

Colonic Stem Cells Expression of Lgr5 and CD133 Proteins as Predictive Markers in Colorectal Cancer among Egyptian Patients

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Abstract

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AIM: Colorectal cancer is the fourth common tumour in Egypt after lymphoid, breast and urinary tumours. The study aims to assess the expression of Lgr5 and CD133 in pre-malignant (adenomatous polyps and IBD), malignant colorectal lesions and normal colonic mucosa by immunohistochemical staining.

MATERIAL AND METHODS: This prospective study was done on 100 patients presenting with colonic symptoms, patients were divided into four groups; group I including 20 patients in the control group, group II including 20 ulcerative colitis (U.C) patients, group III including 20 patients with adenomatous polyps and group IV including 40 patients with colorectal cancer (CRC).

RESULTS: Lgr5 and CD133 expression was significantly higher in carcinoma than in adenomas, IBD and normal mucosa ($P < 0.001$). Lgr5 and CD133 was positively correlated with histological grade ($P = 0.001$), depth of invasion ($P = 0.001$), lymph node metastasis ($P < 0.001$), distant metastasis ($P < 0.004$) and TNM stage ($P < 0.001$).

CONCLUSION: Role of Lgr5 and CD133 as stem cell marker was expressed and presented with different expression in the normal colonic mucosa, adenoma and CRC and showed increased expression in an advanced stage of CRC. This may suggest its possible involvement in colorectal tumorigenesis and invasion.

Introduction

Worldwide, colorectal cancer is the third most commonly diagnosed cancer in males and the second in females [1]. Traditional models of tumorigenesis suggest that every cell within the tumour population is capable of tumour initiation and propagation. The newly discussed cancer stem cell (CSC) model, however, proposes that only a small fraction of cells possess tumour propagation abilities. This hypothesis raises questions regarding the efficiency of current diagnostic and therapeutic measures, suggesting that CSCs are a rational target for the development of robust diagnostic, therapeutic, and follow-up strategies [2]. Many pieces of evidence suggest that cancer is a disease of stem cells [3] [4]. The cancer

stem cell model was described for hematologic malignancies in 1997, and since then evidence has emerged to support it for many solid tumours as well, including colon cancer, this model proposes that certain cells within the tumour mass are pluripotent and capable of self-renewal and have an enhanced ability to initiate distant metastasis. Becker et al., [5] in their study suggest that (1) Lgr5 is a potential marker of intestinal stem cells in humans and (2) loss of restriction to the stem cell niche is an early event in the premalignant transformation of stem cells and may play a role in carcinogenesis. Femia et al., [6] found overexpression of Lgr5 in precancerous lesions and tumours, they support Lgr5 as putative neoplastic stem cell marker, and they identified Lgr5-positive cells are co-expressing nuclear β -catenin (NBC) which could be a subpopulation with the highest stem cell

features. CD133 (Prominin-1), a transmembrane glycoprotein which was first identified as a potential cancer stem cell (CSC) marker for brain tumours [7]. Both O'Brien et al., [8] and Ricci-Vitiani et al., [9] found that the tumours formed from CD133 positive cells injected into severe combined immunodeficiency (SCID) mice resembled a tumour from which they were taken and formed tumours of differentiated cell types that were mostly CD133 negative. The CD133 negative cells from these tumours did not form metastases in mice even when injected at much higher numbers than CD133 positive cells.

This aim of the study is designed to investigate the distribution and expression of immunostaining of Lgr5 and CD 133 proteins in stem cells of human colon in malignant and premalignant colonic lesions in Egyptian patients.

Patients and Methods

This prospective case-control study was conducted on 100 subjects who attended the Gastrointestinal Endoscopy unit in Nasser Institute for research and treatment Hospital, and in collaboration with Theodor Bilharz Research Institute, during the period from March 2015 to March 2017. Patients were categorised into four groups:

Group I: included 20 patients had a normal colonoscopy and served as a control group.

Group II: included 20 patients with IBD (ulcerative colitis) diagnosed endoscopically and pathologically.

Group III: included 20 patients who were found to have colorectal polyps endoscopically and proved to be adenomatous pathologically and served as adenoma group.

Group IV: included 40 patients with colorectal cancer diagnosed endoscopically and pathologically.

Inclusion criteria: Adult patients >18 years old and have eligible indications for colonoscopy as follow:

1. Patient with lower GIT symptoms including chronic diarrhoea, chronic constipation, alternating bowel habits and bleeding per rectum.

2. Cases with relevant alarming symptoms and signs for CRC, e.g. significant unexplained weight loss, unexplained anaemia.

3. Patients with remote metastases proved to be adenocarcinoma and were suspected to have CRC.

4. Patients underwent screening for CRC.

5. Patients who have a family or personal history of CRC, genetic CRC syndrome or adenomas.

6. Patients with inflammatory bowel diseases were diagnosed for more than 8 years.

7. Patients are known to have FAP, family history of FAP or history of previous adenomatous polyps.

Exclusion criteria: Patients previously received chemotherapy for colorectal malignancy.

Patients subjected to the following: Informed written consent, Full history taking and thorough clinical examination (pallor, abdominal masses). Laboratory investigations including Stool analysis, Complete blood count (CBC), erythrocyte sedimentation rate (ESR) and Carcino Embryonic Antigen (CEA). Abdominal ultrasound for scanning masses, metastases and lymphadenopathy. Full colonoscopic examination by using Pentax colonoscope by model number (EC 3840 L).

Tissue Samples (biopsy specimens)

Biopsies were taken from the inspected lesions and the nearby normal colonic mucosa. Biopsies were taken using a standard cold biopsy forceps (CFB-2.5- 230-S, Wilson Cook medical®). In the *Control Group* multiple biopsies were taken from the colonic mucosa in all cases while in *IBD Group* multiple biopsies were taken from the inflamed mucosa and multiple biopsies were taken from the nearby grossly normal colonic mucosa while in *Adenomas Group* polyps will be removed using a polypectomy snare and multiple biopsies were taken from the nearby mucosa and in *CRC Group* multiple biopsies were taken from cancer and multiple biopsies from the nearby mucosa.

Specimens were fixed in 10% buffered formalin. Paraffin blocks were prepared. Histopathologic sections were cut at 4 µm thick. All slides were treated with 3-aminopropyl-triethoxysilane (3APTES/SIGMA-A-3648). These slides were used instead of the ordinary albumenised slides to minimise staining artefacts and for better fixation of sections on the slides.

Immunohistochemical reaction was performed using avidin-biotin complex (ABC) immunoperoxidase technique. Sections were de-waxed in xylene and hydrated in descending grades of ethanol. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide and then in 100% methanol for 20 minutes.

Antigen retrieval was performed by microwaving the sections in citrate buffer (PH 6.0) for 15 minutes at 700 W. Sections were incubated overnight at 40C with the anti-human primary monoclonal antibodies against Lgr5 and CD133 (Santa Cruz Biotechnology Inc.; Santa Cruz, USA)

and diluted at 1:100 and 1:150 respectively. Next day, sections were washed in PBS then incubated with streptavidin-biotin-peroxidase complex and substantiated using a peroxidase/DAB (diaminobenzidine) enzymatic reaction for Lgr5 and CD133. Staining was completed by 5 to 10 minutes incubation with 3, 3'-diaminobenzidine (DAB)+substrate-chromogen which resulted in a brown-coloured precipitate at the antigen sites of Lgr5 and CD133 (cytoplasmic stain). Slides were washed in PBS for five minutes then placed in 70%, 95% and 100% alcohol for five minutes each. The nuclei were counterstained with Mayer's hematoxylin. Coverslips were mounted using Dpx. Positive and negative control slides for each marker were included in each session. As a negative control, liver tissue section was processed in the sequences mentioned above but with the omission of the primary antibodies.

All immunostained slides were analysed using Zeis microscope with high resolution (Axio Scope, Germany) in ten successive high-power fields (HPFs). Both Lgr5 and CD133 antigens were expressed as brown cytoplasmic staining. Two features of immunoreactions were assessed separately on a semi-quantitative basis (H score) as follows: 1) the extent of staining was assessed as the percentage of positively stained cells in 10 HPFs in the highest expression (hot spot) areas in each case. Then, means of percentages were calculated. 2) The intensity of staining of the positive cells was relatively designated as + (mild or weak), ++ (moderate), and +++ (strong) according to Itoi et al., [10]. Then data were converted to immune-histochemical score (ranged from 1 to 6) by multiplying intensity and extent scores. An immunohistochemical score of 5-6 was considered strong immunoreactivity and was given score 3, 3-4 was considered moderate and was given score 2, and 1-2 was considered weak and was given score 1.

The data were analysed using Microsoft Excel 2010 and Statistical Package for Social Science (SPSS version 22.0) for Windows (SPSS IBM., Chicago, IL). Results were expressed as mean \pm SD with 95% confidence interval using mean for quantitative variables, frequencies and percentages for qualitative ones. $P < 0.05$ was considered statistically significant. Quantitative data were analysed by applying the one-way analysis of variance (ANOVA) test for comparison of the mean of more than two groups, while independent-samples t-test was used for comparison of the means of two groups. Chi-square test was used to compare proportions between two qualitative parameters.

Results

The demographic features of the whole studied patients and each group are summarised in (Table 1).

Table 1: Age and sex of the studied groups

Descriptive parameters	Control group N = 20	IBD group N = 20	Adenomas group N = 20	Malignant group N = 40	Total patients N = 100	P. Value
Age Mean \pm SD	55.3 \pm 15.02	30.7 \pm 7.7	40.2 \pm 12.7	53.7 \pm 15.12	44.975 \pm 18.17	0.001**
Sex						0.9
Female	7 (20.0%)	8 (22.9%)	12 (10.9%)	16 (45.7%)	43 (43%)	
Male	13 (23.6%)	12 (21.8%)	8 (11.4%)	24 (43.6%)	57 (57%)	

Bleeding per rectum was the most common presentation in the studied patients; presenting 35% of the total patients, malignant group (37.5%), and adenoma group (50%). Weight loss was statistically highly significant ($P = 0.03$) in patients of the malignant group (80%).

Table 2: Clinical presentation in the studied groups

Clinical presentation	Control group N = 20	IBD group N = 20	Adenomas group N = 20	Malignant group N = 40	Total patients N = 100	P. Value
Abdominal pain	8 (40.0%)	5 (25.0%)	2 (10.0%)	5 (12.5%)	20 (20.0%)	0.04*
Bleeding per rectum	0 (0.0%)	10 (50.0%)	10 (50.0%)	15 (37.5%)	35 (35.0%)	0.001**
Constipation	6 (30.0%)	0 (0.0%)	0 (0.0%)	3 (7.5%)	9 (9.0%)	0.003**
Diarrhea	6 (30.0%)	0 (0.0%)	5 (25.0%)	2 (5.0%)	13 (13.0%)	0.001**
Anemia	0 (0.0%)	2 (10.0%)	1 (5.0%)	7 (17.5%)	10 (10.0%)	0.1
Weight loss	0 (0.0%)	0 (0.0%)	2 (10.0%)	8 (20.0%)	10 (10.0%)	0.03*
Mucorrhea	0 (0.0%)	3 (15.0%)	0 (0.0%)	0 (10.0%)	3 (3.0%)	0.006**

Mucorrhea was statistically highly significant ($P = 0.006$) in IBD group. Constipation and diarrhoea were statistically significant in control patients (Table 2; Figure 1A-F).

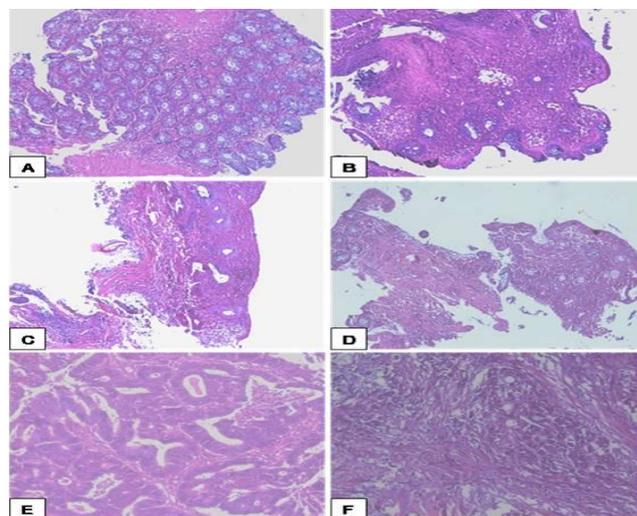


Figure 1: A) Control case of mild colitis (H & E, x 100); B) UC case, (H & E, x 100); C) UC case with mild dysplasia, (H & E, x 100); D) A case of CD (H & E x 100); E) A cases of well-differentiated adenocarcinoma (GII); F) A cases of poorly differentiated (H & E x 400)

Fungating mass was the most the most common endoscopic finding of CRC group (62.5%) while ulceration was present 60% of IBD group and the polypoid lesion was present 100% of adenoma group (Table 3).

CEA was highly elevated in CRC group ($P = 0.001$) with the high significant difference compared with other studied groups (370.4 mg/L vs 6.8, 6.9 and 5.8 mg/L, respectively) (Table 4).

Table 3: Endoscopic findings in the studied groups

	Control group N = 20	IBD group N = 20	Adenomas group N = 20	Malignant group N = 40	Total patients N = 100	P-Value
Site	Rectum	4 (20%)	6 (30%)	5 (25%)	10 (25%)	0.7
	Sigmoid	0 (0.0%)	0 (0.0%)	5 (25.0%)	8 (20%)	0.01*
	Recto-sigmoid	10 (50%)	8 (40%)	4 (20%)	9 (22.5%)	0.08
	Hepatic flexure	6 (30%)	0 (0.0%)	3 (15.0%)	9 (22.5%)	0.07
	Caecum	0 (0.0%)	0 (0.0%)	3 (15.0%)	4 (10.0%)	0.1
	Whole colon	0 (0.0%)	6 (30.0%)	0 (0.0%)	0 (0.0%)	0.001**
	Endoscopic finding	Mass	0 (0.0%)	0 (0.0%)	0 (0.0%)	25 (62.5%)
Angio-dysplasia		2 (10.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0.04*
Polyp		0 (0.0%)	0 (0.0%)	20 (100.0%)	0 (0.0%)	0.001**
Hyperemia		3 (15.0%)	6 (30.0%)	0 (0.0%)	0 (0.0%)	0.001**
Ulceration		0 (0.0%)	12 (60.0%)	0 (0.0%)	5 (12.5%)	0.001**
Stricture		0 (0.0%)	2 (10.0%)	0 (0.0%)	10 (25%)	0.008**
Normal		15 (75.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0.001**

Lgr5 was positive in 37 patients (92.5 %), 12 patients (60%), 10 patients (50%), 3 patients (15%) in malignant, IBD, adenoma, and normal mucosa respectively, high statistically significance difference between groups at P < 0.001 (Table 5; Figure 2 A-E).

Table 4: Laboratory investigations of the studied groups

	Control N = 20 Mean ± SD	IBD N = 20 Mean ± SD	Adenomas N = 20 Mean ± SD	Malignant N = 40 Mean ± SD	Total patients N = 100 Mean ± SD	P. Value	
Lab. Investigation	HB	13.3 ± 1.07	9.9 ± 1.01	10.9 ± 0.59	8.4 ± 16.7	11.4 ± 12.9	0.01*
	TLC	7.13 ± 2.7	8.79 ± 2.7	8.34 ± 2.4	7.94 ± 2.1	8.1 ± 12.3	0.1
	PLT	257.1 ± 111.3	215.6 ± 46.2	247.9 ± 132.9	214.15 ± 87.9	238.7 ± 92.7	0.1
	ESR	43.45 ± 21.8	61.4 ± 25.96	50.0 ± 26.9	66.7 ± 27.9	55.4 ± 24.3	0.02*
	CEA	6.8 ± 3.05	6.9 ± 2.05	5.8 ± 2.09	370.4 ± 395.6	97.5 ± 86.9	0.001**
	Stool Finding	Blood	6 (30.0%)	4 (20.0%)	6 (30.0%)	7 (17.5%)	23 (23%)
Mucous		4 (20.0%)	6 (30.0%)	4 (20.0%)	7 (17.5%)	21 (21%)	0.7
EH		5 (25.0%)	6 (30.0%)	4 (20.0%)	9 (22.5%)	24 (24%)	0.8
Giardia		4 (20.0%)	4 (20.0%)	4 (20.0%)	11 (27.5%)	23 (23%)	0.8
Pus		1 (5.0%)	0 (0.0%)	2 (10.0%)	6 (15.0%)	9 (9%)	0.2

Four cases out of 5 (80%) of GI showed positive Lgr5 expression while 16 cases out of 18 (88.8%) of GII showed positive Lgr5 and 17 cases out of 17 (100%) of GIII showed positive Lgr5 expression. There is a significant difference between GIII compared to GII and GI at P < 0.01 and P < 0.05 respectively (Table 6; Figure 2 A-E).

Table 5: Immunohistochemical staining of Lgr5 (intensity & extent) and CD133 of the studied groups

	Control N = 20	IBD N = 20	Adenomas N = 20	Malignant N = 40	Total patients N = 100	P. Value	
Lgr5 intensity	Negative	17 (85.0%)	10 (50.0%)	8 (40.0%)	3 (7.5%)	38 (38.0%)	0.001**
	Weak	3 (15.0%)	4 (20.0%)	4 (20.0%)	2 (5.0%)	13 (13.0%)	0.2
	Moderate	0 (0.0%)	6 (30.0%)	8 (40.0%)	8 (20.0%)	22 (22.0%)	0.01*
	Strong	0 (0.0%)	0 (0.0%)	0 (0.0%)	27 (67.5%)	27 (27.0%)	0.001**
Lgr5 Extent	Negative	17 (85.0%)	10 (50.0%)	8 (40.0%)	3 (7.5%)	38 (38.0%)	0.001**
	Less5	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (5.0%)	2 (2.0%)	0.3
	5-25	3 (15.0%)	2 (10.0%)	4 (20.0%)	0 (0.0%)	9 (9.0%)	0.05*
	25-50	0 (0.0%)	2 (10.0%)	0 (0.0%)	0 (0.0%)	2 (2.0%)	0.04*
	50-75	0 (0.0%)	6 (30.0%)	8 (40.0%)	8 (20.0%)	22 (22.0%)	0.01*
CD133	75-100	0 (0.0%)	0 (0.0%)	0 (0.0%)	27 (67.5%)	27 (27.0%)	0.001**
	Negative	17 (85.0%)	6 (30.0%)	8 (40.0%)	0 (0.0%)	31 (31.0%)	0.001**
	Mild	3 (15.0%)	8 (40.0%)	6 (30.0%)	3 (7.5%)	20 (20.0%)	0.01*
	Moderate	0 (0.0%)	6 (30.0%)	6 (30.0%)	11 (27.5%)	23 (23.0%)	0.05*
Strong	0 (0.0%)	0 (0.0%)	0 (0.0%)	26 (65.0%)	26 (26.0%)	0.001**	

CD133 was expressed in 40 patients (100%), 12 patients (60%), 14 patients (70%) and 3 patients (15%) in malignant, adenoma, IBD and control patients respectively, with high statistically significance between different groups at P < 0.001. A very significant difference in CD133 expression was found between colorectal carcinoma and normal mucosa (P < 0.001) (Table 5; Figure 3 A-F).

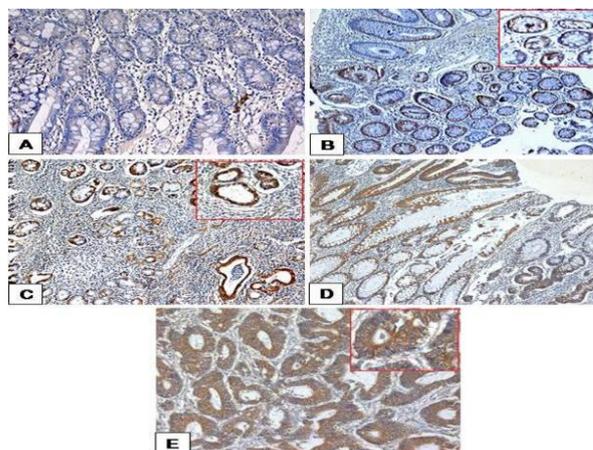


Figure 2: A) Control case of mild colitis, no expression of Lgr5 immunostain in the cytoplasm of cells lining the colonic glands (IHC, DAB, x200), B) Ulcerative Colitis case, with moderate expression of the Lgr5 protein in the cytoplasm of cells lining the colonic gland. (IHC, DAB, x40), C) A case of Crohns Disease, with moderate expression of CD Lgr5 protein in the cytoplasm of cells lining the gland and crypt epithelium (red arrow)(IHC, DAB, x40), D) A cases of tubular adenoma, showing moderate expression of CD Lgr5 protein in the cytoplasm of cells lining the gland(red arrow) and crypt epithelium (black arrow) (IHC, DAB, x100), E) A cases of moderately differentiated adenocarcinoma (GII), showing marked expression of CD Lgr5 protein in the cytoplasm of cells lining the gland(red arrow) and crypt epithelium (black arrow) (IHC, DAB, x100)

As regards this correlation between immunoexpression of CD133 and histopathological grade, the intensity of CD133 was strong in GII (55.7%) and GIII (88.3%). There is a significant difference between GIII compared to GII and GI at P<0.01 and P < 0.05 respectively (Table 7; Fig. 3 A-F).

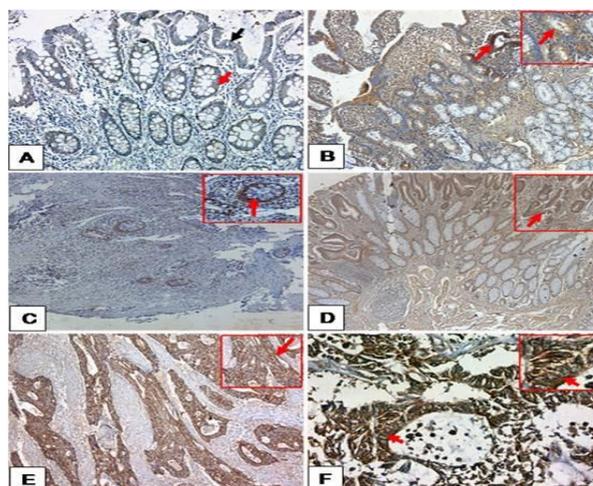


Figure 3: A) Control case of mild colitis, showing mild expression of CD133 immunostain in the cytoplasm of cells lining the crypt epithelium(black arrow) and glands (red arrow)(IHC, DAB, x200), B) Ulcerative Colitis case, showing mild to moderate expression of CD133 immunostain in the cytoplasm of cells lining the colonic glands (red arrow) (IHC, DAB, x100), C) A case of Crohns Disease, showing moderate expression of CD133 immunostain in the cytoplasm of cells lining the colonic glands (red arrow) IHC,DAB, x100), D) A cases of tubulovillous adenoma, showing moderate expression of CD133 immunostain in the cytoplasm of cells lining the colonic glands (red arrow) (IHC,DAB, x40), E)&F A cases of moderately differentiated adenocarcinoma, showing marked expression of CD133 immunostain in the cytoplasm of cells lining the colonic glands (red arrow) (IHC, DAB, x200, x400)

Four cases out of 5 of Dukes' A (80%) were positive for Lgr5, 13 cases out of 15 of Dukes' B (86.6%) and 20 cases out of 20 of Dukes' C (100%) were positive for Lgr5. There is significant difference between Dukes' B and C comparing to Dukes' A at $P < 0.01$ and $P < 0.05$ respectively (Table 8).

Table 6: Correlation of Lgr5 score and histopathological grades in the malignant group

Histopathological grade	Lgr5 score staining		
	+, Mild (Score 1) No %	++, Moderate (score 2) No %	+++, Strong (Score 3) No %
Well differentiated (GI) N=5	1 (25%)	3 (75 %)	0(0%)
Moderately differentiated (GII) N=18	1 (16,6%)**	6 (33.4%)**	9 (50%)**
Poorly differentiated (GIII) N=17	0 (0%)	3 (5.9%)*,^	14(94.1%)*,^

Cross tables, Pearson Chi-Square; $P < 0.05$; * $P < 0.01$ compared to GI group; ^ $P < 0.05$ compared to GII group.

Three cases out of 5 of Dukes' A (60%) were moderately positive for CD133, 10 cases out of 15 of Dukes' B (66.7%) showed strong expression of CD133 and 18 cases out of 20 of Dukes' C (90%) were strongly positive for CD133 (Table 9).

Table 7: Correlation of CD133 immunoexpression and histopathological grades in the malignant group

Histopathological grade	Immunoexpression of CD133 staining		
	+, Mild No %	++, Moderate No %	+++, Strong No %
Well differentiated, (GI) N=5	3 (60%)	2 (40%)	0 (0%)
Moderately differentiated, (GII)N=18	0 (0%)	2 (11.7%)*,^	15(88.3%)*,^
Poorly differentiated, (GIII) N=17	0 (0%)	2 (11.7%)*,^	15(88.3%)*,^

Cross tables, Pearson Chi-Square; $P < 0.05$; * $P < 0.05$ compared to GI group; ** $P < 0.01$ compared to GI group; ^ $P < 0.05$ compared to GII group.

Discussion

Worldwide, colorectal cancer is the third most commonly diagnosed cancer in males and the second in females. Colorectal cancer (CRC) annually affects more than one million men and women and causes more than half a million deaths [1]. CRC is presented as a multistep genetic disorder characterised by specific mutations in signal transduction pathways. The development and progression from adenoma to cancer and metastatic disease require the simultaneous failure of protective mechanisms [11].

Table 8: Correlation of Lgr5 score and histopathological stages in malignant group

Histopathological Stages	Lgr5 score immunoexpression		
	+, Mild (Score 1) No %	++, Moderate (2) No %	+++, Strong (Score 3) No %
Dukes' A N=5	0(0%)	4 (100%)	0(0%)
Dukes' B N=15	0 (0%)	3 (23.1%)*	10(76.9%)*
Dukes' C N=20	0(0%)	2 (10%)*,^	18 (90%)*,^

Cross tables, Pearson Chi-Square; $P < 0.05$; * $P < 0.01$ compared to Duck A group; ^ $P < 0.05$ compared to Duck B group.

Colorectal cancer develops from a dysplastic precursor lesion, regardless of whether it arises

sporadically, in the setting of high-risk hereditary conditions, or in the context of chronic inflammation like inflammatory bowel disease (IBD).

Table 9: Correlation of CD133 immunoexpression and histopathological stages malignant group

Histopathological Stages	Immunoexpression of CD133 staining		
	+, Mild No %	++, Moderate No %	+++, Strong No %
Duke A N = 5	2 (40%)	3 (60%)	0 (0%)
Duke B N = 15	2 (13.3%)	3 (20%)*	10 (66.7%)*
Duke C N = 20	0 (0%)	2 (10%)*,^	18 (90%)*,^

Cross tables, Pearson Chi-Square; $P < 0.05$; * $P < 0.01$ compared to Dukes' A group; ^ $P < 0.05$ compared to Dukes' B group.

In IBD, however, dysplasia can be polypoid or flat. In fact, the rather unusual macroscopic appearance and biologic behaviour of dysplasia in IBD have stimulated a good deal of research into the natural history and molecular pathogenesis of CRC in patients with IBD [12]. Cancer stem cell theory in CRC has been investigated, and it is based on evidence that only a small subset of cells, the CSCs, within the tumour population, can initiate and sustain tumour growth and several stem cell markers have been studied [13].

Metastasis is responsible for approximately 90% of cancer-associated mortality and can be divided into translocation and colonisation. As the CSC population represents the only cells that propagate tumours, it can be extrapolated that these cells are responsible for metastasis formation. It is, therefore, a prerequisite that these cells must be able to detach from a primary tumour, invade, access, and survive at the circulation, disseminate at distant sites, transmigrate across the endothelial lining of the target tissue, and form secondary tumours [14] [15].

Several markers have been identified as solid cancer stem cell markers. CD133 is a transmembrane pentaspan protein which was initially described as a surface antigen specific for human hematopoietic stem cells. Indeed, CD133 alone or in combination with other markers is currently used for the isolation of stem cells from numerous tissues, such as bone marrow, brain, kidney, prostate, liver, pancreas, and skin. Furthermore, investigators have used monoclonal antibodies to CD133 for the identification and isolation of a putative cancer stem cell population from malignant tumours of brain, prostate, liver, pancreas, lung, and colon [16].

On the other hand protein glycoprotein coupled receptors (GCRs) have been investigated to be closely associated with tumorigenesis [17]. Lgr5 which is one of (GCRs) members proved to be a stem cell marker [18].

This prospective study was conducted on 100 patients presenting with colonic symptoms, attending to Gastrointestinal Endoscopy unit in Nasser Institute for research and treatment Hospital. This study was aiming to assess the expression of CD133 and Lgr5 in pre-malignant (adenomatous polyps and IBD), malignant colorectal lesions and normal colonic

mucosa by immunohistochemical staining.

According to the immunohistochemical results, Lgr5 was detected as brown cytoplasmic granules with different expression pattern in the normal colonic mucosa, adenoma and carcinoma. Our results showed that Lgr5 protein was strongly positively (score 3) in 67.7% of cases (27/40), moderately (score 2) in 20% of cases (8/40) and mild (score 1) in 5% of cases (2/40) of colorectal carcinomas respectively, with high statistically significance difference between groups at $P < 0.001$. Statistically speaking a significant correlation was found between a score of Lgr5 expression and the type of the lesion as the score increases with progression of the lesion from adenoma to carcinoma (P -value < 0.01). This was in agreement with Fan et al., [18] who stated that 54% of colorectal carcinoma cases showed score group (3) of Lgr5 expression. This means that Lgr5 expression might be involved in colorectal carcinogenesis.

In the current study, as regards the tumour grading, Lgr5 immunoexpression in CRC group. Three cases (80%) of GI, 16 cases (88.8%) of GII and 17 cases (100%) of GIII with the highly statistically significant difference between GIII compared to GII and GI at $P < 0.01$ and $P < 0.05$ respectively. These results were in disagreement with Fan et al., [18] who found that no correlation was found between Lgr5 score and the grade of CRC as 34% of grade II showed score 1, 14.9% of grade II showed score 2 and 4.5% showed score 3. In grade III 86.6% of cases showed score (1) and 13.4% of cases showed score (2) and no cases showed score (3). This current study was in disagreement with Takeda et al., [19] that found Lgr5 expression was not correlated to the degree of differentiation of CRC cases.

These results were in contrast with Simon et al., [20] that found Lgr5 was correlated significantly with tumour grade in gastric adenocarcinoma (P -value < 0.01).

Concerning the expression of CD133 in CRC cases, the present study revealed a significant difference between colorectal carcinoma and normal mucosa ($P < 0.001$). CD133 was positive in 15% of cases (3/20) of normal mucosa (control cases). CD133 protein was strongly positively in intensity in 65% of cases (26/40), moderately 27.5% of cases (11/40) and mild intensity of CD133 in 7.5% of cases (3/40) of the colorectal carcinomas respectively, with high statistically significance ($P < 0.001$). These findings are in agreement with Takahashi et al., [21] and by Yang et al., [22]. However; our results are in contrary to those of Hongo et al., [23], Choi et al., [24], Horst et al., [25] and Kojima et al., [26]. This can be explained by the difference in the sample size; the studies which disagreed with our results had a larger sample size.

These findings are in agreement with those detected by Wang et al., [27], found that high CD133

expression was significantly associated with moderately and poorly differentiated CRC. In contrast Coco et al., [28], stated that no significant relation between CD133 expression and histologic grade and also with that found by Hongo et al., [23], Takahashi et al., [21], Choi et al., [24], Horst et al., [25] and Saigusa et al., [29].

As regard Dukes' staging and Lgr5 expression our results showed that four cases out of 5 of Dukes' A (60%) were positive for Lgr5, 13 cases out of 15 of Dukes' B (86.6%) showed immunoreactivity for Lgr5, and 20 cases out of 20 of Dukes' C (100%) were positive for Lgr5 with statistically significance difference ($P < 0.01$). There was a significant correlation between a score of Lgr5 and the stage of CRC (P -value < 0.05). These results suggest that Lgr5 expression perhaps play a role not only in tumour initiation but also in the progression of a tumour. These results in agreement with Merlos et al., [30], Takahashi et al., [21], and Uchida et al., [31] found increased Lgr5 expression in advanced stages of CRC cases.

Concerning the expression of CD133 in CRC cases in correlation to Dukes' stage, there are three cases out of 5 of Dukes' A (60%) were moderately positive for CD133, 1 cases out of 15 of Dukes' B (66.7%) showed strong expression of CD133, and 18 cases out of 20 of Dukes' C (90%) were strongly positive for CD133. This is in contrast with results of Wang et al., [27], Takahashi et al., [21], Choi et al., [24], Horst et al., [25] and Kojima et al., [26] found no significant relation between CD133 expression and modified Dukes. Horst et al., [25] reported significant relation with N and M stage. The studies had different results from ours are much larger in the number of patients and also Coco et al., [28] studies have different cut-off used to discriminate low and high expression.

In conclusion, as stem cell marker of cells with intestinal differentiation, Lgr5 and CD133 were presented with significantly increased expression with progression from normal colon towards CRC. Lgr5 expression and CD133 was positively correlated with stage of CRC suggesting its possible involvement in colorectal tumorigenesis progression and patient's outcome.

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