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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.

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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 cells markers and their potential role in cancer.[13] Some of the driver genomic alterations of CRC are associated to the intestinal stem cells, including *sex-determining region y-box 9* (*SOX9*)[5] and leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) through the R-spondins,[4] indicating that the stem cell markers play significant roles in colorectal tumorigenesis. Several intestinal stem cell markers has been identified with LGR5 being the most investigated and established marker.[14] *LGR5*$^+$ cells also correlate to expression of the markers *olfactomedin-4* (*OLMF4*)[15] and *achaete scute complex like 2* (*ASCL2*).[16,17] 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.[18] 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).[19] Several +4 stem cell markers have been suggested, including *B cell–specific Moloney murine leukemia virus insertion site 1* (*Bmi1*),[19] *telomerase reverse transcriptase* (*Tert*),[20] and *homeodomain only protein X* (*Hopx*).[21] *Leucine-rich repeats and immunoglobulin-like domains 1* (*Lrig1*),[22,23] Musashi1 (MSI1),[24-26] and *Sox9*[27,28] 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,[17,29-41] BMI1,[42-49] MSI1,[50-52] and SOX9.[5,28,53-61] This review evaluates the potential clinical implications of these 4 putative intestinal stem cell markers and their potential role in human CRC.

LGR5

LGR5 was initially identified in 1998.[62] 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.[18] *Lgr5* expressing cells are long-lived and have the ability to generate all cell types of the small intestine and colon epithelia.[18] *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.[18] Accordingly, immunohistochemical staining of LGR5 shows that the protein is expressed in cytoplasm and membrane of a few cells, located at the crypt base of human colon tissue.[29-31]

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.[65-67] Because LGR5 has been identified as a Wnt target gene, this indicates a positive feedback loop mechanism.[68] Furthermore, β-catenin has been reported to be positively correlated with LGR5 in human CRC tissue.[29,32] However, later studies have not confirmed this correlation.[17,31] 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[4,65-67,71] 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 vitro* 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.[33,72] 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 mucosa[29,31-38] (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

Table 1 Implications of LGR5 Expression in Human CRC

Main Findings	Study Cohort (No. of Specimens)	AJCC Stage (No. of Patients Analyzed)	Reference
LGR5 expression is not associated with prognosis.	Tumor (891)	NS	Ziskin et al., 2013[17]
Increased LGR5 expression in CRC and correlates with female sex.	Tumor (102) Unpaired healthy (12)	I + II (35) III + IV (67)	Fan et al., 2010[29]
Increased expression of LGR5 in distant metastasis derived from tumors with LGR5 positive cells in tumor buds and vascular compartments of the primary tumor.	Tumor (89)	III (45) IV (44)	Kleist *et al.*, 2011[30]
LGR5 expression at the invasive front is positively correlated to advanced disease. LGR5 expression at the luminal surface is inversely correlated to disease stage.	Tumor (30)	I + II (17) III + IV (13)	Takeda et al., 2011[31]
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.	Tumor (53) Paired healthy (53)	I (9) II (21) III (16) IV (7)	He et al., 2014[32]
LGR5 expression correlates with AJCC stage and TNM stages. High LGR5 expression correlates with poor prognosis.	Tumor (296) Paired healthy (216)	I (60) II (91) III (67) IV (78)	Hsu et al., 2013[33]
LGR5 mRNA is significantly up-regulated in CRC.	Tumor (39) Paired healthy (39)	I to IV (39)	McClanahan et al., 2006[34]
Patients with high LGR5 expression in their primary tumors have a poorer prognosis.	Tumor (180) Paired healthy (180)	0 (21) I (22) II (54) III (61) IV (19)	Takahashi et al., 2011[35]
LGR5 is an independent prognostic marker.	Tumor (192) Paired healthy (80)	I (47) II (70) III (65) IV (10)	Wu et al., 2012[36]
LGR5 correlates with lymph node metastasis, vascular invasion, lymphatic invasion, tumor depth, and tumor grade.	Tumor (50) Paired healthy (50)	II (25) III (25)	Uchida et al., 2010[37]
LGR5 expression is increased in stage IV CRC patients compared to normal matched mucosa	Tumor (42) Paired healthy (42)	IV (42)	Gao et al., 2014[38]
LGR5 homozygous wt genotype in blood associated with a lower time to tumor reoccurrence in CRC patients than *LGR5* heterozygote patients.	Tumor (234)	High risk II (105) III (129)	Gerger et al., 2011[39]
LGR5 gene variation correlates negatively with LGR5 protein expression in CRC.	Tumor (89) Unpaired buccal swaps (72)	III (45) IV (44)	Kleist et al., 2012[40]
LGR5 expression is associated with a favorable prognosis.	Tumor (90)	II (90)	de Sousa E Melo et al., 2011[41]

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[17] and that high expression of Wnt-driven intestinal stem cell markers, including LGR5, within CRC tissue is associated with a favorable prognosis.[41] Interestingly, increased *LGR5* mRNA expression in the peripheral blood of CRC patients has also been associated with a poor outcome.[74] 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.[75,76] 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.[33] 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.[77,78] *Bmi1* was initially identified as an oncogene that, together with *c-myc*, plays a role in initiation of mouse B cell lymphomas.[79] It was later found to be important in hematopoiesis and neural development.[80] BMI1 targets the *Ink4a/Arf* locus, which encodes the critical cell cycle regulators p16 and p19^{ARF} (p14^{ARF} in humans).[81] These are involved in the retinoblastoma protein (Rb) and p53 signaling pathways, regulating cell cycle and apoptosis.[82]

In vivo lineage tracing suggests that *Bmi1* expression marks small intestinal stem cells located at the +4 position from the crypt bottom in mice.[19] These stem cells are functionally distinct from the Lgr5 expressing stem cell population.[83] The +4 putative stem cells are characterized by being quiescent, being resistant to

irradiation, and having regenerative potential after injury or ablation of *Lgr5* expressing cells.[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.[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.[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.[42-47] Human CRC cells have been proposed to require *BMI1* expression for maintenance of tumor growth.[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.[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.[43-45] Inhibition of BMI1 results in growth arrest of the preestablished tumors *in vivo*.[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.[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.[44] BMI1 expression is correlated to cancer stage, suggesting that BMI1 might be associated with colon cancer progression.[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.[47] Furthermore, high BMI1 expression in primary tumors from CRC patients is an independent prognostic factor for disease-free survival and for overall survival.[46,47] However, high BMI1 expression is also correlated with a better prognosis compared to patients with low expression.[48] By combing several biomarkers with the BMI1 expression, prognostic stratification is improved.[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.[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.

MSI1

MSI1 is an evolutionary conserved RNA-binding protein initially identified in *Drosophila* as a protein important for sensory organ development and as a neuronal stem cell marker in mammals.[88-90] MSI1 is one of the first proposed intestinal stem cell markers and may contribute to the undifferentiated state of intestinal stem cells.[24,25,91] MSI1 is mainly expressed in the cytoplasm of human colon epithelia cells positioned between cells 1 and 10 from the bottom of the crypt.[25] Occasionally, MSI1 is also expressed in the nucleus of these cells.[25] Furthermore, *Msi1* expressing cells have been shown to correspond to cells expressing the intestinal stem cell marker *Lgr5* and the +4 stem cell marker *Tert* in mice.[20,26]

Table 2 Implications of BMI1 in CRC Patients

Main Findings	Study Cohort (No. of Specimens)	AJCC Stage (No. of Patients Analyzed)	Reference
BMI1 is overexpressed in CRC.	Tumor (11) Paired healthy (11)	NS	Reinish et al., 2006[42]
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.	Tumor (87) Paired healthy (87)	NS; N and M stage provided	Kim et al., 2004[43]
BMI1 is overexpressed in human low-grade intraepithelial dysplasia, high-grade intraepithelial dysplasia, and cancer.	NS	NS	Tateishi et al., 2006[44]
High expression of BMI1 correlates with metastasis and advanced stage of cancer.	Tumor (43) Paired healthy (43)	I (9) II (18) III (10) IV(6)	Liu et al., 2010[45]
High expression of BMI1 is associated with a lower overall survival.	Tumor (98) Paired healthy (98)	II (29) III (69)	Du et al., 2010[46]
Patients with BMI1 positive tumors have a lower disease-free survival and a lower overall survival.	Tumor (203) Paired healthy (203)	I (24) II (81) III (80) IV (18)	Li et al., 2010[47]
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 (247) Paired healthy (47)	I (52) II (110) III (85)	Benard et al., 2014[48]
Patients with decreased postoperative plasma *BMI1* mRNA levels have a better prognosis than patients with increased postoperative *BMI1* mRNA levels	Tumor (45)	I + II (15) III + IV (12) NS (18)	Pun et al., 2014[49]

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.[92] Additionally, MSI1 compete 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,[96] consistent with an earlier study describing a TCF/LEF binding site on the *Msi1* promoter.[97] This positive feedback loop 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.[97] In accordance, knockdown of *MSI1* in human colon cancer cells leads to inhibition of proliferation and reduced migratory potential.[50] 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 expressing tumor cells of the colon predominantly also, although not exclusively, express MSI1 in the cytoplasm.[52] 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.[50] 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]

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.[53,99-101] 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.[28] 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.[54-56,100] 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.[101] Additionally, the goblet cell lineage of the colon is strongly reduced in the *Sox9* deficient mice.[101] Somewhat contradicting to this a study showed that SOX9 indirectly represses genes associated with goblet cell differentiation, eg, the mucin-encoding gene *MUC2*.[100]

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.[53,55,56,103-105] SOX9 has been shown to be a direct Wnt signaling target of the activated β-catenin−TCF4 complex in human colon carcinoma cells,[100] but another study showed that SOX9 potentially inhibit the β-catenin−TCF4 complex, suggesting a negative feedback loop.[101] Furthermore, *Bmi1* has been identified as a potential SOX9 target in mouse primary cells and transformed cells, hence repressing the tumor suppressors p16 and $p19^{ARF}$, leading to cell cycle progression and bypassing of apoptosis.[53] Overexpression of *SOX9* in human CRC cells induces an increase of BMI1 with a subsequent decrease in p16, whereas the opposite

Table 3 Implications of MSI1 in CRC Patients

Main Findings	Study Cohort (No. of Specimens)	AJCC Stage (No. of Patients Analyzed)	Reference
MSI1 is an independent prognostic marker to predict poor outcome.	Tumor (203) Paired healthy (203)	I (24) II (81) III (80) IV (18)	Li et al., 2011[50]
>2-fold increase in *MSI1* mRNA expression in majority of CRC.	Tumor (15) Paired healthy (15)	NS	Sureban et al., 2008[51]
MSI1 expression correlates to TNM stage.	Tumor (69) Unpaired healthy (8)	I + II (39) III + IV (30)	Fan et al., 2010[52]
MSI1 mRNA expression differs in normal, adenoma, and carcinoma.	Tumor (31) Unpaired healthy (10)	NS	Fan et al., 2010[52]

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.

effect has been observed by *SOX9* knockdown.[53] In contrast, mice with *Sox9* 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 *Sox9* 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.[53] 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.[56] 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.[56] Both the truncated and the full length SOX9 is increased in human colon cancer tissue.[56]

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.[53-55,57-60] 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 adenomatous expression and cancerous expression.[55] 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.[57] 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.[59] In contrast, correlation between down-regulated 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.[54,55,59] When stratified for American Joint

Table 4 Implications of SOX9 in CRC Patients

Main Findings	Study Cohort (No. of Specimens)	AJCC Stage (No. of Patients Analyzed)	Reference
SOX9 is frequently mutated in nonhypermutated tumors.	Tumor (224) Paired healthy/blood (224)	NS	Cancer Genome Atlas Network, 2012[5]
Heterogeneous expression of SOX9 in tumors.	Tumor (3)	NS	Ramalingam et al., 2012[28]
SOX9 is up-regulated in CRC.	Tumor (110) Unpaired healthy (22)	NS	Matheu et al., 2012[53]
SOX9 mRNA is up-regulated in CRC and associated with advanced tumor stage.	Tumor (79) Paired healthy (25)	NS	Matheu et al., 2012[53]
SOX9 overexpression correlates with poorer survival in 5-FU-treated stage III patients.	Tumor (441) Paired healthy (441)	II (280) III (161)	Candy et al., 2013[54]
Strong SOX9 expression is most common in non-mucin-producing CRC. Strong SOX9 expression correlated with lower overall survival.	Tumor (188) Paired healthy (188)	I/II (97) III/IV (86)	Lü et al., 2008[55]
SOX9 is overexpressed in CRC.	Tumor (27) Paired healthy (27)	I (6) II (12) III (5) IV (1) NS (3)	Abdel-Samad et al., 2011[56]
A truncated variant of *SOX9* is overexpressed in CRC.	Tumor (17) Paired healthy (17)	I (5) II (8) III (2) IV (1) NS (1)	Abdel-Samad et al., 2011[56]
SOX9 is up-regulated in CRC.	Tumor (10) Paired healthy (10)	NS	Lü et al., 2006[57]
Increased *SOX9* gene expression in CRC.	Tumor (77) Paired healthy (77)	I/II (39) III/IV (38)	Huang et al., 2013[58]
High levels of SOX9 associated with age and MSI. No significant decrease in survival for the patients with high SOX9 expression.	Tumor (31) Paired healthy (31)	Dukes A (1) Dukes B (11) Dukes C (19)	Panza et al., 2013[59]
SOX9 is up-regulated in CRC. *SOX9* is down-regulated in MSI relative to MSS tumors.	Tumor (424) Paired healthy (20)	I (23) II (340) III (49) IV (12)	Andersen *et al.*, 2009[60]
SOX9 was down-regulated in CRC.	Tumor (10) Paired healthy (10)	NS	Chen *et al.*, 2006[61]

Abbreviations: AJCC = American Joint Committee on Cancer; CRC = colorectal cancer; 5-FU = 5-fluorouracil; MSI = microsatellite instable; MSS = microsatellite stabile; 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.[27,28] 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.[5,39,40,53,56] 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 tumors[106,107] 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.[31] 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

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

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