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Original Article

Single nucleotide polymorphisms of the adult intestinal stem cell marker Lgr5 in primary and metastatic colorectal cancer

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Abstract: Morphological and clinical heterogeneity of advanced colorectal cancer is probably caused by genetic variability in putative cancer stem cell genes, including *Lgr5*. Here, we investigated 23 variants of the *Lgr5* gene in normal tissue, primary tumors, lymph node metastases and distant metastases of stage III and stage IV colorectal cancer patients. These data were compared to results of immunohistochemical Lgr5 expression analysis and to prognostic clinical parameters. No differences were found comparing germline and somatic *Lgr5* genotype in primary tumors, but additional *Lgr5* gene alterations could be demonstrated in lymph node and distant metastases. Significant negative correlation was seen between *Lgr5* allelic variation and Lgr5 protein expression (p=0.0394), which mainly can be attributed to the negative influence of non-coding *Lgr5* gene variations on Lgr5 protein expression (p=0.0166). *Lgr5* gene variants could be found more frequently in primary tumors of stage III patients with increased time to recurrence, in distant metastases of patients with better survival and in lymph node metastases of patients with poorer survival compared to patients with *Lgr5* wild type in primary and metastatic tissues, respectively. However, the analytic power of these prognostic data was low due to small sample size in the investigated groups. In conclusion, our data indicate that *Lgr5* allelic variation affect Lgr5 protein expression in colorectal carcinomas. The somatic *Lgr5* genotype seems to be relatively stable in primary tumors, but becomes vulnerable during the metastatic process of colorectal cancer. This instability has possibly prognostic importance, which has to be further evaluated by large cohort studies.

Keywords: Colorectal cancer, metastases, Lgr5, stem cell like cells, allelic variation

Introduction

Colorectal cancer (CRC) is one of the most common causes of cancer-related death worldwide [1]. Although cure may be achieved by surgery and adjuvant postoperative chemotherapy in approximately two thirds of the patients with early stages of the disease, more than 50% of the patients actually have unresectable locally advanced or metastatic CRC at onset or develop metastases in a later phase [2]. Palliative chemotherapy represents the main treatment option for these patients, but even recently developed combination regimens have relatively limited impact on survival [2]. Such interindividual differences in the behavior of CRC are caused by the heterogeneity of tumors, which may be in part due to the existence of cancer stem cells. These cells harbor key properties as self-renewal by asymmetric division, multilineage potential and resistance to apoptosis [3]. Phenotypic characterization of colorectal cancer stem cells is still a matter of debate and ongoing research studies [4], and therefore, the term stem cell like cells seems to be more appropriate to describe putative stem cells in neoplastic tissues. Definitive markers for cancer stem cells or stem cell like cells should be gene products that are coupled to the function of the stem cells.

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 [5]. Analyses of Wnt/β-catenin signaling in human colorectal cancer [6], Lgr5-EGFP^{+} stem cell transformation in a mice model [7] and the tumor initiating potential of single-cell-cloned cancer stem cells on xenotransplants [8] suggest that stem cell like cells are involved in colorectal carcinogenesis and that these cells can be identified, among others, by the marker Lgr5.

There is substantial germline genetic variability within the genes used as markers to identify stem cell like cancer cells, including single nucleotide polymorphisms (SNP) [9]. These common DNA-sequence variations may alter the gene function and/or activity including transcription, translation or splicing, thereby causing interindividual differences in tumor behavior [10]. A recent study could demonstrate varying tumor recurrence capacity in colorectal cancer patients according to germline genetic variants in a panel of putative cancer stem cell associated genes, including *Lgr5* [9]. However, in the case of *Lgr5*, a link between germline genetic variability and protein expression has not been reported so far. In addition, the evidence and role of potentially cofounding somatic changes in the *Lgr5* gene during CRC development and metastasizing is still poorly understood.

To address these issues, we investigated 23 polymorphisms of the *Lgr5* gene in normal tissue, primary tumors, lymph node metastases and distant metastases of stage III and stage IV colorectal cancer patients. Furthermore, we searched for a possible link of immunohistochemical Lgr5 expression to germline and somatic *Lgr5* genotype, respectively.

Material and methods

Patients, controls 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 Southern 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 [11] and tumor stage was determined according to the criteria proposed by the International Union Against Cancer (UICC) [12]. Clinicopathological details of patients and specimens are shown in **Table 1A**. Fifteen lymph node metastases and seven distant metastases were suitable for both, immunohistochemical and molecular genetic analysis. Supplementary information for the distant metastases is displayed in **Table 1B**.

A control group of 72 healthy individuals (38 women and 34 men, aged 22 to 50 years) was investigated for establishment and validation of the SNaPshot analysis method (see below). Buccal swabs and data were obtained and used according to the ethic and data security guidelines of the Medical Faculty at University Essen (Germany).

Candidate polymorphisms

Common and putatively functional polymorphisms of the *Lgr5* gene were selected using the NCBI database-PubMed [13].

Stringent and predefined selection criteria were used in a modified approach according to Greger *et al*. [9]: (a) minor allele frequency 1% or more in Caucasians, (b) polymorphism that could alter the function of the gene in a biologically relevant manner (either published data or predicted function using Functional-Single-Nucleotide-Polymorphism (F-SNP) database, http://compbio.cs.queensu.ca/F-SNP [14, 15], and (c) published clinical associations (e.g. cancer risk).

DNA extraction and quantification

DNA extraction from buccal swabs (healthy controls) and tumor tissue or non-neoplastic tissue

Table 1A. Clinicopathological details of patients and specimens.

Parameter		Number of cases (%)
Clinical Stage	III	45 (50.6%)
	IV	44 (49.4%)
Response to chemotherapy	Yes	57 (64.0%)
	No	32 (36.0%)
Type of chemotherapy	FLV / Xeloda	62 (69.7%)
	FLIRI[a]	12 (13.5%)
	FLOX[b]	15 (16.8%)
Large intestine tumor site	Coecum	19 (21.3%)
	Ascending	16 (18.0%)
	Transverse	11 (12.4%)
	Descending	8 (9.0%)
	Sigmoid	21 (23.6%)
	Rectum	14 (15.7%)
pT stage	≤ 2	4 (4.5%)
	3	69 (77.5%)
	4	16 (18.0%)
pN stage	0	15 (16.8%)
	1	48 (54.0%)
	2	26 (29.2%)
Histological Grading	1 (highly differentiated)	0 (0%)
	2 (moderately differentiated)	69 (77.5%)
	3 (poorly differentiated)	20 (22.5%)

[a]Including one case with FLIRI+Avastin; [b]Including two cases with FLOX+Erbitux.

Table 1B. Details of patients with distant metastases (n = 7).

Parameter		Number of cases
Clinical stage	III	4
	IV	3
Distant anatomic site	Liver	2
	Abdominal wall[a]	2
	Small intestine[a]	1
	Peritoneum[a]	1
	Ovary	1

[a]In the case of intraabdominal metastatic sites, continuous tumor growth from the primary site could be excluded by re-evaluation of the slides.

(colorectal carcinoma patients) was done using innuPREP® DNA Mini Kit (analytikjena®, Jena). Manual microdissection was performed before DNA extraction from primary and metastatic colorectal cancer tissue: A sufficient amount of neoplastic tissue was identified on hematoxylin- and eosin stained slides using a scaled optical adjustment. This same area was then re-identified on the unstained 10 µm dewaxed, rehydrated and air-dried tissue section and separately isolated under microscopic control with a cannula, predominantly without adherent non-neoplastic tissue. Separately embedded resection margins without evidence of tumor were used as normal tissue for colorectal cancer patients.

DNA amplification and electrophoresis

Multiplex PCR was done in a volume of 12.5 µl in the GeneAmp® PCR system 9700 (Applied Biosystems) with 2-5 ng of DNA as template in 15 mM Tris/HCl, 50 mM KCl, with 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.1 µM primers, and 1.5 Units AmpliTaq Gold Polymerase (Applied Biosystems). PCR conditions were initial denaturation and activation step of 8 min at 95° C, 30 cycles of 1 min at 94° C, 1 min at 58° C and 2

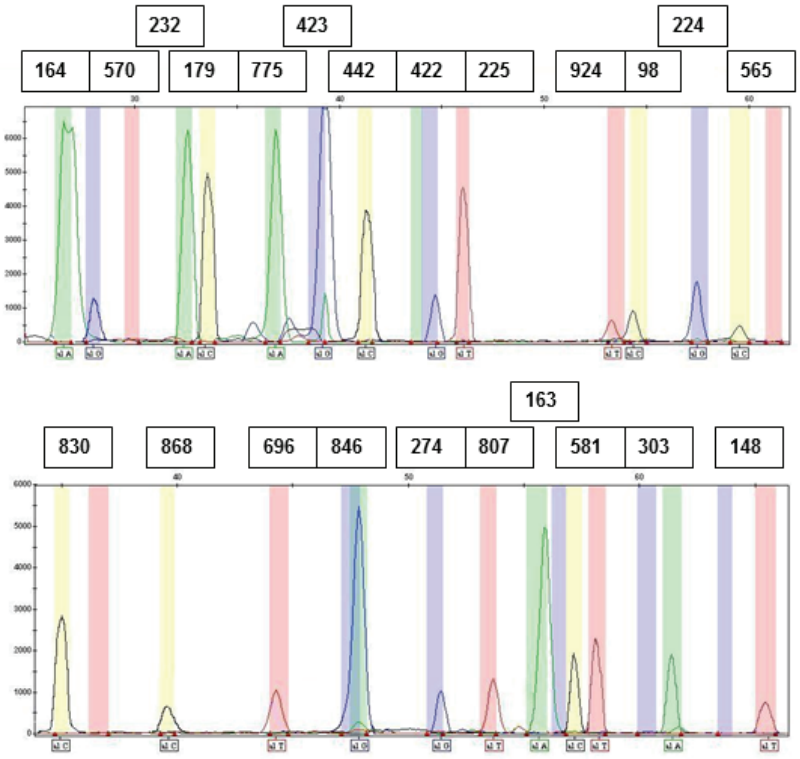

Figure 1. Electropherograms of the two SNaPshot assays for the analysis of *Lgr5* variants. Each single nucleotide polymorphism is coded with the last three positions of its rs-number. See Table 2 for full answer.

min at 72° C, and a 60 min final elongation step at 60° C. SNaPshot analyses were performed in accordance with the manufacturer's instructions and evaluated on ABI310 or ABI3130 Genetic Analyzers. Electrophoresis results were analyzed using the GeneMapper® ID Software v3.2 with self designed panels and bins sets. Electropherograms of the two SNaPshot assays for the analysis of *Lgr5* variants are displayed in **Figure 1**.

Immunohistochemistry

Status of immunohistochemical Lgr5 expression in 89 primary tumors and seven distant metastases were investigated in our previous study [16], and the same immunohistochemical procedures were currently applied on formalin-fixed, paraffin-embedded archival tissues obtained from 15 lymph node metastases.

Lgr5 expression in the lymph node metastases was quantified according to a modified method established by Maeda et al. [17] and used for our previous immunohistochemical analysis of primary tumors and distant metastases [16].

Statistical analysis

Comparison between frequency of *Lgr5* allelic variation in healthy controls and patient tissue was performed using unconditional logistic regression model and Akaike information criterion was applied to determine the best model of inheritance. The association between *Lgr5* genotype and immunohistochemical Lgr5 expression was compared using the Wilcoxon test. Log-rank test was applied to the analyses between various variables and survival end points (overall survival, time to relapse), and Cox's proportional hazards regression analysis was used to calculate hazard ratio (HR). 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

Genotyping

Table 2 displays the frequency of 12 coding and 11 non-coding *Lgr5* single nucleotide polymorphisms (SNPs) in healthy individuals and non-neoplastic tissue (germline genotype), primary tumors, distant metastases and lymph node metastases (somatic genotypes) in colorectal cancer patients. Major differences in the allelic frequency of germline genotype between healthy individuals and patients were seen only for two SNPs: rs113809442 and rs10879303. This difference was not significant for rs113809442 (*p*-value in the dominant model: 0.089), but statistically significant for SNP rs10879303 (*p*-value in the dominant model: 0.0095, *p*-value for log-additive comprising

Table 2. Frequency of *Lgr5* single nucleotide polymorphisms in primary colorectal cancer, distant metastases and lymph node metastases.

rs number	Geno-type	Controls Germline (n=72)	CRC[a] patients (n=89) Germline	CRC patients (n=89) Primary tumors	CRC Metastases Distant (n =7)	CRC Metastases Lymphatic (n=15)	Published frequency[b]
rs117535164	AA	72 (100%)	88 (98.9%)	88 (98.9%)	7 (100%)	15 (100%)	n.d[c]
	AG	0 (0%)	1 (1.1%)	1 (1.1%)	0 (0%)	0 (0%)	
rs35021570	GG	72 (100%)	87 (97.8%)	87 (97.8%)	7 (100%)	15 (100%)	n.d.
	GT	0 (0%)	2 (2.2%)	2 (2.2%)	0 (0%)	0 (0%)	
rs116452232	AA	72 (100%)	89 (100%)	89 (100%)	7 (100%)	15 (100%)	n.d.
rs117504830	CC	71 (98.6%)	87 (97.8%)	87 (97.8%)	6 (85.7%)	15 (100%)	n.d.
	CT	1 (1.4%)	2 (2.2%)	2 (2.2%)	1 (14.3%)	0 (0%)	
rs12303775	AA	72 (100%)	89 (100%)	89 (100%)	7 (100%)	15 (100%)	n.d.
rs73339868	CC	72 (100%)	89 (100%)	89 (100%)	7 (100%)	15 (100%)	n.d.
rs761115696	TT	71 (98.6%)	87 (97.8%)	87 (97.8%)	7 (100%)	15 (100%)	96% T
	TC	1 (1.4%)	2 (2.2%)	2 (2.2%)	0 (0%)	0 (0%)	4% C
rs61737423	GG	72 (100%)	89 (100%)	89 (100%)	7 (100%)	15 (100%)	98% G
	GA	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2% A
rs17109924	TT	64 (88.9%)	79 (88.8%)	79 (88.8%)	6 (85.7%)	15 (100%)	93% T
	TC	8 (11.1%)	10 (11.2%)	10 (11.2%)	1 (14.3%)	0 (0%)	7% C
rs113809442	CC	67 (93%)	88 (98.9%)	88 (98.9%)	7(100%)	15 (100%)	n.d.
	CG	4 (5.6%)	1 (1.1%)	1 (1.1%)	0 (0%)	0 (0%)	
	GG	1 (1.4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
rs113487098	CC	72 (100%)	89 (100%)	89 (100%)	7 (100%)	15 (100%)	n.d.
rs61737422	GG	72 (100%)	89 (100%)	89 (100%)	7 (100%)	15 (100%)	n.d.
rs7961581	TT	41 (56.9%)	51 (57.3%)	51 (57.3%)	4 (57.1%)	10 (66.7%)	77% T
	TC	26 (36.1%)	34 (38.2%)	34 (38.2%)	2 (28.6%)	4 (26.6%)	23% C
	CC	5 (7%)	4 (4.5%)	4 (4.5%)	1 (14.3%)	1 (6.7%)	
rs77053565	CC	68 (94.4%)	82 (92.1%)	82 (92.1%)	6 (85.7%)	15 (100%)	96% C
	CT	4 (5.6%)	7 (7.9%)	7 (7.9%)	1 (14.3%)	0 (0%)	4% T
rs11178846	GG	68 (94.4%)	84 (94.4%)	84 (94.4%)	7 (100%)	14 (93.3%)	93% G
	GA	4 (5.6%)	5 (5.6%)	5 (5.6%)	0 (0%)	1 (6.7%)	7% A
rs2304274	GG	65 (90.3%)	80 (89.9%)	80 (89.9%)	7 (100%)	15 (100%)	92% G
	GA	7 (9.7%)	9 (10.1%)	9 (10.1%)	0 (0%)	0 (0%)	8% A
rs75061163	AA	72 (100%)	88 (98.9%)	88 (98.9%)	7 (100%)	15 (100%)	99% A
	AG	0 (0%)	1 (1.1%)	1 (1.1%)	0 (0%)	0 (0%)	1% G
rs74101179	CC	72 (100%)	89 (100%)	89 (100%)	7 (100%)	15 (100%)	99% C
	CT	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1% T
rs73146224	GG	71 (98.6%)	89 (100%)	89 (100%)	7 (100%)	14 (93.3%)	97% G
	GA	1 (1.4%)	0 (0%)	0 (0%)	0 (0%)	1 (6.7%)	3% A
rs10879303	AA	65 (90.3%)	88 (98.9%)	88 (98.9%)	7 (100%)	15 (100%)	92% A
	AG	5 (6.9%)	1 (1.1%)	1 (1.1%)	0 (0%)	0 (0%)	8% G
	GG	2 (2.8%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
rs7307225	TT	57 (79.2%)	72 (80.9%)	72 (80.9%)	5 (71.4%)	14 (93.3%)	90% T
	TC	15 (20.8%)	17 (19.1%)	17 (19.1%)	2 (28.6%)	1 (6.7%)	10% C
rs76736148	TT	72 (100%)	88 (98.9%)	88 (98.9%)	7 (100%)	15 (100%)	99% T
	TG	0 (0%)	1 (1.1%)	1 (1.1%)	0 (0%)	0 (0%)	1% G
rs79665807	TT	71 (98.6%)	89 (100%)	89 (100%)	7 (100%)	15 (100%)	97% T
	TC	1 (1.4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3% C

[a]CRC = Colorectal carcinoma; [b]Publshed frequencies according to NCBI database [13]; [c]n.d. = no data; grey background = coding (functional) single nucleotide polymorphisms.

codominant, dominant, recessive and overdominant model: 0.0071). However, this significant result is derivate from Hardy-Weinberg-Equilibrium in controls (p = 0.018). The allelic frequencies for rs113809442 and rs7961581 (another SNP with homozygous variant genotype) were within the probability limits of Hardy-Weinberg-Equilibrium (data not shown).

Analysis of primary tumor tissue (somatic genotype) did not show additional genetic alterations compared to non-neoplastic tissue (germline

Table 3A. Comparison of immunohistochemical Lgr5 expression between genetic *Lgr5* wild type and *Lgr5* variant colorectal carcinomas.

Parameter	CRC[a] with *Lgr5* wild type genotype (n=25)	CRC with variant *Lgr5* genotype (n=64)	*p*-value[b]
Lgr5 expression (%) Mean ± SD[c] (Median)	8.94 ± 8.41 (6.1)	5.76 ± 7.04 (3.0)	0.0394

[a]CRC = colorectal carcinoma (primary tumors); [b]*p* = Wilcoxon test; [c]SD = standard deviation

Table 3B. Comparison of immunohistochemical Lgr5 expression between colorectal carcinomas with genetic wild type and variants in non-coding polymorphisms and coding polymorphisms of *Lgr5* gene.

Parameter	CRC[a] with *Lgr5* wild type genotype (n=25)	CRC with non-coding *Lgr5* SNP[b] (n=48)	CRC with coding *Lgr5* SNP (n=16)
Lgr5 expression (%) Mean ± SD[c] (Median)	8.94 ± 8.41 (6.1)	5.18 ± 6.84 (2.70)	7.52 ± 7.56 (5.65)
Comparison of all groups *p*-value	*p*=0.047[d]		
Pairwise comparison *p*-value	*p*= 0.0166[e,f]		
		p=0.237[e]	
		p=0.640[e]	

[a]CRC = colorectal carcinoma (primary tumors); [b]SNP = single nucleotide polymorphism; [c]SD = standard deviation; [d]*p* = Kruskal-Wallis test; [e]*p* = Wilcoxon test; [f]*p* = significant after Bonferroni correction; black box = group excluded from pairwise comparison

Table 3C. Comparison of immunohistochemical Lgr5 expression between colorectal carcinomas with genetic wild type *Lgr5* gene and variants in non-coding polymorphism rs7961581 and coding polymorphism rs17109924.

Parameter	CRC[a] with *Lgr5* wild type genotype	CRC with variant *Lgr5* genotype	*p*-value[b]
Lgr5 expression (%) Mean ± SD[c] (Median)	rs7961581-T/T (n=51) 7.69 ± 8.30 (4.5)	rs7961581-T/C+C/C (n=38) 5.27 ± 6.20 (3.0)	0.250
	rs17109924-T/T (n=79) 6.56 ± 7.56 (4.1)	rs17109924-T/C+C/C (n=10) 7.42 ± 7.71 (5.6)	0.688

[a]CRC = colorectal carcinoma(primary tumors); [b]*p* = Wilcoxon test; [c]SD = standard deviation

genotype) of the patients (**Table 2**). The number of patients with SNPs in the primary tumors (somatic = germline genotype) is shown in **Tables 3A-C**.

Results of SNP analysis for those patients with available material in both, primary tumors and metastases are displayed in **Tables 4A** and **4B**. Changes in the somatic compared to germline *Lgr5* genotype were found in three out of seven (42.9%) distant metastases (**Table 4A**): Two distant metastases (28.6%) showed the variant allele in coding SNPs in addition to germline genetic variants, in one of these metastasis was also seen loss of the wild type allele in a non-coding SNP. One distant metastasis (14.3%) showed loss of the variant allele in a non-coding SNP. Changes in the somatic compared to germline *Lgr5* genotype were found in four out of 15 (26.7%) lymph node metastases (**Table 4B**): Two lymph node metastases (13.3%) showed somatic variations in non-coding SNPs. In two other lymph node metastases (13.3%), we found loss of the variant allele in coding SNPs. Four out of eight (50%) patients with *Lgr5* allelic variation in lymph node metastases and five out of seven (71.4%) patients with *Lgr5* wild type in lymph node metastases showed either synchronous distant metastases or tumor recurrence (data not displayed in the Table).

Comparison of Lgr5 genotype and immunohistochemical Lgr5 expression

Results of Lgr5 expression in the 89 primary tumors were obtained from our previous immunohistochemical study [16]. Primary colorectal carcinomas with variant *Lgr5* genotype showed

Table 4A. Relationship between *Lgr5* genotype and immunohistochemical Lgr5 expression in distant metastases.

Case ID[a]	*Lgr5* genotype (GT) in distant metastases			Lgr5 expression (%)		
	Germline GT	Somatic GT		Comparison of genotype (GT) groups		
		Mutation	LOH	Ind.[b]	SNP somatic GT	Change somatic GT compared to germline GT
5	wild type	No	No	9.9	No (n=3): 3.3 ± 5.71 [0][c]	No (n=4): 3.52 ± 3.78 [2.1]
26	wild type	No	No	0		
41	rs77053565-CT	No	rs77053565 loss of variant allele	0		
4	rs796581-TC rs7307225-TC	rs17109924-CT	rs796581-C loss of wild type allele	0	Yes (n=4): 5.95 ± 9.15 [2.1]	Yes (n=3): 6.53 ± 9.24 [0]
19	rs77053565-CT	rs117504830-CT	No	19.6		
33	rs796581-TC	No	No	2.2		
38	rs796581-TC rs7307225-TC	No	No	2.0		

[a]ID = identification number; [b]Ind. = individual value; [c]mean ± standard deviation [median]

Table 4B. Relationship between *Lgr5* genotype and immunohistochemical Lgr5 expression in lymph node metastases.

Case ID[a]	*Lgr5* genotype (GT) in lymph node metastases			Lgr5 expression (%)		
	Germline GT	Somatic GT		Comparison of genotype (GT) groups		
		Mutation	LOH	Ind.[b]	SNP somatic GT	Change somatic GT compared to germline GT
5	wild type	No	No	23.6	No (n=7): 6.15 ± 8.54 [3.1][c]	No (n=11): 3.7 ± 6.68 [1.1]
6	wild type	No	No	3.1		
9	wild type	No	No	1.2		
15	wild type	No	No	0		
53	wild type	No	No	4.5		
55	wild type	No	No	0		
75	rs17109924-CT rs35021570-GT	No	rs17109924 rs35021570, both loss of variant allele	10.7		
21	rs796581-TC	No	No	7.2	Yes (n=8): 4.69 ± 5.79 [1.1]	Yes (n=4): 8.8 ± 5.16 [11.0]
23	wild type	rs73146224-GA	No	11.4		
25	rs7307225-TC	No	No	0		
61	rs35021570-GT	rs11178846-GA	rs35021570 loss of variant allele	0		
64	rs796581-CC	No	No	0		
68	rs117504830-CT rs796581-CT	No	rs117504830 loss of variant allele	13.1		
74	rs796581-CT	No	No	1.1		
82	rs796581-CT	No	No	0		

[a]ID = identification number; [b]Ind. = individual value; [c]mean ± standard deviation [median]

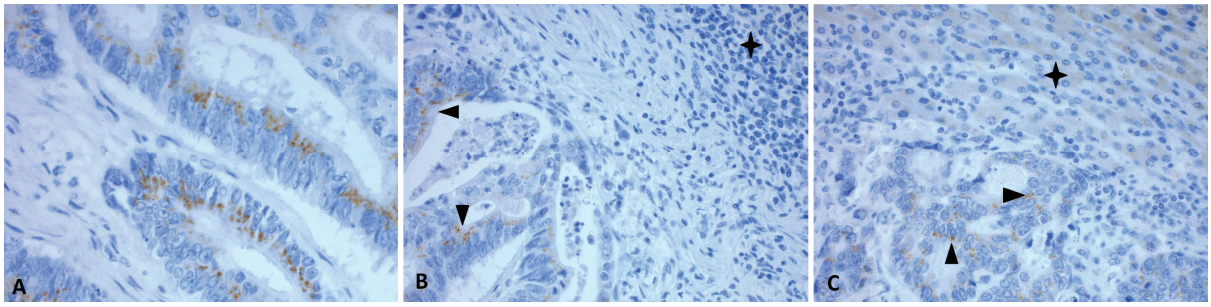

Figure 2. Immunohistochemical Lgr5 expression in primary and metastatic colorectal cancer tissue (x 400). A: Lgr5 hotspot within primary colon carcinoma. Lgr5 immunostaining has a granular appearance. B: Lymph node metastases from colorectal cancer with focal Lgr5 expression (arrows), whereas the normal lymphatic tissue (star) is negative. C: Liver metastasis from colorectal cancer with focal Lgr5 expression (arrows), whereas normal hepatocytes (star) are negative.

a significantly lower immunohistochemical Lgr5 expression compared to carcinomas with wild type genotype (p = 0.0394) (**Table 3A**). After stratification into tumors with non-coding and coding SNPs (**Table 3B**), only carcinomas with non-coding SNPs showed significantly different (lower) Lgr5 expression compared to carcinomas without genetic *Lgr5* variants (p=0.0166, significant after Bonferroni correction). In contrast, carcinomas with coding SNPs showed Lgr5 expression, which was only little lower than in carcinomas with genetic wild type, but approximately twice as high as in carcinomas with non-coding SNPs, defined by median of Lgr5% (not significant with p=0.234 and p=0.640, respectively). Separate comparison for rs7961581 and rs17109924, which had higher frequency of allelic variation than the other SNPs, did not show significant differences of Lgr5 expression between tumors with wild type and those with variant genotype (p= 0.250 and p=0.688, respectively, **Table 3C**).

Immunostaining results for the seven distant metastases included in the current study were also obtained from our previous work [16]. Immunohistochemical Lgr5 expression status of these metastases is displayed in **Table 4A**. The median of Lgr5 expression was slightly higher in distant metastases with variant alleles (regardless, whether these alleles represented germline genotype or were acquired as mutation) compared to distant metastases with only wild type alleles (regardless, whether absence of variant alleles represented germline genotype or loss in somatic genotype). Considering only the stability of a given (germline) genotype, distant metastases with instable somatic *Lgr5* genotype (i.e. changes compared to germline genotype) showed a lower median of Lgr5 expression than distant metastases with stable somatic genotype (**Table 4A**).

Lgr5 expression was detected in 9 out of 15 (60%) lymph node metastases, showing heterogeneous distribution pattern within the individual metastases. The proportion of Lgr5 positive cells in lymph node metastases showed considerable variability among individual patients (range 0 – 23.6%). The median of Lgr5 expression was lower in lymph node metastases with variant alleles (regardless, whether these alleles represented germline genotype or were acquired as mutation) compared to lymph node metastases with wild type alleles (regardless, whether absence of variant alleles represented germline genotype or loss in somatic genotype) (**Table 4B**). Considering only the stability of a given (germline) genotype, lymph node metastases with an instable somatic *Lgr5* genotype (i.e. changes compared to germline genotype) showed a 10-fold higher median of Lgr5 expression than lymph node metastases with a stable somatic genotype (**Table 4B**). Examples of Lgr5 expression in primary and metastatic colorectal cancer tissue are displayed in **Figure 2**.

Comparison of Lgr5 genotype and prognostic clinical data

Results of survival analysis are displayed in **Table 5A**. In the case of primary tumors, patients with variant alleles in SNPs of the *Lgr5* gene showed similar survival as patients with wild type *Lgr5*. Although no significant results were observed, patients with *Lgr5* variant alleles in distant metastases showed a numeric better survival than patients with wild type *Lgr5* in dis-

Table 5A. Survival of patients with wild type genotype compared to variant genotype in tumor tissue.

Parameter	Survival time in months		
	Mean ± SD[a] [Median]	Multivariate analysis[b] HR[c] [95% CI[d]]	p-value
Primary colorectal carcinomas wild type genotype (n=25)	50.12 ± 28.37 [52]	1.27 [0.67 – 2.43]	0.467
Primary colorectal carcinomas variant genotype (n=64)	46.59 ± 33.14 [43.5]		
Distant metastases, Wild type genotype (n=3)	25.67 ± 11.15 [30]	n.d.[de]	0.997
Distant metastases, Variant genotype (n=4)	65 ± 26.23 [63]		
Lymph node metastases, Wild type genotype (n=7)	62 ± 23.6 [62]		
Lymph node metastases, Variant genotype (n=8)	44 ± 27.8 [49.5]	5.20 [0.78 – 34.70]	0.089

[a]SD = standard deviation multivariate analysis stratified by tumor stage and response to chemotherapy; [c]HR = hazard ratio; [d]CI = confidence interval; [e]n.d.= no data, because of small sample size in one group, HR and CI can not be assessed.

Table 5B. Recurrence of stage 3 patients with wild type genotype compared to variant genotype in primary carcinomas.

Parameter	Stage 3 (n=45)			
	Wild type genotype (n=13)		Variant genotype (n=32)	
	Recurrence (n= 3)	No recurrence (n=10)	Recurrence (n=12)	No recurrence (n=20)
Time to recurrence Mean ± SD[a] [Median] months	12.7 ± 4.64 [11]	----	36.1 ± 32.1 [24.5]	-----
Univariate analysis HR[b] [95% CI[c]]	0.27 [0.06 – 1.16], *p*-value = 0.078			

[a]SD = standard deviation; [b]HR = hazard ratio; [c]CI = confidence interval

tant metastases, whereas patients with *Lgr5* variant alleles in lymph node metastases showed a numeric worse survival compared to patients with wild type alleles in lymph node metastases.

Recurrence data for stage III patients are displayed in **Table 5B**. The proportion of patients with recurrent tumor was slightly higher in patients with *Lgr5* variant alleles in primary colorectal carcinomas (12/32, 37.5%) compared to patients with wild type *Lgr5* (3/13, 23.1%). However, patients with *Lgr5* variant alleles in the carcinomas showed more than 2-fold longer time to recurrence (as demonstrated by median of survival in months) than patients with *Lgr5* wild type in the carcinomas.
Due to limited sample size, we do not have enough power to explain, whether the differences between the investigated groups are real or by chance.

Discussion

Currently, the adult intestinal stem cell marker Lgr5 (GPR49) [5] is one of the most promising candidates to mark cancer stem cells or at least stem cell like cells in colorectal cancer [6]. In this study, we investigated 23 single nucleotide polymorphisms (SNP) of the *Lgr5* gene in primary and metastatic colorectal cancer to determine the stability of a germline genotype during metastasizing, its relationship to immunohistochemical Lgr5 expression and its prognostic impact.

The variant allele frequencies found in healthy controls and non-neoplastic tissues of the pa-

tients (germline genotypes) were mainly within the range of frequencies published in the NCBI database [13], so far reference data were available. However, the frequency of allelic variation in the non-coding SNPs rs796518 and rs7307225 was currently more than 10% higher compared to the reference frequencies. These discrepancies could be due to differences in size and composition of the investigated populations. Recently, the type 2 diabetes SNP rs7961581 (*TSPAN8/LGR5*) was found to be associated with colorectal cancer risk [18], but there was no evidence for this association in our database. Surprisingly, we registered a lower variant allele frequency assigned to SNP rs10879303 in colorectal cancer patients compared to healthy individuals, which could be a possible hint at a protective function of this SNP against colorectal cancer. However, our analysis was primary not designed as case-control-study and the healthy individuals were only included to obtain reference data for those SNPs with unknown (i.e. so far not published) frequency in normal populations. Considering this fact and noting that the significant result is derivate from Hardy-Weinberg-Equilibrium, the possibility of sampling bias cannot be excluded. Nevertheless, it might be worthy to elucidate the association of this SNP with colorectal cancer by future large cohort studies.

In primary colorectal carcinomas, germline and somatic Lgr5 genotype were exactly the same. The absence of additional mutations in high stage carcinomas can hint at stability in a putative cancer stem cell associated gene during carcinogenesis. We can only speculate, but not prove, whether this result reflects the ability of stem cell like cancer cells to maintain their genomic integrity by strategies as for example postulated in the "immortal strand hypothesis" [19] for normal stem cells. However, in the context of cancer, this hypothesis might be contradictive to the idea that cancer stem cells originate from normal stem cells, which have accumulated oncogenic mutations [20]. Regardless of which theory is right, a possibly existing mechanism to protect genomic integrity must be vulnerable in both, normal and neoplastic stem cells, because Greger *et al*. [9] could demonstrate germline genetic variability in a panel of colonic putative cancer stem cell associated genes as we did for *Lgr5*, and additionally, we detected at least few mutations (or loss of heterozygosity) at the *Lgr5* locus in the metastases analyzed here.

It is assumed that DNA sequence variations may alter the gene function and/or its activity including transcription, translation or splicing [10]. The current study could demonstrate that primary colorectal carcinomas (CRC) with allelic variation in the *Lgr5* gene showed a significant lower immunohistochemical Lgr5 expression than primary tumors with wild type *Lgr5* gene. More detailed analysis revealed that this result has to be attributed to the panel of non-coding SNPs included in this study, because only primary CRC with variant alleles in non-coding SNPs showed significantly different immunohistochemical Lgr5 expression compared to *Lgr5* wild type tumors. One explanation for this result could be that these type of DNA sequence alterations might impair splicing, alter DNA structures and influence interaction of Lgr5 with components, which are assumed to control its expression, for example Wnt, c-myc, p21CIP1/WAF1/CDKN1A, hsa-mir-23a/b, Gls [21] or members of other pathways as Hedgehog [22]. The combination of these impairments and dysregulations could have a stronger effect on Lgr5 expression than alteration of coding DNA sequences.

Performing correlation analysis between *Lgr5* genotype and immunohistochemical Lgr5 expression on metastatic colorectal cancer, lymph node metastases seem to reflect tendentiously the results found for primary tumors, whereas the relationship between these two parameters was inversely in distant metastases. However, in contrast to primary tumors, these results were not statistically significant in metastases. Interestingly, this relationship was inversely again in distant metastases compared to lymph node metastases, when evaluating Lgr5 expression in metastases with and without additional *Lgr5* gene alteration compared to the corresponding primary tumors. Even if we have to consider small sample size as an important factor for these diverging results in metastatic tissue, a biological background cannot be excluded. Recent investigation figured out that *Lgr5* is silenced by CpG island methylation during progression of tumorigenesis, but can be reexpressed [23]. It can be hypothesized that the biological consequences of allelic variation could depend on the silencing status of *Lgr5*, which might be different in colorectal primary tumors, lymph node metastases and distant metastases. In addition, allelic alteration could possibly also affect the mechanism of silencing

or reactivating of the *Lgr5* gene.

Tumorigenesis and the metastatic process depend probably on different Lgr5 functions, which could be variable suppressed or stimulated by silencing or reactivation of *Lgr5.* This could be one reason for our finding that a variant *Lgr5* genotype showed tendentiously different impact on survival depending on its occurrence in primary tumors, lymph node metastases or distant metastases, respectively. A recent study on colorectal cancer cell lines [24] demonstrated that ablation of Lgr5 induces increased invasion and anchorage-independent growth, and enhances tumourigenicity in xenografts experiments. Conversely, overexpression of Lgr5 augments cell adhesion, reduces clonogenicity and attenuates tumourigenicity. Assuming that patients die from growing metastatic tumors, ineffective silencing of *Lgr5* due to allelic variation and consecutive reduced tumor growth could be a possible explanation, why patients with variant alleles in distant metastases seem to have better survival than patients with wild type alleles in distant metastases. The association between silenced Wnt target genes (including *Lgr5*) and good prognosis due to reduced tumor growth has recently been discovered [23]. However, size of metastases is usually not the limiting factor for survival in patients with lymph node metastases. Therefore, impaired control of other *Lgr5* functions by allelic alteration might be responsible for differences in survival between patients with *Lgr5* variant genotype and patients with *Lgr5* wild type in their lymph node metastases. Assuming that in patients with regional lymph node metastases hematogenic metastatic spread has already taken place, reduced cell adhesion due to impaired *Lgr5* function caused by allelic alteration could favor the hematogenic metastatic process with its disadvantages for survival. However, we were not able to prove this theory in our small cohort of analyzed lymph node metastases, because all patients with *Lgr5* variant alleles in these lymph node metastases showed less evidence of synchronous or metachronous metastases (i.e. relapse) (50%) than patients with wild type *Lgr5* (71.4%).

Even if our survival data were not statistically significant, they hint at a possible prognostic importance of *Lgr5* DNA sequence alteration and further research in this direction could be useful. A recent study on germline polymorphisms in putative cancer stem cell genes [9] figured out that the minor allele rs17109924 T>C was significantly associated with increased time to tumor recurrence. In accordance with this discovery, we noted also longer time to tumor recurrence for patients with *Lgr5* variant alleles in primary colorectal cancer compared to patients with *Lgr5* wild type primary tumors. However, the analytic power of our prognostic results was low due to small sample size in the investigated groups.

In conclusion, our data indicate that *Lgr5* allelic variation affect Lgr5 protein expression in colorectal carcinomas. The somatic *Lgr5* genotype seems to be relatively stable in primary tumors, but becomes vulnerable during the metastatic process of colorectal cancer. This instability has possibly prognostic importance, which needs to be further evaluated by large cohort studies.

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