Original Article
Expression of the adult intestinal stem cell marker Lgr5 in the metastatic cascade of colorectal cancer

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Abstract: The recently advanced cancer stem cell model postulates that progression and metastasis of cancer are mainly driven by tumor cells with stem cell properties. Intestinal cancer stem cells are difficult to study due to the lack of reliable markers, but expression of the *Wnt* target gene Lgr5 is promising to define at least stem cell like cells in intestinal and colorectal cancer. The aim of this study was to find a possible link of stem cell like cancer cells to the metastatic process of colorectal cancer. To this end, we evaluated immunohistochemical Lgr5 expression in 31 distant metastases and in primary tumor compartments with relevance for metastasizing, comprising 89 colorectal carcinomas. Lgr5 expression was seen in 51.6% of distant metastases. 12.9%, 14.8% and 26.7% of primary tumors with histologically confirmed tumor buds, angioinvasion and perineural infiltrates, respectively, showed evidence of Lgr5 expression in these tumor compartments. However, distant metastases, which were derived from carcinomas with such Lgr5 positive tumor compartments, showed 6- to 11.5-fold higher median value of Lgr5 expression compared to those metastases derived from tumors without Lgr5 expressing cells in these compartments. These differences between the metastases were statistically significant, if being related to tumor buds (all tumors; p = 0.047) and to vascular infiltrates (stage IV tumors; p = 0.007). In conclusion, our results point to rare evidence of Lgr5 positive stem cell like cells in the metastatic cascade of colorectal cancer, but these few cells might be biologically powerful in the metastatic process of cancer subsets. Clonal analysis is necessary to proof this hypothesis.

Keywords: Lgr5, stem cell like cells, tumor compartments, metastasis

Introduction

Improvement of diagnosis and therapy in patients with colorectal cancer depends highly on the understanding of tumor biology. Currently, two models are used to explain onset, local progression and metastasizing of malignant tumors [1]. According to the more traditional stochastic concept, all cells in a tumor have the capacity to propagate the cancer. A new tumor growth paradigm is given by the cancer stem cell model, which postulates that stem cell like cells (cancer stem cells or tumor initiating cells) are the primary cellular tumor component that drives disease progression and metastasis. These cells harbor key properties as self-renewal by asymmetric division, multilineage potential and resistance to apoptosis [2]. The required combination of these traits makes cancer stem cells rare and difficult to detect, but highly tumorigenic [3].

A recent study on intestinal *Wnt* target genes qualified Lgr5 (leucin-rich repeat-containing G protein-coupled receptor 5) as an intestinal stem cell marker, which can identify stem cells in adult tissues [4]. Aberrant Wnt/β-catenin signaling is proposed to trigger early intestinal carcinogenesis and tumor progression as indicated by analyses on human colorectal cancer [3]. Barker et al. demonstrated in a mice model that transformation of Lgr5-EGFP+ stem cells drives adenoma formation in both the small intestine and colon, and postulated that transformation of stem cells constitutes the principal route towards intestinal cancer [5]. This idea is supported by analysis on spheroid cultures and freshly isolated tumor cells from multiple colon carcinomas, which provided compelling evidence that single-cell-cloned cancer stem cells, characterized by several markers including Lgr5, can form an adenocarcinoma on xenotransplantation [6].

Based on knowledge from these studies [3-6], it can be hypothesized that stem cell like cells are involved in colorectal carcinogenesis and it is reasonable to assume that these cells can be identified by Lgr5 expression.

Recent analyses on human tissues indicate a possible role of Lgr5 expressing cells in development of gastrointestinal and colorectal tumors [7-9] and propose possible prognostic importance of these cells [8,10]. However, the relationship of Lgr5 expressing cells to crucial events in tumor progression, especially in metastasizing, is poorly understood.

Therefore, we searched for immunohistochemical evidence of Lgr5 antigen in 89 primary colorectal carcinomas and 31 corresponding distant metastases, focusing on the following questions:

1. Do distant metastases contain stem cell like cancer cells and, if yes, is the burden of these cells related to the load of stem cell like cells in the primary tumors?

2. Do primary tumor compartments with known relevance for metastasizing, i.e. tumor buds at the invasive margins [11], vascular [12] and perineural spaces [13], show evidence of migrating stem cell like cancer cells?

3. If stem cell like cells can be detected in the named primary tumor compartments and in distant metastases: Is there a relationship between them?

Material and methods

Patients and specimens

Eighty-nine patients (48 males and 41 females, mean age: 62.6 years, range: 32 – 82 years) who underwent surgical treatment and either adjuvant or palliative postoperative chemotherapy between January 1999 and December 2005 at Soerlandet Hospital Trust, Kristiansand, Norway, for carcinoma of the colon and rectum, respectively, were included in the study. Archival cancer tissue and patient data were obtained and used after approval of the Regional Ethics Committee (REK) of Southern Norway in accordance with the declaration of Helsinki and the International Conference of Harmonization – Good Clinical Practice. The anonymity of the patients investigated was preserved corresponding to rules of data protection of the National Data Protection Commission (NSD) of Norway and the institutional guidelines of our hospital. All patients underwent surgery for their primary tumor. No preoperative chemotherapy or radiotherapy was administered. All specimens underwent additional independent histopathological review (B.K.). Tumor differentiation was graded according to the World Health Organization (WHO) classification system corresponding to the least differentiated area independent of quantitative extent [14]: 69 (78%) tumors were moderately and 20 (22%) tumors were poorly differentiated. Tumor stage was determined according to the criteria proposed by the International Union Against Cancer (UICC) [15]: The samples comprised two (2%) pT1, two (2%) pT2, 69 (78%) pT3 and 16 (18%) pT4 tumors. Lymph node metastases were diagnosed in 74 (83%) patients. At time of diagnosis, 45 (51%) patients had a clinical stage III and 44 (49%) patients a clinical stage IV. The available 31 distant metastases comprised 22 synchronous metastases from stage IV patients and 9 metachronous metastases from stage III patients. Metastatic tissue was obtained from the liver (12 patients), from small and large intestine (five patients), from the peritoneum (five patients), from the ovaries (four patients), from the abdominal wall (two patients), from the retroperitoneum (one patient), from the spleen (one patient) and from the lung (one patient). In the case of intraabdominal metastatic sites, continuous tumor growth from the primary site could be excluded by re-evaluation of the slides.

Quantification of tumor budding

Quantification of tumor buds was performed according to a method published by Prall et al. [11]. Hematoxylin and eosin (HE) stained slides of the primary tumors were viewed at scanning magnification and the area with maximal budding was located and marked to identify this area on the slides for immunohistochemistry. Buds were highlighted using immunohistochemistry with the pan-cytokeratin antibody AE1/AE3 (see section *Immunohistochemistry*). All separate microclusters of tumor cells with five or fewer nuclei or single tumor cells were counted in one 0.942 mm^2 field of vision at x 200 magnification using a Zeiss Axioplan microscope.

Immunohistochemistry

Formalin-fixed, paraffin-embedded archival tis-

sues obtained from 89 primary colorectal carcinomas and 31 distant metastases were cut into 3 μm thick sections. Sections were mounted, deparaffinized in xylene, and rehydrated through descending concentrations of ethanol.

For analysis of Lgr5 expression in primary tumors and distant metastases, antigen retrieval was performed using a preheated (65°C) citrate buffer (Target Retrieval Solution Citrate pH6, Dako Cytomation, Glostrup, Denmark) heated in a water bath for 20 minutes at 97°C followed by a resting time of 34 minutes. Blocking of endogenous peroxidases was accomplished by incubating sections in 3% hydrogen peroxide (Dako) for 5 minutes. Lgr5 polyclonal antibody against the C-terminal unit (AP2745b, also listed as LS-A1236, 1:50, Nordic BioSite, Täby, Sweden as Scandinavian distributor for MBL International) was incubated with sections for 30 minutes at room temperature.

For pan-cytokeratin AE1/AE3 analysis in primary tumors, antigen retrieval was performed using a preheated (65°C) Tris-ethylenediamine-tetraacetic acid buffer (pH 9) for 20 minutes at 97 °C followed by a resting time of 30 minutes. The slides were incubated for 30 minutes at room temperature with the monoclonal mouse anti-human AE1/AE3 antibody (clone AE1/AE3, 1:400, Dako).

Immunostaining of Lgr5 and AE1/AE3, respectively, was performed using the Envision+ System HRP and visualized by diaminobenzidine (Dako), according to the manufacturer`s instructions, followed by counterstaining with hematoxylin. Small and large bowel tissue obtained from patients without tumor diagnosis were used as positive controls for Lgr5, and normal tonsil tissue were used as positive controls for AE1/AE3. Negative controls were performed by omitting the primary antibodies.

Lgr5 expression in primary tumors and distant metastases was quantified according to a modified method established by Maeda et al. [16]. Slides were examined with low power (x 50) microscopy to identify the regions containing the highest burden of Lgr5 positive cells (hotspots) in the cancer nests. Slides without clearly detectable hotspots at low power microscopy were re-examined with higher magnification (x 100) to identify areas with weakly stained cells. Ten fields of hotspots in the tumor

tissue were selected, and expression of Lgr5 was evaluated in 1000 tumor cells (100 cells per field) with high power (x 400; 0.273 mm^2 per field) microscopy, using a raster ocular lens. Tumor cells focally as well as completely stained by the Lgr5 antibody were defined as positive for Lgr5, irrespective of staining intensity. In primary tumors, additional quantifying procedures were performed. The number of Lgr5-positive cells was counted in all visible tumor buds, thus determining an Lgr5/tumor bud ratio. Furthermore, the evidence of Lgr5-positive cells within intravascular and perineural tumor cell clusters was documented.

Statistical analysis

The association between Lgr5 expression in general, in different tumor compartments and in distant metastases were compared using the Spearman rank test (Spearman`s rho) and Wilcoxon test. A *p*-value of less than 0.05 (two-tailed) was considered statistically significant. All data were analyzed by using SAS 9.1.5 (SAS Institute Inc., Cary, NC, USA).

Results

Detection of tumor budding, angioinvasion and perineural infiltration

Tumor budding counts ranged from 0 to 114 buds with 17 as median and a mean of 21.5 in all colorectal carcinomas. For stage III carcinomas, the range was 0 to 63, median 17 and mean 19.27. In stage IV carcinomas, the number of tumor buds ranged from 0 to 114 with a median of 16.5 and mean of 23.72.
Vascular invasion was detected in 54 carcinomas. Lymphatic permeation was found in 43 primary carcinomas (23 stage III tumors, 20 stage IV tumors). Venous angioinvasion was seen in 11 primary tumors (one stage III tumor, 10 stage IV tumors).

Perineural tumor infiltration was seen in 15 primary carcinomas (six stage III and nine stage IV tumors).

Lgr5 expression in primary tumors and distant metastases

Correct immunoreaction was indicated by rare Lgr5 positive cells at the base of the crypts in small and large bowel tissue of the positive con-
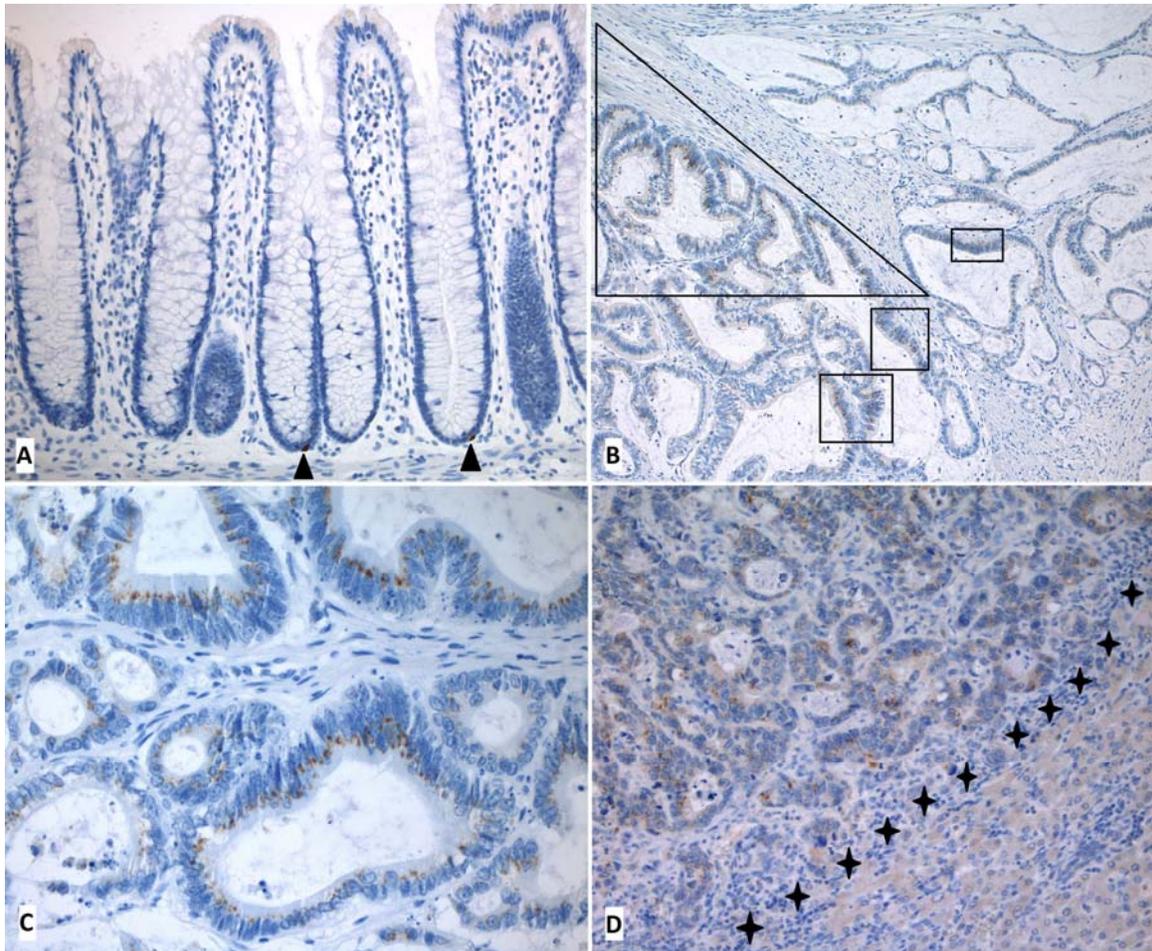
Figure 1. A. Large bowel tissue of a positive control shows only single Lgr5 positive cells at the base of the crypts (indicated by triangles, x 200). B. Primary colon carcinoma with heterogeneous distribution of Lgr5 expression. The triangular and the rectangular frames mark several hotspots of Lgr5 expression (x 100). C. Higher magnification of one Lgr5 hotspot within primary colon carcinoma. Lgr5 immunostaining has a granular appearance and is predominately localized in the luminal and perinuclear part of the cytoplasm in tumor cells (x 400). D. Liver metastasis showing Lgr5 expression in the tumor cells (left part), whereas the normal hepatocytes are negative (right part), x 200. The slightly brownish appearance of hepatocytes is due to lipofuscin pigment and does not represent immunoreaction. The stars mark the border between metastatic and non-neoplastic tissue.

trols (**Figure 1A**).

Lgr5 expression was detected in 82 out of 89 (92.1%) primary colorectal carcinomas and 16 out of 31 (51.6%) distant metastases, showing heterogeneous distribution pattern within the individual tumors tissues. Examples for Lgr5 expression in primary tumors and metastases are displayed in **Figure 1B-D**. The proportion of Lgr5 positive cells in primary tumors (range 0 – 33.6%) and metastases (range 0-33.6%) showed considerable variability among individual patients as displayed in **Figure 2**. Percentage of Lgr5 in distant metastases and primary tumors correlated significantly in all patients (p = 0.04), but was not significantly associated after stratification into stage III and IV tumors (p = 0.33 and 0.09, respectively) (**Table 1**).

Examples for Lgr5 expression in tumor buds, vascular and perineural spaces are displayed in **Figure 3** (with documentation of tumor budding in **Figure 3A**). 12.9%, 14.8% and 26.7% of primary tumors with histologically confirmed tumor buds, angioinvasion and perineural infiltrates, respectively, showed evidence of Lgr5 expression in these tumor compartments (**Figure 4A**). Similar low and varying evidence of Lgr5-

Table 1. Relationship between percentage of Lgr5 expression in primary tumors and percentage of Lgr5 expressing cells in distant metastases

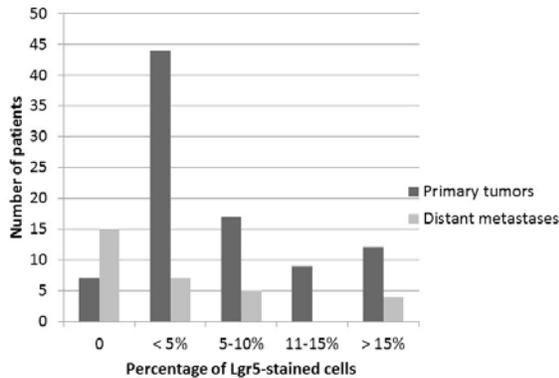|	Number of patients with metastases	%Lgr5 in primary tumors Mean ± SD (Median)	% Lgr5 in metastases Mean ± SD (Median)	$r_{Spearmen}$	p-value
All	31	7.00 ± 6.24 (5.6)	4.64 ± 7.58 (1.0)	0.372	0.04
Stage III	9	6.91 ± 5.43 (7.2)	3.42 ± 5.58 (0.0)	0.367	0.33
Stage IV	22	7.04 ± 6.54 (5.3)	5.15 ± 8.20 (1.2)	0.375	0.09

Figure 2. Proportion of Lgr5 positive cells in primary tumors and metastases, showing considerable variability among patients.

positive cells in these compartments was seen, if considering separately stage III tumors (4.8%, 13%, 20%, respectively) (**Figure 4B**) and stage IV tumors (20.9%, 16.1% and 30%, respectively) (**Figure 4C**).

At more detailed analysis of Lgr5 expression in tumor buds, the ratio between Lgr5 positive tumor buds and total bud count in the primary tumors (i.e. ratio Lgr5/bud) ranged from 0 to 0.09 with median 0 and mean 0.006 in all tumors. In stage III tumors, the ratio Lgr5/bud ranged from 0 to 0.08 with median 0 and mean 0.003. In stage IV tumors, the ratio Lgr5/bud ranged from 0 to 0.09 with median 0 and mean 0.009.
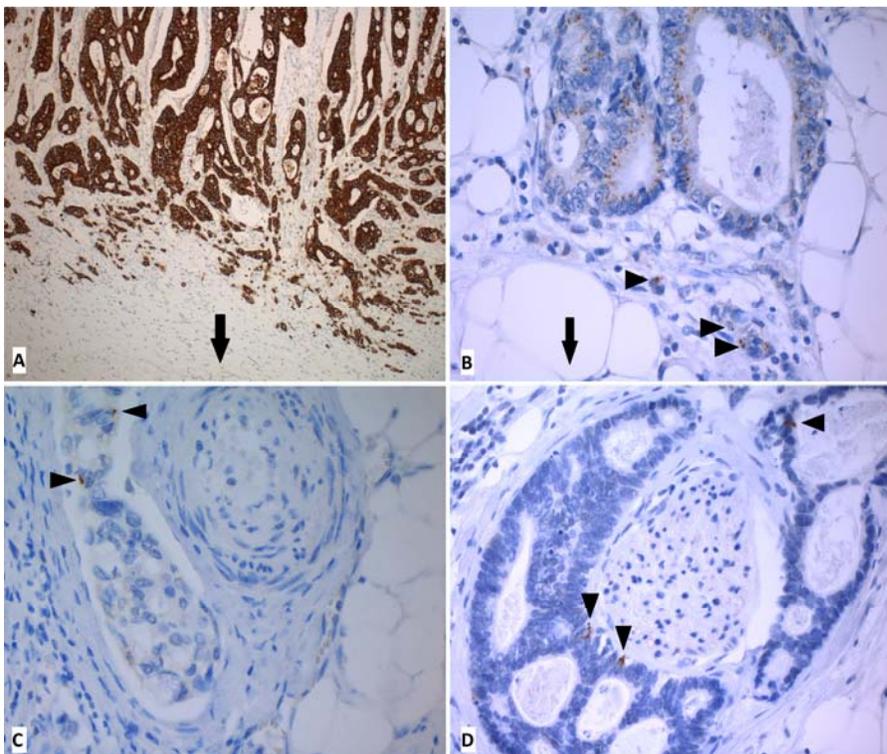

Figure 3 A. Pan-cytokeratin (AE1/AE3) immunostaining highlights tumor cell detachments (tumor buds) at the invasion front of a primary colon carcinoma (x 100). The arrow marks the direction of invasion. **B.** Tumor budding at the invasion front at higher magnification. The triangles indicate tumor buds composed of one, four and five cells, respectively, with positive Lgr5 immunostaining (x 400). The arrow marks the direction of invasion. **C.** Intravascular tumor thrombus with rare Lgr5-positive cells (indicated by triangles, x 400). **D.** Perineural tumor infiltrate with rare Lgr5-positive cells (indicated by triangles, x 400).
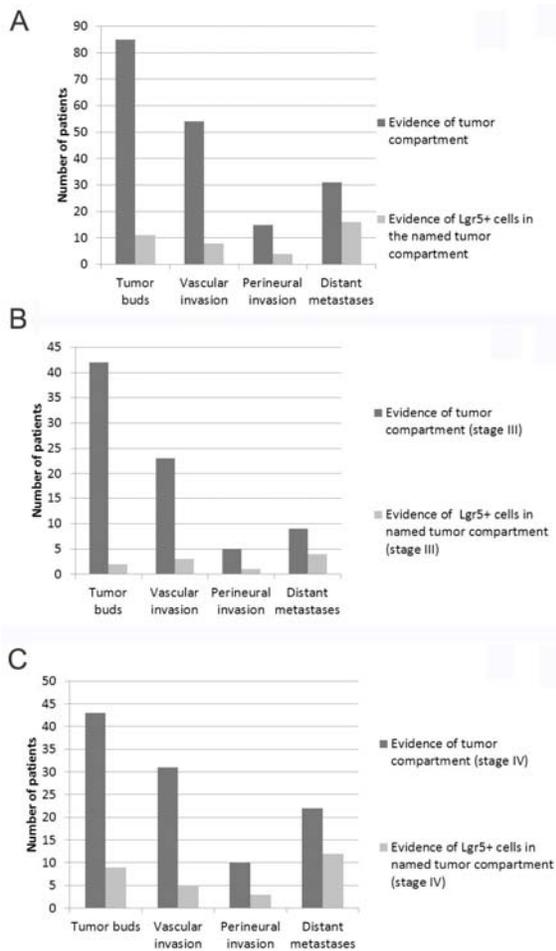
Figure 4. Evidence of Lgr5-positive cells in tumor buds, vascular spaces and perineural spaces related to the frequency of these tumor compartments in primary colorectal carcinomas. Evidence of Lgr5 expression in distant metastases is displayed for comparison. (A = all, B = stage III, C = stage IV tumors).

Relationship between Lgr5 expression in distant metastases and in individual primary tumor compartments

Remarkably higher median Lgr5% values were found in metastases, which were derived from primary tumors with Lgr5-positive cells in tumor buds, vascular and perineural spaces, compared to those metastases derived from tumors without Lgr5-positive cells in the named primary tumor compartments (**Table 2**). Such differences between median Lgr5 percentages in metastases were found comparing evidence and non-evidence of Lgr5 expressing cells in all three analyzed primary tumor compartments.

However, these differences showed statistical significance only for comparison of Lgr5-positive and Lgr5-negative tumor buds (marginal significance; $p = 0.047$) and vascular spaces (confined to stage IV tumors; $p = 0.007$), respectively (**Table 2**). Analysis of the ratio Lgr5/tumor bud related to Lgr5 percentages in distant metastases suggests a trend towards correlation between Lgr5/tumor bud ratio and Lgr5 expression in distant metastases (**Table 3**).

Due to limited sample size, statistical analysis with regard to Lgr5 expression in primary tumor compartments and distant metastases could not be performed for stage III tumors.

Discussion

This study is based on the main hypothesis that cancer stem cells could play an important role in metastasizing of colorectal cancer. To evaluate this assumption, we performed immunohistochemical expression analysis of the adult intestinal stem cell marker Lgr5 on 89 primary colorectal carcinomas, comprising 45 stage III tumors with high risk for distant metastases and 44 stage IV tumors with evidence of distant metastases. Lgr5 expression in the total primary tumor cell compartment as well as in primary tumor compartments with known relevance for metastasis, i.e. tumor buds at the invasive margins [11], vascular [12] and perineural spaces [13], were related to Lgr5 expression in 31 distant metastases.

The cohort comprising all cases showed a significant relationship between Lgr5 expression in primary tumors and corresponding distant metastases, which is in accordance to results of Horst et al. [17], who found similar expression of the putative cancer stem cell marker CD133 in primary colorectal carcinomas and liver metastases.

These results raise the question, if stem cell like cells in metastases could have their origin in primary tumors. If this is the case and Lgr5 expression is assumed to be a characteristic of stem cell like colorectal cancer cells, which might have the biological potential of cancer stem cells, it could be hypothesized that Lgr5 expressing tumor cells need to become detached as so called tumor buds from the primary tumor site and migrate to the metastatic site via vascular and perineural spaces. These

Table 2. Relationship between percentage of Lgr5 expressing cells in distant metastases and evidence of Lgr5 expressing cells in primary tumor compartments

Evidence of Lgr5+ tumor cells in primary tumor compartments	% Lgr5+ tumor cells in distant metastases					
	All patients (stage III + IV)			Stage IV patients		
	n[1]	Mean ± SD[2] (Median)	P[3]	n	Mean ± SD (Median)	p
Vascular spaces Lgr5(+) tumor cells: Yes	6	13.95 ± 11.98 (12.70)		5	16.74 ± 11.00 (16.10)	
			0.07			0.007
Lgr5(+) tumor cells: No	13	4.13 ± 5.14 (2.00)		9	2.79 ± 3.32 (1.40)	
Perineural spaces Lgr5(+) tumor cells: Yes	2	17.85 ± 2.47 (17.85)		2	17.85 ± 2.47 (17.85)	
			0.052			0.11
Lgr5(+) tumor cells: No	7	2.27 ± 2.76 (2.00)		4	3.48 ± 3.14 (3.30)	
Tumor buds Lgr5(+) tumor cells: Yes	6	11.02 ± 12.54 (7.50)		5	11.24 ± 14.00 (5.10)	
			0.047			0.13
Lgr5(+) tumor cells: No	20	2.69 ± 4.50 (0.00)		13	2.53 ± 3.64 (0.00)	

[1]N = number of patients, [2]SD = standard deviation, [3]p = p-value according to Wilcoxon test.
Correlation analysis was not performed for stage III patients, because of low case numbers in the individual primary tumor compartments.

Table 3. Relationship between ratio Lgr5/tumor bud in primary tumors and percentage of Lgr5 expressing cells in distant metastases

Number of patients with metastases and tumor buds	Ratio Lgr5/tumor bud Mean ± SD (Median)	% Lgr5 in metastases Mean ± SD (Median)	$r_{Spearmen}$	p-value
29	0.009 ± 0.02 (0)	4.29 ± 7.26 (1)	0.366	0.051

are main prerequisites for initiation of the metastatic process, according to the migrating stem cell theory [3]. In contrast to expectations from our hypothesis, we found relatively low frequency of Lgr5 expressing cells among tumor buds as well as in vascular and perineural spaces, even in stage IV tumors with clinically confirmed metastases at time of diagnosis (20.9%, 16.1% and 30%, respectively). To the best of our knowledge, the load of stem cell like cells in vascular and perineural primary tumor compartments has not been investigated so far. Considering recent knowledge about Lgr5 as Wnt target gene [4] and strong nuclear β-catenin accumulation as reflection of aberrant Wnt signaling in colorectal cancer progression [18], our results, especially the small evidence of tumor buds with Lgr5 expression and the low Lgr5/tumor bud ratio, might be in contrast to results from previous investigation. These authors demonstrated accumulation of nuclear β-catenin in dedifferentiated tumor cells at the tumor-host interface and regarded these cells as potential migrating cancer stem cells [19]. However, a recent study pointing to expression

of Lgr5 in human colorectal carcinogenesis and its potential correlation with β-catenin expression, did not find different expression of Lgr5 between center and invasive margins of the tumors [20]. These discrepancies between results for β-catenin and Lgr5 expression at the tumor-host interface could possibly be attributed to the fact that Lgr5 expression can alternatively or additionally be stimulated by other mechanisms than Wnt signaling, for example JNK activation [21].

Furthermore, a low detection rate of Lgr5 expressing, i.e. stem cell like, cells in the tumor-host interface seems not automatically exclude a role of these cells for metastasizing. Studies on colon and pancreatic cancer figured out that only a distinct subpopulation of cancer initiating cells is sufficient competent to evade the primary tumor and spread through the invasive and metastatic cascade [22,23]. With the current methodical approach, we were not able to differentiate between subpopulations of cancer initiating cells. However, our results could give reason to speculate, whether the small number

of Lgr5 expressing cells detected among tumor buds and in vascular as well as perineural spaces of our primary tumor cohort might represent a population of biologically powerful stem cell like cancer cells, hypothetically with the potential to initiate metastases. Distant metastases, which were derived from primary tumors with Lgr5-positive tumor buds, vascular and perineural tumor infiltrates showed an approximately 6- to 8-fold higher median value of Lgr5 expression compared to those metastases, which were related to primary tumors without Lgr5 expressing cells in the named tumor compartments. Considering only stage IV tumors, the median value of Lgr5 expression in distant metastases was even 11.5-fold higher, if Lgr5 expressing cells were found in vascular spaces of the primary tumor. Due to small sample size in the investigated subgroups, probably caused by the low detection rate of Lgr5-positive cells in the primary tumor compartments, the statistical power to confirm these differences of Lgr5 expression between distant metastases was only strong enough for results obtained from analysis of vascular spaces in stage IV tumors and tumor buds in the entire patient cohort. Limited number of cases was also the reason to waive statistical analysis of stage III tumors, but it can`t be excluded that the above named differences occurred also in this cohort or have at least contributed to statistical significant differences in the entire ("all") patient cohort. To avoid tumor groups with even smaller sample size, the vascular tumor compartment was not subdivided into lymphatic and venous spaces, which seems to be an appropriate approach due to the known close relationship between lymphatic and vascular invasion [24].

Challenging above named considerations about a role of migrating stem cell like cells for metastasizing, our analysis revealed Lgr5 expression in 16 out of 31 (51.6%) distant metastases, which could be attributed to both, primary tumors with and without evidence of Lgr5 positive tumor cells in tumor buds, vascular and perineural spaces. This result might reflect the known heterogeneity and clonal evolution of colorectal tumor cells resulting from mutations. This implies that differentiated tumor cells can revert to cancer stem cells or stem cell like cells after having gained a clonal advantage over the original cancer stem cells [1,25] and that an acquired stem cell or stem cell like phenotype can get lost due to requirements of tumor development. Therefore, further investigations, mainly clonal analyses, are necessary to evaluate a possible causal relationship between stem cell like cells in primary tumors, in the tumor-host interface and in metastases. Confirmation of the migrating cancer stem cell theory in colorectal cancer will also require the use of markers, which not only detect stem cell like cells, but definite intestinal cancer stem cells. Currently, such markers are hardly to identify, because until now it has been unclear, whether the published markers CD44, EpCAM, CD166 [26] really mark cancer stem cells or only lead to their enrichment [3], and CD133, another promising marker, is not restricted to stem cells [27].

In conclusion, our results point to rare evidence of Lgr5 positive stem cell like cells in the metastatic cascade of colorectal cancer, but these few cells might be biologically powerful in the metastatic process of cancer subsets. Further investigation is necessary to proof this hypothesis and to explore its importance for prevention and therapy of colorectal cancer metastasis.

Acknowledgment

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