EXPRESSION OF LGR5 AND BETA-CATENIN IN BENIGN AND MALIGNANT COLORECTAL LESIONS IN ZAGAZIG UNIVERSITY HOSPITAL.

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ABSTRACT

Background: Lgr5 -(GPR49) a Wnt target gene- is one of the most important cancer stem cell markers that have been isolated in colorectal cancer (CRC). The potentiation of Wnt/Beta-catenin signaling is now believed to mediate the self-renewal and proliferation of Lgr5 stem cells.

Aim: to evaluate the expression of Lgr5 and Beta-catenin in colorectal cancer and benign colorectal lesions.

Methods: Representative sections from thirty benign lesions [22 colorectal adenomas, 8 ulcerative colitis (UC)], and 30 CRC cases were stained by immunohistochemical technique using anti-Lgr5 and anti-Beta-catenin.

Results: High Lgr5 expression was found in 0%, 60%, and 70% of normal mucosa, benign lesions, and CRC, respectively. Higher grades of Beta-catenin expression compared to normal expression was found in benign lesions as 23.3% for grade II and 6.7% for grade III and in CRC as 73.3% for grade II and 20% for grade III. Significant statistical differences between control groups, benign and malignant lesions in both Lgr5 and Beta-catenin expression were found.

Conclusion: Lgr5 is highly expressed in CRC. That may suggest a role in carcinogenesis based on the CSC hypothesis. Also, Beta-catenin is activated in most CRC.

Keywords: Cancer stem cell, Lgr5, Beta-catenin, CRC.

INTRODUCTION

Colorectal cancer is considered the third most common cancer in men and the second in women worldwide [(1)]. In Egypt the estimated incidence of colorectal cancer is 3.7% in both sexes and the mortality is 3.6% [(2)].

The cancer stem cell (CSC) hypothesis set that CSCs are responsible for tumor initiation, metastases and treatment resistance leading to disease relapse following surgery and/or chemo-radiotherapy [(3)]. CSCs own the ability to self-renew and to differentiate into diverse lineages [(4)].

Lgr5 (also called GPR49) is one of the most important cancer stem cell markers that have been reported [(5)]. Overexpression of Lgr5 protein in CRC has been reported in several studies and its relation with tumor development, resistance for 5-FU-based chemotherapy, and recurrence was demonstrated [(6,7,8)]. Therefore, high level of Lgr5 protein is thought to be a poor prognostic factor in CRC.

The Wnt signaling pathway is essential for normal intestinal growth and development [(9)]. Inappropriate activation of this pathway, most commonly caused by mutations in the adenomatous polyposis coli (APC) gene, is associated with colorectal cancer [(10)]. The potentiation of Wnt/Beta-catenin signaling is now believed to mediate the self-renewal and proliferation of Lgr5 stem cells [(11)]. But little is known about the relation between Lgr5 and Beta-catenin expression in CRC.

MATERIAL AND METHODS

The present study is based upon archival paraffin blocks and fresh samples from benign and malignant colorectal lesions collected in Sharkia governorate. Colorectal adenomas (n=22) and ulcerative colitis (UC) (n=8)

[benign lesions group: group 2] and colorectal carcinomas (n=30) [malignant lesions group: group 3] with the adjacent normal mucosa (n=30) [control group: group 1] were included in this study. Based on the TNM classification of WHO (2010) [(12)], the pathological diagnosis of CRC was set.

- ***Immunohistochemical staining***

The primary antibodies used were rabbit anti-Lgr5 monoclonal antibody (EPR3065Y, Novus Biologicals, CO, USA), and mouse anti-Beta-catenin monoclonal antibody (M3539, Dako, North America) with dilution concentration at (1:100-1:200), and (1:200), respectively.

- ***Technique***

Positively charged slides at thickness 3-4 microns were embedded in xylene for 5 minutes. Series of xylene and alcohol were done, and then tissue sections were microwaved in 0.01 M sodium citrate (pH 6.0) for antigen retrieval for 25 minutes. Incubation for 10 minutes with 3% hydrogen peroxide was done, then in 1.5% bovine serum albumin at room temperature for 1 h. Primary antibodies (anti-Lgr5 or anti-Beta-catenin) were incubated at room temperature for 30 minutes, and then a secondary antibody from a streptavidin biotin complex peroxidase kit was used [(13)] with the substrate 3,3′-diaminobenzidine tetrahydrochloride (DAB; Dako) for 10 minutes in DAB. Lastly, sections were washed with distilled water and counterstained with Mayer's hematoxylin.

- ***Immunohistochemical evaluation***

Evaluation of Lgr5 expression

Lgr5 expression was considered to be positive if a uniform membranous and/or cytoplasmic staining was present. The scoring system of Lgr5 includes the evaluation of both intensity and extent. The intensity was graded as (0= negative staining, 1= mild staining, 2= moderate staining and 3= strong staining). The extent was evaluated as (0= negative, 1= 1-25%, 2= 26-50% and 3= >50%). The scoring system is calculated by adding the intensity and the extent of Lgr5 staining (score = intensity+ extent), and then scores are evaluated as (negative=0, low=1-2, high=3-6) [(14)].

Evaluation of Beta-catenin expression

Beta-catenin expression was evaluated according to the distribution of Beta-catenin within the cell membrane (0–1), cytoplasm (0–2), and nuclei (0–2). Beta-catenin scoring system was evaluated by adding the nuclear score (0 = no expression; +1 = weak expression; +2 = positive expression) to the cytoplasmic score (0 = no expression; +1 = weak expression; +2 = positive expression) and membranous score (0 = positive membrane expression; +1 = negative membrane expression) [(15)]. Positive nuclear Beta-catenin staining was considered, if there is a strong staining for Beta-catenin in more than 10% of the tumor cell nuclei [(16)]. Three grades were categorized: Grade I includes total score (0-1), Grade II includes total score (2-3) and Grade III includes total score (4-5).

STATISTICAL ANALYSIS

The collected data were computerized and statistically analyzed using SPSS program (Statistical Package for Social Science) version 18.0. Qualitative data were represented as frequencies and relative percentages. Chi square test was used to calculate difference between qualitative variables. Fisher's exact test was used to calculate difference between qualitative variables. The significance level for all mentioned tests was done. P value of <0.05, <0.01 indicates significant and highly significant results, respectively.

RESULTS

Lgr5 expression in control, benign and malignant lesions group

Lgr5 protein was expressed in a single cell up to several cells at the crypt base of normal colonic mucosa **(figure 1A)**, however negative expression was seen in numerous crypts. The pattern of Lgr5 cellular staining was presented as granular material in the cytoplasm or at the cell membrane. The pattern of Lgr5 expression was variable in cases of colorectal adenomas and CRC, may be, patchy with variable intensity or diffuse **(figure 1B)**.

Colorectal adenomas and inflammatory lesions exhibited more cytoplasmic expression of Lgr5 protein than normal colonic mucosa. High expression of Lgr5 was found in 72.7% of adenomas **(figure 1C)**, and 25% of ulcerative colitis. In cases of colorectal adenomas, Lgr5 protein was highly expressed in 75% of tubular adenomas, and in 66.7% of tubulovillous adenomas, 75% low grade adenomas, and 71.4% high grade adenomas.

Lgr5 protein overexpression was found in CRC cases in the cytoplasm of cancer cells and/or their cell membrane **(figure 1D)**. In some cases of CRC, glandular secretions were also immunoreactive. According to the TNM classification 85% of N1-2 and 80% of stage III showed high Lgr5 expression.

Beta-catenin expression in control, benign and malignant lesions group

Normal mucosa of control group showed diffuse strong membranous Beta-catenin staining, grade I (score 0) in all cases (100%) **(figure 2A)**. In UC cases, 5 cases showed grade I Beta-catenin expression (62.5%). About 27% of adenomas exhibited a higher grade of Beta-catenin staining (gradeII or gradeIII) **(figure 2B)**. No grade III in any of all tubular adenomas, while tubulovillous adenomas exhibited equal percentage (33.3%) of Beta-catenin expression for grade I, II, and III. Low grade adenomas showed equally grade II and III Beta-catenin expression in 25%, while high grade adenomas showed grade II Beta-catenin expression in 14.3%.

Beta-catenin expression in cases of CRC, showed higher grades (22 grade II and 6 grade III) than normal colonic mucosa **(figure 2C and 2D)**. Only 5.6% of low grade CRC showed grade III Beta-catenin expression, while 41.7% of high grade CRC showed grade III Beta-catenin expression.

Lgr5 and Beta-catenin expression in all studied groups

Expression of Lgr5 protein was high in 0%, 60%, and 70% of normal colonic mucosa, benign lesions (adenomas and UC cases), and CRC, respectively. Grades of Beta-catenin was gradually increased as grade II in 23.3% of benign lesions, and 73.3% of CRC, and as grade III in 6.7% of benign lesions, and 20% of CRC **(Table 3)**. In benign lesions, low and high Lgr5 expression showed relatively similar association with grade I and II Beta-catenin expression. However, grade III Beta-catenin expression was only associated with high Lgr5 expression. In CRCs, low and high Lgr5 expression showed relatively similar association with grade II and III Beta-catenin expression **(Table 4).**

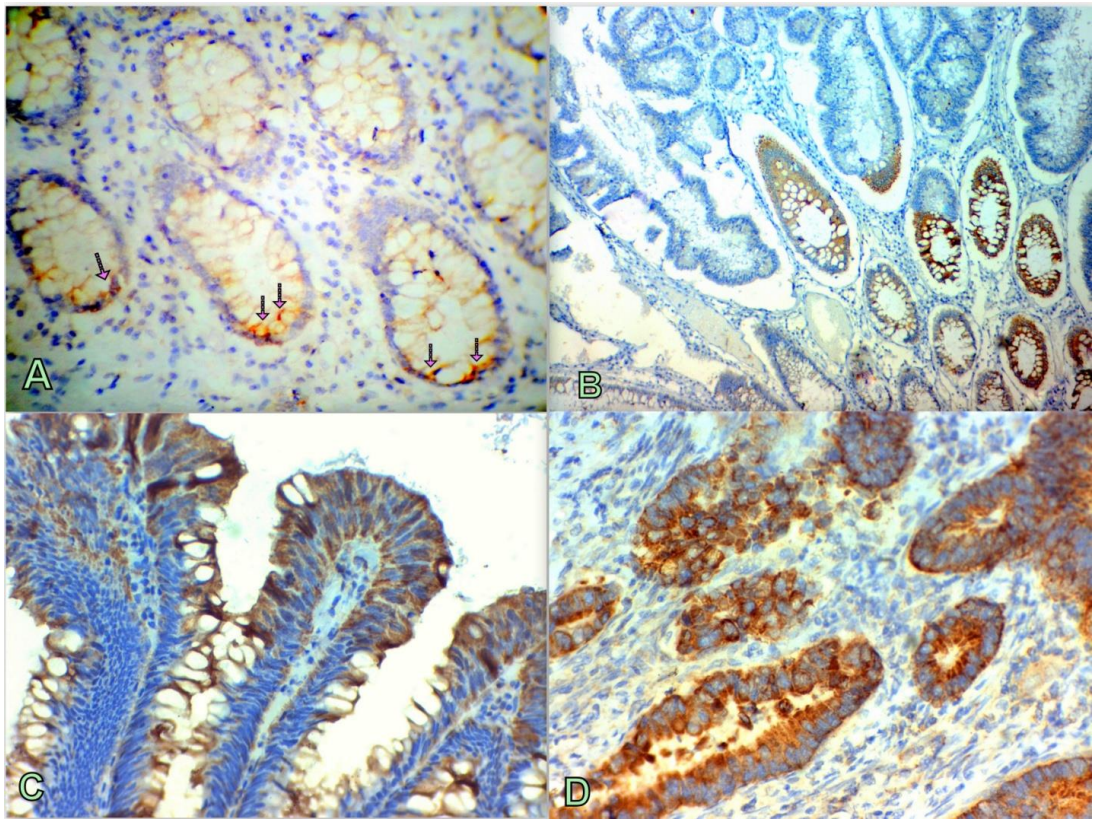

Figure 1: **A)** Normal colonic mucosa shows individual cells immunoreactive for Lgr5 at the crypt base (arrows).**B)** A case of high grade tubular adenoma shows strong patchy Lgr5 staining (score 5).**C)** A case of low grade tubulovillous adenoma shows strong cytoplasmic Lgr5 expression (score 5). **D)** A case of low grade adenocarcinoma, NOS, stage IIIB, shows strong differentiated malignant glands with diffuse strong cytoplasmic Lgr5 expression (score 6). (A, C, D at original magnification X400 and B at X100).

Table 1: Expression of Lgr5 and Beta-catenin in benign lesions

Variable	N	Lgr5				χ^2	P	Beta-catenin						χ^2	P
		Low		High				GI		GII		GIII			
		N	%	N	%			N	%	N	%	N	%		
Diagnosis:															
UC	8	6	75	2	25	**5.57**	**0.02***	5	62.5	3	37.5	0	0	1.75	0.42
Adenoma	22	6	27.3	16	72.7			16	72.7	4	18.2	2	9.1		NS
Adenoma Type:															
Tubular	16	4	25	12	75	0.15	0.70	14	87.5	2	12.5	0	0	**8.14**	**0.02***
Tubulovillous	6	2	33.3	4	66.7		NS	2	33.3	2	33.3	2	33.3		
Adenoma Grade:															
Low	8	2	25	6	75	0.03	0.86	4	50	2	25	2	25	4.71	0.10
High	14	4	28.6	10	71.4		NS	12	85.7	2	14.3	0	0		NS

Table (1) shows that there was a statistical significance increase in high Lgr5 among adenoma cases compared to inflammatory (p value 0.02). No significant relation was found between Lgr5 expression and the adenoma type or grade. A significant relation was found between Beta-catenin expression and the type of colorectal adenomas (tubular and tubulovillous) (p value 0.02). No significant relation was found between Beta-catenin expression and the grading of adenomas.

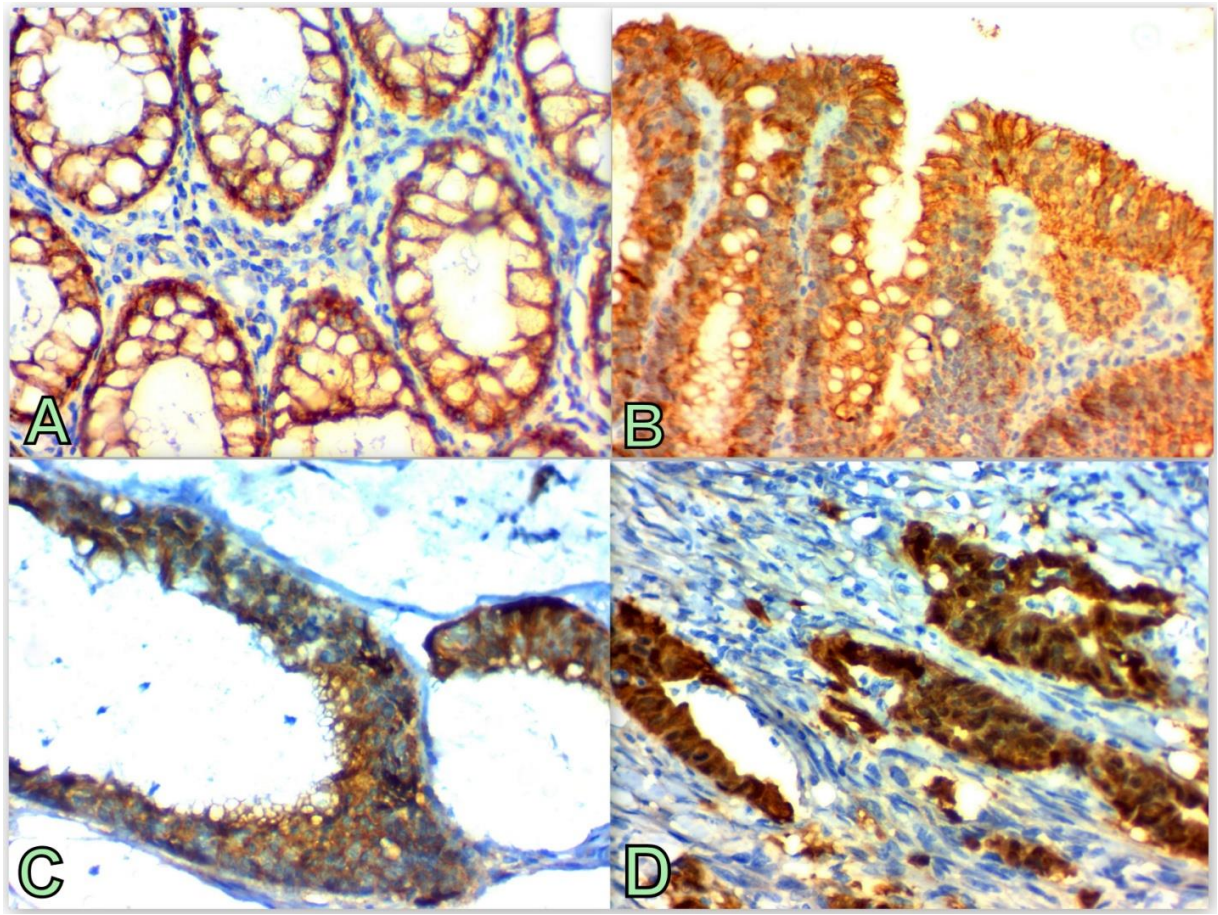

Figure 2: A) Normal colonic mucosa shows membranous Beta-catenin expression (score 0).**B)** A case of low grade tubulovillous colonic adenoma shows reduced membranous Beta-catenin expression and diffuse cytoplasmic Beta-catenin staining grade II (score 3).**C)** A case of mucinous adenocarcinoma, stage IIIC showing loss of membranous expression and presence of diffuse moderate cytoplasmic Beta-catenin expression (score 3).D) A case of low grade adenocarcinoma, NOS, stage IIIC, shows moderately differentiated malignant glands with grade III cytoplasmic and nuclear Beta-catenin staining (score 5). (original magnification X400).

Table 2: Expression of Lgr5 and Beta-catenin in colorectal carcinomas

Variable	N	Lgr5				χ^2	P	Beta-catenin						χ^2	P
		Low(n=9)		High(n=21)				G I		GII		GIII			
		No	%	No	%			No	%	No	%	No	%		
Site:															
Rt	14	9	64.3	5	35.7			0	0	11	78.6	3	21.4		
Lt	15	0	0	15	100	**14.7**	**0.001****	1	6.7	11	73.3	3	20	15	**0.005****
Multi	1	0	0	1	100			1	100	0	0	0	0		
Diagnosis:															
Adeno-carcinoma	20	6	30	14	70	0.08	0.69	2	10	15	75	3	15	3.47	0.48
Mucinous	6	2	33.3	4	66.7		NS	0	0	5	83.3	1	16.7		NS
Signet	4	1	25	3	75			0	0	2	50	2	50		
Grade:															
Low	18	4	22.2	14	77.8	1.30	0.26	2	11.1	15	83.3	1	5.6	6.64	**0.04***
High	12	5	41.7	7	58.3		NS	0	0	7	58.3	5	41.7		
T:															
T2	9	3	33.3	6	66.7			1	11.1	8	88.9	0	0		
T3	18	5	27.8	13	72.2	0.11	0.95	1	5.6	11	61.1	6	33.3	5.46	0.24
T4a	3	1	33.3	2	66.7		NS	0	0	3	100	0	0		NS
N:															
N0	10	6	60	4	40	6.43	**0.01***	1	10	8	80	1	10	1.09	0.58
N1-2	20	3	15	17	85			1	5	14	70	5	25		NS
Stage:															
I	5	2	40	3	60			1	20	4	80	0	0		
II	5	4	80	1	20	8.33	**0.02***	0	0	4	80	1	20	3.09	0.54
III	20	3	15	17	85			1	5	14	70	5	25		NS

Table (2) shows that there was a statistical significance increase in high Lgr5 among left-sided CRCs compared to other sites (p value 0.001), and Also there was a statistical significance increase in high Lgr5 expression among CRC with lymph node metastasis (p value 0.01), and stage III (p value 0.03) compared to CRC with no lymph node metastasis and with other stages, respectively. No significant relation was found between Lgr5 expression and the histopathological diagnosis, grading, and primary tumor (T). Beta-catenin expression showed a high significant relation with tumor site (0.005), and a significant relation with tumor grade (p 0.04). No significance was found between Beta-catenin expression and tumor variants, primary tumor (T), lymph node metastasis (N), or tumor stage.

Table 3: Comparison of the three studied groups in the expression of Lgr5 and Beta-catenin

Variable	Control (n=30)		Benign lesions (n=30)		Malignant lesions (n=30)		χ^2	P
	N	%	N	%	N	%		
Lgr5:								
Low	30	100	12	40	9	30	Fisher	**<0.001****
High	0	0	18	60	21	70		
Beta-catenin:								
G I	30	100	21	70	2	6.7	Fisher	
G II	0	0	7	23.3	22	73.3		**<0.001****
G III	0	0	2	6.7	6	20		

Table (3): There were highly statistical significance differences between control, benign and malignant lesions in both Lgr5 (p **<0.001**) and Beta-catenin expression (p **<0.001**). The difference was found between control group and both benign and malignant groups and also between benign and malignant groups.

Table 4: Relation between Lgr5 and Beta-catenin in both benign and malignant cases:

Group	Variable	Lgr5				χ^2	P
		Low		High			
		No	%	No	%		
Benign	**Beta-catenin:**	(n=12)		(n=18)			
	G I	8	66.7	13	72.2	Fisher	0.33
	G II	4	33.3	3	16.7		NS
	G III	0	0	2	11.1		
Malignant	**Beta-catenin:**	(n=9)		(n=21)			
	G I	0	0	2	9.5	Fisher	
	G II	7	77.8	15	71.4		0.63
	G III	2	22.2	4	19		NS

Table (4): There was no statistical significance relation between Lgr5 and Beta-catenin expression in both benign and malignant cases.

DISCUSSION

Normally colonic mucosa expresses Lgr5 protein as individual cell up to several cells at the crypt base similarly as identified by **Barker et al (2007)** [17] in mouse colon and as shown by **Baker et al (2015)** [18] who used chromogenic ISH in human colon and reported Lgr5 mRNA expression with the same pattern in small intestine. The pattern of Lgr5 expression was variable between focal and diffuse, patchy and whole gland involvement, high and low intensity within the same gland. These different patterns may be attributed to abnormal regulation of the stem cell niche. The 'patchy' expression of Lgr5 has previously been reported in colonic adenomas in various studies using Lgr5 antibodies [18-20]. **Barker et al (2015)** [18] noted that within the same adenoma, regions of high-grade dysplasia showed a generally higher level of Lgr5 expression than regions of low-grade dysplasia. Overexpression of Lgr5 protein in colorectal adenomas in comparison with few Lgr5 positive cells in normal colonic mucosa was supported by results of higher Lgr5 expression in all studied sporadic colonic adenomas when compared with matched normal mucosa [8]. Expansion of Lgr5 mRNA was found in an adenoma in contrast with non-adenomatous crypts [21]. Lgr5 was markedly overexpressed in most cases of advanced CRCs in comparison with normal mucosal tissue [8, 22]. Also, as shown by **Hirsch et al (2014)** [23] who used real-time qRT–PCR, Lgr5 was overexpressed in CRC cell lines Caco-2, HT-29, SW480, SW620 and T84, but not in the HCT 116 cell line, this was explained by absence of a stem-like cell fraction in this cell line.

Agreeing with our results, significant relation was observed between Lgr5 expression and lymph node metastasis, and stage [7, 8, 22]. **Fan et al (2010)** [20] showed only a significant relation with stage, but not with lymph node metastasis. Also, no significant relation was found between Lgr5 expression and the site, and the histopathological diagnosis [7, 8] and grading [7, 8, 20, 24]. However, **Wu et al (2012)** [22] found that Lgr5 expression was significantly correlated to histological grade and depth of invasion. Therefore, Lgr5 overexpression correlates with poor prognosis.

The majority of cases of CRC (about 90%) harbors a mutational change in the genes of Wnt/Beta-catenin pathway, mainly APC or Beta-catenin, thus leads to Wnt/Beta-catenin pathway activation. Beta-catenin accumulates in the nuclear compartment of nearly 80% of the tumors [25-27]. **Kobayashi et al (2000)** [28] revealed that cytoplasmic staining intensity was significantly higher ($P < 0.001$) in adenomas and cancers than in normal mucosa, in both sporadic and FAP cases. Also, **Kobayashi et al (2000)** [28] found that grade III expression was

in 6%, 42% of adenomas, and CRCs, respectively. **Kazem et al (2014)** [29] showed that grade II and III Beta-catenin expression in CRCs was found in 60%, and 40%, respectively. **Wangefjord et al., (2013)** [30] reported that MSI screening status was inversely associated with Beta-catenin overexpression in 557 cases of CRC studied in a large, prospective cohort study. Therefore, overexpression of Beta-catenin was found in benign and malignant colorectal lesions, indicating this protein activation.

Colorectal carcinomas develop in cases of ulcerative colitis, especially in those who have longstanding disease of pancolitis [31]. Focal up-regulation of Beta-catenin was observed in ulcerative colitis (50%), and no nuclear expression was found in any case of ulcerative colitis [32]. **Cooper et al (2000)** [33] found that Beta-catenin is translocated to the cytoplasm and/or nucleus in dysplasia associated with lesion or mass in dextran sulfate sodium mouse colitis model. Confirming the previous results of different studies about Beta-catenin overexpression in colorectal lesions compared to normal mucosa.

Lgr5 and its related receptor family members Lgr4 and Lgr6 have now all been considered as receptors for the R-spondin -family of ligands, which act to potentiate Wnt/β-catenin signaling by complexing with Frizzled/LRP receptors [34-36]. Lgr5 overexpression was positively correlated with Beta-catenin expression in CRC [20] and in hepatocellular carcinomas [37]. However, our results showed that there was no statistical significant relation between Lgr5 and Beta-catenin expression in benign and malignant colorectal lesions. This was supported by **Takeda et al (2011)** [38] who also found no association between both proteins. Our opposing results against other studies [20, 37] may be related to the larger sample (n=102) and higher stages (T1-4) included in CRC cases (20). Also, the use of real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis for evaluation of Lgr5 expression and PCR with sequencing for Beta-catenin mutation detection [37] may have role in the diverse results.

CONCLUSION

In summary, Lgr5 as a cancer stem cell marker is overexpressed in CRC, suggesting its role in the steps of carcinogenesis based on the CSC hypothesis. Therefore, follow up of CRC cases and Lgr5 immunostaining of the recurrent cases are recommended. Also, Beta-catenin is activated in most CRC. Both proteins showed significant expression in CRC, but the relation between them needs further evaluation.

Abbreviation: CRC: colorectal cancer, UC: ulcerative colitis, CSC: cancer stem cell, APC: adenomatous polyposis coli.

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