Cancer stem cell marker expression and methylation status in patients with colorectal cancer

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Abstract. The number of individuals diagnosed with colorectal cancer (CRC) has been on an alarming upward trajectory over the past decade. In some countries, this cancer represents one of the most frequently diagnosed types of neoplasia. Therefore, it is an important demand to study the pathology underlying this disease to gain insights into the mechanism of resistance to treatment. Resistance of tumors to chemotherapy and tumor aggressiveness have been associated with a minor population of neoplastic cells, which are considered to be responsible for tumor recurrence. These types of neoplastic cells are known as cancer stem cells, which have been previously reported to serve an important role in pathogenesis of this malignant disease. Slovakia has one of the highest incidence rates of CRC worldwide. In the present study, the aim was to classify the abundance of selected stem cell markers (CD133, CD166 and Lgr5) in CRC tumors using flow cytometry. In addition, the methylation status of selected genomic regions of CRC biomarkers (*ADAMTS16, MGMT, PROM1 (CD133), LGR5 and ALCAM*) was investigated by pyrosequencing in a cohort of patients from Martin University Hospital, Martin, Slovakia. Samples from both primary tumors and metastatic tumors were tested. Analysis of DNA methylation in the genomic regions of indicated five CRC biomarkers was also performed, which revealed the highest levels of methylation in the *A disintegrin and metalloproteinase with thrombospondin motifs 16* and *O6-methyguanine-DNA methyl transferase* genes, whereas the lowest levels of methylation were found in genes expressing *prominin-1*, *leucine-rich repeat-containing G-protein-coupled receptor* 5 and *activated leukocyte cell adhesion molecule*. Furthermore, tumor tissues from metastases showed significantly higher levels of $CD133^+$ cells compared with that in primary tumors. Higher levels of $CD133^+$ cells correlated with TNM stage and the invasiveness of CRC into the lymphatic system. Although relatively small number of samples was processed, CD133 marker was consider to be important marker in pathology of CRC.

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Introduction

Colorectal cancer (CRC) is a prevalent neoplastic disease (1). The incidence of patients with CRC aged <50 years has increased worldwide substantially over the past decade (1). The 5-year overall survival rate from CRC ranges from 90% if diagnosed at early stages), which declines to 10% if diagnosed at late metastatic stages (2). Therefore, earlier detection of this disease is paramount. However, difficulty remains in achieving this due to the late manifestation of symptoms from CRC (1). Therefore, enhancing the understanding into the pathological mechanism of colorectal cancer coupled with optimized national screening programs would serve a key role in facilitating the early detection of CRC.

Epigenetic changes, in particular DNA methylation, has been reported to serve a vital role in colorectal carcinogenesis (3) probably through modification of gene expression. Aberrant DNA methylation regulates the carcinogenesis of CRC, since

unmethylated CpG islands located in the gene can become strongly methylated (3,4). Previous studies have proposed the concept of the involvement of the colon cancer stem cells (cCSCs) during the early stages of carcinogenesis and during relapse (5). cCSCs are multipotent neoplastic cells that can regulate tumor growth, recurrence (6) and, in some cases, resistance to chemotherapeutic agents (7). Detection of CSCs is mostly performed by investigating the expression profile of surface CD markers. In CRC, several CD markers have been previously documented to be relevant for cancer stem cell identification: CD133, CD166 and leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5). Although CD133 expression is an important parameter for the identification and characterization of CSCs (8), its functional role in CRC physiology remains unclear. CD133 can activate the Wnt/β-catenin pathway, which lead to cancer cell proliferation (9,10). A number of studies have attempted to assess the role of CD133 (8-10). However, doubts remain regarding the association between CD133 and tumor recurrence (7), tumor size and tumor differentiation (8). Tumors with high expression levels of CD133 were found to be more likely to be resistant to standard chemotherapy (11). By contrast, it remains controversial whether CD133 expression can be used as an indicator for liver metastasis and overall survival (6,12-15). It was also previously reported that methylation of CpG islands in the *CD133* promotor region has significant effects towards protein expression on primary tumors in colorectal cancer, GIST stromal tumors and glioblastomas (16-18). Therefore, further studies are required for the characterization of CD133 function in cancer.

CD166 [(activated leukocyte cell adhesion molecule (ALCAM)] is a transmembrane type-1 glycoprotein (19). Due to its adhesive properties, it has been previously associated with CRC tumor growth (19). Several studies have revealed the role of CD166 in CRC stem cells (20,21). Detection of CD166, epithelial cellular adhesion molecule (EpCAM) and CD44 expression together was shown to be viable for identifying colorectal cancer stem cells more precisely (22) compared with to other markers, such as CD133. Other studies have also suggested that CD166 can be used as another stem cell marker in cancer stem cells from various types of cancer e.g., colorectal, breast, prostate and non-small cell lung cancer (20-23). The *ALCAM* gene also harbors several CpG islands that can be regulated by DNA methylation, which appear to represent the typical mechanism involved in repressing the expression of stemness markers in non-stem cancer cells (24). CD166 has been previously shown to be an adequate marker for CSCs from various types of malignancies, including digestive (gastric, pancreatic) and non-digestive cancers, where they are epigenetically regulated (25). However, the role of CD166 in CRC remains unclear due to inconclusive results reported by a number of previous studies (23,26-28).

Leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) belongs to the G protein-coupled receptors family and is located on the surfaces of intestinal stem cells (29). In total, ~80% CRC tissues express Lgr5 (17). In addition, the expression of *Lgr5* could be found in both tumor and normal colorectal tissues (17), the overexpression of which has been associated with advanced stages of CRC, distant metastases, 5-fluorouracil resistance and recurrence (30-32). Lgr5 appears to be a possible predictor of advanced CRC (33), such that high levels of Lgr5 expression at stage IV CRC have been associated with poor prognosis (34,35). The expression of Lgr5 is tightly regulated by methylation in its promotor region (36). A previous study has reported that positive *Lgr5* methylation is inversely associated with higher tumor grades and invasiveness, which is consistent with the observations that the overexpression of *Lgr5* is associated with the severity of malignancy and CRC invasiveness (36).

O6-methyguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme that removes mutagenic and cytotoxic adducts from O6-guanine molecules in the DNA (37,38). Hypermethylation of this gene can be used as a clinical biomarker for the early diagnosis and prognostic assessment of patients with CRC (37,38). Hypermethylation of its promoter region results in the silencing of *MGMT* gene, which has been previously observed in various types of CRC (37-39). CRC-associated adenomas are predicted to have worse prognosis, whilst hypermethylation in the *MGMT* gene in malignant CRC is associated with favorable therapeutic responses following treatment with alkylating agents (39). The A disintegrin and metalloproteinase with thrombospondin motifs (*ADAMTS*) family of proteins is a key component of CRC and other epithelial cancer carcinogenesis. Information on the effects of *ADAMTS16* hypermethylation on CRC remain scarce, though a previous study has suggested that DNA methylation at its CpG loci can be found in CRC and other epithelial tumors, which reduces its expression (40).

Therefore, the aim of presented study was to determine the abundance of selected stem cell markers (CD133, CD166 and Lgr5) on the surfaces of CRC cancer cells isolated from surgically resected colorectal tissues from patients using flow cytometry. In addition, the methylation status in the promoter regions of selected genes (*ADAMTS16, MGMT, PROM1, LGR5* and *ALCAM*) was determined. A secondary aim of the present study was to determine the statistical relationship among the expression levels of stem cell markers and their methylation status, grade (G), TNM staging (TNM), lymphatic (L), perineural (Pn) and venous (V) invasion and other factors (e.g., sex, age, tumor sidedness).

Materials and methods

Patients and tissue specimens. Primary (PT) and metastatic tumor (MTS) samples were obtained during surgical resection from 30 patients with diagnosed CRC in collaboration with The Department of Pathological Anatomy and Clinic of Surgery and Transplant Center, Martin University Hospital, Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin (Martin, Slovakia). Sample collection and processing protocols were reviewed and approved by The Ethics Committee of Jessenius Faculty of Medicine in Martin (approval no. EK 1856/2016). All patients signed the informed consent document prior to surgery. The patient's clinical protocols were reviewed for clinical data, diagnosis, sex and age (Table I). A total of 30 samples [sex, 17 males and 13 females; origin, 21 PT and 9 MTS, age, 68.7±9.7 years (mean ± SD)] were analyzed by flow cytometry and pyrosequencing. Tissue samples were collected between April 2018 and June 2020. Inclusion criteria for recruitment of patients into the study were i) diagnosis [colorectal cancer (stage I-IV)], ii) written informed consent and ii) the

Table I. Patient characteristics.

Parameter	N (%)
Sex	
Male	17 (56)
Female	13 (44)
Age	
MTS	9 (30)
PT	21 (70)
Side of primary tumor	
R	11 (37)
L	10 (33)
N/A (metastasis)	9 (30)
TNM stage	
1	7 (23)
2	4 (14)
3	9 (30)
4	10 (33)
Grade	
Low	22 (73)
High	8 (27)
Lymphatic invasion	
No	14 (47)
Yes	16 (53)
Venous invasion	
No	20 (67)
Yes	10 (33)
Perineural invasion	
No	24 (80)
Yes	6 (20)
Mismatch repair	
Proficient	19 (63)
Deficient	5 (17)
N/A	6 (20)

Data are presented as N (%).

tissue sample size, provided by pathologist [tumors >0.5 cm (or 5 grams of tissue) were processed].

Histology. Paraffin blocks were generated from the CRC tissues and fixed in 10% formalin. (room temperature, 24 h). Tissue was dehydrated by passing the tissue through series of alcohol [70% ethanol (1x), 96% ethanol (4x) and xylene (3x)] and finally placed in warm paraffin wax. Tissue sections (5 μm) rehydrated with decreasing strengths of alcohol and finally with water were then submitted for hematoxylin-eosin staining. The hematoxylin nuclei staining step (8 min, room temperature) was followed by washing with tap water and then distilled water. The histology sections were counterstained with 1% eosin alcoholic solution (1 min, room temperature) and washed again. The slides were then observed and images were recorded using a BX53 light microscope (Olympus Corporation). Representative images are shown in Fig. 1A-C, showing primary colonic adenocarcinoma with (A) cribriform (B) papillary and (C) tubular/tubulocystic growth pattern with typical 'dirty' necrosis and peritumoral desmoplastic reaction. Fig. 1D-F shows metastatic colorectal adenocarcinoma in liver with 'high-grade' architectural morphology (D) glandular structures with abortive luminas, (E) solid trabecular growth pattern and 'high-grade' histocytologic morphology (F) enlarged pleomorphic nuclei with high nucleus/cytoplasmic ratio and hyperchromasia. Scale bars represent 100 μm.

Single cell suspension preparation. After surgical resection, the tumors were transferred to the Department of Pathological Anatomy and dissected for histological evaluation. Small pieces (~0.5 cm) of tissue were stored in RNAlater buffer (cat. no. AM7020; Thermo Fisher Scientific, Inc.) at -80˚C for future DNA analysis. Any residual tissues were placed into a 50-ml Falcon tube containing DMEM/F-12 + GlutaMAX™ medium (cat. no. 10565-018; Gibco; Thermo Fisher Scientific, Inc.) supplemented with Penicillin-Streptomycin (cat. no. 15070063; Gibco; Thermo Fisher Scientific, Inc.) and 10% FBS (cat. no. 10082-147, Gibco; Thermo Fisher Scientific, Inc.) and stored at 4˚C until further analysis. Before analysis, tumor samples were sterilized with 30-50% ethanol for 10 sec (at room temperature) as described previously (41) and processed further in a sterile environment (BSL-2 laminar hood). Briefly, tumor samples were washed with sterile PBS, pH 7.4 (cat. no. 10010-031; Gibco; Thermo Fisher Scientific, Inc.) and ~5 g tissue was minced into smaller pieces (1.5-3.5 mm^3) with a surgical razor and incubated for 1 h at 37˚C in 1X Hank's balanced salt solution (cat. no. 14025-050; Gibco; Thermo Fisher Scientific, Inc.) containing 1 mg/ml collagenase, type IV (cat. no. 17104019; Gibco; Thermo Fisher Scientific, Inc.). After incubation, the single cell suspension was isolated with trituration using a 10-ml Pasteur pipette and filtered through a sterile 70-μm nylon cell MACS Smart Strainer (cat. no. 130-098-462; Miltenyi Biotec GmbH), washed with 10 ml 1X PBS and centrifuged at 400 x g (room temperature) for 3 min. Cell count was then performed using a TC10 Automated Cell Counter (Bio-Rad, Laboratories, Inc.) and ~$1x10^6$ -$3x10^6$ of cells were aliquoted for freezing. The cell suspensions were then frozen in 1 ml freezing medium (culture medium containing 10% DMSO) and inserted into a Mr. Frosty cell freezer before being stored at -80˚C until flow cytometry analysis (Table SI).

Flow cytometry analysis. After the collection of samples, frozen aliquots were quickly defrosted, washed 2X with sterile PBS and counted. Cells were first blocked using a FACS buffer [PBS, pH 7.4; 0.001 M EDTA; and 5% mouse serum (cat. no. ab7486, Abcam)] for 30 min on ice. After blocking, ~100,000 primary cancer cells were again re-suspended in 100 μl FACS buffer, transferred into 5-ml FACS tubes and stained with fluorescently-labeled antibodies [PE-anti-human Lgr5 (GPR49) antibody, PE-anti-human CD166 antibody and APC-anti-human CD133/2 antibody, see Table SI for manufacturers] according to manufacturer's protocols for 1 h on ice in the dark. Cells that were not stained served as negative controls and $1x10^6$ cells/test were used (1 test was equal to 100 μl of cell suspension, for the amount of antibody/test

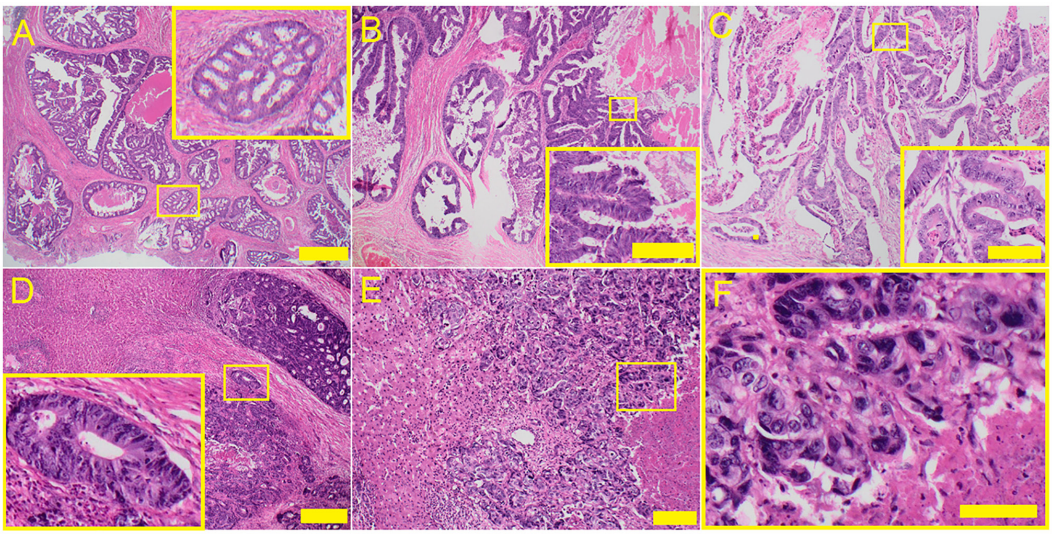

Figure 1. Pathological analysis. Primary colonic adenocarcinoma tissues with (A) cribriform, (B) papillary or (C) tubular/tubulocystic growth patterns, showing typical 'dirty' necrosis and peritumoral desmoplastic reactions. (D-F) Metastatic colorectal adenocarcinoma in the liver with 'high-grade' architectural morphology. (D) Glandular structures with abortive luminas. (E) Solid trabecular growth pattern and 'high-grade' histocytologic morphology. (F) Enlarged pleomorphic nuclei of (E) with high nucleus/cytoplasmic ratio and hyperchromasia. Scale bars, 100 μm.

see Table SI). Subsequently, cells were washed with PBS and analyzed using the BD FACSARIA II flow cytometer (BD Biosciences) and processed using the BD FACSDiva software v9.0 (BD Biosciences). Cell debris and dead cells were excluded from measurement by gating and 7-Aminoactinomycin D staining. Fig. S1 shows the representative histograms from the flow cytometry analyses of isolated tumor cells. Data from flow cytometry analyses were uploaded to Mendeley (https://data.mendeley.com/datasets/vknp983sbf/1). High levels of auto-fluorescence is common for primary cancer cells. Therefore, to avoid artifacts associated with fluorescence bleed-through into non-specific channels, single-factor (CD marker) measurements were performed per test.

Extraction of DNA and bisulfite treatment of DNA. From the total of 30 CRC samples, three samples were excluded due to insufficient DNA concentration (Table II). Nucleic acids were extracted using a DNase Blood and Tissue kit (Qiagen GmbH) according to the manufacturer's protocol. Subsequently, 1-2 μg DNA was bisulfite-treated using an EpiTect Bisulfite kit (Qiagen, Inc.) and ≤1 μg DNA was used in a total reaction volume of 20 μl. Previously described protocols were then followed (42). DNA was placed into a thermal cycler with the following program: denaturation (95˚C, 5 min), incubation (60˚C, 25 min), denaturation (95˚C, 5 min), incubation (60˚C, 85 min), denaturation (95˚C, 175 min), incubation (60˚C, 25 min), incubation (20˚C). After bisulfite modification (in accordance to manufacturer's protocol), genomic DNA was stored at -20˚C until PyroMark PCR analysis.

Pyrosequencing and CpG assays. Bisulfite-converted DNA was amplified using the PyroMark PCR kit (Qiagen, Inc.) in accordance with manufacturer's protocol. For the analysis of the selected regions of *CD133* (4 CpG), *CD166* (4 CpG), *LgR5* (4 CpG), *ADAMTS16* (3 CpG) and *MGMT* (7 CpG), commercially available CpG assays [PyroMark CpG Assay (200), URL address:https://geneglobe.qiagen.com/product-groups/pyromark-cpg-assays: Hs_PROM1_05_PM (cat. no. PM00110194), Hs_ALCAM_03_PM (cat. no. PM00108717), Hs_LGR5_01_PM (cat. no. PM00052416), Hs_ADAMTS16_01_PM (cat. no. PM00022106), Hs_MGMT_01_PM (cat. no. PM00149702)] were used. Protocol and PCR reaction conditions were as follows: DNA polymerase activation (95˚C, 15 min), followed by a three-step cycle of denaturation (94˚C, 30 sec), annealing (56˚C, 30 sec) and extensions (72˚C, 30 sec), a process repeated 45 cycles in a row. The final extension was carried out (72˚C, 10 min).

PCR products were visualized by electrophoretic analysis (1.75% agarose gel) under UV light. Analyses were conducted according to the manufacturer's protocol, which was described previously (42). Data (% methylation for the indicated gene) from analyses were uploaded to Mendeley (https://data.mendeley.com/datasets/vknp983sbf/1). Completely methylated and unmethylated DNA were used as control samples (EpiTect Control DNA, methylated (cat. no. 59655)/EpiTect Control DNA, unmethylated (cat. no. 59665); Qiagen GmbH.

Statistical analysis. The data were explored and analyzed in R ver. 4.0.5 (43). The clinicopathological parameters were presented as N (%), whereas continuous variables were presented as the median (lower and upper quartiles). The null hypothesis of independence between two categorical factors was tested using Fisher's exact test. The null hypothesis of equality of the population medians was tested using the Wilcoxon-Mann-Whitney test. Kruskal-Wallis test was used if there were more than two populations. In cases of significance, Dunn's post hoc test was used. $P<0.05$ was considered to indicate a statistically significant difference, whereas $0.05< P<0.1$ was considered to indicate a weakly significant difference. The tests were two-sided. If the P-value from the two-sided alternative testing was <0.05, the direction (increase, decrease) was reported.

Random forest (RF) data analysis. The discriminative ability of the studied markers for discriminating between

Table II. Patient characteristics after the exclusion of three samples due to insufficient DNA concentration.

Parameter	N (%)
Sex	
Male	16 (60)
Female	11 (40)
Age	
MTS	8 (30)
PT	19 (70)
Side of primary tumor	
R	10 (37)
L	9 (33)
N/A (metastasis)	8 (30)
TNM Stage	
1	6 (22)
2	4 (15)
3	8 (30)
4	9 (33)
Grade	
Low	19 (70)
High	8 (30)
Lymphatic invasion	
No	11 (41)
Yes	16 (59)
Venous invasion	
No	17 (63)
Yes	10 (37)
Perineural invasion	
No	22 (81)
Yes	5 (19)
Mismatch repair	
Proficient	16 (60)
Deficient	5 (20)
N/A	6 (22)

PT and MTS was assessed using the RF machine learning algorithm as implemented in R library randomForestSRC (https://luminwin.github.io/randomForestSRC/). RF was trained without TNM staging. Predictors were then ranked by the variable importance measure (vimp); see Fig. S2B. The predictive power of the trained RF was measured using the receiver operating characteristic curve (see Fig. S2A) obtained from the Out-Of-Bag data and quantified by the area under curve (AUC). Due to the imbalanced representation of the two groups, the imbalanced RF was used.

Results

Flow cytometry. Expression of CD133, CD166 and Lgr5 markers was detected with fluorescent antibodies by flow cytometry on the surface of the cells isolated from tumor samples in a cohort of patients diagnosed with CRC and

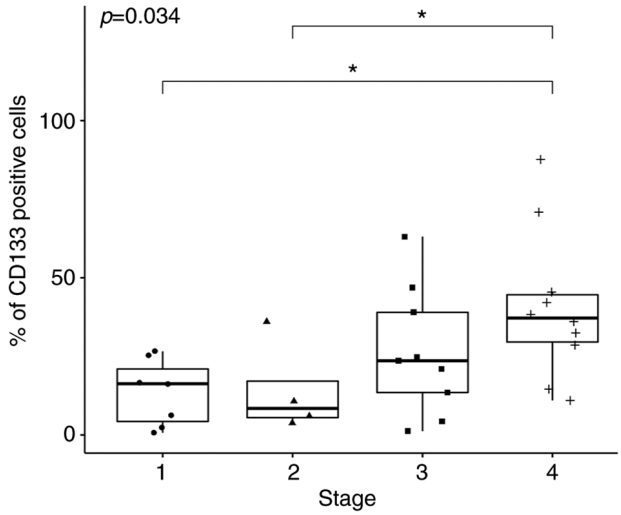

Figure 2. CD133 expression in the cells among the different TNM stages. The boxplot was overlaid with swarmplot. *P<0.05 (Dunn's post hoc test).

the percentage of the cells positive for these markers was recorded. All 30 tissue samples were analyzed for the expression of CD133 and Lgr5 markers. However, only 24 samples were analyzed for the expression of CD166, due to the insufficient number of cells in the six clinical samples. Tumor cells showed the highest average rate of CD166 (63. 7% of positive cells), followed by Lgr5 (31%) and CD133 (26.5%). For Lgr5 and CD166 expression, the difference were not found to be significant between the MTS and PT subgroups. Kruskal-Wallis test of the median expression of CD133 at stages 1, 2, 3 and 4 [Stage 1 represents (T1-T2, N0 and M0), Stage 2 (T3-T4,N0,M0), Stage 3 (any T, N1-N2, M0) and Stage 4 (any T, any N, M1) on TNM scale] revealed significance (P=0.034; Fig. 2). In total, two of the Dunn's post hoc comparisons revealed statistical significance. Specifically, expression of CD133 at Stage 4 was significantly higher compared with that at Stage 1 (P=0.01) whereas the median expression in Stage 4 was significantly higher compared with that in Stage 2 (P=0.03; Fig. 2; Table III). For Lgr5 and CD166, the ANOVA null hypothesis was not rejected. Using the Wilcoxon-Mann-Whitney test with the one-side alternative, the percentage of CD133, CD166 and Lgr5 positive cells in all samples was compared between PTs and MTS. The percentage of $CD133^+$ cells (Fig. 3A) was found to be significantly higher in MTS compared with that in PTs (P=0.007). For CD166 and Lgr5 (Fig. 3B and C), although percentage of positive cells was also markedly higher in MTS compared with that in PTs, statistical significance could not be reached (Fig. 3B and C). Subsequently, using the Wilcoxon-Mann-Whitney test, the median expression of all three markers was compared within each category of grade (low vs. high), lymphatic invasion (yes vs. no), venous invasion (yes vs. no), perineural invasion (yes vs. no) and sidedness of tumor (right vs. left). CD133 showed statistical significance within the lymphatic invasion category (P=0.019; Fig. 3D). A weak statistical significance was found for $CD166^+$ cells within the grade category (P=0.087), sex compared with venous invasion (P=0.017), sex compared with sidedness of tumor (P=0.008) and venous invasion compared with

Table III. P-values from the Dunn's post hoc comparisons of CD133 expression, *CD133* DNA methylation and MGMT DNA methylation among the four TNM stages.

TNM stage comparison	CD133 expression (flow cytometry)	*CD133* DNA methylation	*MGMT* DNA methylation
1 vs. 2	0.96	0.06	0.02
1 vs. 3	0.21	0.05	0.83
1 vs. 4	0.01	0.01	0.06
2 vs. 3	0.27	0.86	0.03
2 vs. 4	0.03	0.82	0.44
3 vs. 4	0.16	0.61	0.07

MGMT, O6-methyguanine-DNA methyltransferase.

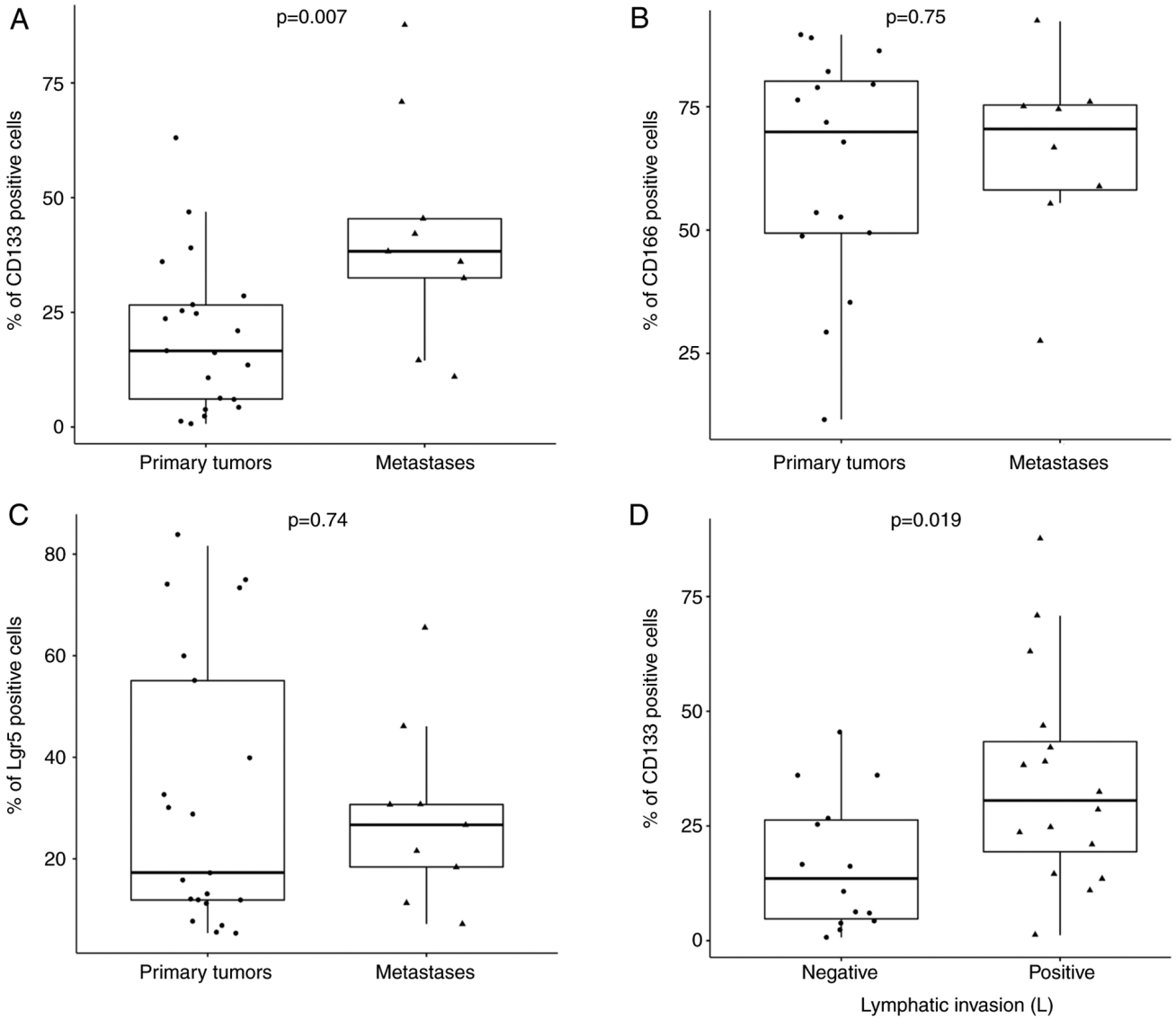

Figure 3. Comparison of marker expression between primary tumor and metastatic tumor in addition to between the presence and absence of lymphatic invasion. Boxplots were overlaid using swarm plot. (A) CD133, (B) CD166 and (C) Lgr5 expression in MTS and PT samples. (D) CD133 expression between patients showing tumor propagation in lymphatic vessels and those negative for this. Lgr5, leucine-rich repeat-containing G-protein-coupled receptor 5.

lymphatic invasion (P=0.058). However, for the other factors the differences in the median expression were not statistically significant (Table IV). For the comparison of CD166 and Lgr5 with the TNM staging category, one-way ANOVA test was used. However, for these markers (CD166 and Lgr5) no statistical significance could be detected. As mentioned

Table IV. Comparisons of each marker among each clinicopathological parameter.

A, Grade

				Venous invasion		Sex	
Category	CD133	CD166	Lgr5	Positive	Negative	Male	Female
Low (n=22)	19 (11,38)	67 (49,76)	20 (11,32)	8 (36)	14 (64)	13 (59)	9 (41)
High (n=8)	26 (19,37)	79 (64,86)	38 (15,56)	2 (25)	6 (75)	4 (50)	4 (50)
P-value	0.8	0.087	0.3	0.7		0.7	

B, Lymphatic invasion

						Sex	
Category	CD133	CD166	Lgr5	Venous invasion		Male	Female
Negative (n=14)	14 (5,26)	70 (50,76)	15 (8,32)	2 (14)	12 (86)	7 (50)	7 (50)
Positive (n=16)	31 (19,43)	71 (54,85)	29 (15,53)	8 (50)	8 (50)	10 (62)	6 (38)
P-value	0.026	0.6	0.12	0.058		0.5	

C, Venous invasion

					Sex	
Category	CD133	CD166	Lgr5	Venous invasion	Male	Female
Negative (n=20)	24 (10,37)	73 (50,81)	23 (11,48)	-	8 (40)	12 (60)
Positive (n=10)	25 (12,37)	63 (53,76)	24 (14,31)	-	9 (90)	1 (10)
P-value	>0.9	0.6	0.8	-	0.017	

D, Perineural invasion

						Sex	
Category	CD133	CD166	Lgr5	Venous invasion		Male	Female
Negative (n=24)	25 (11,40)	68 (49,78)	20 (11,41)	7 (29)	17 (71)	14 (58)	10 (42)
Positive (n=6)	22 (8,30)	75 (59,80)	29 (19,63)	3 (50)	3 (50)	3 (50)	3 (50)
P-value	0.5	0.4	0.4	0.4		>0.9	

E, Sidedness of tumor

						Sex	
Category	CD133	CD166	Lgr5	Venous invasion		Male	Female
Right (n=11)	21 (9,32)	78 (64,81)	33 (13,67)	4 (36)	7 (64)	8 (73)	3 (27)
Left (n=10)	16 (3,24)	52 (45,74)	14 (9,30)	1 (10)	9 (90)	1 (10)	9 (90)
P-value	0.3	0.4	0.14	0.3		0.008	

Data represents the N (%) or the median (interquartile range) within each clinicopathological category. Fisher's exact test or Wilcoxon-Mann-Whitney test (where normality was not tenable) with the one-side alternative were used for categorical and continuous variables, respectively. Lgr5, leucine-rich repeat-containing G-protein-coupled receptor 5.

above, only CD133 was significantly associated with TNM staging. Representative flow cytometry diagrams for all groups quantified in corresponding graphs in Figs. 2 and 3 are shown in Fig. S1.

To assess the ability of the studied markers for discriminating between PT and MTS, the imbalanced RF machine learning algorithm was trained before the importance measure was used to rank the predictors. Since TNM staging appeared to be the most important predictor, another RF was trained without TNM staging. The discriminative ability of the RF without TNM staging was found to be substantially lower (AUC=64%, Fig. S2A). CD133 was found to be the most important predictor, followed by other factors, including Lgr5, tumor sidedness, ADAMTS16_1 and ADAMTS16_2, which had lower importance compared with CD133 (Fig. S2B). Furthermore, imbalanced RF with CD133, CD166, LGR5, sex and age were also trained.

Pyrosequencing. Samples treated with sodium bisulfite were used for pyrosequencing analysis. In the PCR reaction, cytosine was converted to uracil or thymine in the PCR product, whereas methylated cytosines remained unchanged. In this part of the study detection of methylated regions in the sequences of five genes, *ALCAM*, *PROM1*, *LgR5*, *MGMT* and *ADAMTS16*, were focused upon by pyrosequencing. The average percentage of methylation for *ALCAM* was found to be 3% (3% in CpG1, 2% in CpG2 and 4% in CpG3), whereas for *PROM1* it was 6.5% (4% in CpG1, 13% in CpG2, 3% in CpG3 and 6% in CpG4). For *Lgr5*, it was 6.25% (8% in CpG1, 6% in CpG2, 6% in CpG3 and 5% in CpG4). These three stem cell markers showed hypomethylation in their selected regions according to pyrosequencing. The average percentage of methylation for unmethylated and methylated DNA was 6.25% (unmethylated) and 76% (methylated) for *ALCAM*, 7.5% (unmethylated) and 88% (methylated) for *PROM1* and 8% (unmethylated) and 90% (methylated) for *LgR5*. The other two CRC biomarkers *MGMT* and *ADAMTS16* showed a degree of hypermethylation in their selected regions according to pyrosequencing analysis. The average percentage of methylation for *MGMT* was 22.14% (24% in CpG1, 20% in CpG2, 17% in CpG3, 18% in CpG4, 20% in CpG5, 21% in CpG6 and 33% in CpG7) and for *ADAMTS16* it was 75% (79% in CpG1, 72% in CpG2 and 74% in CpG3). The unmethylated and methylated DNA showed average methylation in *MGMT* to be 1 unmethylated) and 94% (methylated), and to be 3.3 (unmethylated) and 93% (methylated) in *ADAMTS16*, respectively. *ADAMTS16* methylation was found to be markedly higher in MTS samples compared with that in the PT subpopulations. In addition, *PROM1* methylation was significantly higher in the subpopulation without venous invasion compared with that in the subpopulation with venous invasion (P=0.049). *PROM1* methylation was also significantly higher in the subpopulation without perineural invasion compared with that in samples with perineural invasion (P=0.012). In the subpopulation without lymphatic invasion, *PROM1* methylation was markedly higher compared with that in the population with lymphatic invasion. For ALCAM methylation, only markedly higher levels were found in the subpopulation without lymphatic invasion compared with that in the subpopulation with lymphatic invasion. Similarly, markedly higher methylation levels of ALCAM were found in the subpopulation without perineural invasion compared with those in samples with perineural invasion. No statistical significance could be found for other categories (Table V).

Following the application of Kruskal-Wallis test on the median expression levels of *MGMT* at TNM stages 1-4, the null hypothesis was rejected (P=0.036; Fig. 4). In total, two of the Dunn's post hoc comparisons were statistically significant. Specifically, *MGMT* methylation at TNM stage 1 was statistically significantly higher compared with that at Stage 2 (P=0.02) whereas the extent of *MGMT* methylation at Stage 3 was significantly higher compared with that at Stage 2 (P=0.03; Table III). For *PROM1*, Kruskal-Wallis test of the four TNM stages only yielded a weak significance (P=0.070; Fig. 5). According to Dunn's post hoc test, the median methylation of *PROM1* at Stage 1 was significantly higher compared with that at Stages 3 (P=0.05) and 4 (P=0.01; Table III).

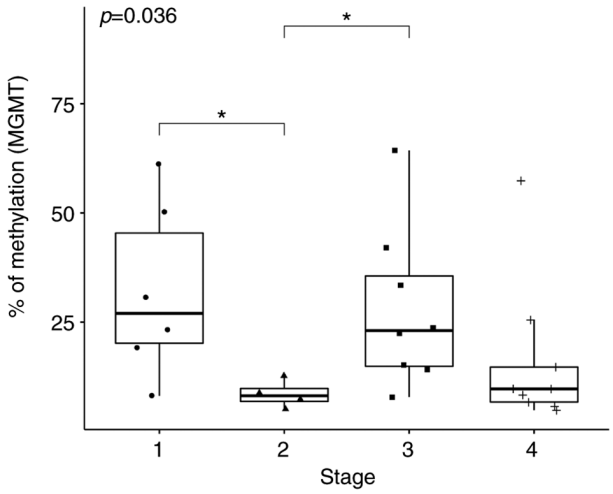

Figure 4. Comparison of MGMT methylation among the four TNM stages. The boxplot was overlaid using swarmplot. P-value is from Kruskal-Wallis test. *P<0.05 (Dunn's post hoc test). MGMT, O6-methyguanine-DNA methyltransferase.

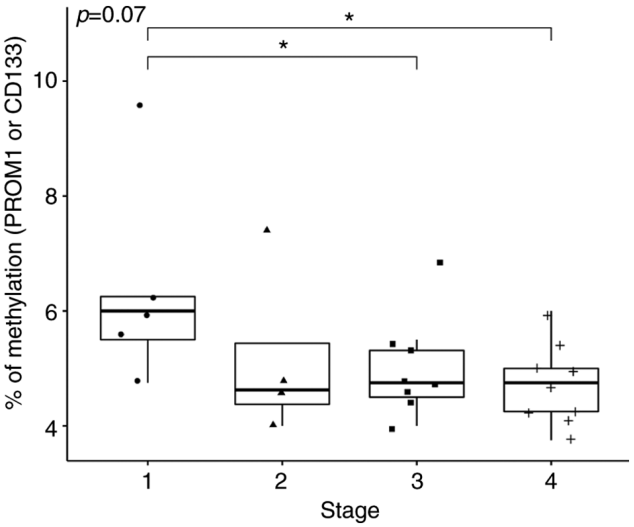

Figure 5. Comparison of CD133 methylation among the four TNM stages. The boxplot was overlaid with swarmplot. P-value is from Kruskal-Wallis test. *P<0.05 (Dunn's post hoc test). A gross outlier in stage 1 was removed from the plot.

Patients/tissue specimens. There was a significant association between Sex and the origin of the tumor (P=0.042; Table VI). The median population age was also found to be

Table V. Comparisons of each marker among each clinicopathological parameter.

A, Grade

Category	ALCAM	CD133	Lgr5	MGMT	ADAMTS16
Low (n=22)	3.00 (2.75,3.25)	5.00 (4.50,5.75)	6.00 (5.50,6.75)	15 (8,24)	77 (63,82)
High (n=8)	3.12 (2.69,3.38)	4.75 (4.44,5.25)	6.38 (5.88,7.06)	22 (9,46)	81 (77,86)
P-value	0.9	0.6	0.3	0.4	0.2

B, Lymphatic invasion

Category	ALCAM	CD133	Lgr5	MGMT	ADAMTS16
Negative (n=14)	3.25 (3.00,3.38)	5.50 (4.75,6.88)	6.25 (5.88,6.75)	13 (8,27)	78 (76,84)
Positive (n=16)	3.00 (2.75,3.25)	4.75 (4.25,5.31)	6.12 (5.50,7.00)	15 (9,27)	77 (63,83)
P-value	0.085	0.063	0.6	0.6	0.5

C, Venous invasion

Category	ALCAM	CD133	Lgr5	MGMT	ADAMTS16
Negative (n=20)	3.00 (3.00,3.25)	5.25 (4.75,6.25)	6.25 (5.50,7.00)	23 (8,42)	77 (73,83)
Positive (n=10)	2.88 (2.75,3.25)	4.62 (4.06,4.94)	6.00 (4.94,6.25)	11 (8,15)	80 (66,84)
P-value	0.6	0.049	0.2	0.1	0.7

D, Perineural invasion

Category	ALCAM	CD133	Lgr5	MGMT	ADAMTS16
Negative (n=24)	3.00 (2.81,3.25)	5.12 (4.75,6.00)	6.25 (5.56,7.00)	15 (8,25)	78 (68,83)
Positive (n=6)	2.75 (2.50,3.00)	4.25 (4.00,4.50)	5.50 (4.75,6.25)	14 (10,42)	81 (73,83)
P-value	0.078	0.012	0.2	0.8	>0.9

E, Sidedness of tumour

Category	ALCAM	CD133	Lgr5	MGMT	ADAMTS16
Left (n=11)	3.12 (3.00,3.25)	5.25 (4.75,6.62)	6.00 (5.31,6.50)	14 (10,23)	76 (61,79)
Right (n=10)	3.00 (3.00,3.25)	4.75 (4.50,5.50)	6.25 (5.75,6.25)	33 (15,50)	77 (73,79)
P-value	>0.9	0.6	0.6	0.11	0.6

Data are presented as the median (interquartile range). Wilcoxon-Mann-Whitney test was used. ALCAM, activated leukocyte cell adhesion molecule; lgr5, leucine-rich repeat-containing G-protein-coupled receptor 5; MGMT, O6-methyguanine-DNA methyltransferase; ADAMTS, A disintegrin and metalloproteinase with thrombospondin motifs 16.

significantly higher in the PT group compared with that in the MTS group (P=0.041; Table VI). Frequency distribution of tissues among the TNM stages was also significantly associated with that of tumor origin (P<0.001; Table VI). Sidedness of tumors (Left or Right) was significantly associated with sex (P=0.008; Table VI) and with MMR status (P=0.012; Table VI). In addition, the four TNM histological stages were found to significantly associate with sex (P=0.021; Table VII), with lymphatic invasion (P<0.001) and with venous invasion (P=0.048) but not with age (Table VII). TNM staging also associated significantly with the origin of tumor (P<0.001), CD133 (P=0.034) and MGMT (P=0.036) but not with PROM1 (P=0.070; Table VII).

Discussion

The main aim of the present study was to measure the expression of three previously reported cancer stem cell markers CD133, CD166 and Lgr5 in addition to assessing the methylation status of specific regions of selected genes *ALCAM, PROM1,*

Table VI. Assessment of association between origin of tumor and each clinicopathological parameter.

Parameter	Metastasis (n=9)	Primary tumor (n=21)	P-value	Right-side tumor (n=11)	Left-side tumor (n=10)	P-value
Sex			0.042			0.008
Male	8 (89)	9 (43)		8 (73)	1 (10)	
Female	1 (11)	12 (57)		3 (27)	9 (90)	
Age	68 (60,71)	73 (65,77)	0.041	73 (65,76)	74 (70,78)	0.5
Stage			<0.001			0.4
1	0 (0)	7 (33)		2 (18)	5 (50)	
2	0 (0)	4 (19)		3 (27)	1 (10)	
3	0 (0)	9 (43)		5 (45)	4 (40)	
4	9 (100)	1 (4.8)		1 (9.1)	0 (0)	
Mismatch repair			>0.9			0.012
Deficient	0 (0)	5 (24)		0 (0)	5 (50)	
Proficient	3 (100)	16 (76)		11 (100)	5 (50)	

Data are presented as either N (%) or the median (interquartile range). Fisher's exact test was used for comparing N (%) variables whereas Wilcoxon-Mann-Whitney test was used for comparing median (interquartile range) variables.

Table VII. Association between each parameter and TNM staging.

	TNM				
Parameters	1	2	3	4	P-value
Sex					0.021
Male	2 (29)	3 (75)	3 (33)	9 (90)	
Female	5 (71)	1 (25)	6 (67)	1 (10)	
Age	71 (64,76)	73 (70,76)	76 (71,79)	66 (61,70)	0.068
Origin of tumour					<0.001
Metastasis	0 (0)	0 (0)	0 (0)	9 (90)	
Primary tumour	7 (100)	4 (100)	9 (100)	1 (10)	
Lymphatic invasion					<0.001
Positive	0 (0)	0 (0)	8 (89)	8 (80)	
Negative	7 (100)	4 (100)	1 (11)	2 (20)	
Venous invasion					0.048
Positive	0 (0)	2 (50)	2 (22)	6 (60)	
Negative	7 (100)	2 (50)	7 (78)	4 (40)	

Data are presented as N (%) or the median (interquartile range). Fisher's exact test or Kruskal-Wallis test (for continuous variables) was used for comparison.

LgR5, MGMT and *ADAMTS1* in a cohort of 30 patients with CRC. According to the American Institute for Cancer Research (44), Slovakia ranks second in terms of the incidence of colon cancer worldwide, after Hungary. In addition, >1.93 million new cases were reported worldwide in 2020 (44). CRC remains to be one of the most common malignancies worldwide (44). Therefore, deepening the understanding of CRC physiology and the mechanism underlying CRC tumor growth and progression is in urgent demand for the development of novel therapies. A previous 'stochastic' model predicted that every cancer cell in the tumor is equally tumorigenic (17). However, subsequent studies have led to the establishment of a more hierarchical model of cancer, in particular highlighting the presence of a core population of CSCs (17,33,35). This model predicts that only specific cell types are responsible for tumor growth, such that the unsuccessful elimination of this cell population increases the risk of tumor relapse and formation of metastases (17). CSCs have been extensively studied, since they are considered to be the cancer cell population that is responsible for resistance to therapy and tumor recurrence. Several cell surface markers were previously identified alongside stem-like transcriptional factors for cCSCs (45-47). Their

expression was verified mostly on histological levels and their biological impact was supported *in vitro* using tumor sphere formation assays (48,49). However, CRC carcinogenesis is a complex process that is poorly understood. This process has been reported to include both genetic and epigenetic alterations, with DNA methylation serving a particularly key role in tumor growth and cancer progression (38,50). Therefore, studies into CRC on multiple molecular levels are required.

In present study, flow cytometry analysis of selected surface markers on tumor tissues isolated by surgery was performed, to measure their levels of expression in CRC. In addition, the extent of DNA methylation of five selected genes that have been previously documented to serve a role in CRC carcinogenesis was measured. The percentage of cells, positive for CD133, a known stem cell marker for colon CSCs (51), was found to be significantly higher in cells isolated from metastases compared with that in cells isolated from primary tumors. The percentage of $CD133^+$ cells detected in tumor tissue (primary or metastatic) was also higher compared with that reported previously (52). Although the role of CD133 was initially found to be controversial, a number of clinical studies reported its value as a possible prognostic marker for the survival rates of patients with CRC (14,53). However, more recently *CD133* was identified to be a pivotal if not one of the most prominent cCSC cell markers (53). Park *et al* (53) examined a cohort of 303 patients and found that the overall 5-year survival and disease-free survival were inversely associated with *CD133* expression, such that poorer survival was associated with higher levels of *CD133*$^+$ cells. In the present study, the percentage of $CD133^+$ cells expression also showed statistically significant differences between the two groups of samples from lymphatic invasion (samples with positive lymphatic invasion have significantly higher percentage of $CD133^+$ cells). Therefore, the nature of the regulation of CD133 expression on an epigenetic level was assessed. Although a statistically significant association could not be detected between *CD133* gene methylation and CD133 protein expression, the results did indicate that higher (3 and 4) stages of CRC were associated with increased expression levels of CD133 compared with those in stages 1 and 2. In addition, methylation of CD133 was significantly decreased from stages 1 to 4. Yi *et al* (18) previously suggested that the potential epigenetic mechanism involved in the regulation of *CD133* expression in CRC was by the dysregulation of DNA hypermethylation of the *CD133* gene in CRC cells. In another study, a high degree of *CD133* methylation was found in GIST48b and GIST882 cells by bisulfite pyrosequencing (16). This previous study also interrogated human gastrointestinal stromal tumor samples, which found lower mean *CD133* gene methylation percentages in primary tumors compared with those in the cell lines (16). However, further studies are necessary to verify this form of regulation of *CD133* expression, since other authors have also shown that $CD133^+$ cells lacked methylation on their corresponding promoter CpG islands, but were methylated in the cultured cell lines isolated from human tissues (18,54). According to study performed by Yi *et al* (18) hypermethylation patterns of CpG islands are preserved in cultured cells but are highly heterogeneous in intact tumors (18). Pellacani *et al* (54) revealed that CD133 expression is regulated by DNA methylation only in cell lines *in vitro*, where methylation of its promoter correlated inversely with gene expression (54). The relationship between DNA methylation and its dynamics is therefore a field that requires additional experimental exploration to gain further insights into its mechanism.

Differences in the expression of CD166, another marker that has been previously found to be associated with CSCs in CRC (19), was found to be greater, but not to a level of statistical significance between primary tumors and metastatic tumors. Similar results were obtained for Lgr5, another potential stem cell marker (55). Compared with results from previous studies that utilized flow cytometry for the detection of $Lgr5^+$ cells (51,55,56), higher levels of positive cells could be detected in all samples in the present study. Leng *et al* (56) previously showed that the tumorigenicity of isolated cancer cells was restricted to $Lgr5^+$ populations. In particular, the $Lgr5^+$ $CD44^+EpCAM^+$ cell population exhibited more characteristics typical of the CSC-like phenotype, as predicted from results from colony formation assays, tumor sphere formation, tumorigenicity and expression of stem cell markers (Lgr5, CD44, EpCAM), compared with those in other cell populations ($Lgr5^+CD44^+EpCAM^-$, $Lgr5^+CD44^-EpCAM^+$, $Lgr5^-CD44^+EpCAM^+$ and $Lgr5^-CD44^-$EpCAM-) within CRC (56).

Subsequently, the methylation status of two biomarkers, *MGMT* and *ADAMTS16*, which are frequently reported to be methylated in CRC (40,57,58), was examined. Several previous studies have reported that transcriptional silencing of the *MGMT* gene in various tumor types may be one of the causes of hypermethylation in the CpG islands in a specific promotor region (57-59). Hypermethylated *MGMT* genomic regions have been previously observed in adenomas, in addition to in the non-malignant colonic mucosa of patients with CRC, where it was found to be associated with more favorable therapeutic responses following treatment with alkylating agents (39,60). The present study found that the *MGMT* methylation fluctuated among the stages and no clear trend could be deduced even at higher stages. Lower methylation of this gene could be found at stages 2 and 4, however. Indeed, the prognostic role of *MGMT* remains controversial (38,59,61).

Cancer-specific promoter hypermethylation of *ADAMTS16* has also been proposed to be a viable biomarker for CRC (40). It is predicted that changes in DNA methylation contribute to the downregulation of *ADAMTS16* expression, which can result in development of CRC (40). However, further epigenetic analyses and functional studies of this component in CRC tumors are required.

To conclude, flow cytometry analysis of three cancer stem cell markers was performed in a cohort of 30 patients with CRC. Positivity for each of the three individual marker was found to be associated with age, sex, PTs and MTS, TNM stage, grade, presence of invasions (lymphatic, venous and perineural invasion) and the sidedness of tumor. Analysis of DNA methylation in the specified genomic regions of the five CRC biomarkers was also performed, which revealed the highest level of methylation in the *ADAMTS16* and *MGMT* genes whilst the lowest level of methylation was found in the *PROM1*, *Lgr5* and *ALCAM* genes. This decrease of methylation in the *CD133* gene from stages 1 to 4 was found to be associated with the trend of increased CD133 protein expression. Furthermore, tumor tissues from metastases showed significantly higher expression of CD133 protein compared

with that in primary tumors. Higher levels of $CD133^+$ cells were associated with TNM stage and the invasiveness of CRC into the lymphatic system. Although the significant limitation of the present study was the relatively small number of samples processed, CD133 marker can be considered to be important marker in pathology of CRC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JS, SM and ZL designed the study. SM, JS and KJ performed the experiments. MG conducted the statistical analysis. MK, JM, MV, RK, MP, JJ, PM, LL and EG obtained and handled colorectal specimens and clinical data. JS, SM, MG, ZL and EM analyzed the data and were major contributors in writing the manuscript. MK and JM performed the histological examination of the samples and clinical data. ZL, JS and EH supervised the entire study and participated in study design and coordination. SJ and MS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Sample collection and processing protocols were reviewed and approved by The Ethics Committee of Jessenius Faculty of Medicine in Martin (approval no. EK 1856/2016; Martin, Slovakia). All patients signed the informed consent document prior to surgery.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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