Patients who died during follow-up were censored. Overall survival was defined as time from surgery of the primary tumor to death of any cause within the 5-year follow-up period. Survival analysis was investigated as
elaborated in Paper II and Paper III. All two-sided p-values ≤0.05 were considered significant. A more elaborate description including model validations are presented in Paper II and Paper III.

2.6 ETHICS (PAPER II AND III)

The study was approved by the Data Protection Agency of the Capital Region of Denmark (2007-58-0015) and by the Scientific Ethics Committee of the Capital Region of Denmark (H-1-2013-028).
3. SUMMARY OF RESULTS

The following section included the most important results obtained during the PhD study period. Furthermore, some additional results which were not included in the published papers are also presented.
3.1 PATIENTS AND CLINICAL CHARACTERISTICS (PAPER II AND III)

Among the 144 stage II CC patients, who were included in the studies, 37 (25.7%) patients relapsed (Figure 7) with median time to relapse of 21 months ranging from 4 to 84 months. Overall 58 (40.3%) patients had died during follow-up period. By the clinical establishment of relapse, the majority of the patients (24 patients corresponding to 64.9% of the patients with relapse) had relapsed in two or more sites. Following surgery all the patients had tumor cell free margins. Furthermore, 70 (48.6%) of the patients had one or more of the histological risk factors present in their primary tumor. An overview of the patient characteristics are further presented in Paper II and Paper III.

Mismatch Repair Status

Overall 33 patients (22.9%) had tumors with loss of MLH1, MSH2, PMS2, or MSH6. An overview of the patients with relation to tumor side and dMMR are presented in Figure 7 and Figure 8.

Figure 7 Overview of the patient cohort with relation to MMR status, location of tumor, and relapse.

The majority of the dMMR tumors had loss of MLH1 and therefore also PMS2 (Figure 8). Furthermore, a total of 5 out of the 144 (3.5%) patients had primary tumors presenting dMMR but without promoter hyper-methylations. No promoter hyper-methylations were detected in tumors with MSH2 and/or MSH6 deficiency.
Figure 8 Summary of the MMR status of the 144 stage II CC patients included in the cohort. The distribution of protein loss of the four MMR proteins: MLH1, PMS2, MSH2, and MSH6 and the frequency of MLH1 promoter hyper-methylations are presented.

3.2 BMI1 EXPRESSION IN STAGE II COLON CANCER PATIENTS (PAPER II)

BMI1 Protein Expression

Based on pretreatment experiments and dilution experiments the BMI1 antibody from Santa Cruz Technology was excluded as none of the optimizations led to a specific staining. Thus, the clone F6 BMI1 antibody from Merck Millipore was superior in specificity in this regard. When applying the BMI1 antibody from Merck Millipore, the BMI1 antigen was not affected by the differences in formalin fixation time. As expected BMI1 was expressed in the nuclei of the epithelial cells located at the base of the crypts in the colon tissue with decreasing expression level towards the lumen (Figure 9). However, BMI1 was also highly expressed in a number of other cell types, including lymphocytes and stromal cells. The BMI1 expression varied widely both in number of positive cells, in the intensity within the individual tumor, and in intensity in-between the tumors. Examples of BMI1 expression in the tumors are presented in Figure 9.
Figure 9 Examples of BMI1 expression in normal colon tissue and stage II CC tissue (x10 magnification). (A) BMI1 protein expression in normal colon tissue. (B) Low BMI1 protein expression at the tumor invasive front. (C) High BMI1 protein expression at the tumor invasive front (Modified from Paper II)[113].
The Prognostic Value of BMI1

The BMI1 expression correlated with a number of different clinicopathological features. The BMI1 expression at the invasive front correlated significantly with both MMR status (p=0.01, Mann-Whitney U test) and age of the patients (p=0.01, Spearman rank correlation). However, both correlations were weak. Furthermore, the tumor location (p=<0.01, Mann-Whitney U test), T-stage status (p=<0.01, Mann-Whitney U test) and the histological subtypes (p=0.02, Kruskal-Wallis test) correlated significantly with BMI1 expression at the luminal surface. No significant correlations were found between the BMI1 expression at the invasive front or the luminal surface and the collective histological risk factor variable, the remaining histological risk factors, or gender of the patients.

There was no significant association between risk of relapse of stage II CC patients and BMI1 expression at neither the invasive front nor the luminal surface (Table 3). Similarly, the BMI1 expression was neither associated with 5-year overall survival (Table 3).

Table 3 Univariate Cox regression analysis exploring the association between BMI expression of primary tumors of stage II CC patients, risk of relapse and 5-year overall survival (Paper II)[113]. Hazard ratios (HR) and 95% confidence intervals (95% CI) are presented.

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Hazard Ratio (95% CI)</th>
<th>p-value</th>
<th>Hazard Ratio (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Univariate Analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endpoint: Relapse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI1 Expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive front</td>
<td>144</td>
<td>1.12 (0.78-1.60)</td>
<td>0.53a</td>
<td>1.12 (0.80-1.56)a</td>
<td>0.46a</td>
</tr>
<tr>
<td>Luminal surface</td>
<td>141</td>
<td>1.06 (0.75-1.48)</td>
<td>0.70b</td>
<td>1.16 (0.86-1.60)b</td>
<td>0.33b</td>
</tr>
<tr>
<td>Univariate Analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endpoint: 5-Year Overall Survival</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

aBMI1 as a continuous score. The hazard ratio is presented with a difference of 3 in BMI1 units.
Invasive front (Expression at the invasive front of the tumor)
Luminal surface (Expression at the luminal surface of the neoplastic glands)
n (Number of patients)
3.3 SOX9 EXPRESSION IN STAGE II COLON CANCER PATIENTS (PAPER III)

SOX9 Protein Expression

The SOX9 antigen was not affected by the formalin fixation time of the tissue. In the colon the highest expression of SOX9 was observed in the nuclei of the epithelial cells located at the bottom of the crypts. Like the BMI1 expression, the intensity of SOX9 protein expression in the colon epithelial cells decreased towards the luminal surface with no expression at the surface. Very rarely a few stromal cells also expressed SOX9. Interestingly, SOX9 was not detected in 12 (8.3%) and 11 (7.6%) of the tumors at the luminal surface and the invasive front, respectively. However, when SOX9 was present in the tumors it was almost exclusively detected in all tumor cells with only a variation in the intensity of the expression. Thus, SOX9 was detected in >75% of the tumor cells at either the invasive front or the luminal surface. Intratumoral heterogeneity was present in some cases where SOX9 could be detected at the invasive front but not at the luminal surface or vice versa. Examples of high and low SOX9 expression in stage II colon tumors and SOX9 expression in normal colon tissue are presented in Figure 10.

Figure 10 SOX9 expression in normal colon tissue and stage II CC. (A) SOX9 expression in normal colon tissue (x2.7 magnification and x20 magnification in the left corner). (B) High SOX9 expression at the invasive front of a tumor (x1.25 magnification and x20 magnification in the left corner). (C) Low SOX9 at the invasive front a tumor (x1.25 magnification and x20 magnification in the left corner). (Modified from Paper III)[86].
In one case a peculiar expression of SOX9 was observed in the tumor (Figure 11, unpublished). Two clearly distinct subclones with different morphologies were present within the same tumor. In addition to the morphology, the subclones could also be separated according to the SOX9 expression (Figure 11, unpublished). Several tumor islands with a glandular structure and high SOX9 expression in the tumor cell nuclei were observed. However, several tumor islands with low differentiated morphology and undetected SOX9 expression in the tumor cells were also present.
Figure 11 Distinct SOX9 expression in a stage II CC case tumor islands presenting glandular morphology and high SOX9 expression (marked by red arrow) and tumor islands presenting low differentiation histology and undetectable levels of SOX9 (black arrow). (A) x2.5 magnification. (B) x10 magnification.
Bmi1 has previously been proposed as a potential SOX9 target in mice[95]. To investigate whether the expression of BMI1 and SOX9 correlated a two-tailed Spearman rank correlation was conducted. At the invasive front BMI1 and SOX9 was not correlated (p=0.10, r=0.138). A significant correlation was found between BMI1 and SOX9 expression at the luminal surface (p=<0.01, r=0.36). Though, it should be noted that the r-value is quite low, suggesting a very weak correlation. A Cohen’s weighted κ analysis exploring inter-observer agreement of the intensity score of SOX9, showed a high concordance with a κ-coefficient of 0.84 (95% CI: 0.77-0.91).

SOX9 Expression Predicts Relapse of Stage II Colon Cancer Patients
The SOX9 expression at neither the invasive front nor the luminal surface correlated with any of the clinicopathological data including the histological risk factor variable. The SOX9 expression was divided into high and low expression. This was based on whenever SOX9 was detectable, it was present in >75% of the tumor cells in almost all cases. Kaplan-Meier survival plots compared by log-rank test showed that patients with a low SOX9 expression in their primary tumors had a significantly lower relapse free survival than patients with high expression of SOX9 (Figure 12). There was no significant difference in relapse free survival and the level of SOX9 expression at the luminal surface.

Figure 12 Kaplan-Meier survival plots of relapse free survival over time (months) with high and low SOX9 expression as variables and compared by the log-rank test. (A) SOX9 expression at the luminal surface of the tumors. (B) SOX9 expression at the invasive front of the tumors. HR and 95% CI from univariate Cox proportional hazard models are presented (Paper III)[86].

Univariate Cox proportional hazards analysis confirmed that patients with low expression of SOX9 at the invasive front of their primary tumors were statistically associated with 2.32 higher risk of relapse when including SOX9 expression as a dichotomous variable (Table 4). This was also found in multivariate analysis adjusting for dMMR
and histological risk factors. Conversely, high expression of SOX9 was associated with a lower risk of relapse in univariate Cox proportional hazard analysis when including the SOX9 expression as a continuous variable (Table 4). This remained statistical significant in a multivariate analysis adjusting for dMMR and histological risk factors. In both the univariate and multivariate analysis dMMR and the histological risk factor variable was statistically associated with a lower and higher risk of relapse, respectively (Table 4).

**Table 4 Univariate and multivariate Cox proportional hazards analysis of SOX9 expression, dMMR and histopathological risk factors of relapse (Modified from Paper III) [86].**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate Analysis</th>
<th>Multivariate Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard Ratio (95% CI)</td>
<td>p-value</td>
</tr>
<tr>
<td>SOX9 Expression at the Invasive Front</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuous</td>
<td>0.73 (0.56-0.94)</td>
<td>0.01†</td>
</tr>
<tr>
<td>High</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>2.32 (1.14-4.69)</td>
<td>0.02</td>
</tr>
<tr>
<td>MMR Status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dMMR</td>
<td>0.19 (0.05-0.80)</td>
<td>0.02</td>
</tr>
<tr>
<td>pMMR</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Histopathological Risk Factor†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3.01 (1.48-6.08)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

†SOX9 included as a continuous variable. HR is presented with a difference of 3 in SOX9 units. †SOX9 expression included as a dichotomous variable (High/Low). The histological risk factor variable was defined as present if either of the risk factors was present in the tumor: T4 stage, nerve and/or vein infiltration, low differentiated histology (unless the tumor was dMMR), tumor perforation, or less than 12 lymph nodes sampled at primary resection.

A significant association between higher risk of relapse and T4 tumors, nerve infiltration, or less than 12 sampled lymph nodes at primary resection was found (Paper III). There was no association between risk of relapse and gender, tumor location, the histological subtype, or vein infiltration. Furthermore, a univariate Cox proportional hazards analysis revealed that there was no association between low SOX9 expression and 5-year overall survival (HR: 1.56; 95% CI: 0.79-3.09; p=0.2). These data indicate that SOX9 is an independent predictor of the risk of relapse in stage II CC patients.
3.4 TNF-α AND NF-κB-MEDIATED REGULATION OF SOX9 (PAPER IV)

Due to the results of SOX9 in stage II CC patients, the regulation of SOX9 was further investigated in human CC cell lines. The research of SOX9’s role in CC has so far mainly focused on the effect of overexpressing or repressing SOX9 in CC and the genes of which the transcription factor regulates. Limited studies have focused on the exact regulation of SOX9 in CC. We had preliminary data suggesting that TNF-α potentially regulated SOX9[114]. Therefore, the following project was initiated.

**TNF-α Increases Endogenous SOX9 mRNA Expression Levels in CC Cells**

To elucidate the effect of TNF-α on endogenous SOX9 mRNA expression in human CC cell lines, SW480, DLD1, HT29, and Caco-2 was stimulated with TNF-α. TNF-α stimulation of HT29 and Caco-2 increased the endogenous expression of SOX9 mRNA (Figure 13). In Caco-2 cells the effect of TNF-α was dose-dependent. TNF-α had no effect on the SOX9 mRNA expression level in SW480 and DLD1 cells (Figure 13). These results suggest that the TNF-α affects the endogenous SOX9 mRNA expression but the effect is confined to certain CC cell lines. Due to the dose-response demonstrated in Caco-2 cells, this cell line was used as a model in the subsequent experiments.

*Figure 13 The effect of 10 nM of TNF-α on the endogenous SOX9 mRNA expression level in CC cell lines in DLD1, SW480, and HT29. The effect of increasing TNF-α concentrations on the endogenous SOX9 mRNA expression level in Caco-2 cells (n=6).*

\[ p ≤ 0.05; \quad **p < 0.01 \]

\( n \) (Number of replicates)
NF-κB Transcription Factors Affects SOX9 Promoter Activity

To further investigate TNF-α regulation of SOX9, a SOX9 promoter reporter assay was conducted. A luciferase reporter construct driven by the SOX9 promoter was generated. The promoter reporter plasmid was transiently transfected into Caco-2 cells with or without combinations of NF-κB (p50, p52, or p65) overexpression plasmids. Overexpression of p50 alone or in combination with overexpression of p65 led to an increase of SOX9 promoter activity (Figure 14). Likewise, overexpression of p52 together with p65 overexpression or alone resulted in increased SOX9 promoter activity (Figure 14). No significant effect on the SOX9 promoter was found when overexpressing p65 alone (Figure 14). These studies suggest that SOX9 may be regulated through the NF-κB transcription factors.

Figure 14 The SOX9 promoter activity when Caco-2 cells were transiently transfected with overexpression plasmids of p50, p52, and/or p65. The values are presented as means of luciferase activity relative to the promoter construct pGL4-SOX9 (n=4).

*p≤0.05; **p<0.01
n (Number of replicates)
EV (empty vector)
4. DISCUSSION, PERSPECTIVES, AND CONCLUSION

This section includes discussions and perspectives of the results obtained. Finally, conclusions of the studies are presented.
The prospect of increased numbers of early stage CRC patients and a persistent group of early stage CRC patients that relapse of their cancer regardless of the current stratification emphasizes the need for biomarkers to improve the high-risk stratification. At present, the TNM classification system is the gold standard within clinical pathology practice and it successfully guides the late TNM stages (stage III and IV) for further adjuvant treatment. However, some patients with lower TNM stages have similar adverse prognosis as patients with higher TNM stages. Currently, stage II CC patients are stratified according to certain high-risk histopathological characteristics; however, it remains a challenge to identify the 20% of the patients in whom the disease will recur as there at present are no biomarker to identify the remaining high-risk patients[3]. Thus, the primary objective of this PhD project was to investigate novel biomarkers to improve the stratification of stage II CC patients into high- and low-risk groups. Based on the cancer stem cell theory the proposed intestinal stem cell markers BMI1 and SOX9 were investigated in primary tumors of stage II CC patients following the REMARK guidelines[115]. To our knowledge BMI1 or SOX9 has not previously been investigated as prognostic biomarkers for relapse in stage II CC patients. Interestingly, SOX9 was found to independently predict risk of relapse of stage II CC and therefore the regulation of the $\text{SOX9}$ gene was further investigated. Our data suggests that SOX9 might be regulated by TNF-α potentially through the downstream NF-κB signaling pathway in Caco-2 cells.

4.1 THE ROLE AND REGULATION OF SOX9 IN COLON CANCER

The low expression of SOX9 as a predictor of adverse prognosis of stage II CC patients could illustrate that SOX9 is a tumor suppressor important for regulation of proliferation and expansion of colon epithelial cells. Indeed, Sox9 deficiency has been shown to cause hyperplasia and dysplasia of the intestinal epithelium in mice[92,96], which might be attributed its function as inhibitor of the β-catenin/TCF4 complex in the canonical Wnt signaling pathway[32,92]. Furthermore, the Cancer Genome Atlas reported that SOX9 was frequently mutated in human CRC and these mutations exclusively were nonsense and frameshift mutations, suggestive of a tumor suppressive role of SOX9[27]. These mutations could be the cause of the absent SOX9 protein expression in the investigated tumors of the present cohort. In Paper III we used an antibody directed against the C-terminal of SOX9. In a previous study, Abdel-Samad et al. reported a truncated variant of SOX9 with absent C-terminal (MiniSOX9)[89]. Thus, it is plausible that tumors with undetectable SOX9 in our study, might express MiniSOX9, and not the full length SOX9. It is also possible that the undetectable SOX9 expression is caused by SOX9 promoter hyper-methylations. Accordingly, hyper-methylations of the SOX9
promoter have previously been reported to be associated with loss of SOX9 expression and to have a prognostic significance in bladder cancer[116].

It can be speculated that tumor cells with low expression of SOX9 represent a clone of tumor cells with a metastatic advantage compared to tumor cells expressing SOX9. In one case we observed a tumor presenting two subclones which could be separated based on morphology and SOX9 expression. It would indeed be interesting to separate the two subclones and analyze them genetically to fully elucidate the differences. Additionally, analyzing the SOX9 expression in the recurrent tumors could provide useful information to whether SOX9 is transiently silenced to reach another stage of malignancy, such as epithelial-mesenchymal transition, or whether SOX9 is permanently lost. The fact that we found a potential more malignant subclone of cancer cells without SOX9 expression could support the model suggested by Rozhok et al. arguing that different cancer clones outcompete each other depending on the microenvironment they reside in and are subjected to[51]. Further molecular genetic analyses of the tumors are necessary to elucidate the underlying cause of low or undetectable SOX9 expression.

Since the SOXE proteins are environment and context dependent the low SOX9 expression might also be due to stimulus from the microenvironment. This may to some extent explain the heterogeneous expression of SOX9 we observed in the investigated tumors. We observed in two out of four cell lines that the pro-inflammatory cytokine TNF-α was associated with an increase of endogenous SOX9 mRNA expression. In Caco-2 cells the effect was observed to be directly related to the concentration of TNF-α. Data from a previous study investigating TNF-α responsive promoters identified the SOX9 promoter as potentially responsive to TNF-α stimulations in Caco-2 cells[114]. The NF-κB signaling pathway is a common downstream effector pathway of TNF-α. In line with the previous mentioned study, several potential NF-κB binding sites has been identified in the SOX9 promoter[117]. Some NF-κB binding sites in the SOX9 promoter region appear more active than others[117]. Furthermore, p65 has been proposed to regulate several intestinal stem cell genes together with β-catenin including Sox9, and this regulation was partly dependent on TNF-α[50]. We demonstrated that transient transfections with either p50 or p52 significantly increased the SOX9 promoter activity, while co-transfections of p50/p65 or p52/p65 caused a minor decrease compared to transfecting with p50 or p52 alone. Previous studies have reported homodimerization of p50 and p52 and the function exerted by the transcription factors depends on their binding to other regulatory proteins such as the NF-κB co-factor B-cell CLL/lymphoma 3(BCL-3)[42,43]. Based on the present studies we cannot exclude that TNF-α or NF-κB signaling regulates SOX9 indirectly or in concert with other regulatory proteins.

Another explanation to why p50 or p52 overexpression leads to increased promoter activity and to a lesser extent when p65 was overexpressed as well could be that
the cells are overloaded when co-transfecting with multiple overexpression plasmids. However, we tried to overcome this by adjusting the overall DNA level equally in all transfected cells. Furthermore, it cannot be excluded that the p50 and p52 overexpression alone might take use of the endogenous levels of p65 in the cells to some extent. Overexpression of p65 alone did not affect the SOX9 promoter activity which can be due to the fact that the endogenous p50 (p105) and p52 (p100) needs further processing to become actively functional transcription factors and is kept inactive by the IκB in the cytoplasm. It should be noted that overexpressing the NF-κB subunits in colon carcinoma cell lines should be interpreted with caution since this is an artificial system and may not reflect reality. In addition, the function of the NF-κB subunits might be concentration sensitive, thus upon high expression they may interact with other proteins and genes than in moderate or low concentrations.

The contradicting evidence of SOX9’s role in CRC can be attributed to the fact that different variants of the gene might exist as reported previously[89]. Furthermore, the use of different cell lines and model systems is also a contributing factor. We used the Caco-2 cell line which can spontaneously differentiate if the cells become too confluent. In hindsight the cells should have been cultured identically in our experiments. With the current setup we cannot exclude that the higher confluency of the cells when harvesting endogenous mRNA, had an effect on the SOX9 expression, especially since SOX9 mainly is expressed in less differentiated epithelial populations[91]. Additional experimental work needs to be performed to show the direct connection between TNF-α stimulated NF-κB regulation of SOX9.

A study have shown that persistent activation of the NF-κB signaling pathway in intestinal epithelial cells of mice slowly induces adenomas[118]. Loss of APC is an early event in colon tumorigenesis which can result in constitutive Wnt signaling. The combination of Apc loss and persistent NF-κB activation potentially enhance tumorigenesis[118]. This could suggest that SOX9 expression might be important in the early phase of tumorigenesis and might be lost during progression in more aggressive colon carcinomas. Potentially, SOX9 exert different roles at different levels of the carcinogenic process; therefore, while the regulation by TNF-α or NF-κB might only be relevant at a certain stage, inactivation or loss of SOX9 might be associated with a later stage of disease.

The inconsistency in clinical studies can also be due to differences in the experimental methods. A discussion of the experimental considerations for Paper II and Paper III will be further addressed in the following.

4.2 METHODOLOGICAL CONSIDERATIONS (PAPER II AND III)

Within a relative short period of time several intestinal stem cell markers has been proposed. Some of these have been reported to have a potential prognostic significance. However, a limitation when investigating some of these markers is the lack of
sufficient detection methods. An example is the most investigated intestinal stem cell marker, LGR5. Even though it was one of the first proposed markers in 2007[55], there is still no antibody that can sufficiently detect the protein in human tissue. Thus, most studies are conducted using in situ hybridization.

Today the discipline of molecular pathology is gaining ground in the clinical diagnostics and may be preferred in certain cases. Due to the higher costs, complexity and time consuming analysis, IHC will usually be applied as first line diagnostic method if possible. In comparison to molecular pathology techniques, the IHC analysis is simple and costs less. Furthermore, decades of experience precede the IHC analysis, whereas molecular pathology is a relative new discipline in pathology and experience is still being generated as the field is evolving in a fast pace. Even though the IHC technique is simple it can be more subjective than many molecular pathology techniques. One of the main biases that follow the IHC technique is the antigen stability and detection. This can be affected at numerous steps before and during the IHC analysis. Prior to the analysis, the handling of the tissue specimen (time until processing) and the subsequent tissue processing (e.g. fixation) can affect the antigen accessibility and stability. During the IHC process the antigen detection depends mainly on the antigen retrieval method, the primary antibody, and the secondary antibody applied. To limit undesired technical issues the studies conducted in Paper II and Paper III followed the clinical diagnostic routine using the fully automated EnVision system. Furthermore, the slides were partially digitally scored, marking the areas within the tumors that were analyzed. For the SOX9 analysis the inter-observer concordance was high, indicating that the SOX9 analysis is robust.

A limitation to our SOX9 study is that we only applied an antibody which targets the C-terminal region which has previously been shown to be absent in CC patients and cell lines[89]. Optimally we should have tested several antibodies to both investigate the SOX9 antigen robustness and reproducibility. To test antibodies targeting other regions of the SOX9 protein could also has provided additional information concerning SOX9 as a reliable biomarker for stage II CC relapse. In the BMI1 study we tested two different antibodies where the Millipore antibody was more reliable for detecting the BMI1 antigen. Discrepancies between our studies (Paper II and Paper III) and others can be the use of different antibodies. Thus, to fully exclude BMI1 as a biomarker or apply SOX9 as a biomarker, additional studies are needed to ensure reproducibility and consensus. In some cases different formalin fixation levels within the tumor might be falsely mistaken for heterogeneity due to decreased antigenicity. To ensure this was not the case for the BMI1 and SOX9 antigen, we tested the effect of different formalin fixation times. Both antigens were independent of the formalin fixation when applying the selected antibodies.

Due to the lack of international guidelines and the heterogeneity of the SOX9 and BMI1 protein expression within the investigated tumors, both the luminal surface and the
invasive front of the tumors were scored. In previous studies the different regions has not been distinguished in this way, even though several studies have reported a heterogeneous expression at least with regards to SOX9 expression[87,91], including our study presented in **Paper III**. The heterogeneous expression of the intestinal stem cell proteins can compromise the use of TMA, which is a popular technique for large-scale patient studies due to the low cost and efficiency. A limitation to TMAs is that they only provide a minor reflection of the tumor. Thus, the analysis of interest should be thoroughly validated before using of TMA. A recent Norwegian study investigated the prognostic value of SOX9 using TMA. The study included a large number of CRC patients (n=761) but they found no prognostic value of SOX9[104]. This could be due to random selection of cores within the tumors and not at the invasive front. Another explanation is the difference in antigen retrieval and the applied antibody.

Both BMI1 and SOX9 expression was applied as continuous variables in the statistical analysis. For SOX9 high and low expression levels were distinguishable since almost all cells had the highest score of percentage of positive cells even if the intensity of the expression was weak. With regards to BMI1 the expression was more heterogeneous both in terms of percent positive cells and intensity, thus no valid cut-off could be set for high or low expression. Presently, the majority of the other studies have not discussed the choice of the cut-point set in their studies[65,73–75]. Furthermore, none of the studies investigating the potential of SOX9 or BMI1 as biomarkers in CRC has applied the expressions of the proteins as continuous data. Additionally, out of all the studies conducted investigating SOX9 and BMI1 in clinical settings, only the previous mentioned Norwegian study tried to report their study according to the REMARK guidelines[104]. Therefore, it is difficult to compare the outcome of our studies with the previous conducted studies.

The primary endpoint of both **Paper II** and **Paper III** was risk of relapse. A limitation of the study cohort is that we excluded patients diagnosed with other primary cancer diagnoses prior to or after their primary stage II CC diagnosis. This comprises a selection bias which does not reflect all patients seen in the clinic. In hindsight the patients could have been included and censored. Furthermore, we did not have access to disease specific death, thus we have not included deaths associated with relapse as events unless the relapse was noted in the pathology registry due to an autopsy. Knowledge of the cause of death could have strengthened the studies further. Additionally, it would have been interesting to explore the predictive value of SOX9 with regards to cancer-related death as outcome as well. Unfortunately, we did not retrieve information on whether the patients have adjuvant therapy or not which optimally should have been adjusted for, especially since the guidelines for the risk factor associated to number of lymph nodes were changed from 10 to 12 retrieved lymph nodes during our study inclusion period. Furthermore, it would have been of value to investigate whether the patients who received adjuvant therapy had an improved long term prognosis.
4.3 SOX9 AND BMI1 AS BIOMARKERS OF RELAPSE (PAPER II AND III)

The REMARK guidelines is an initiative to improve the transparency of biomarker research[115]. However, insufficient reporting in clinical studies remains an issue. When comparing the clinical studies investigating BMI1 and SOX9 as biomarkers, it is evident that there are several issues that makes the studies difficult to compare and also makes them difficult to translate into clinical use. This includes the technical approaches used (i.e. IHC analysis, in situ hybridization etc.), type of specimen (snap-frozen, FFPE tissue, TMA etc.), patient cohort (inclusion and exclusion criterias, potential genetic differences between populations etc.), and choice of statistical endpoints and analysis.

Even though BMI1 and SOX9 was overexpressed in the majority of the investigated stage II colon tumors, as previously reported (reviewed in Paper I), this does not necessarily indicate a higher level of malignancy. BMI1 had limited prognostic value in the investigated cohort as the protein expression was associated with neither overall survival nor relapse as presented in Paper II. The BMI1 expression correlated with tumor location and various histopathological characteristics; however this has limited clinical relevance. The prognostic value of BMI1 is contradicting in the literature as BMI1 has been reported both to be associated with a good and poor prognosis (Paper I). Due to the fact that BMI1 is part of a larger protein complex to exert its function, it is possible that the protein might not be relevant as a singular biomarker but rather in combination with the remaining PRC1 complex proteins as investigated in a previous study[76]. Since the present patient cohort only contained tumors from stage II CC patients it cannot be excluded that BMI1 may have a prognostic significance in later stages of CC.

SOX9 expression at the invasive front was shown to predict relapse of stage II CC patients in Paper III. In contrast to the initial hypothesis, low SOX9 expression was associated with a high risk of relapse and high SOX9 expression level was associated with a low risk of relapse. The data is opposite of what would be expected based on the cancer stem cell hypothesis. Whether the SOX9 expressing tumor cells can be defined as cancer stem cells can be debated, as cancer stem cells are expected to constitute a minority of the tumor cells. One explanation to why SOX9 at the invasive front of the tumor was associated with risk of relapse and not the expression at the luminal surface could be the microenvironment present at the two different areas. The luminal surface of the neoplastic glands tend to have a higher level of immune infiltrate and necrotic areas which affects the surrounding cells but can also have an effect on the IHC staining. Another explanation might be that the luminal area itself does not provide the same malignant potential as the invasive part of the tumor does.

The prediction of relapse by SOX9 was independent of both MMR status and the histopathological risk factors, which both were significantly associated with a low and high
risk of relapse, respectively. The majority of histopathological risk factors were associated with relapse. However, no association was found between risk of relapse and the histological tumor subtype or vein infiltration. This can be due to the low numbers of those events. However, especially the prognostic significance of vein infiltration has been debated due to inconsistent results but it remains included as a risk factor in guidelines[119]. Furthermore, the majority of dMMR tumors were deficient due to MLH1 promoter hyper-methylations as expected[22]. These data suggest that SOX9 may have an additive value to predicting relapse to the already applied risk factors in the clinical histopathological diagnostics.

Several attempts have been done to identify stage II CC patients who have a high risk of relapse by gene signatures. However, many of the molecular panels have issues being translated into clinical use[120]. This is partly due to requirement of sensitivity and specificity of the tests and the starting tissue materials. Many of the genetic signature assays requires fresh frozen tissue as patient material whereas the main available specimen in the clinical diagnostic practice is FFPE tissue[120]. Optimally, a biomarker for high risk of relapse should also include whether the stage II CC patients actually benefit from adjuvant therapy they would receive. A recent retrospective study reported that absent CDX2 expression in stage II CC was associated with relapse and that these patients benefitted from adjuvant therapy[121]. Indeed, it would be interesting to investigate whether SOX9 could either contribute to or have a similar prognostic value as CDX2. Due to the design of the present studies we cannot direct stage II CC patients for adjuvant therapy. However, the SOX9-directed stratification of high risk stage II CC patients may provide information for more intense follow-up of the high risk stage II CC patients.

4.4 CONCLUDING REMARKS

While SOX9 was demonstrated to be an independent predictor of relapse in the investigated comprehensive cohort of stage II CC patients, BMI1 had limited prognostic value. MMR status and the majority of the histopathological risk factors also had a prognostic value in terms of risk of relapse. Furthermore, our studies indicated that SOX9 may be regulated by TNF-α through the NF-κB signaling pathway in Caco-2 cells. However, additional studies are needed to confirm these indications. Intestinal stem cell markers may have a prognostic or predictive role in CRC. With regards to BMI1 and SOX9 we advocate for studies to confirm our prognostic findings. Moreover, investigation of the cause of SOX9 deficiency in the tumors by promoter methylation assay and DNA sequencing would be a natural next step. Even though high risk stage II CC patients currently are stratified by histopathological risk factors and receive adjuvant therapy, there are still many unknown factors and improvements to be made. Thus, further investigation of the molecular mechanisms of CC and improvement of personalized treatment strategies of the stage II CC patients are important steps forward.
4. REFERENCES


5. ENCLOSED PAPERS
Clinical Implications of Intestinal Stem Cell Markers in Colorectal Cancer.


Clinical Implications of Intestinal Stem Cell Markers in Colorectal Cancer

Maiken Lise Markes Espersen,1,2 Jesper Olsen,2,3 Dorte Linnemann,1 Estrid Høgdall,1 Jesper T. Troelsen2

Abstract

Colorectal cancer (CRC) still has one of the highest incidence and mortality rate among cancers. Therefore, improved differential diagnostics and personalized treatment are still needed. Several intestinal stem cell markers have been found to be associated with CRC and might have a prognostic and predictive significance in CRC patients. This review provides an overview of the intestinal stem cell markers leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), B cell–specific Moloney murine leukemia virus insertion site 1 (BMI1), Múshashí (MSI1), and sex-determining region y-box 9 (SOX9) and their implications in human CRC. The exact roles of the intestinal stem cell markers in CRC development and progression remain unclear; however, high expression of these stem cell markers have a potential prognostic significance and might be implicated in chemotherapy resistance.

Keywords: Biomarkers, BMI1, LGR5, MSI1, SOX9

Introduction

Colorectal cancer (CRC) is one of the most common cancers in the developed world and carries the second highest mortality rate.1 Thus, there is a great need for improved differentiated diagnosis and personalized treatment of CRC patients.

Sporadic CRC arises as a consequence of lacking homeostatic control of proliferation and apoptosis within colon epithelial cells, driving the cells toward immortality and enhanced proliferation. This deregulation is caused by genetic and epigenetic alterations impairing essential pathways involving p53, PI3K, epidermal growth factor receptor (EGFR), and the canonical Wnt-signaling pathway. The Wnt signaling pathway is a major driver of CRC initiation and progression. Upon activation of the Wnt signaling pathway, β-catenin is translocated from the cytoplasm into the nucleus, where it associates with TCF/LEF transcription factors, thus regulating downstream Wnt target genes, such as C-MYC.2,3

The essential Wnt-associated gene adenomatous polyposis coli (APC) is one of the most frequently mutated genes in early neoplastic transformation. Other Wnt signaling–associated genes have additionally been described as altered in CRC, including the ring finger protein 43 (RNF43) gene, which recently was described to be one of the most commonly mutated genes in CRC.4–7 Moreover, TPS5 and the KRAS oncogene are also commonly altered in CRC, with the mutational status of KRAS oncogene being predictive for anti-EGFR monoclonal antibody therapy.8

Another hallmark of CRC is DNA mismatch repair (MMR) deficiency, which is reported in approximately 15% of all cases of CRC. The most commonly affected MMR genes are MLH1, MSH2, and MSH6. MMR deficiency causes accumulation of mutations and microsatellite instability (MSI), where microsatellite sequences in the genome are altered. MSI tumors are further subdivided according to the frequency of MSI into high frequency of MSI or low frequency of MSI. Colorectal tumors with impaired MMR are predominantly associated with right-sided colon tumors and correlate to a favorable prognosis.9

The traditional stochastic model of cancer development argues that in principle, all tumor cells are biologic equivalents and have the potential to proliferate and drive tumor growth.10 Within recent years, the traditional cancer model has been challenged by another model, the cancer stem cell model. The cancer stem cells model proposes that tumors are composed of a hierarchy of cells that are biologically distinct.10,11 Cells with stem cell properties reside within the tumor and are responsible for tumor initiation, progression, metastasis, recurrence, and resistance to chemotherapy.12
Stem Cell Markers in CRC

Similar to stem cells, cancer stem cells are able to both self-renew and can differentiate into progenitors. They are largely believed to be the result of acquired epigenetic and genetic changes in the stem cells. The adult stem cells already possess critical characteristics such as self-renewal capacity and long-term replicative potential, but during normal homeostasis, these capacities are tightly regulated. Because of the properties of the stem cells, the number of genetic alterations before transformation is hypothetically fewer than what more differentiated cells need to acquire to transform. Furthermore, the longevity of the stem cells provides the necessary time to accumulate oncogenic alterations.

Extensive studies have been performed to identify putative intestinal stem cell markers and their potential role in cancer. Some of the driver genetic alterations of CRC are associated to the intestinal stem cells, including sex-determining region Y-box 9 (SOX9) and leucine-rich-repeat-containing G-protein-coupled receptor 5 (LGR5) through the R-spondins, indicating that the stem cell markers play significant roles in colorectal tumorigenesis. Several intestinal stem cell markers have been identified with LGR5 being the most investigated and established marker. LGR5+ cells also correlate to expression of the markers olfactomedin-4 (OLMF4) and achete scute complex like 2 (ASCL2). The Lgr5+ stem cell population marks the population of crypt base columnar cells located interspersed between the Paneth cells at the bottom of the small intestinal crypts, which previously was suggested as the stem cell population. Simultaneously, another stem cell population in the small intestine has been identified at position +4 (+4 referring to the location of stem cells approximately 4 cells from the bottom of the crypts). Several +4 stem cell markers have been suggested, including B cell-specific Moloney murine leukemia virus insertion site 1 (Bmi1), telomerase reverse transcriptase (Tert), and homeodomain only protein X (Hippo). Leucine-rich repeats and immunoglobulin-like domains 1 (Lrig1), Musashi 1 (MS1), and Sec9 have been suggested as more general markers marking both stem cell populations.

Most of the studies have focused on the adult stem cells of the small intestine. However, within recent years, several of these stem cell markers have been linked to CRC, and an increase in their expression level in the primary tumors of CRC patients has been correlated to a poor prognosis and chemotherapy resistance. Some of these markers have been more extensively investigated than others. The most investigated intestinal stem cell markers in a clinical setting are LGR5, Bmi1, Msi1, and Sox9. This review evaluates the potential clinical implications of these putative stem cell markers and their potential role in human CRC.

LGR5

LGR5 was initially identified in 1998. The receptor did not receive much attention until 2007, where it was reported to be a potential stem cell marker of the small intestine and colon in mice. Lgr5 expressing cells are long-lived and have the ability to generate all cell types of the small intestine and colon epithelia. Lgr5 is expressed in cells at the bottom of the colonic crypts and in crypt base columnar cells interspersed between the Paneth cells at the crypt bottom of the small intestine in mice. Accordingly, immunohistochemical staining of LGR5 shows that the protein is expressed in cytoplasm and membranes of a few cells, located at the crypt base of human colon tissue.
Table 1  Implications of LGR5 Expression in Human CRC

<table>
<thead>
<tr>
<th>Main Findings</th>
<th>Study Cohort (No. of Specimens)</th>
<th>AJCC Stage (No. of Patients Analyzed)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGR5 expression is not associated with prognosis.</td>
<td>Tumor (691)</td>
<td>NS</td>
<td>Akdin et al., 2013</td>
</tr>
<tr>
<td>Increased LGR5 expression in CRC and correlates with female sex.</td>
<td>Tumor (102)</td>
<td>I + II (65)</td>
<td>Fan et al., 2010</td>
</tr>
<tr>
<td>Increased expression of LGR5 in distant metastasis derived from tumors with LGR5-positive cells in tumor buds and vascular compartments of the primary tumor.</td>
<td>Tumor (89)</td>
<td>III (46)</td>
<td>Kleist et al., 2011</td>
</tr>
<tr>
<td>LGR5 expression at the invasive front is positively correlated to advanced disease.</td>
<td>Tumor (20)</td>
<td>I + II (7)</td>
<td>Takeda et al., 2011</td>
</tr>
<tr>
<td>LGR5 expression at the luminal surface is inversely correlated to disease stage.</td>
<td>Tumor (63)</td>
<td>I (3)</td>
<td>He et al., 2014</td>
</tr>
<tr>
<td>LGR5 expression correlates with TNM stage, lymph node metastasis, and vascular invasion.</td>
<td>Tumor (236)</td>
<td>II (92)</td>
<td>He et al., 2015</td>
</tr>
<tr>
<td>High levels of LGR5 correlate to poor prognosis.</td>
<td>Tumor (90)</td>
<td>III (57)</td>
<td>Hsu et al., 2013</td>
</tr>
<tr>
<td>LGR5 is an independent prognostic factor.</td>
<td>Tumor (15)</td>
<td>III (5)</td>
<td>McDougal et al., 2006</td>
</tr>
<tr>
<td>LGR5 mRNA is significantly up-regulated in CRC.</td>
<td>Tumor (180)</td>
<td>I to IV (39)</td>
<td>Takeda et al., 2013</td>
</tr>
<tr>
<td>Patients with high LGR5 expression in their primary tumors have a poorer prognosis.</td>
<td>Tumor (180)</td>
<td>II (23)</td>
<td>Wu et al., 2012</td>
</tr>
<tr>
<td>LGR5 is an independent prognostic marker.</td>
<td>Tumor (152)</td>
<td>II (54)</td>
<td>Uchida et al., 2010</td>
</tr>
<tr>
<td>LGR5 correlates with lymph node metastasis, vascular invasion, lymphatic invasion, tumor depth, and tumor grade.</td>
<td>Tumor (236)</td>
<td>I (19)</td>
<td>Guo et al., 2014</td>
</tr>
<tr>
<td>LGR5 expression is increased in stage IV CRC patients compared to normal matched mucosa.</td>
<td>Tumor (254)</td>
<td>II (70)</td>
<td>Geiger et al., 2011</td>
</tr>
<tr>
<td>LGR5 homozygous wt genotypes in blood associated with a lower time to tumor recurrence in CRC patients than LGR5 heterozygous patients.</td>
<td>Tumor (254)</td>
<td>II (70)</td>
<td>Geiger et al., 2011</td>
</tr>
<tr>
<td>LGR5 gene variation correlates negatively with LGR5 protein expression in CRC.</td>
<td>Tumor (89)</td>
<td>II (41)</td>
<td>Kleist et al., 2012</td>
</tr>
<tr>
<td>LGR5 expression is associated with favorable prognosis.</td>
<td>Tumor (152)</td>
<td>II (54)</td>
<td>de Souza E Molo et al., 2011</td>
</tr>
</tbody>
</table>

Abbreviations: AJCC — American Joint Committee on Cancer; CRC — colorectal cancer; LGR5 — leucine-rich repeat-containing 5 protein-coupled receptor 5; NS — Not specified. Paired healthy — healthy colon mucosa from same individual as investigated tumor; TNM — tumor, node, metastasis classification system; wt — wild type.

prognostic significance and that high expression of Wnt-driven intestinal stem cell markers, including LGR5, within CRC tissue is associated with a favorable prognosis. Interestingly, increased LGR5 mRNA expression in the peripheral blood of CRC patients has also been associated with a poor outcome. This might reflect circulating cancer cells with stem cell-like properties playing a role in the metastatic event.

CRC cell lines studies describe LGR5 cells to be associated with chemotherapeutic resistance and resistance mechanisms. Accordingly, patients with low levels of LGR5 within their primary tumors have a significant better response rate to 5-fluorouracil (5-FU)-based therapy than patients with high levels of LGR5. Studies on the implications of LGR5 in CRC patients are summarized in Table 1.

These studies suggest that LGR5 might be of relevance as a prognostic and predictive marker.

**BMP1**

BMP1 is a component of the polycomb repressive complex 1, which plays an important role in gene silencing by chromatin modification in cells, including, among others, embryonic and adult stem cells. BMP1 was initially identified as an oncogene that, together with c-myc, plays a role in initiation of mouse B cell lymphomas. It was later found to be important in hematopoietic and neural development. BMP1 targets the Idh2/Arf locus, which encodes the critical cell cycle regulators p16 and p19ARF (p14ARF in humans). These are involved in the retinoblastoma protein (Rb) and p53 signaling pathways, regulating cell cycle and apoptosis.

In vivo lineage tracing suggests that Bmp1 expression marks small intestinal stem cells located at the +4 position from the crypt bottom in mice. These stem cells are functionally distinct from the Lgr5 expressing stem cell population. The +4 putative stem cells are characterized by being quiescent, being resistant to
Stem Cell Markers in CRC

irradiation, and having regenerative potential after injury or ablation of Lgr5 expressing cells.\textsuperscript{19,83,84} Whether two functionally distinct intestinal adult stem cell populations exist and whether expression of Bmi1 actually marks +4 stem cells are still controversial.\textsuperscript{85,86}

Bmi1 has been described to be low expressed or absent in the nucleus of human colon epithelial cells at the very bottom of the crypt.\textsuperscript{82-84} The exact role of Bmi1 in the normal colon and in CRC is unclear. Table 2 lists studies that have investigated the implications of Bmi1 in human CRC. Several studies report overexpression of the Bmi1 at the protein and mRNA levels in CRC relative to healthy colon tissue.\textsuperscript{82-87} Human CRC cells have been proposed to require Bmi1 expression for maintenance of tumor growth.\textsuperscript{87} Furthermore, knockdown of Bmi1 severely affects the self-renewal capacity \textit{in vitro} and impairs the cancer-initiating potential of human colon cancer cells in mice.\textsuperscript{87,88} Bmi1 expression might be inversely correlated to various cell cycle proteins, eg, p14 and p16, and positively correlated to c-MYC expression, although findings are contradictory.\textsuperscript{83,84} Inhibition of Bmi1 results in growth arrest of the preestablished tumors \textit{in vivo}.\textsuperscript{89} These results suggest Bmi1 as a relevant therapeutic target of CRC.

Bmi1 expression has also been correlated to several clinicopathologic factors, such as tumor size, serum carcinoembryonic antigen levels, and histologic differentiation grade.\textsuperscript{83,84} A gradient of Bmi1 expression can be observed in human colon precancerous and cancerous tissue. Here, low-grade intraepithelial dysplastic tissue has the lowest expression and high-grade dysplastic and cancerous tissue has the highest.\textsuperscript{84} Bmi1 expression is correlated to cancer stage, suggesting that Bmi1 might be associated with colon cancer progression.\textsuperscript{84-87}

The prognostic significance of Bmi1 expression in colorectal tumors is conflicting. More patients with Bmi1 positive tumors have tumor recurrence or metastasis compared to patients with Bmi1 negative tumors.\textsuperscript{87} Furthermore, high Bmi1 expression in primary tumors from CRC patients is an independent prognostic factor for disease-free survival and for overall survival.\textsuperscript{46,87} However, high Bmi1 expression is also correlated with a better prognosis compared to patients with low expression.\textsuperscript{48} By combining several biomarkers with the Bmi1 expression, prognostic stratification is improved.\textsuperscript{49} In addition, another recent study reported that patients with decreased postoperative plasma mRNA levels of Bmi1 compared to patients with increased postoperative mRNA levels correlated with a favorable prognosis in CRC.\textsuperscript{49} These studies suggest that Bmi1 may be of relevance as a prognostic indicator in CRC. However, the exact directionality of its prognostic utility remains to be elucidated.

\section*{MSH}

MSH1 is an evolutionary conserved RNA-binding protein initially identified in \textit{Drosophila} as a protein important for sensory organ development and as a neuronal stem cell marker in mammals.\textsuperscript{88,90} MSH1 is one of the first proposed intestinal stem cell markers and may contribute to the undifferentiated state of intestinal stem cells.\textsuperscript{24,25,91} MSH1 is mainly expressed in the cytoplasm of human colon epithelial cells positioned between cells 1 and 10 from the bottom of the crypt.\textsuperscript{35} Occasionally, MSH1 is also expressed in the nucleus of these cells.\textsuperscript{25} Furthermore, MSH1 expressing cells have been shown to correspond to cells expressing the intestinal stem cell marker Lgr5 and the +4 stem cell marker Terr in mice.\textsuperscript{90,92}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Main Findings} & \textbf{Study Cohort (No. of Specimens)} & \textbf{AJCC Stage (No. of Patients Analyzed)} & \textbf{Reference} \\
\hline
Bmi1 is overexpressed in CRC. & Tumor (1) & NS & Rekhtin et al., 2006\textsuperscript{87} \\
Bmi1 expression correlates with gender, histologic tumor differentiation, tumor size, and serum CEA levels. & Tumor (87), Pooled healthy (87) & NS, N and M stage provided & Kim et al., 2004\textsuperscript{85} \\
Bmi1 expression has an inverse correlation to the expression of p16 and p14. & Tumor (45) & NS & Tatish et al., 2006\textsuperscript{86} \\
Bmi1 is overexpressed in human low-grade intraepithelial dysplasia. High-grade intraepithelial dysplastic, and cancer. & Tumor (98), Pooled healthy (98) & NS & Du et al., 2010\textsuperscript{88} \\
High expression of Bmi1 correlates with metastasis and advanced stage of cancer. & Tumor (233), Pooled healthy (203) & NS & Li et al., 2010\textsuperscript{89} \\
High expression of Bmi1 is associated with a lower overall survival & Tumor (98), Pooled healthy (98) & NS & Du et al., 2010\textsuperscript{88} \\
Patients with Bmi1 positive tumors have a lower disease-free survival and a lower overall survival. & Tumor (24), Pooled healthy (24) & NS & Benard et al., 2014\textsuperscript{84} \\
High expression of Bmi1 is associated with a better prognosis. Combination of Bmi1 with other biomarkers improves the prognostic stratification when compared to applying the biomarkers individually. & Tumor (24), Pooled healthy (24) & NS & Benard et al., 2014\textsuperscript{84} \\
Patients with decreased postoperative plasma Bmi1 mRNA levels have a better prognosis than patients with increased postoperative Bmi1 mRNA levels & Tumor (45) & NS & Pan et al., 2014\textsuperscript{89} \\
\hline
\end{tabular}
\caption{Implications of Bmi1 in CRC Patients}
\end{table}

Abbreviations: AJCC - American Joint Committee on Cancer; Bmi1 - B cell-specific Moloney murine leukemia virus insertion site 1; CEA - carcinoembryonic antigen; CRC - colorectal cancer; NS - not specified; Pooled healthy - healthy colon mucosa from same individual as investigated tumor.
MSI1 functions as suppressor by binding to its target mRNA, thus repressing translation of its downstream targets.92 Additionally, MSI1 competes with eukaryotic initiation factor 4G (eIF4G) for binding to the poly(A)-binding protein (PABP), thereby inhibiting translation initiation.93 Two of the most recognized RNA targets of MSI1 are the genes encoding the Notch antagonist Numb and p21, an inhibitor of cyclin-dependent kinases.94,95 MSI1 was also found to negatively regulate APC translation in human cultured colonocytes.96 Interestingly, reduced APC expression leads to increased levels of MSI1, suggesting that MSI1 itself is a target of the Wnt signaling pathway,97 consistent with an earlier study describing a TCF/LEF binding site on the Msi1 promoter.98 This positive feedback loop might be important for regulating homeostasis of colon tissue, and if disturbed, it could lead to tumor formation.

Intestinal epithelial cells overexpressing Msi1 increase proliferation and acquire tumorigenic features in xenografts.99 In accordance, knockdown of Msi1 in human colon cancer cells leads to inhibition of proliferation and reduced migratory potential.100 Furthermore, knockdown of Msi1 in xenografts results in tumor growth arrest, suggesting that Msi1 may play a role in tumor progression.51

Studies investigating the implications of Msi1 in human CRC are listed in Table 3. The level of Msi1 mRNA expression has been reported to be significantly increased in human colorectal adenocarcinomas, and the expression level varies in normal, adenoma, and carcinoma of colon tissues.50-52 MSI1 expression tumor cells of the colon predominantly also, although not exclusively, express Msi1 in the cytoplasm.53 MSI1 is often focally expressed in adenomas, whereas the expression pattern in carcinomas is more diffuse.52 Moreover, MSI1 overexpression is significantly associated with the proliferation marker Ki-67, advanced cancer stage, and a more aggressive disease phenotype.50-52 When adjusted for American Joint Committee on Cancer stage, vessel infiltration, histologic type, and grade, MSI1 appears as an independent prognostic marker for prediction of poor outcome in stage III and IV disease (but not stage I and II disease).50 Furthermore, positive MSI1 expression in the primary tumor is associated with a nearly 5.4-fold increased risk of distant metastasis.20 This poor outcome in patients with stage III and IV cancers, who generally receive adjuvant chemotherapy, may be explained by a study in mice showing that MSI1 positive cells are insensitive to 5-FU.98

Table 3 Implications of MSI1 in CRC Patients

<table>
<thead>
<tr>
<th>Main Findings</th>
<th>Study Cohort (No. of Specimens)</th>
<th>AJCC Stage (No. of Patients Analyzed)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSI1 is an independent prognostic marker to predict poor outcome.</td>
<td>Tumor (203)</td>
<td>I (24)</td>
<td>Li et al., 2011103</td>
</tr>
<tr>
<td></td>
<td>Paired healthy (253)</td>
<td>II (81)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>III (80)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV (14)</td>
<td></td>
</tr>
<tr>
<td>&gt;2-fold increase in MSI1 mRNA expression in majority of CRC.</td>
<td>Tumor (19)</td>
<td>NS</td>
<td>Sartoretti et al., 2009101</td>
</tr>
<tr>
<td></td>
<td>Paired healthy (15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSI1 expression correlates to TNM stage.</td>
<td>Tumor (68)</td>
<td>I + II (19)</td>
<td>Fan et al., 2010102</td>
</tr>
<tr>
<td></td>
<td>Unpaired healthy (8)</td>
<td>III + IV (59)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>MSI7 mRNA expression differs in normal, adenoma, and carcinoma.</td>
<td>Tumor (31)</td>
<td>NS</td>
<td>Fan et al., 2010102</td>
</tr>
<tr>
<td></td>
<td>Unpaired healthy (18)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: AJCC — American Joint Committee on Cancer; CRC — colorectal cancer; MSI1 — MutaSIFT; NS — not specified; Paired healthy — healthy colon mucosa from same individual as investigated tumor; TNM — tumor, node, metastasis classification system.

These studies suggest that MSI1 might be of relevance as both a negative prognostic marker and a predictive marker.

SOX9

SOX9 is a transcription factor involved in numerous developmental processes and is required for regulation of cell proliferation, senescence, and lineage commitment.53,59-101 A Sox9 expressing population of cells has been shown to exert multipotency and self-renewal capacity, as well as to have the ability to repopulate the intestinal crypts in mice.102 Similarly, a study of colon epithelial stem cells describes that cells expressing high levels of Sox9 are associated with a more undifferentiated cell population having stem cell characteristics in vitro and these cells are furthermore enriched for Lgr5 mRNA.103 Cells with low Sox9 expression accordingly have a gene expression profile consistent with a more differentiated phenotype.28 This is in agreement with the expression observed in human colon epithelia, where SOX9 is described as being primarily expressed in the nucleus of cells in the lower proliferative part of the colonic crypts and with a weaker expression in cells toward the luminal surface.59-60,104 Furthermore, inactivation of Sox9 in mice results in aberrant structure of the colon tissue with villus-like protrusions into the lumen, similar to the small intestinal morphology, emphasizing the importance of Sox9 in the small intestinal and colon morphology.105 Additionally, the goblet cell lineage of the colon is strongly reduced in the Sox9 deficient mice.106 Somewhat contradicting to this a study showed that Sox9 indirectly represses genes associated with goblet cell differentiation, eg, the mucin-encoding gene MUC2a.107

The exact role of SOX9 in carcinogenesis and cancer progression is, however, controversial because both oncogenic and tumor-suppressing functions of the protein have been described.55,56,59,60,103-105 SOX9 has been shown to be a direct Wnt signaling target of the activated β-catenin-TCF4 complex in human colon carcinoma cells.108 But another study showed that Sox9 potentially inhibit the β-catenin-TCF4 complex, suggesting a negative feedback loop.109 Furthermore, Bmi1 has been identified as a potential SOX9 target in mouse primary cells and transformed cells, hence repressing the tumor suppressors p16 and p19ARF, leading to cell cycle progression and bypassing of apoptosis.55 Overexpression of SOX9 in human CRC cells induces an increase of BMI1 with a subsequent decrease in p16, whereas the opposite
Stem Cell Markers in CRC

effect has been observed by SOX9 knockdown.23 In contrast, mice with Snail deficiency show extensive hyperplasia of the colon with numerous enlarged crypts and some with cystic appearances, indicating increased proliferation.101 However, no malignant transformation was seen, which implies that Snail deficiency alone cannot induce malignancy.101

One possible explanation for the discrepancy of results with regard to SOX9 could be that different variants of the protein exist. One study identified SOX9 as frequently mutated in CRC,5 and others have described a low copy number gain of chromosome 17, where the SOX9 gene is located.102 This could be a possible mechanism explaining the SOX9 overexpression in some patients. Interestingly, a truncated variant of SOX9 lacking its transactivation domain has been described in human CRC cell lines and tumors.106 The variant activated the canonical Wnt signaling pathway, thus having oncogenic properties, whereas the fully transcribed and translated SOX9 protein repressed the Wnt signaling pathway.106 Both the truncated and the full length SOX9 is increased in human colon cancer tissue.106

Several studies have also described increased expression of SOX9 at both mRNA and protein level in human CRC specimens and cell lines compared to healthy colon epithelia.55-57,59-60 Only one small study (n = 10) has described a decrease in SOX9 expression in colorectal adenocarcinomas.61 There is no significant difference in SOX9 expression when comparing adenomas and cancerous expression.59 SOX9 is expressed in a random heterogeneous manner throughout colorectal tumors.28,55 Moreover, a strong expression of SOX9 is more common in non-mucin-producing CRC than mucinous or signet ring carcinomas.56 One study describes that SOX9 overexpression correlates with vascular invasion in the primary tumor.54 Others find that high SOX9 expression correlates with age, female sex, and MSI tumors, especially MSI-high tumors.56 In contrast, correlation between downregulated SOX9 expression and MSI relative to microsatellite stable tumors has also been described.60 Table 4 lists the studies on SOX9 in human CRC.

Correlation between SOX9 expression levels and patient survival is inconsistent.24-55,59 When stratified for American Joint

<table>
<thead>
<tr>
<th>Main Findings</th>
<th>Study Cohort (No. of Specimens)</th>
<th>AJCC Stage (No. of Patients Analyzed)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOX9 is frequently mutated in nonhypermutated tumors.</td>
<td>Tumor (224) Pair end healthy (224)</td>
<td>NS</td>
<td>Cancer Genome Atlas Network, 201225</td>
</tr>
<tr>
<td>SOX9 is up-regulated in CRC.</td>
<td>Tumor (118) Unpaired healthy (22)</td>
<td>NS</td>
<td>Ramalingam et al., 201226</td>
</tr>
<tr>
<td>SOX9 mRNA is up-regulated in CRC and associated with advanced tumor stage.</td>
<td>Tumor (70) Unpaired healthy (26)</td>
<td>NS</td>
<td>Mathe et al., 201230</td>
</tr>
<tr>
<td>SOX9 overexpression correlates with poorer survival in 5-FU-treated stage III patients.</td>
<td>Tumor (41) Unpaired healthy (41)</td>
<td>II (11), III (20)</td>
<td>Cady et al., 201341</td>
</tr>
<tr>
<td>Strong SOX9 expression is most common in non-mucin-producing CRC. Strong SOX9 expression correlated with lower overall survival.</td>
<td>Tumor (166) Unpaired healthy (166)</td>
<td>II (97), III (69), IV (6)</td>
<td>Lu et al., 200655</td>
</tr>
<tr>
<td>SOX9 is overexpressed in CRC.</td>
<td>Tumor (27) Unpaired healthy (27)</td>
<td>I (6), II (12), III (10), IV (3)</td>
<td>Abdel-Samed et al., 201137</td>
</tr>
<tr>
<td>A truncated variant of SOX9 is overexpressed in CRC.</td>
<td>Tumor (7) Unpaired healthy (17)</td>
<td>I (9), II (6), III (6), IV (1), NS (3)</td>
<td>Abdel-Samed et al., 201137</td>
</tr>
<tr>
<td>SOX9 is up-regulated in CRC.</td>
<td>Tumor (10) Unpaired healthy (10)</td>
<td>NS</td>
<td>Lu et al., 200657</td>
</tr>
<tr>
<td>Increased SOX9 gene expression in CRC.</td>
<td>Tumor (77) Unpaired healthy (77)</td>
<td>II (39), III (28), IV (3)</td>
<td>Panag et al., 201348</td>
</tr>
<tr>
<td>High levels of SOX9 associated with age and MSI. No significant decrease in survival for the patients with high SOX9 expression.</td>
<td>Tumor (51) Unpaired healthy (31)</td>
<td>Degree A (1), Degree B (11), Degree C (19)</td>
<td>Panag et al., 201348</td>
</tr>
<tr>
<td>SOX9 is up-regulated in CRC. SOX9 is down-regulated in MSI relative to MSS tumors.</td>
<td>Tumor (42) Unpaired healthy (20)</td>
<td>II (23), III (3), IV (49)</td>
<td>Andreasen et al., 200940</td>
</tr>
<tr>
<td>SOX9 was down-regulated in CRC.</td>
<td>Tumor (19) Unpaired healthy (19)</td>
<td>NS</td>
<td>Chen et al., 200649</td>
</tr>
</tbody>
</table>

Abbreviations: AJCC = American Joint Committee on Cancer; CRC = colorectal cancer; 5-FU = 5-fluorouracil; MSI = microsatellite unstable; MSS = microsatellite stable; NS = not specified; Paired healthy = healthy colon mucosa from same individual as investigated tumor; SOX9 = sex-determining region Y-box 9.
Committee on Cancer stage, SOX9 protein overexpression is associated with a lower survival in 5-FU–treated stage III cancers. This is not the case for 5-FU–untreated stage III or stage II CRC patients, suggesting that SOX9 may be a prognostic indicator in patients receiving 5-FU adjuvant chemotherapy. It should be noted, however, that the study did not adjust for MMR deficiency, which may have both a prognostic value and a predictive value with regard to 5-FU resistance.106,107

Discussion

It is evident that all the 4 stem cell markers are overexpressed at the protein and mRNA levels in primary tumors of CRC patients compared to normal mucosa and that this may have prognostic significance. The exact mechanisms and functions of the increased expression of the intestinal stem cell markers remain to be elucidated, as studies are contradictory with respect to the oncogenic or tumor-suppressive functions. Apparently the proteins play important roles in essential signaling pathways, such as Rho, p53, Notch, and Wnt signaling, in which deregulation of these often are involved in the carcinogenic process. Most studies identifying and investigating adult stem cells of the intestines focus on the small intestine, with little attention paid to the actual colon stem cells. The evidence of Lgr5 marking both colon and small intestine stem cells is convincing. However, this is less established for the other 3 markers, with Smo and Smo studies suggesting that the stem cell populations of the small intestine and the colon differ considerably with respect to the Sox9 expression level. This could extend the example of different carcinogenic functions observed in CRC cell lines using small intestinal stem cell markers. In addition, pooling right-sided and left-sided colon tumors, and rectum tumors with different mutational profiles in the same investigations might further add to the diversity of expression signatures.

Another potential mechanism to the opposing results could be an alternating expression along the carcinogenic progression such that the stem cell–associated pathways or intestinal stem cell proteins are silenced during certain stages of progression and reexpressed at other stages. It has also been suggested that a more primitive stem cell program than the intestinal stem cell signatures might play a role in the progression of CRC.41

Variants of the different stem cell proteins might also add to the contradicting results of the intestinal stem cell markers’ implications in cancer development and progression. Few studies indicate that variants and mutations of the stem cell genes might be of importance from a prognostic perspective. Thus, further studies on the functional role and clinical significance of these variants and mutations are needed.

LGR5, MSI1, SOX9, and BM11 expression correlates to various clinicopathologic features of primary colorectal tumors. However, there are some discrepancies between the studies. This could be due to the relatively rare event of some of the features and the relatively small numbers of included patients in some of the studies. However, common to all stem cell markers is that their expression level has been correlated to more advanced disease stage. Furthermore, tumors with MMR deficiency have low expression levels of LGR5 and SOX9, which is in accordance with the favorable prognosis of patients with MMR deficient tumors and the poor prognosis associated with an increased LGR5 or SOX9 expression in some studies.57,68

A hallmark of cancer stem cells is their potential resistance to chemotherapeutic drugs. Interestingly, low expression of LGR5 in primary tumors from CRC patients correlates with improved response to 5-FU–based chemotherapy, and SOX9 overexpression correlates with short survival in stage III 5-FU–treated CRC patients, suggesting that these markers might be relevant for predicting chemotherapy resistance. However, larger patient studies are needed to clarify these indications. Another strategy in trying to improve the prognostic and predictive value could be to combine several of the stem cell markers in a panel rather than using only one stem cell marker, as is seen in other studies focusing on other genes and proteins.58,109

Furthermore, adjusting for known predictive and prognostic factors, such as MMR deficiency, is necessary to further clarify the significance of the stem markers as prognostic and predictive biomarkers.

The expression of the 4 stem cell markers has been described as heterogeneous within tumor tissue. Interestingly, the location of LGR5 expressing tumors within the tumor might be relevant in relation to disease stage. Thus, it can be speculated that increased expression of stem cell–like cells at the invasive front of the tumor might be associated with a more aggressive cancer phenotype. Several studies have thus implied a role of the stem cell markers in metastasis. However, a heterogeneous expression pattern potentially compromises the use of tissue microarrays, an emerging technique for large-scale patient studies. This technique has been used by several of the studies. If the intestinal stem cell markers are introduced to routine settings, a low-cost, simple analysis using full slides—e.g., immunohistochemistry with clearly defined cutoff values—would be preferred to tissue microarrays. Another challenge is the current lack of proper antibodies targeting LGR5, which precludes a proper investigation of LGR5 in human tissue.

In conclusion, the intestinal stem cell markers LGR5, BM11, MSII, and SOX9 are overexpressed in human CRC. The high expression of these stem cell markers might have a prognostic significance and may be associated with chemotherapeutic resistance. However, further extensive studies are needed to elucidate whether these intestinal stem cell markers can be used as predictive and prognostic biomarkers in a clinical setting.

Acknowledgments

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Disclosure

The authors have stated that they have no conflicts of interest.

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The prognostic value of polycomb group protein B-cell-specific moloney murine leukemia virus insertion site 1 in stage II colon cancer patients

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The prognostic value of polycomb group protein B-cell-specific moloney murine leukemia virus insertion site 1 in stage II colon cancer patients

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1Department of Pathology, Herlev University Hospital, Herlev; 2Department of Science and Environment, Roskilde University, Roskilde; and 3Department of Surgery, Køge University Hospital, Køge, Denmark


The aim of this study was to investigate the prognostic value of B-cell-specific moloney murine leukemia virus insertion site 1 (BMI1) protein expression in primary tumors of stage II colon cancer patients. BMI1 protein expression was assessed by immunohistochemistry in a retrospective patient cohort consisting of 144 stage II colon cancer patients. BMI1 expression at the invasive front of the primary tumors correlated with mismatch repair status of the tumors. Furthermore, BMI1 expression at the luminal surface correlated with T-stage, tumor location, and the histological subtypes of the tumors. In a univariate Cox proportional hazard analysis, no statistically significant association between risk of relapse and BMI1 protein expression at the invasive front (HR: 1.12; 95% CI 0.78–1.60; p = 0.52) or at the luminal surface of the tumor (HR: 1.06; 95% CI 0.75–1.85; p = 0.70) was found. Likewise, there was no association between 5-year overall survival and BMI1 expression at the invasive front (HR: 1.12; 95% CI 0.80–1.56; p = 0.46) or at the luminal surface of the tumor (HR: 1.16; 95% CI 0.86–1.60; p = 0.33). In conclusion, BMI1 expression in primary tumors of stage II colon cancer patients could not predict relapse or overall survival of the patients, thus having a limited prognostic value in stage II colon cancer patients.

Key words: B-cell-specific moloney murine leukemia virus insertion site 1; biomarkers; colon cancer; recurrence.

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Colorectal cancer is one of the most common cancers and accounts for the second highest mortality rate among cancers (1). Approximately one third of the patients are diagnosed with stage II colon cancer (2). The main treatment of stage II colon cancer is surgical resection of the tumor. The patients are offered adjuvant therapy if they are considered in high risk of relapse. The stratification of high-risk patients is based on histopathological features composed of depth of invasion (T4 tumor), low differentiation, presence of vein or perineural invasion, margin involvement, tumor perforation, and number of sampled lymph nodes (<12 lymph nodes). Despite of proper surgical intervention and stratification of the patients, approximately 20% of the stage II colon cancer patients have relapse of their cancer. Thus, prognostic and predictive markers for stage II colon cancer relapse are highly desired.

One of the hallmarks of cancer is genomic instability and the mismatch repair (MMR) system has gained attention in relation to colon cancer. Germline mutations in central MMR genes, including MutL homolog 1 (MLH1), Postmeiotic Segregation Increased 2 (PMS2), MutS protein homolog 2 (MSH2), and MutS protein homolog 6 (MSH6) are associated with Lynch Syndrome. However, loss of MMR genes is not limited to Lynch Syndrome but is also found in 15% sporadic colorectal cancers, mainly due to MLH1 promoter hyper-methylations (3). MMR status of sporadic colorectal tumors has been reported to have a prognostic significance (4).

Received 25 December 2015. Accepted 8 March 2016
Tumor tissue and immunohistochemistry

The tumor tissue was processed as part of the diagnostic routine as formerly described (15). 3 μm full slides were incubated for 45 min at 60 °C. The staining was performed by the EnVision™ FLEX, High pH detection system (Dako, Glostrup, Denmark) using the automated Autostainer Link 48 (Dako) according to manufacturer’s instructions. Both a monoclonal BM11 antibody (Mouse, clone F6, cat no. 05-637, Merck Millipore, Darmstadt, Germany) and polyclonal BM11 antibody (Rabbit, E17-9, cat no. sc-10745, Santa Cruz Biotechnology, Heidelberg, Germany) were tested for detection of BM11 protein expression. The BM11 antibody from Millipore [diluted 1:200 with EnVision Flex Linker (Dako)] was selected as the most optimal antibody and therefore used further in the study. Mayer’s hematoxylin was used for counterstaining by the automated slide stainer TissueTek®Prisma™/Filtra® (Sakura, Alphen aan den Rijn, Netherlands). For each run a control tissue slide consisting of normal tissue from colon, small intestine, testis, venticle, and breast was included. The stability of the epitope was tested by staining normal colon tissue which had been subjected to 10% neutral buffered formalin for 3, 27, 51, and 123 h, respectively.

The BM11 protein expression was evaluated both at the invasive front and at the luminal surface independently by a specialized pathologist and a trained molecular biologist. While the invasive front was defined as the area where the tumor periphery invades deepest into the tissue, the luminal surface was considered the luminal surface of the neoplastic glands.

Five random areas at the invasive front and the luminal surface of the tumors were selected using the image analysis software Visiopharm Integrator System (version 4.5.6.516, Visiopharm, Hoersholm, Denmark). The immunohistochemical staining reaction was scored as previously described (15) evaluating both percent positive tumor cells and intensity for a final overall score by multiplying the intensity score with the percent score. Tumors with overall score 0 were rerun for confirmation. The positive stromal cells and lymphocytes were used as an internal control for the staining for each tissue slide. In cases of interobserver disagreement a consensus score was generated by evaluating the slides once more and the pathologist made the final decision. All analysis was conducted blinded to patient outcome.

Statistics

Due to a low number of some of the histopathological risk factors, the patients were grouped as having a risk factor if either of the following histological risk factors were present: T4 tumor grade, low differentiated histology (unless the tumor was dMMR), tumor perforation, vein infiltration, nerve infiltration, or <12 lymph nodes sampled at primary resection.

In all statistical analysis, BM11 was analyzed as a continuous variable. Correlations between clinicopathological variables and BM11 expression were investigated at the invasive front and at the luminal surface. Spearman rank correlation was used to investigate the association between age and the BM11 expression level. Associations between BM11 expression levels and categorical variables were...
THE PROGNOSTIC VALUE OF BM11 IN STAGE II COLON CANCER

explored by rank test for location (Mann–Whitney U and Kruskal–Wallis). The median, range, and interquartile range (Tukey’s Hinge) was presented to improve the overview of potential differences in the clinicopathological subgroups and the BM11 expression.

Time to relapse was the primary endpoint and was analyzed by univariate Cox proportional hazards models containing the BM11 expression at the invasive front or at the luminal surface as continuous variable. Time to relapse was defined as time from surgical resection of the primary colon tumor to local relapse or distant metastasis. Patients who died during follow-up were censored. The secondary endpoint was 5-year overall survival which was investigated by univariate analysis as well. 5-year overall survival was defined as time from surgery to death of any cause. The hazard ratio is presented with a difference of three in BM11 units. The clinicopathological variables were not tested in the models since this has been published in a previous study (15). The assumptions for the Cox proportional hazards model were assessed using cumulative sums of martingale residuals.

The statistical analysis was performed using SPSS Statistics 22 (IBM, Armonk, NY, USA) and SAS (version 9.3, SAS Institute, Cary, NC, USA). P-values of ≤0.05 were considered significant.

RESULTS

Patient characteristics

The basic patient characteristics of the cohort and the MMR status has been previously described (15). Table 1 provides an overview of patient characteristics of the study. The invasive front of the primary tumors was evaluated in all of the 144 stage II colon cancers. However, the luminal surface of the tumors was only accessible for evaluation from 141 of the stage II colon cancers.

BM11 expression

We initially tested two antibodies targeting the BM11 protein. The monoclonal BM11 antibody (Mouse, clone F6, cat. no. 05-637, Millipore) was superior to the polyclonal BM11 antibody (Rabbit, HT-99, cat no. sc-10745, Santa Cruz Biotechnology) in terms of specificity. Thus, the former was used for all subsequent analysis. Additionally, differences in fixation time did not affect the BM11 protein staining using the selected antibody.

High expression of BM11 was observed in the nuclei of epithelial cells at the bottom of the colon crypts with a decreasing expression toward the lumen and with no expression at the luminal surface (Fig. 1). The endothelial cells, smooth muscle cells, and perineural cells also expressed nuclear BM11. Additionally, a number of lymphocytes and stromal cells such as fibroblasts and/or myofibroblasts were positive for BM11. An example of the expression of BM11 in normal colon tissue is presented in Fig. 1.

The expression of BM11 in stage II colon cancer tissues was heterogeneous at both intratumoral and intertumoral levels. The number of BM11 positive cells and the intensity of the staining varied widely in the tumors. Within the individual tumor the BM11 expression could vary from highly positive at the lumen and very low expression at the invasive front or vice versa. Examples of high and low expression of BM11 are presented in Fig. 1.

The prognostic value of BM11

BM11 expression at the invasive front correlated significantly with MMR status and age (Table 1). However, the correlation between dMMR and BM11 was weak. Moreover, the r-value of the Spearman rank correlation was quite low indicating a very weak correlation between BM11 expression and age of the patients. At the luminal surface BM11 correlated significantly with tumor location, T-stage, and the histological subtype of the tumors (Table 1). This correlation was not significant at the invasive front. There were no significant correlations between gender, the histological risk factor variable, or the remaining histological risk factors and BM11 expression at neither the invasive front nor the luminal surface (Table 1).

Univariate Cox proportional hazards analysis showed no significant association between risk of relapse and BM11 expression at the invasive front or at the luminal surface of the tumors (Table 2). Likewise, there was no significant association between 5-year overall survival and the BM11 expression at two sites in the tumors (Table 2).

DISCUSSION

Within the last decade, the stem cells and their role in cancer has been focused for much attention. Meanwhile, several potential intestinal stem cell markers has been reported and investigated in clinical prognostic settings (9). One of the potential stem cell markers of the intestine is BM11. We set to investigate the prognostic value of the expression of BM11 in primary tumors from a comprehensive cohort of patients diagnosed with stage II colon cancer.

Since no current national or international guidelines are present for BM11 protein expression analysis, we sought to score BM11 in what was the most informative manner in our opinion, by evaluating both the invasive front and the luminal surface. We have previously reported that dMMR was
### Table 1. Patient baseline characteristics

<table>
<thead>
<tr>
<th>Total no. of patients</th>
<th>No. of patients</th>
<th>BMI1 invasive front (n = 144)</th>
<th>BMI1 luminal surface (n = 141)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median (range)</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td>75 (50-90) 4%</td>
<td>8 (0-12) 4-8</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>Female 74 (51.4%)</td>
<td>8 (0-12) 8-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Male 70 (48.6%)</td>
<td>8 (0-12) 8-8</td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td>Right 73 (50.7%)</td>
<td>8 (0-12) 8-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Left 71 (49.3%)</td>
<td>8 (0-12) 8-8</td>
</tr>
<tr>
<td>Histological risk factors</td>
<td></td>
<td>Yes 70 (48.6%)</td>
<td>8 (0-12) 8-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No 74 (51.4%)</td>
<td>8 (0-12) 8-8</td>
</tr>
<tr>
<td>T-stage</td>
<td></td>
<td>T3 123 (85.4%)</td>
<td>8 (0-12) 6-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T4 21 (14.6%)</td>
<td>8 (2-8) 8-8</td>
</tr>
<tr>
<td>Histological subtype</td>
<td></td>
<td>High 112 (77.8%)</td>
<td>8 (0-12) 8-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low differentiation 15 (10.4%)</td>
<td>8 (3-12) 5-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mucinous 17 (11.8%)</td>
<td>8 (0-12) 4-8</td>
</tr>
<tr>
<td>Vein infiltration</td>
<td></td>
<td>Yes 29 (20.1%)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>No 115 (79.9%)</td>
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</tr>
<tr>
<td>Nerve infiltration</td>
<td></td>
<td>Yes 15 (10.0%)</td>
<td>8 (0-12) 4-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No 131 (91.0%)</td>
<td>8 (0-12) 4-8</td>
</tr>
<tr>
<td>Lymph nodes sampled</td>
<td></td>
<td>&lt;12 27 (18.8%)</td>
<td>8 (0-12) 6-10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥12 117 (81.3)</td>
<td>8 (0-12) 8-8</td>
</tr>
<tr>
<td>Tumor perforation</td>
<td></td>
<td>Yes 2 (1.4%) 5.5 (3-8)</td>
<td>3-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No 142 (98.6%)</td>
<td>8 (0-12) 8-8</td>
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<tr>
<td>MMR status</td>
<td></td>
<td>pMMR 111 (77.1%)</td>
<td>8 (0-12) 8-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dMMR 33 (22.9%)</td>
<td>8 (0-12) 3-8</td>
</tr>
</tbody>
</table>

dMMR, mismatch repair deficient; MMR, mismatch repair; n, number of patients analyzed; pMMR, mismatch repair proficient; BMI1 invasive front, BMI1 expression at the invasive front of the tumor; BMI1 Luminal Surface, BMI1 expression at the luminal surface of the neoplastic glands.

¹Mann–Whitney U test.
²Kruskal–Wallis test. The 'Histopathological risk factor' variable is based on the presence of one or more of the risk factors in italics. Left sided tumors include tumors of the left flexur, descendens, or sigmoidum. Right sided tumors include tumors of the cecum, ascendens, right flexur, or transversum.

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**Fig. 1.** Immunohistochemical staining of B-cell-specific moloney murine leukemia virus insertion site 1 (BMI1) at the invasive front (x10 magnification). (A) BMI1 expressed in normal colon. (B) Low expression of BMI1 in stage II colon cancer tissue. (C) High expression of BMI1 in stage II colon cancer tissue.
associated with a low risk of relapse (15). In the present study, we found that the BMI1 expression at the invasive front correlated with MMR status; however, the correlation was weak, suggesting that the correlation is of less importance. We also found that the BMI1 expression at the luminal surface correlated with T-stage, tumor location, and histological subtype of the tumor. None of the other studies investigating BMI1 as a prognostic marker has found correlations between BMI1 and the histological subtype or tumor location (11–13, 16). However, none of the other studies have investigated both the luminal surface and the invasive front of the individual tumors. A study found a correlation between BMI1 expression and T-stage investigating the BMI1 expression by tissue microarray (12). The study differs from ours by the use of tissue microarray. This could be an explanation to the discrepancies in results, as the tissue microarray provides a minor reflection of the tumor. Moreover, none of the other published studies have included MMR status as a variable. Conclusively, the correlation between BMI1 and the specific clinicopathological features is contradicting.

The primary objective of our study was to investigate if the protein could predict relapse of the stage II colon cancer and as a secondary endpoint investigate if it was associated with overall survival of the patients. We found that the BMI1 was not associated with neither of the prognostic endpoints, suggesting that BMI1 is not feasible as a prognostic marker for stage II colon cancer patients. To our knowledge, this is the first study investigating the prognostic value of BMI1 expression in only stage II colon cancer patients. Other studies have included all colon cancer stages (11–13), therefore it cannot be excluded that BMI1 might only be relevant in less or more advanced stages than stage II. Therefore, our study should optimally be verified in another cohort before a final consensus of the prognostic value of BMI1 can be presented.

The BMI1 expression was analyzed as a continuous variable in the study since we had no valid cut point for high, moderate, and low BMI1 expression. Thus, we did not find a rational argument for grouping the expression of BMI1 in certain subgroups based on the retrieved data. Other studies have dichotomized the expression of BMI1 into high and low expression or positive and negative staining which might be a contributing cause to the discrepancies seen in the studies in between and compared to our study. Another contributing cause could be the different antibodies used in the studies and the staining protocols applied (12, 13). We tested two antibodies to ensure the most optimal staining of BMI1 and found that one of the antibodies was superior with respect to specificity compared to the other antibody.

Optimally, a biomarker identifying high-risk patients should also provide information on the benefit of adjuvant therapy. Unfortunately, we did not have data on adjuvant therapy. It would have been interesting to further explore whether the patients included in the study had benefitted from adjuvant therapy. A limitation to our study is that patients were excluded from the cohort if they had had another primary cancer diagnosis prior to or after the primary stage II colon cancer diagnosis. This constitutes a selection bias of the patient cohort, posing another explanation of why our results might differ from previous studies. Insufficient reporting of patient materials and methods including patient inclusion and exclusion criteria, antibody specifications, and statistical considerations of the different cohorts further complicates the comparison across studies.

The understanding of BMI1 as a biomarker appear to be complicated with our study showing no association with overall survival or relapse; another study showing an association between positive BMI1 expression of primary colon tumors and lower overall survival of the patients (12); and a third study reporting that high BMI1 expression in colon tumors is associated with a longer survival than patients with low BMI1 expression (13). Since BMI1 acts in a complex with other polycomb proteins, the latter authors constructed a variable consisting of several polycomb proteins, including BMI1 and observed that the best survival and longest recurrence free period was found when all of these polycomb proteins combined were highly expressed in the tumor samples compared to using

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them as singular biomarkers (13). This indicates that BMI1 might not be optimal as a singular biomarker but may have a prognostic significance in combination with other markers. Unfortunately, in this study, it was not possible to also investigate the remaining polycomb proteins. We have previously shown that the transcription factor sex-determining region y-box 9 (SOX9) can predict relapse of stage II colon cancer patients. Additional studies are necessary to confirm the prognostic value of SOX9 and to explore whether other biomarkers together with SOX9 could improve stratification of high-risk stage II colon cancer patients (15).

In conclusion, we could not demonstrate that BMI1 expression in primary tumors of stage II colon cancer patients predicts relapse of cancer nor have a significant effect on overall survival of the patients. Further studies are needed to find optimal biomarkers for prediction of relapse to improve the personalized treatment of stage II colon cancer patients.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

BMI1: B-cell-specific moloney murine leukemia virus insertion site 1; dMMR: mismatch repair deficiency; IHC: immunohistochemistry; MLH1: MutL homolog 1; MMR: mismatch repair; MSH2: MutS protein homolog 2; MSH6: MutS protein homolog 6; PMS2: postmeiotic Segregation Increased 2; SOX9: sex-determining region y-box 9.

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SOX9 Expression Predicts Relapse of Stage II Colon Cancer Patients

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SOX9 expression predicts relapse of stage II colon cancer patients

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Keywords:
SOX9 transcription factor; Biomarkers; Colon cancer; Recurrence; Mismatch repair deficiency

Summary
The aim of this study was to investigate if the protein expression of sex determining region y-box 9 (SOX9) in primary tumors could predict relapse of stage II colon cancer patients. One hundred forty-four patients with stage II primary colon cancer were retrospectively enrolled in the study. SOX9 expression was evaluated by immunohistochemistry, and mismatch repair status was assessed by both immunohistochemistry and promoter hypermethylation assay. High SOX9 expression at the invasive front was significantly associated with lower risk of relapse when including the SOX9 expression as a continuous variable (from low to high expression) in univariate (hazard ratio [HR], 0.73; 95% confidence interval [CI], 0.56–0.94; \( P = .01 \)) and multivariate Cox proportional hazards analyses (HR, 0.75; 95% CI, 0.58–0.96; \( P = .02 \)), adjusting for mismatch repair deficiency and histopathologic risk factors. Conversely, low SOX9 expression at the invasive front was significantly associated with higher risk of relapse, when including SOX9 expression as a dichotomous variable, in univariate (HR, 2.32; 95% CI, 1.14–4.46; \( P = .02 \)) and multivariate analyses (HR, 2.52; 95% CI, 1.14–4.46; \( P = .02 \)), adjusting for histopathologic risk factors and mismatch repair deficiency. In conclusion, high levels of SOX9 of primary stage II colon tumors predict low risk of relapse, whereas low levels of SOX9 predict high risk of relapse. SOX9 may have an important value as a biomarker when evaluating risk of relapse for personalized treatment.

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1. Introduction

Colorectal cancer is one of the most frequent malignancies in the Western world [1]. Approximately one-fourth of patients with colon cancer are diagnosed as having stage II colon cancer [2]. Today, a minority of patients with stage II colon cancer is offered adjuvant therapy based on high-risk histopathologic features (T4 stage, vein invasion, perineural invasion, margin involvement, number of sampled lymph nodes, perforation, and low differentiated histology) [2,3].

Despite proper surgical intervention, approximately 20% of all patients with stage II colon cancer have relapse of their cancer. Yet still no optimal biomarker has been established in the clinic to identify high-risk patients and predict relapse of stage II colon cancer. Thus, the incentive for novel prognostic and predictive markers to identify the patients, who most likely will benefit from additional treatment, is extensive.

One of the hallmarks of cancer is genomic instability [4]. MSI can occur as a consequence of dMMR [5]. Germline mutations in MMR genes are associated with the Lynch syndrome, whereas MLH1 promoter hypermethylation are primarily found in sporadic colon cancer cases [6].

The cancer stem cell theory proposes that cancer stem cells are involved in initiation, progression, recurrence of cancer, and treatment response. SOX9 is a transcription factor involved in several developmental processes and is important for cell proliferation, senescence, and lineage commitment [7–10]. We hypothesized that there may be an association between the SOX9 expression in primary tumors of patients with stage II colon cancer and their risk of relapse. To test our hypothesis, we investigated the SOX9 expression by IHC in primary tumors of patients with stage II colon cancer.

2. Materials and methods

2.1. Patient cohort

The study was performed as a retrospective cohort study. Formalin-fixed, paraffin-embedded primary tumors from 144 patients diagnosed as having and treated for stage II colon cancer at Glostrup University Hospital, Gentofte University Hospital, and Herlev University Hospital in Denmark, were included consecutively from January 2005 to August 2008 using the national pathology registry of Denmark and patient medical records. The patients had undergone complete surgical resection as primary treatment. The inclusion period was based on the desire of a follow-up period of at least 5 years. Registered data and inclusion and exclusion criteria can be found in the Appendix. Enrollment and exclusion of patients is shown in Fig. 1. The study was approved by the Scientific Ethics Committee of the Capital Region of Denmark (H-1-2013-028) and by the Data Protection Agency of the Capital Region of Denmark (2007-58-0015).

2.2. Tumor tissue

The tumor tissue had been processed as part of the diagnostic routine after curative surgery. The tumor tissue was fixated in 10% neutral-buffered formalin for at least 48 hours prior to paraffin embedding. From each patient, 2 tissue blocks of the primary tumor were obtained. Full slides of the tumor were used for IHC against SOX9. TMA s were constructed with four 1-mm cores from each secondary tissue block as previously described [11]. A fifth 1-mm core in a tumor cell-enriched area (>50% tumor cells) was punched for DNA extraction.

2.3. IHC analysis

All analyzes and assays were conducted blinded to patient outcome.

2.3.1. SOX9 IHC and evaluation

Three-micrometer slides were cut and incubated for 45 minutes at 60°C. IHC was performed using the EnVision FLEX, High pH detection system (Dako, Glostrup, Denmark) and the automated Autostainer Link 48 (Dako Glostrup, Denmark) according to the manufacturer’s instructions. Anti-SOX9 antibody (1:10,000; Merck Millipore, Darmstadt, Germany) was used for SOX9 detection. Specification of the antibody can be found in the Supplementary Table. The tissue slides were counterstained with Mayer hematoxylin using the automated slide stainer TissueTek PrismaFilm (Sakura, Copenhagen, Denmark). Finally, the slides were scanned using the Nanocmoomer 2.0 HT (Hamamatsu, Hentschin, Germany).

The stability of the SOX9 antigen was evaluated by staining normal colon tissue that had been subjected to formalin fixation for 3, 27, 51, and 123 hours. A control slide was included for each run and consisted of normal tissue from the colon, small intestine, tests, ventricle, and breast. The selection of the control tissues was based on previous reports of SOX9 staining [12–17].

SOX9 expression was evaluated at the invasive front of the tumor and at the luminal surface independently by a specialized pathologist and a trained molecular biologist supervised by a specialized pathologist. We defined the invasive front as the area where the tumor periphery invades deepest into the colonic tissue. The luminal surface refers to the luminal surface of the neoplastic glands.

Five random areas were selected for each region, using the image analysis software Visiopharm Integrator System (version 4.5.6.516; Visiopharm, Horsholm, Denmark). Percent positive and negative tumor nuclei were counted and given a score: score 0 (0–5% positive nuclei), score 1 (5%–25% positive nuclei), score 2 (25%–50% positive nuclei), score 3 (50%–75% positive nuclei), and score 4 (>75% positive nuclei).
Consecutively included stage II colon cancer patients registered in the national pathology registry of Denmark from January 2005 to August 2008 (N = 282)

- Excluded: Not stage II cancer
  - n = 15

- Excluded: <50 years of age at diagnosis
  - n = 113
  - Died <1 month after surgery
    - n = 6
  - Metastasis <3 months after diagnosis
    - n = 13
  - Diagnosed with other cancers
    - n = 75
  - Chronic inflammation / IBD
    - n = 2

- Excluded: Synchronous tumors
  - n = 10

- Inadequate tissue
  - n = 8
  - n = 2

Final population: n = 144 (100%)
- Relapse: n = 37 (25.7%)
- Deaths: n = 58 (40.3%)

Fig. 1 Flowchart showing enrollment and exclusion of patients from the study. Abbreviations: n, number of patients; IBD, inflammatory bowel diseases.

2.3.2. MMR IHC status

TMA sections (3 μm) were stained with monoclonal antibodies against MLH1 (Ready-To-Use; Dako, Glostrup, Denmark), MSH2 (1:400; Cell Marque, Rocklin, CA), MSH6 (Ready-To-Use; Dako), and PMS2 (Ready-To-Use; Dako, Glostrup, Denmark) using the EnVision detection system described above. Specifications of the antibodies can be found in the Supplementary Table. The tumor was considered dMMR if 1 or more of the 4 MMR proteins were absent in the tumor nuclei. If the tumor nuclei stained positive for the 4 MMR proteins, the tumor was pMMR. Nuclear staining of stromal cells was used as an internal control. A positive control slide containing normal tonsil, appendix, pancreas, and liver was included in each run.

2.4. Promoter hypermethylation assay

DNA was extracted from the 1-mm core as previously described [19]. Promoter hypermethylation of MLH1, MSH2, MSH6, and PMS2 was assessed by the SALSA MS-MLPA ME011 Mismatch Repair genes kit (cat. no.
SOX9 predicts relapse of stage II colon cancer patients

EK1-FAM; MRC-Holland, Amsterdam, the Netherlands) and performed according to manufacturer’s instructions. Using the enclosed software, the methylation ratio was analyzed as “hypermethylated” or “unmethylated.” All unmethylated cases were analyzed twice to confirm status of case.

2.5. Statistics

The primary end point of the study was whether the protein expression of SOX9 in primary tumors from stage II colon cancer patients could predict relapse. A secondary end point was to investigate the prognostic value of dMMR in stage II colon cancer patients with relapse.

If the tumor was positive for any of the risk factors—T4 stage, nerve infiltration, vein infiltration, low-differentiated histologic appearance (unless the tumor was dMMR), tumor perforation, or less than 12 lymph nodes sampled at primary resection—the patient was grouped as having a histopathologic risk factor. This was done due to the relatively small numbers of some of the histopathologic risk factors.

Association between SOX9 expression levels and age was explored by the Spearman rank correlation. Rank test for location (Mann-Whitney U and Kruskal-Wallis) was applied to investigate associations between SOX9 expression levels and categorical variables.

Survival probabilities were estimated by Kaplan-Meier plots and compared by the log-rank test. Univariate and multivariate analyses adjusting for the histopathologic risk factor variable and dMMR were done using the Cox proportional hazards model containing the SOX9 expression as a dichotomous variable (high/low) or as a continuous variable, with a hazard ratio of 3 differences in scores and with relapse as outcome. The model assumptions were validated by sensitivity testing and Martingale residuals.

A 5-year overall survival univariate model with SOX9 expression at the invasive front and the luminal surface as a dichotomous variable was conducted. Weighted Cohen κ statistics were applied for investigating interobserver agreement. All statistical analyses were conducted in IBM SPSS Statistics 22 (IBM, Armonk, NY) and SAS (version 9.3; SAS Institute, Cary, NC). P values of .05 or less were considered significant.

3. Results

3.1. Patients and clinical characteristics

The median follow-up period was 92 months (range, 69-111 months). Among the 144 patients included in the study, 37 patients (25.7%) relapsed during follow-up and 58 patients (40.3%) had died (Fig. 1). The median time to relapse was 21 months (range, 4-84 months). Of the patients that relapsed, 31 (83.8%) had died. Most patients with relapse (24 patients; 64.9%) had recurrence in 2 or more sites.

Overall, 70 (48.6%) of the patients had 1 or more histopathologic risk factors associated with their primary tumor. All of the patients had a tumor cell–free resection margin. The luminal surface of the tumor was not available from 2 patients. Thus, the luminal surface was only analyzed on tumor samples from 142 patients. Overall patient characteristics are presented in Table 1.

A total of 33 (22.9%) patients had dMMR tumors, whereas 30 (90.9%) were MLH1 and PMS2 deficient. MSH2 and MSH6 deficiency was seen in 1 (3.0%) tumor, and 2 (6.1%) tumors demonstrated solely MSH6 deficiency. Promoter hypermethylation assay was conducted to further characterize the patient cohort as most sporadic colon cancer cases are caused by MLH1 promoter hypermethylation [6]. Of the dMMR tumors, 28 (84.8%) were MLH1 promoter hypermethylated. No promoter hypermethylation was observed in tumors with MSH2 and/or MSH6 deficiency. Overall, 5 (3.5%) of 144 patients had dMMR tumors without promoter hypermethylation.

3.2. SOX9 expression

The SOX9 staining was not not affected by the fixation or the storage time of the tissue. The SOX9 expression in the normal tissue can be found in Supplementary Figure. Both intratumoral and intertumoral heterogeneous SOX9 expressions were observed in the colon cancer tissue (Fig. 2). In most cases, SOX9 stained positive in more than 75% (score 4) of the tumor nuclei; however, the staining intensity ranged from highly intense to very low. Interestingly, SOX9 protein expression was not detected in 11 (7.6%) and 12 (8.3%) of the tumors at the invasive front and luminal surface, respectively (Fig. 2).

3.3. SOX9 and risk of relapse

There was no significant correlation between SOX9 expression at either the invasive front or the luminal surface of the tumors and age, sex, tumor side, or histopathologic risk factors (Table 1). Kaplan-Meier survival plots showed a significant difference in relapse-free survival of patients with low SOX9 expression at the invasive front of their primary tumor compared with patients with high SOX9 expression (Fig. 3). SOX9 expression levels at the luminal surface showed no significant difference in relapse-free survival time (Fig. 3).

Univariate Cox proportional hazards analysis showed that low SOX9 expression at the invasive front in the tumor was statistically associated with higher risk of relapse (Table 2). Similarly in multivariate analysis, low SOX9 expression at the invasive front was significantly associated with 2.3 higher-risk relapse (Table 2). For univariate and multivariate survival analyses, including SOX9 as a continuous variable, the risk of relapse decreased by 25% when there was a difference of 3 in the SOX9 score from low SOX9 expression to high expression at the invasive front (Table 2).
Table 1  Patient baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>No. of patients</th>
<th>SOX9 invasive front (n = 144)</th>
<th>SOX9 luminal surface (n = 142)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (range)</td>
<td>Median (range)</td>
<td>P</td>
</tr>
<tr>
<td><strong>Total no. of patients</strong></td>
<td>144</td>
<td></td>
<td>0.95 (R = 0.01)</td>
</tr>
<tr>
<td>Age (y), median (range)</td>
<td>73 (50-90)</td>
<td>8 (0-12)</td>
<td>0.78</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>74 (51.4%)</td>
<td>8 (0-12)</td>
<td>0.36</td>
</tr>
<tr>
<td>Male</td>
<td>70 (48.6%)</td>
<td>8 (0-12)</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor location</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>73 (50.7%)</td>
<td>8 (0-12)</td>
<td>0.33</td>
</tr>
<tr>
<td>Left</td>
<td>71 (49.3%)</td>
<td>8 (0-12)</td>
<td></td>
</tr>
<tr>
<td><strong>Histologic risk factor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>70 (48.6%)</td>
<td>8 (0-12)</td>
<td>0.18</td>
</tr>
<tr>
<td>No</td>
<td>74 (51.4%)</td>
<td>8 (0-12)</td>
<td></td>
</tr>
<tr>
<td><strong>T stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>123 (85.4%)</td>
<td>8 (0-12)</td>
<td>0.39</td>
</tr>
<tr>
<td>T4</td>
<td>21 (14.6%)</td>
<td>8 (0-12)</td>
<td></td>
</tr>
<tr>
<td><strong>Histologic subtype</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High differentiation</td>
<td>112 (77.8%)</td>
<td>8 (0-12)</td>
<td>0.47</td>
</tr>
<tr>
<td>Low differentiation</td>
<td>15 (10.4%)</td>
<td>8 (3-12)</td>
<td></td>
</tr>
<tr>
<td>Mucinous</td>
<td>17 (11.8%)</td>
<td>8 (0-12)</td>
<td></td>
</tr>
<tr>
<td><strong>Vein infiltration</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>29 (20.1%)</td>
<td>8 (0-12)</td>
<td>0.11</td>
</tr>
<tr>
<td>No</td>
<td>115 (79.9%)</td>
<td>8 (0-12)</td>
<td></td>
</tr>
<tr>
<td><strong>Nerve infiltration</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>13 (9.0%)</td>
<td>8 (0-12)</td>
<td>0.41</td>
</tr>
<tr>
<td>No</td>
<td>131 (91.0%)</td>
<td>8 (0-12)</td>
<td></td>
</tr>
<tr>
<td><strong>Lymph nodes sampled</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;12</td>
<td>27 (18.8%)</td>
<td>8 (0-12)</td>
<td>0.49</td>
</tr>
<tr>
<td>≥12</td>
<td>117 (81.2%)</td>
<td>8 (0-12)</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor perforation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2 (1.4%)</td>
<td>10 (8-12)</td>
<td>0.55</td>
</tr>
<tr>
<td>No</td>
<td>142 (98.6%)</td>
<td>8 (0-12)</td>
<td></td>
</tr>
<tr>
<td><strong>pMMR status</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>pMMR</td>
<td>111 (77.1%)</td>
<td>8 (0-12)</td>
<td>0.55</td>
</tr>
<tr>
<td>dMMR</td>
<td>33 (22.9%)</td>
<td>8 (0-12)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Left-sided tumor includes tumors of left flexur, descends, and sigmoidum. Right-sided tumor includes tumor of cecum, ascendens, right flexur, and transversum.

Abbreviations: dMMR, mismatch repair deficient; pMMR, mismatch repair proficient; SOX9 invasive front, SOX9 expression at the invasive front of the tumor; SOX9 luminal surface, SOX9 expression at the luminal surface of the neoplastic glands.

- Spearman rank correlation.
- Mann-Whitney U test.
- The patient was considered to have a histopathologic risk factor if either of the risk factors was present in the tumor. T4 stage, nerve and/or vein infiltration, low differentiated histology (unless the tumor was dMMR), tumor perforation, or less than 12 lymph nodes sampled at primary resection.
- Kruskal-Wallis test.

For univariate analysis, tumor perforation was not included due to the low number of events in the patient cohort. Of the histopathologic risk factors, T stage, nerve infiltration, or less than 12 sampled lymph nodes increased the risk of relapse significantly 2- to 4-fold (Table 2). In multivariate analysis, the occurrence of 1 or more histopathologic risk factors was associated with an approximate 3-fold risk of relapse (Table 2). Furthermore, dMMR was significantly associated with an approximately 80% reduced risk of relapse both in univariate and multivariate analyses (Table 2). There was no association between low SOX9 expression and 5-year overall survival by univariate Cox proportional hazards analysis (hazard ratio, 1.56; 95% confidence interval, 0.79-3.09; P = .2). The interobserver agreement of the intensity score of SOX9 at the invasive front showed a high concordance with a Cohen weighted κ coefficient of 0.84 (95% confidence interval, 0.77-0.91).

4. Discussion

To our knowledge, this is the first study investigating the protein expression of SOX9 as a potential biomarker of
Fig. 2  Immunohistochemical staining of SOX9 in normal colon tissue (A and B) and in stage II colon cancer tissue (C–F). A, SOX9 expression by a ×2.7 magnification. C and E, SOX9 expression by a ×1.25 magnification. Panels B, D, and F are ×20 magnification of marked areas of panels A, C, and E. A and B, SOX9 is expressed in nuclei of the epithelial cells at the bottom of the crypts. C and D, High expression of SOX9 in tumor cells. E and F, Undetected SOX9 expression in tumor cells, including internal stromal control for the SOX9 staining.

Fig. 3  Relapse-free survival of low versus high SOX9 expression at the luminal surface (A) and at the invasive front (B) of the primary tumors from patients with stage II colon cancer. Abbreviations: HR, hazard ratio (Cox proportional hazards model, univariate); CI, confidence interval.
Table 2  Univariate and multivariate analyses of prognostic factors for relapse

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard ratio (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.72 (0.38-1.38)</td>
<td>.32</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>1.47 (0.76-2.83)</td>
<td>.25</td>
</tr>
<tr>
<td>Right</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOX9 expression at the invasive front</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuous score</td>
<td>0.73 (0.56-0.94)</td>
<td>.01 *</td>
</tr>
<tr>
<td>High</td>
<td>2.32 (1.14-4.69)</td>
<td>.02</td>
</tr>
<tr>
<td>Low</td>
<td>0.19 (0.03-0.80)</td>
<td>.02</td>
</tr>
<tr>
<td>MMR status</td>
<td>dMMR</td>
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<td></td>
<td>pMMR</td>
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</tr>
<tr>
<td>Histopathologic risk factor</td>
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<td>&lt;.01</td>
</tr>
<tr>
<td></td>
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<td></td>
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<tr>
<td>T stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>2.74 (1.34-5.66)</td>
<td>.01</td>
</tr>
<tr>
<td>T3</td>
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<td></td>
</tr>
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<td>.46</td>
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<td>.64</td>
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<tr>
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<tr>
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<tr>
<td>Lymph nodes sampled</td>
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<td>≥12</td>
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Abbreviations: CI, confidence interval; dMMR, mismatch repair deficiency; MMR, mismatch repair; pMMR, mismatch repair proficient.

* Cox proportional hazards model with SOX9 expression as a continuous score. The hazard ratio is based on a difference of 3 in the SOX9 score.

b Cox proportional hazards model with SOX9 expression as a dichotomous score (high/low).

* If either of the risk factors was present in the tumor—T4 stage, nerve and/or vein infiltration, low differentiated histology (unless the tumor was dMMR), tumor perforation, or less than 12 lymph nodes sampled at primary resection—the patient was considered to have a histopathologic risk factor.

Relapse in patients diagnosed as having a stage II colon cancer. By investigating a comprehensive characterized patient cohort with stage II colon cancer and following the REMARK guidelines [20], we found that low SOX9 expression at the invasive front of their primary tumors was significantly associated with relapse.

The SOX9 expression pattern was very heterogeneous in the tumors, especially in the central tumor region. In an effort to adjust for the heterogeneity, the luminal surface and the invasive front within each tumor were scored separately. Our results emphasize the importance of histopathology in diagnostics and validation of the biomarker expression patterns. The interobserver concordance was high even with 2 evaluators with different professions, indicating that this analysis is robust and reproducible and could be implemented as part of the clinical routine.

The underlying molecular mechanisms for the association of decreased SOX9 expression with risk of relapse are not fully solved. Together with SOX8 and SOX10, SOX9 is part of the SOX family group called SOXE [21]. The function of the SOXE transcription factors is highly context dependent, and they bind and regulate differently depending on the environment they are subjected to. Therefore, the regulation of SOX9 and the exact function of SOX9 are complex. The transcription factor has been reported as having both oncogenic and tumor-suppressive effects in colorectal cancer [7,9,10,22].

A recent article by The Cancer Genome Atlas provided a comprehensive molecular analysis of colorectal carcinomas
and reported that SOX9 was frequently mutated [23]. The mutations consisted of frameshift and nonsense mutations, suggesting that SOX9 may play a tumor-suppressive role [23]. This has further been supported by studies reporting widespread hyperplasia and dysplasia in the intestinal epithelium of Sox9-deficient mice [10,24]. In addition, SOX9 has been reported to be a canonical Wnt/β-catenin target gene in human colon cancer cells [9], exerting a negative feedback loop by inhibiting the signaling pathway as well [10]. Abdel-Samad and colleagues [25] reported a truncated variant of SOX9 lacking its transactivation domain. The variant stimulated the canonical Wnt pathway but also inhibited wild-type SOX9. This could be a plausible explanation for the conflicting roles of SOX9 reported in the literature.

A low or nondetectable expression of SOX9 at the invasive front and an associated higher risk of relapse, together with a lower risk of relapse, the higher the SOX9 expression detected, could illustrate that SOX9 is a crucial tumor suppressor governing proliferation and expansion of the cells. The lack of expression can be triggered by mutations but also by promoter hypermethylation. Indeed, SOX9 promoter hypermethylation has been previously reported to be associated with SOX9 expression loss and bladder cancer progression [26].

Decreased expression and the heterogeneous expression of the transcription factor in the colon tumors might be dependent on extracellular signaling from the stroma. It could be speculated that the tumor cells with low or absent SOX9 at the invasive front are a subclone gaining properties to enter the epithelial-mesenchymal transition. Accordingly, this provides the tumor cells with the ability to migrate and invade further into the tissue, thereby gaining increased metastatic potential and reaching another stage of cancer progression.

That we did not find any association with risk of relapse at the luminal surface of the neoplastic glands could be that loss of SOX9 in the tumor cells of this area does not have the same metastatic potential as deeper in the tissue. However, further studies are necessary to shed light on the exact functions of the epigenetic and mutational effects on SOX9 protein expression and the potential association with cancer progression and risk of relapse.

SOX9 has previously been reported as overexpressed in colorectal cancer, which we also observed in most cases [7,15,25,27–32]. Currently, there are no consensus guidelines nationally or internationally regarding scoring of SOX9. Thus, we decided to score most informatively by scoring at the invasive front and at the luminal surface instead of an average score of the whole tumor slide. However, this also makes our study differ from other prognostic studies of SOX9 in colorectal cancer [7,15,30].

We did not find any correlation between high expression of SOX9 and poorer prognosis as previously reported [15], nor could Panza et al in their study [30]. The description of the patient cohort is sparse, which makes it difficult to point out differences from the present study and thereby plausible explanations for the discrepancies of the studies. Interestingly, SOX9 protein overexpression has been found to predict reduced overall survival in stage III and not stage II colon cancer patients, which provides a plausible explanation as to why we did not find any correlation between SOX9 expression and overall survival [27]. Furthermore, the use of different antibodies can influence the results, as these potentially recognize different epitopes of the SOX9 protein. This also constrains our study, as we only investigated one antibody which was the most applied in the literature. Optimally, we could have investigated and validated our findings more thoroughly by applying antibodies from additional suppliers, but unfortunately, this was not possible.

Both high and low SOX9 expression levels have been associated with MSI [30,31]. However, we did not find any correlation between SOX9 expression levels and MMR status. As expected, dMMR in our study was significantly associated with a lower risk of relapse, which has also been reported by other studies [33,34]. Most of the dMMR tumors were hypermethylated in the MLH1 promoter, indicating sporadic colon cancer [6]. MMR deficiency was investigated by IHC as part of the diagnostic routine. The MMR deficiency could have been verified by MSI testing; however, this was unfortunately not possible. Instead, the methylation profile of the MMR genes was tested to provide additional information to the cohort because MLH1 promoter hypermethylation is most commonly found in sporadic colon cancer cases. However, it should be noted that a few cases of patients harboring both MLH1 promoter hypermethylation and MLH1 mutations have been reported [6,35]. The likelihood of this is small in our study because we only selected patients 50 years or older. It is uncertain whether the patients with dMMR tumors without hypermethylation had a hereditary factor. Unfortunately, in our study, these tumors were not genetically investigated because this has not been the general procedure.

The SOX9 expression level was associated with neither sex nor age as others have previously described [30]. Of the conventional histopathologic risk factors, vein infiltration and the histologic subtypes were not associated with relapse which may be due to the relatively small size of our study.

An important limitation of this study is the exclusion of patients with other primary cancers before or after their primary diagnosis of stage II colon cancer. This comprises a selection bias of the patients not reflecting all patients seen in the clinic. Because of the setup of the study, we cannot argue that our findings can be directly translated into recommending adjuvant therapy to SOX9 expression–indicated high-risk patients. We believe that a larger randomized clinical trial is needed for answering that present issue. However, the stratification of high-risk patients by their SOX9 expression may provide information for more intense follow-up of these patients, and we believe that a hazard ratio of 2.32 would be high enough to justify this.
5. Conclusion

In conclusion, this is the first study to report that low SOX9 protein expression at the invasive front of primary stage II colon tumors predicts high risk of relapse. Conversely, a high SOX9 protein expression level at the invasive front of primary stage II colon tumors predicts low risk of relapse. SOX9 expression may have an important value as a biomarker for prediction of relapse. If our findings are further validated, SOX9 analysis has a potential to enter the clinical pathology routine in evaluating risk of relapse for further personalized treatment.

Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.humpath.2015.12.026.

References

Appendix

Registered data and patient inclusion and exclusion criteria

Histopathological risk factors (vein infiltration, nerve infiltration, T-stage, number of sampled lymphnodes, histological subtype (mucinous adenocarcinoma, low differentiated adenocarcinoma, or high differentiated adenocarcinoma), and tumor perforation), age, and gender was registered for data analysis. The primary site of the tumor was registered as right-sided if the tumor was located in cecum, ascendens, right flexur, or the transversum. The tumor was registered as left-sided if it was located in the left flexur, descendens, or sigmoideum. Only patients $\geq 50$ years of age at time of diagnosis was included. Patients with a history of other primary cancers prior to or after the primary colon cancer diagnosis were excluded; likewise patients with a history of chronic inflammation or inflammatory bowel diseases were excluded. None of the included patients had received neoadjuvant chemotherapy, and patients with multiple or synchronous tumors at diagnosis were excluded. Patients who died less than a month after primary surgical procedure and patients who had metastasis within 3 months after the primary diagnosis were not included in the study.
**Supplementary Table 1:**

Supplementary Table 1: Antibody specifications.

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<th>Antibody</th>
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<th>EnVision Flex+ Linker</th>
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<td>Rabbit</td>
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<td>Rabbit</td>
<td>-</td>
<td>Polyclonal</td>
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**Supplementary Figure**

In the crypt epithelium of normal small intestine, colon, and ventricle tissue, SOX9 was primarily expressed in the nuclei of epithelial cells located in the proliferation zone. Nuclear SOX9 expression was also detected in a few stromal cells, perineural cells, smooth muscle cells, and lymphocytic cells mainly located in the lymphatic follicle proliferative center of the gastrointestinal tissue. In normal breast tissue SOX9 expression was present in the nuclei of glandular and ductal epithelial cells and in a few stromal cells. Furthermore, SOX9 was expressed in the nuclei of sertoli cells.

**Supplementary Figure.** Immunohistochemical staining of SOX9 in control tissues (×10 magnification). (A) SOX9 expressed in normal small intestinal tissue. (B) SOX9 expression in normal ventricle tissue. (C) SOX9 expression in normal breast tissue. (D) SOX9 expression in normal testis.
Tumor Necrosis Factor-α Induces the Expression of SOX9 through NF-κB Activation in Caco-2 Cells

Maiken Lise Marcker Espersen, Mehmet Coskun, Dorte Linnemann, Estrid Høgdall, and Jesper T. Troelsen

Manuscript in preparation
Tumor Necrosis Factor-α Induces the Expression of SOX9 through NF-κB Activation in Caco-2 Cells
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Conflicts of interests
The authors declare no conflicts of interest.

Manuscript in preparation
Abstract
The transcription factor SOX9 plays an important role for intestinal epithelial homeostasis including proliferation, lineage commitment, and regeneration. SOX9 has been indicated to have a clinical prognostic value in colon cancer patients. Yet, little is known about the regulation of SOX9 in colon cancer. In the present study we investigated the effect of the pro-inflammatory cytokine TNF-α on SOX9 expression through the downstream effector pathway NF-κB in colon carcinoma cell lines.

SW480, DLD1, HT29, and Caco-2 cells were stimulated with TNF-α. RNA were harvested for quantitative RT-PCR to assess the effect of TNF-α on the endogenous SOX9 expression. The effect of NF-κB subunits on the SOX9 promoter activity was investigated by transient transfections and luciferase promoter reporter assay in Caco-2 cells.

While HT29 and Caco-2 cells had a significant increase of endogenous SOX9 mRNA expression upon TNF-α stimulation, the SOX9 expression of SW480 and DLD1 cells was not affected by TNF-α. In Caco-2 cells the effect was TNF-α dose dependent. p50 or p52 exogenous overexpression significantly up-regulated the SOX9 promoter activity. Moreover, overexpression of p65 together with overexpression of p50 or p52 constructs significantly induced the SOX9 promoter activity.

These findings indicate that TNF-α affects the endogenous expression level of SOX9 and the effect might be mediated by the NF-κB subunits. Further studies are necessary to fully elucidate the regulation of SOX9 by the TNF-α and NF-κB and the clinical implications in colon cancer.

Keywords
Colon cancer, Tumor necrosis factor alpha, Nuclear factor kappa B, SOX9, Gene expression regulation

Abbreviations
CRC, Colorectal cancer; DMEM, Dulbecco’s modified eagle medium; NF-κB, Nuclear factor kappa B; RPLP0, Ribosomal Protein Large P0; SOX9, Sex-determining region y-box 9; TNF-α, Tumor necrosis factor alpha
Introduction

Colorectal cancer (CRC) is one of the most common cancers with 1.2 million new cases per year[1]. CRC arises due to transformation of normal epithelial cells into malignant cells. This involves dysregulation of essential signaling pathways leading to cellular longevity, self-sufficiency in growth signaling and invasive and metastasizing properties. A major driver of colorectal carcinogenesis is aberrant canonical Wnt-signaling. Furthermore, the nuclear factor kappa B (NF-κB) signaling pathway regulates numerous key processes associated to the hallmarks of cancer but also regulates differentiation and proliferation of intestinal stem cells in concert with the canonical Wnt-signaling pathway[2,3]. Within the last decade stem cells or stem cell like cells has been proposed to play a crucial role in carcinogenesis and progression of cancer.

Sex-determining region y-box 9 (SOX9) is a transcription factor and potential intestinal stem cell marker. The transcription factor has been shown to exert an important role in governing regeneration of the intestinal tissue upon irradiation and tightly regulating proliferation of the tissue[4,5]. SOX9 expression is not restricted to the intestinal stem cells but are also expressed in transit-amplifying cells and Paneth cells of the small intestine[6–8]. The transcription factor regulate the canonical Wnt-signaling pathway by inhibiting β-catenin[6] but has also been shown to be a target of the β-catenin/TCF4 complex[8], suggesting a negative feedback loop.

Several studies report increased SOX9 expression in tumors from CRC patients and that the transcription factor may have a prognostic value in CRC[9–13][14]. Yet, little is known about the actual regulation of SOX9 in CRC. We have previously shown that SOX9 protein expression at the invasive front of primary tumors from stage II colon cancer patients is associated to risk of relapse[15]. This indicates that SOX9 may have an important clinical value. Thus, we sought to further investigate how SOX9 was regulated in colon cancer. We have previously reported an increase of SOX9 expression in the colon carcinoma cell line Caco-2 upon treatment with the pro-inflammatory cytokine Tumor necrosis factor alpha (TNF-α) [16]. Furthermore, a recent study suggested that p65 subunit of the NF-κB signaling pathway might be important for Sox9 regulation in the intestines of a mouse model[2]. In the present study we investigated the TNF-α-mediated downstream effect on SOX9 regulation through the canonical NF-κB signaling pathway.

Materials and Methods

Cell cultures and treatment

The human colonic cell lines Caco-2, HT29, DLD1, and SW480 were cultured as monolayers in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and passaged twice weekly as previously described[17]. For TNFα-stimulation experiments, 1x106 cells were seeded in 24-well plates (NUNC Brand, Thermo Fisher Scientific, Waltman, MA, USA), grown to >95% confluence, and
stimulated in medium with or without TNF-α (R&D Systems, Minneapolis, MN, USA) for 24 hours as previously described [18]. While HT29, DLD1, and SW480 cells were stimulated with 10 nM TNF-α, Caco-2 cells was stimulated with increasing concentrations of TNF-α (0.1, 1, or 10 nM) [17].

**RNA extraction and quantitative RT-PCR**

Total RNA from Caco-2, HT29, DLD1, or SW480 cells was extracted using the NucleoSpin columns (Macherey-Nagel, Düren, Germany) following the manufacturer’s protocol. 200 ng of each RNA sample was used for cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen, Paisley, UK). All qRT-PCR reactions were performed on a LightCycler 480 (Roche Diagnostics, Indianapolis, IN, USA) using the Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Pittsburgh, PA, USA) according to manufacturer’s instructions. The following primers were used to amplify SOX9 transcripts: forward: 5’-GATTACAAGTACCAGCCCGC-3’ and reverse: 5’-GGATTGCCCGAGTGCTC-3’ (Eurofins, Ebersberg, Germany). Target gene expressions were normalized to the expression of human Ribosomal Protein Large P0 (RPLP0) serving as reference gene.

**Construction of SOX9 promoter reporter plasmid**

The SOX9 promoter region was cloned into the firefly luciferase reporter vector pGL4.10 (pGL4-SOX9) by the HindIII restriction site using the In-Fusion® HD Cloning Kit (Clontech Laboratories, Mountain View, CA, USA) according to manufacturer’s instructions. The 703 bp upstream region of the SOX9 gene (from position chr17:70,116,683-70,117,385, the GRCh37/hg19 Assembly) was amplified using the primer sequences: forward: 5’-ctcggcggccaagcttCGTAAACTCGGCTACGCATT-3’ and reverse: 5’-ccggattgccaagcttCGGCGAGCACTTAGGAAG-3’ (lower cases: sequence overhang complementary to pGL4.10 vector; higher cases: SOX9 primer sequences). The plasmid was sequenced to ensure the plasmid was constructed correctly.

**Transient transfections**

For transfections Caco-2 cells were grown in DMEM containing 4.5 g/L glucose with ultraglutamine (Lonza, Basel, Switzerland), 10% heat inactivated HyClone™ Fetal Bovine Serum (Thermo Fisher Scientific), and 100 U/ml Penicillin Streptomycin (Gibco, Thermo Fisher Scientific). 70% confluent Caco-2 cells were trypsinized using 0.05% 1× trypsin-EDTA (Gibco, Thermo Fisher Scientific) and seeded in a 24-well plate in a density of 5×10^4 cells/ml medium overnight. 0.2 µg of the constructed promoter reporter pGL4-SOX9 plasmid was co-transfected with pCMV4-p50 (plasmid #21965 [19], Addgene, Cambridge, MA, USA), pCMV4-p52 pCMV4-p52 (plasmid #23289 [20], Addgene), pCMV4-p65 (plasmid #21965[19], Addgene), or a combination of these overexpression plasmids in combination with the empty pcDNA3.1+ and 0.1 µg pCMV-LacZ plasmid (functions as internal transfection control). Finally, pBluescript SK+ plasmid was added to normalize the DNA mix to a total amount of 0.3 µg DNA before mixing.
with 2 µM polyethylenimine (PEI25, Alfa Aesar, Karlsruhe, Germany) dissolved in 150 mM NaCl. The mixture was incubated for one hour at room temperature. Subsequently, 49 µl of the mixture was added to each well and spun down. Transfections were carried out in four replicates. The cells were incubated for 4 hours hereafter the media was changed. After two days the cells were harvested and the luciferase and β-galactosidase levels were measured using the Dual-Light® reporter gene assay system (Thermo Fisher Scientific). The luciferase levels of the pGL4-SOX9 promoter reporter construct was used to normalize the luciferase levels. This was done as the overexpression plasmids stimulated the β-galactosidase expression. All transfections were repeated three times.

Statistical analysis
Values are presented as mean ± standard deviation (SD). Groups were compared using two-tailed Student’s t-test and P-values were considered statistical significant if P≤0.05.

Results
The effect of TNF-α on endogenous SOX9 expression in colon carcinoma cell lines
To reveal the effect of TNF-α on endogenous SOX9 expression in different human colonic epithelial cells, we stimulated SW480, DLD1, HT29, and Caco-2 cells with TNF-α and measured SOX9 mRNA levels. While TNF-α did not affect the endogenous mRNA expression level of SOX9 in SW480 and DLD1 cells (Figure 1), a significantly increased expression of SOX9 mRNA was observed in HT29 (P<0.01) cells stimulated with TNF-α (Figure 1). In Caco-2 cells the effect of TNF-α on the endogenous SOX9 mRNA was dose-dependent with maximal effect observed at 10 nM (P<0.01) (Figure 1). These results indicate that TNF-α has a different effect on the endogenous SOX9 mRNA expression in the included cell lines and that the effect might be cell line dependent. Due to the dose-response demonstrated in Caco-2 cells, the Caco-2 cell line was applied for the subsequent experiments.

NF-κB subunits regulate the SOX9 promoter activity
To further investigate TNF-α-mediated regulation of SOX9, we generated a luciferase reporter plasmid driven by the SOX9 promoter (pGL4-SOX9). The NF-κB signaling pathway is a downstream effector of TNF-α[21]. Therefore, we wanted to explore the effect of the NF-κB signaling pathway on the SOX9 promoter activity. The pGL4-SOX9 construct was either transiently transfected into Caco-2 cells alone or with individually overexpressed NF-κB subunits (p50, p52, or p65), or in combination with two different overexpression constructs of NF-κB subunits (p65 together with p50 or p52). While constructs containing the p52 or p50 subunits significantly induced the SOX9 promoter activity (P<0.01), overexpression of p65 subunit alone had no effect on the SOX9 promoter activity as compared to pGL4-SOX9. Exogenous overexpression of p65 subunit together with overexpression of p52 or p50 overexpression constructs significantly upregulated the SOX9 promoter activity (P<0.05 and P<0.01, respectively)
Discussion

We and others have previously reported that SOX9 may have a clinical value for CRC patients[14,15]. A recent paper by Rozhok et al.[22] extended the classical evolutionary cancer model of oncogenes and tumor suppressor genes by arguing that the microenvironment affects certain clones to outcompete other clones. As the SOXE proteins generally are affected by their surrounding environment we investigated the effect of the pro-inflammatory cytokine TNF-α on the SOX9 expression in colon carcinoma cell lines. In the present study we found that TNF-α was associated with an increase of the endogenous mRNA expression of SOX9. The effect was confined to two (HT29 and Caco-2) cell lines out of four. No effect was observed in SW480 and DLD1 cells. Interestingly, the effect of TNF-α was dose-dependent in Caco-2 cells, therefore this cell line was used for further investigations. In line with these results, a previous study indicated that the SOX9 promoter might be responsive to TNF-α stimulation in Caco-2 cells[18].

One of the downstream effector-pathways of TNF-α is the NF-κB signaling pathway[21]. Several potential NF-κB binding sites in the SOX9 promoter region has been reported with some being more active than others[23]. Furthermore, a partial regulation of Sox9 by TNF-α and binding of p65 to the Sox9 promoter was demonstrated in a recent study in mice[2]. By transient transfections of a SOX9 promoter reporter plasmid in combination with the NF-κB subunits p50 or p52, we found that the SOX9 promoter activity increased significantly. This was also observed when co-transfecting with p50/p65 and p52/p65, however not to the same extent as transfections with the p50 and p52 alone. Neither p50 nor p52 has a transactivation domain like p65, however homodimerization of p50 and p52 has been previously reported[24,25]. The homodimerization most likely also depend on binding to other regulatory proteins such as the NF-κB co-factor B-cell CLL/lymphoma 3 (BCL-3)[24,25]. Based on the present data we cannot exclude that other regulatory proteins together with p50 or p52 play a role in regulating SOX9. Furthermore, it is also possible that p50 and p52 exploit the endogenous levels of p65 in the cells to some extent. Thus, further studies are needed to elucidate the exact regulation of SOX9 by the NF-κB subunits.

Overexpression of p65 alone did not affect the activity of the SOX9 promoter. A reason could be that endogenous p50 and p52 subunits only become active upon further proteolytic processing, thus they might not be available for the overexpressed p65. Additionally, the transcription factors needs to be released from the inhibitor of NF-κB to undergo processing which might also be a cause of the limiting effect. Generally, the transient overexpression of the NF-κB subunits should be interpreted with caution as the functions exerted by the transcription factors may be concentration dependent. Hence, the effect of the NF-κB subunits on the SOX9 promoter activity might be differ-
ing in reality. Furthermore, when cotransfecting with several overexpression plasmids the cells might be overloaded. This could be a plausible explanation for why the effect on SOX9 promoter is not as high when co-transfecting with either p50/p65 or p52/p65 compared to overexpression of p50 or p52 alone.

Persistent moderate NF-κB signaling in intestinal epithelial cells has been demonstrated to slowly induce adenomas in mice[26]. The tumorigenesis was enhanced when the persistent NF-κB signaling was combined with loss of Apc, a critical tumor suppressor of the Wnt-signaling pathway. Another study has also indicated that p65 potentiates the transcriptional effect of β-catenin on the Sox9 promoter and other intestinal stem cell genes[2]. Moreover, this effect might partially be due to TNF-α[2]. We previously showed that SOX9 deficient primary tumors of stage II colon cancer patients is associated with a high risk of relapse compared to patients with primary tumors containing high expression of SOX9[15]. These studies suggest that SOX9 expression might be important in the early stages of tumorigenesis and inactivation or loss of SOX9 might be associated with a later stage of progression. Thus, the SOX9 expression and thereby regulation by TNF-α and NF-κB signaling pathways might play different roles at different stages of cancer progression.

In conclusion, our results point toward that the endogenous SOX9 mRNA expression is affected by TNF-α and this effect might be mediated by NF-κB. As SOX9 might have prognostic value for colon cancer patients, further understanding of the regulation of SOX9 and the implications in CRC are necessary.

References


Figures

Figure 1 The effect of 10 nM tumor necrosis factor (TNF)-α on the endogenous SOX9 mRNA expression in DLD1, SW480 and HT29 cell lines relative to the SOX9 mRNA expression of the untreated cell lines. The effect of 0.1 nM, 1 nM, and 10 nM TNF-α on the endogenous SOX9 mRNA expression in Caco-2 cells relative to the SOX9 mRNA expression of the untreated cell line. (n=6; *P≤0.05; **P<0.01).

Figure 2 Promoter activity analysis of SOX9. The promoter construct (pGL4-SOX9) was transfected alone or together with exogenous overexpression plasmids expressing p65 and p50, or p52. The values are presented as means of luciferase activity relative to the promoter construct pGL4-SOX9. (n=4; *P≤0.05; **P<0.01).