Clinical Implications of Intestinal Stem Cell Markers in Colorectal Cancer

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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), Musashi1 (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.

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 CMYC.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.2–7 Moreover, TP53 and the KRAS oncogene are also commonly affected 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
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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.3 Some of the driver genomic alterations of CRC are associated to the intestinal stem cells, including sex-determining region y-box 9 (SOX9),4 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.14 LGR5 and/or other cell markers have been suggested to be linked to CSCs.5 Extensive studies have been performed to identify putative intestinal stem cell markers and their potential role in cancer.13 Some of these markers have been more extensively investigated than others.13

LGR5 expression in primary tumors from CRC patients and 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.14 LGR5 and/or other cell markers have been suggested to be linked to CSCs.5

Increased protein and mRNA expression of LGR5 has been reported in tumors compared to MMR intact tumors.63-64 LGR5 expressing cells are proposed to mark actively cycling stem cells exerting a homeostatic role in the small intestine of mice.18 Furthermore, spheroid cultures derived from human primary tumors are enriched for LGR5 expression, and the receptor has been suggested to be a selective cancer stem cell marker.63-64

In 2011, Wnt signaling agonists, R-spondins, were identified as the ligands for the LGR5 receptor in human embryonic kidney cells.65-67 The binding of the R-spondins to the receptor enhanced downstream Wnt signaling.56-66 Because LGR5 has been identified as a Wnt target gene, this indicates a positive feedback loop mechanism.67 Furthermore, β-catenin has been reported to be positively correlated with LGR5 in human CRC tissue.69,70 However, later studies have not confirmed this correlation.71 Knockdown of LGR5 in human CRC cell lines has also been linked to a gene expression profile consistent with an activated Wnt signaling pathway, suggestive of a negative feedback loop of LGR5 regulation.69 Furthermore, a recent study reported Wnt signaling inhibition and antioncogenic effects of R-spondin 2 in human CRC cells and noted that this might be dependent on LGR5 expression.70

This is somewhat contradictory to studies showing that R-spondins potentiate Wnt signaling.66,67-71 and studies linking LGR5 expression to tumorigenic properties.34,35,72 A study reported that silencing of LGR5 in human CRC cells resulted in reduced proliferation, migration, and colony formation in vivo as well as in reduced tumorigenicity in vivo.73 This has been supported by others, who have additionally described increased apoptosis after knockdown of LGR5 in human CRC and adenoma cell lines.34,72 These studies suggest that LGR5 and the R-spondin ligands might play opposing roles in different contexts that remain to be elucidated.

Increased protein and mRNA expression of LGR5 has been reported in adenomas and CRC tissue compared to normal colon mucosa29,31-38 (Table 1). LGR5 is localized in the membrane and cytoplasm of tumor cells in adenoma and CRC tissue.29,31,32-36 The expression pattern of LGR5 has been reported to be heterogeneous throughout the tumor or as a local expression pattern with a patchy distribution.29,30,32,37 In addition, allelic variations of LGR5 affect the LGR5 protein expression negatively, and an association between LGR5 polymorphisms and increased time to tumor recurrence has been reported in CRC patients.39,40

LGR5 expression in primary tumors from CRC patients and correlations to clinicopathologic features, such as histologic grade, depth of invasion, tumor differentiation, and histologic subtype, are contradictory.17,31,32,36,37 One study found a lower expression of LGR5 in MMR deficient tumors compared to MMR intact tumors.17 Furthermore, LGR5 expression might also correlate to lymph node and distant metastasis.30,33,36,37 A positive association between high expression of LGR5 at the invasive front of the tumor and advanced disease stage has been reported.31 In addition, LGR5 expression at the luminal surface was inversely correlated to the progression of disease.31 However, others have found no significant impact of the distribution of LGR5 expressing cells within CRC.29

High LGR5 expression in CRC might correlate to lower disease-free survival, overall survival, and cancer-specific survival, indicating that LGR5 is a potential prognostic marker.32,33,36 However, this is inconsistent, with other studies finding that LGR5 does not have a
LGR5 expression is not associated with prognosis. Increased LGR5 expression in CRC and correlates with female sex. Increased expression of LGR5 in distant metastasis derived from tumors with LGR5 positive cells in tumor buds and vascular compartments of the primary tumor. LGR5 expression at the invasive front is positively correlated to advanced disease. LGR5 expression at the luminal surface is inversely correlated to disease stage. LGR5 expression correlates with TNM stage, lymph node metastasis, and vascular invasion. High levels of LGR5 correlate to poor prognosis. LGR5 is an independent prognostic factor. LGR5 expression correlates with AJCC stage and TNM stages. High LGR5 expression correlates with poor prognosis. LGR5 mRNA is significantly up-regulated in CRC. Patients with high LGR5 expression in their primary tumors have a poorer prognosis. LGR5 is an independent prognostic marker. LGR5 correlates with lymph node metastasis, vascular invasion, lymphatic invasion, tumor depth, and tumor grade. LGR5 expression is increased in stage IV CRC patients compared to normal matched mucosa. LGR5 homozygous wt genotype in blood associated with a lower time to tumor reoccurrence in CRC patients than LGR5 heterozygote patients. LGR5 gene variation correlates negatively with LGR5 protein expression in CRC. LGR5 expression is associated with a favorable prognosis.

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 (891)</td>
<td>I to IV (39)</td>
<td>McClanahan et al., 2006</td>
</tr>
<tr>
<td>Increased LGR5 expression in CRC and correlates with female sex.</td>
<td>Tumor (102)</td>
<td>I + II (35)</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 (45)</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 (30)</td>
<td>I + II (17)</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 (53)</td>
<td>I (9)</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 (296)</td>
<td>I (60)</td>
<td>Hsu et al., 2013</td>
</tr>
<tr>
<td>High levels of LGR5 correlate to poor prognosis.</td>
<td>Tumor (9)</td>
<td>I (91)</td>
<td>Takahashi et al., 2011</td>
</tr>
<tr>
<td>LGR5 is an independent prognostic factor.</td>
<td>Tumor (180)</td>
<td>II (54)</td>
<td>Wu et al., 2012</td>
</tr>
<tr>
<td>LGR5 correlates with lymph node metastasis, vascular invasion, lymphatic invasion, tumor depth, and tumor grade.</td>
<td>Tumor (50)</td>
<td>II (54)</td>
<td>Uchida et al., 2010</td>
</tr>
<tr>
<td>LGR5 expression is increased in stage IV CRC patients compared to normal matched mucosa.</td>
<td>Tumor (42)</td>
<td>IV (42)</td>
<td>Gao et al., 2014</td>
</tr>
<tr>
<td>LGR5 homozygous wt genotype in blood associated with a lower time to tumor reoccurrence in CRC patients than LGR5 heterozygote patients.</td>
<td>Tumor (234)</td>
<td>High risk II (105)</td>
<td>Gerger et al., 2011</td>
</tr>
<tr>
<td>LGR5 gene variation correlates negatively with LGR5 protein expression in CRC.</td>
<td>Tumor (89)</td>
<td>III (45)</td>
<td>Kleist et al., 2012</td>
</tr>
<tr>
<td>LGR5 expression is associated with a favorable prognosis.</td>
<td>Tumor (90)</td>
<td>II (93)</td>
<td>de Sousa E Melo et al., 2011</td>
</tr>
</tbody>
</table>

Abbreviations: AJCC = American Joint Committee on Cancer; CRC = colorectal cancer; LGR5 = leucine-rich-repeat-containing G-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 line 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-fluouracil (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.

BMI1

BMI1 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. BMI1 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 hematopoiesis and neural development. BMI1 targets the Ink4a/Arf locus, which encodes the critical cell cycle regulators p16 and p19ARF (p14ARF in humans), which are involved in the retinoblastoma protein (Rb) and p53 signaling pathways, regulating cell cycle and apoptosis. In vivo lineage tracing suggests that BMI1 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

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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{35,86}

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{42-44} 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{42-47} 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 in vitro and impairs the cancer-initiating potential of human colon cancer cells in mice.\textsuperscript{87} 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{43-45} Inhibition of Bmi1 results in growth arrest of the preestablished tumors in vivo.\textsuperscript{87} 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{43,46} 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{43} Bmi1 expression is correlated to cancer stage, suggesting that Bmi1 might be associated with colon cancer progression.\textsuperscript{45-47}

The prognostic significance of Bmi1 expression in colorectal tumors is conflicting. More patients with Bmi1 positive tumors have tumor recurrence or metastases compared to patients with Bmi1 negative tumors.\textsuperscript{47} Furthermore, high Bmi1 expression in primary tumors from CRC patients is an independent prognostic factor for disease-free survival and for overall survival.\textsuperscript{46,47} 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{48} 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.

**Table 2 Implications of BMI1 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>BMI1 is overexpressed in CRC.</td>
<td>Tumor (11) Paired healthy (11)</td>
<td>NS</td>
<td>Reinish et al., 2006\textsuperscript{42}</td>
</tr>
<tr>
<td>BMI1 expression correlates with gender, histologic tumor differentiation, tumor size, and serum CEA levels. BMI1 expression has an inverse correlation to the expression of p16 and p14.</td>
<td>Tumor (87) Paired healthy (87)</td>
<td>NS; N and M stage provided</td>
<td>Kim et al., 2004\textsuperscript{43}</td>
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<tr>
<td>BMI1 is overexpressed in human low-grade intraepithelial dysplasia, high-grade intraepithelial dysplasia, and cancer. High expression of BMI1 correlates with metastasis and advanced stage of cancer.</td>
<td>NS</td>
<td>NS</td>
<td>Tateishi et al., 2006\textsuperscript{44}</td>
</tr>
<tr>
<td>High expression of BMI1 is associated with a lower overall survival.</td>
<td>Tumor (98) Paired healthy (98)</td>
<td>II (29) I (9) III (10) IV (6)</td>
<td>Du et al., 2010\textsuperscript{45}</td>
</tr>
<tr>
<td>Patients with BMI1 positive tumors have a lower disease-free survival and a lower overall survival.</td>
<td>Tumor (203) Paired healthy (203)</td>
<td>I (24) II (81) III (80) IV (18)</td>
<td>Li et al., 2010\textsuperscript{46}</td>
</tr>
<tr>
<td>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.</td>
<td>Tumor (247) Paired healthy (47)</td>
<td>I (52) II (110) III (65)</td>
<td>Benard et al., 2014\textsuperscript{47}</td>
</tr>
<tr>
<td>Patients with decreased postoperative plasma BMI1 mRNA levels have a better prognosis than patients with increased postoperative BMI1 mRNA levels</td>
<td>Tumor (45)</td>
<td>I + II (15) III + IV (12) NS (18)</td>
<td>Pun et al., 2014\textsuperscript{48}</td>
</tr>
</tbody>
</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; Paired 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. Additionally, MSI1 competes with eukaryotic initiation factor 4G (eIF4G) for binding to the poly(A)-binding protein (PABP), thereby inhibiting translation initiation. 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. MSI1 was also found to negatively regulate APC translation in human cultured colonocytes. Interestingly, reduced APC expression leads to increased levels of MSI1, suggesting that MSI1 itself is a target of the Wnt signaling pathway, consistent with an earlier study describing a positive feedback loop that might be important for regulating homeostasis of colon tissue, and if disturbed, it could lead to tumor formation. 

Intestinal epithelium cells overexpressing Msi1 increase proliferation and acquire tumorigenic features in xenografts. In accordance, knockdown of MSI1 in human colon cancer cells leads to inhibition of proliferation and reduced migratory potential. Furthermore, knockdown of MSI1 in xenografts results in tumor growth arrest, suggesting that MSI1 may play a role in tumor progression. 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. MSI1 expression tumor cells of the colon predominantly also, although not exclusively, express MSI1 in the cytoplasm. MSI1 is often focally expressed in adenomas, whereas the expression pattern in carcinomas is more diffuse. Moreover, MSI1 overexpression is significantly associated with the proliferation marker Ki-67, advanced cancer stage, and a more aggressive disease phenotype. 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). Furthermore, positive MSI1 expression in the primary tumor is associated with a nearly 5.4-fold increased risk of distant metastasis. 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.

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. 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. Similarly, a study of colon epithelial stem cells describes that cells expressing high levels of Sox9 are associated with a more undifferentiated population of cells expressing stem cell characteristics in vitro and these cells are furthermore enriched for Lgr5 mRNA. Cells with low Sox9 expression accordingly have a gene expression profile consistent with a more differentiated phenotype. 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 with a weaker expression in cells toward the luminal surface. 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 colonic morphology. Additionally, the goblet cell lineage of the colon is strongly reduced in the Sox9 deficient mice. Somewhat contradicting to this a study showed that SOX9 indirectly represses genes associated with goblet cell differentiation, e.g., the mucin-encoding gene MUC2.

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. SOX9 has been shown to be a direct Wnt signaling target of the activated β-catenin–TCF4 complex in human colon carcinoma cells, but another study showed that SOX9 potentially inhibits the β-catenin–TCF4 complex, suggesting a negative feedback loop. 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.

Overexpression of SOX9 in human CRC cells induces an increase of BMI1 with a subsequent decrease in p16, whereas the opposite

<table>
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<th>Table 3 Implications of MSI1 in CRC Patients</th>
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<tr>
<td><strong>Main Findings</strong></td>
</tr>
<tr>
<td>MSI1 is an independent prognostic marker to predict poor outcome.</td>
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<tr>
<td>&gt;2-fold increase in MSI1 mRNA expression in majority of CRC.</td>
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<td>MSI1 expression correlates to TNM stage.</td>
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<td>MSI1 mRNA expression differs in normal, adenoma, and carcinoma.</td>
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<tr>
<td><strong>Study Cohort (No. of Specimens)</strong></td>
</tr>
<tr>
<td>Tumor (203) Paired healthy (203)</td>
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<tr>
<td>Tumor (15) Paired healthy (15)</td>
</tr>
<tr>
<td>Tumor (69) Unpaired healthy (8)</td>
</tr>
<tr>
<td>Tumor (31) Unpaired healthy (10)</td>
</tr>
<tr>
<td><strong>AJCC Stage (No. of Patients Analyzed)</strong></td>
</tr>
<tr>
<td>I (24) II (81) III (80) IV (18)</td>
</tr>
<tr>
<td>NS</td>
</tr>
<tr>
<td>I + II (39) III + IV (30)</td>
</tr>
<tr>
<td>NS</td>
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<tr>
<td><strong>Reference</strong></td>
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<tr>
<td>Li et al., 2011</td>
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<tr>
<td>Sureban et al., 2008</td>
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<tr>
<td>Fan et al., 2010</td>
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<tr>
<td>Fan et al., 2010</td>
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</tbody>
</table>

*Abbreviations: AJCC = American Joint Committee on Cancer; CRC = colorectal cancer; MSI1 = Musashi1; NS = not specified; Paired healthy = healthy colon mucosa from same individual as investigated tumor; TNM = tumor, node, metastasis classification system.*
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.\textsuperscript{53-55,57-60} Only one small study (n = 10) has described a decrease in SOX9 expression in colorectal adenocarcinomas.\textsuperscript{61} There is no significant difference in SOX9 expression when comparing adenomatous expression and cancerous expression.\textsuperscript{55} SOX9 is expressed in a random heterogeneous manner throughout colorectal tumors.\textsuperscript{28,55} Moreover, a strong expression of SOX9 is more common in non-mucin-producing CRC than mucinous or signet ring carcinomas.\textsuperscript{57} One study describes that SOX9 overexpression correlates with vascular invasion in the primary tumor.\textsuperscript{54} Others find that high SOX9 expression correlates with age, female sex, and MSI tumors, especially MSI-high tumors.\textsuperscript{59} In contrast, correlation between downregulated SOX9 expression and MSI relative to microsatellite stable tumors has also been described.\textsuperscript{60} Table 4 lists the studies on SOX9 in human CRC.

Correlation between SOX9 expression levels and patient survival is inconsistent.\textsuperscript{54,55,59} When stratified for American Joint

Table 4 Implications of SOX9 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>SOX9 is frequently mutated in nonhypermutated tumors.</td>
<td>Tumor (224) Paired healthy/blood (224)</td>
<td>NS</td>
<td>Cancer Genome Atlas Network, 2012\textsuperscript{5}</td>
</tr>
<tr>
<td>Heterogeneous expression of SOX9 in tumors.</td>
<td>Tumor (3)</td>
<td>NS</td>
<td>Ramalingam et al., 2012\textsuperscript{58}</td>
</tr>
<tr>
<td>SOX9 is up-regulated in CRC.</td>
<td>Tumor (110) Unpaired healthy (22)</td>
<td>NS</td>
<td>Matheu et al., 2012\textsuperscript{14}</td>
</tr>
<tr>
<td>SOX9 mRNA is up-regulated in CRC and associated with advanced tumor stage.</td>
<td>Tumor (79) Paired healthy (25)</td>
<td>NS</td>
<td>Matheu et al., 2012\textsuperscript{14}</td>
</tr>
<tr>
<td>SOX9 overexpression correlates with poorer survival in 5-FU-treated stage III patients.</td>
<td>Tumor (441) Paired healthy (441)</td>
<td>II (280) III (161)</td>
<td>Candy et al., 2013\textsuperscript{14}</td>
</tr>
<tr>
<td>Strong SOX9 expression is most common in non-mucin-producing CRC.</td>
<td>Tumor (188) Paired healthy (188)</td>
<td>I/I (97) IV/I (86)</td>
<td>Lü et al., 2008\textsuperscript{55}</td>
</tr>
<tr>
<td>Strong SOX9 expression correlated with lower overall survival.</td>
<td>Tumor (27) Paired healthy (27)</td>
<td>I (6) II (12) III (5) IV (1) NS (3)</td>
<td>Abdel-Samad et al., 2011\textsuperscript{56}</td>
</tr>
<tr>
<td>A truncated variant of SOX9 is overexpressed in CRC.</td>
<td>Tumor (17) Paired healthy (17)</td>
<td>I (5) II (8) III (2) IV (1) NS (1)</td>
<td>Abdel-Samad et al., 2011\textsuperscript{56}</td>
</tr>
<tr>
<td>SOX9 is up-regulated in CRC.</td>
<td>Tumor (10) Paired healthy (10)</td>
<td>NS</td>
<td>Lü et al., 2006\textsuperscript{57}</td>
</tr>
<tr>
<td>Increased SOX9 gene expression in CRC.</td>
<td>Tumor (77) Paired healthy (77)</td>
<td>I/II (39) III/IV (38)</td>
<td>Huang et al., 2013\textsuperscript{54}</td>
</tr>
<tr>
<td>High levels of SOX9 associated with age and MSI.</td>
<td>Tumor (31) Paired healthy (31)</td>
<td>Dukes A (1) Dukes B (11) Dukes C (19)</td>
<td>Panza et al., 2013\textsuperscript{55}</td>
</tr>
<tr>
<td>SOX9 is up-regulated in CRC.</td>
<td>Tumor (424) Paired healthy (20)</td>
<td>I (23) II (340) III (49) IV (12)</td>
<td>Andersen et al., 2009\textsuperscript{50}</td>
</tr>
<tr>
<td>SOX9 is down-regulated in MSI relative to MSS tumors.</td>
<td>Tumor (10) Paired healthy (10)</td>
<td>NS</td>
<td>Chen et al., 2006\textsuperscript{51}</td>
</tr>
</tbody>
</table>

Abbreviations: AJCC = American Joint Committee on Cancer; CRC = colorectal cancer; 5-FU = 5-fluorouracil; MSI = microsatellite instable; 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.54 This is not the case for 5-FU—untreated stage III or stage II CRC patients,54 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 Rb, 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.18 However, this is less established for the other 3 markers, with Sox9 studies suggesting that the stem cell populations of the small intestine and the colon might differ completely with respect to the Sox9 expression level.77,78 This could to some extent explain the somewhat 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.108

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.3,39,40,53,56 Thus, further studies on the functional role and clinical significance of these variants and mutations are needed.

LGR5, MSI1, SOX9, and BMI1 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 defect tumors106,107 and the poor prognosis associated with an increased LGR5 or SOX9 expression in some studies.17,60

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.33,54 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.48,109

Furthermore, adjusting for known predictive and prognostic factors, such as MMR deficiency, are 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 tumor cells within the tumor might be relevant in relation to disease stage.33 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—eg, immunohistochemistry with clearly defined cutoff values—would be preferred to tissue microarray. Another challenge is the current lack of proper antibodies targeting LGR5, which precludes a proper investigation of LGR5 in human tissue.85

In conclusion, the intestinal stem cell markers LGR5, BMI1, MSI1, 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 chemotherapy 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.

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Disclosure

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