Significance of Stem Cell Marker Lgr5 Expression in Colorectal Carcinogenesis

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Abstract

Background: Lgr5 (Leucine rich repeat containing G-protein-coupled receptors) also known as G-protein-coupled receptor (Gpr-49). It belongs to the large, G-protein-coupled, 7-transmembrane (7TM) family of proteins. In order to determine whether Lgr5 is a potential marker of cancer stem cells, we investigated Lgr5 expression in normal colorectal mucosa, adenoma and carcinoma and compared the results with the clinicopathological data and prognosis.

Methods: This is a retrospective study carried upon 40 Egyptian patients with different types of colorectal lesions, 6 cases of adenoma and 43 carcinoma (20 cases were cribriform adenocarcinoma, 6 signet ring carcinoma and 8 mucoid carcinoma). Six cases were taken as control from adjacent normal mucosa in patients with intestinal infarction. Lgr5 expression was evaluated by immunohistochemistry in the collected colorectal lesions.

Results: Lgr5 was detected as cytoplasmic brown granules with different expression patterns in normal colonic mucosa, adenoma and carcinoma. Lgr5 expression was significantly higher in carcinoma than in adenoma (p<0.05). Statistical analysis revealed a significant increase in Lgr5 expression with progression from normal colon towards carcinoma (p<0.01). A significant statistical correlation was also found between Lgr5 expression and stage of colorectal carcinoma including depth of tumor invasion, lymph node metastasis and distant metastasis (p<0.05).

Conclusions: Lgr5 may play an important role in colorectal tumorigenesis and invasion, and may be potential therapeutic target for the treatment of patients with colorectal cancer. It may also be considered as a potential marker for colorectal cancer stem cells (CSCs).

Key Words: Colorectal carcinogenesis – Lgr5 expression – Stem cell marker.

Introduction

CANCER is a stem cell disease because only stem cells have the ability to self-renewal and neoplasia is essentially dysregulated self-renewal [1]. “Cancer stem cells” model is based on evidence that only a small subset of cells, the cancer stem cells (CSCs), within the tumor population, can initiate and sustain tumor growth. Tumor progression may be related to the alteration of genes that regulate stem cell renewal. Although cancers are targeted as homogenous tissues by many therapies, recent evidence suggests that considering the cancerous tissue to be composed of heterogeneous cells, including cancer stem cells (CSCs), may be more effective. It has been suggested that tumors are generated and maintained by a small subset of cancer cells capable of multi-differentiation and self-renewal, which are known as CSCs [2].

Colorectal cancer (CRC) is one of the most common cancers and is the second most common cause of cancer mortality in Western societies [3] In Egypt, it accounts for 4.34% of total malignancies and 15.87% of digestive system tumors ranking the forth most common tumor after lymphoid tumors, breast and urinary tumors [4]. Many treatment protocols have been applied to colorectal cancer, but they have not resulted in a complete cure. This may be due to colorectal cancer stem cells (CSCs) that are resistant to radiation therapy and chemotherapy, and may enable the recurrence of cancers. Therefore, it is important to use therapies that target not only proliferating cells but also stem cells in order to cure cancer [5]. Therefore, in order to seek out and eliminate colon CSCs, a specific biomarker is needed [6].

Lgr5 also known as G-protein- coupled receptor (Gpr-49). These receptors belong to the large, G-protein-coupled, 7-transmembrane (7TM) family of proteins [7]. They have a large N-terminal extracellular (ecto-) domain that contains a series of leucine-rich repeats. They are structurally similar to glycoprotein receptors, including thyroid stimulating hormone (TSH) receptors, follicle stimu-
lating hormone receptor (FSH) and leutinizing hormone receptors (LH) \[8\]. Lgr5/Gpr49 is a Wnt target gene as well as a cancer gene; it was on the original list of Wnt/Tcf4 targets that are active in colorectal cancers \[9\]. Previous studies demonstrated that Lgr5 is overexpressed in hepatocellular carcinoma, ovarian cancer, likely because of the mutational activation of the Wnt pathway in these tumors. Lgr5 expression was observed in esophageal adenocarcinoma \[10\], basal cell carcinomas and in healthy cyclic endometrium \[11\]. However, role of Lgr5 in targeting CRC is still unclear.

**Material and Methods**

This retrospective study included 40 cases of colorectal lesions (22 males and 18 females) including six cases of colonic adenoma (15%) and 34 (85%) colorectal carcinoma (20 cases (58.8%) of them were cribriform adenocarcinoma, 8 (23.5%) mucoid carcinoma and 6 (17.7%) signet ring carcinoma). Six cases of apparently normal colonic mucosa were taken as control. Malignant cases were graded into 21 cases (61.7%) of grade II (18 cases adenocarcinoma, 3 mucoid carcinoma) and 13 (38.3%) grade III (2 adenocarcinoma, 5 mucoid carcinoma and 6 signet ring carcinoma). Regarding the depth of tumor invasion, 2 (5.9%) were T 1, 3 (8.8%) T2, 9 (26.5%) T3 and 20 (58.8%) T4. Lymph node metastasis was detected in 25 cases (73.5%). Distant metastasis was detected in 19 cases (55.9%). According to TNM staging system, 9 cases (26.5%) stage II, 6 cases (17.6%) stage III and 19 (55.9%) stage IV. Collected malignant colorectal tumors were evaluated for the type and graded according to Jass \[12\] into moderately differentiated and poorly differentiated tumors and tumor stage was determined according to criteria proposed by TNM staging system according to Edge, et al. \[13\].

The material included archival formalin fixed paraffin embedded blocks processed during the years 2009-2011 as well as stained H&E slides for review. Blocks were collected from Benha Faculty of Medicine, Pathology Department. Cases were selected on the basis of availability of demographic data (age and sex) and clinical data (lymph node status and presence of distant metastasis). Six control cases were taken from adjacent normal mucosa from patients with intestinal infarction.

From each block three sections of 4 micron thickness were obtained from each case. One section was H&E stained for diagnosis and grading. Other sections were mounted on positively-charged slides, immunohistochemically stained with antibodies against Lgr5 (Labvision corporation/USA, 0.1 micron concentrated) using the Ultra Vision Detection System (Anti-polyvalent, HRP/DAB, ready-to-use, Lab Vision corporation).

**Immunohistochemical staining:** Paraffin-embedded tissue sections, 3-4 micron thick were mounted on positively-charged slides. Paraffin sections were put in xylene 5min. to ensure proper deparaffinization, and then the sections were rehydrated through a series of xylene and alcohol before staining. After antigen retrieval with microwave treatment in 10mM citrate buffer (Neo-Markers, Cat. _ AP-9003), pH 6.0, endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 minutes. Sections were washed 3 times with phosphate buffered saline (PBS), after blocking with 10% normal rabbit serum, sections were incubated in humid chamber overnight at 4oc with polyclonal antibody against Lgr5 at dilution of 1:200. The prepared DAB-substrate-chromogen solution was applied and incubated for 3-5 minutes until color intensity has been reached. Lastly, sections were counterstained with Mayer’s hematoxylin. Dako positive control slides including sections of a human normal colonic tissue for positive expression of Lgr5. Negative control was done by replacing the antibody by normal non-immune serum.

**Interpretation and evaluation of Lgr5 immunohistochemical staining:** Lgr5 was detected as cytoplasmic brown granules with different expression patterns in different colonic specimens. Sections were screened on low power, scoring system was used that incorporates both intensity of staining (1 pale brown, 2 brown and 3 dark brown) and extent scores. An immunohistochemical score of (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.

**Statistical analysis:**

Results were analysed using SPSS version 16 statistical package for microsoft software. Pearson correlation coefficient was used for statistical analysis. \(p\)-value \(<0.05\) was considered significant and \(<0.01\) was highly significant.

**Results**

**Immunohistochemical results:**

Cytoplasmic Lgr5 immunostaining has been found expressed in all colonic neoplasms (benign and malignant). However, in normal colon, scattered, rare single crypt epithelial cells were Lgr5
immunoreactive which were deemed negative because of the paucity of immunopositive cells according to the formentioned scoring criteria.

All 6 cases (100%) of adenoma showed Lgr5 expression score 1. While, 8 (23.5%) out of 34 malignant cases showed score 1, 8 (23.5%) out of 34 malignant cases showed score 2 and 18 (53%) out of 34 malignant cases showed score 3, which revealed statistically significant relation (p<0.05).

In relation to tumor grade, 4 out of 21 cases (19%) of G II showed Lgr5 expression score 1 and 13 out of 21 cases (62%) of G II showed score 3. Four out of 13 cases (30.8%) of G III showed score 1 and 5 out of 13 cases (38.4%) of G III showed score 3, but this relation is statistically insignificant (p>0.05).

Regarding the stage, 6 out of 9 cases (66.7%) of Stage II showed Lgr5 expression score 1 and 1 out of 9 cases (11.1%) of Stage II showed score 3. One out of 6 cases (16.7%) of Stage III showed score 1 and 3 out of 6 cases (50%) of Stage III showed score 3. While, 1 out of 19 cases (5.3%) of Stage IV showed score 1 and 14 out of 19 cases (73.7%) of Stage IV showed Lgr5 expression score 3, with statistically significant relation (p<0.05).

| Table: Correlation between Lgr5 expression and clinicopathological data in examined patients. |
|---------------------------------|----------------|--------|----------------|
| **No.** | **Lgr5 score** | **p-value** |
| **Type:** Adenoma | 6 | 6 (100%) | 0 | 0 | <0.05 |
| Carcinoma | 34 | 8 (23.5%) | 8 (23.5%) | 18 (53%) | |
| **Histopathological type:** Cribriform adenocarc. | 20 | 4 (20%) | 2 (10%) | 14 (70%) | >0.05 |
| Mucoid carcinoma | 8 | 1 (12.5%) | 5 (62.5%) | 2 (25%) | |
| Signet ring carcinoma | 6 | 3 (50%) | 1 (16.7%) | 2 (33.3%) | |
| **Grade:** GII | 21 | 4 (19%) | 4 (19%) | 13 (62%) | >0.05 |
| GIII | 13 | 4 (30.8%) | 4 (30.8%) | 5 (38.4%) | |
| **Stage:** Stage II | 9 | 6 (66.7%) | 2 (22.2%) | 1 (11.1%) | |
| Stage III | 6 | 1 (16.7%) | 2 (33.3%) | 3 (50%) | <0.05 |
| Stage IV | 19 | 1 (5.3%) | 4 (21%) | 14 (73.7%) | |

*N.B.:* Statistical analysis revealed a significant increase in Lgr5 expression with progression from normal colon towards carcinoma (p<0.01). There is significant correlation between Lgr5 expression and types of disease (p<0.05). A significant statistical positive correlation was found between score of Lgr5 expression and stage of colorectal carcinoma including depth of tumor invasion, lymph node metastasis and distant metastasis (p<0.05).
**Discussion**

Cancer stem cell theory has become an intensely investigated topic, even though the origin of cancer stem cells (CSCs) remains elusive and isolation, identification of CSCs has not been achieved for many types of human tumors. CSCs are defined as a small side population of tumor cells with the ability to self-renew and potentially promote the formation of tumors [14,15]. Conventional cancer treatments indiscriminately kill proliferating cells and always unsuccessful due to survival of quiescent CSCs. Therefore, therapies could be designed to target cancer stem cells by inducing their differentiation or to eliminate them by inhibiting maintenance of stem-cell state [5,16]. CSCs can survive radiation therapy and chemotherapy and then return to a proliferative growth state, making them good targets for biomarker identification [10]. The cellular origin of cancer stem cells has not been clearly determined. G-protein-coupled receptors (GPCRs) have been hypothesized to be closely associated with CSCs during tumorigenesis [17,18]. Lgr5 is a member of the GPCRs superfamily. Lgr5 over-expression has been reported in a few cancers, including hepatocellular carcinoma, colorectal cancer, ovarian cancer, basal cell carcinoma, and esophageal adenocarcinoma [19-21].

Recent studies suggested that Lgr5 may be involved in colorectal carcinogenesis as a target of Wnt signaling Van der Flier, et al., [22] and Segditsas, et al., [23] and may be an ideal marker of colorectal CSCs Takeda, et al., [24] and Takahashi, et al., [25]. In this study, Lgr5 was significantly overexpressed in majority of CRCs (53% were score 3) compared with cases of adenoma (all cases were score 1) while all examined normal mucosa were negative for Lgr5 expression. Statistical speaking, there is significant increase in Lgr5 expression with progression from normal colon towards carcinoma (p<0.01). This indicates increased expression of the stem cell marker Lgr5 in premalignant and malignant lesions than in normal mucosa and suggesting its role in CRC initiation.

These results were in agreement with Fan, et al., [26] who found that Lgr5 immunoreactivity in normal control colonic crypts was scattered and rare whereas 54% of colorectal carcinoma cases were score 3. Lewis, et al., [27], also noticed that 27% of colorectal adenoma cases showed Lgr5 expression score 1 and 54% of CRC cases showed score 3. Becker, et al., [10], found that Lgr5 expression was detected in 70% of barret's esophageus cases. On the other hand, Lgr5 expression was detected in between 90 and 100% of advanced dysplastic lesions and esophageal adenocarcinoma. In gastric lesions, it was found that 18.2% of premalignant lesions showed Lgr5 expression score 1 and about 80% of gastric carcinoma showed score 3, Schmuck, et al., [28]; Wang, et al., [29] and Simon, et al., [30]. There was an increase in the number and intensity of lgr5+ cells from non-neoplastic epithelium to gastric cancer.

Insignificant statistical correlation was found between Lgr5 score and the grade of CRC. Lgr5 expression was significantly correlated with depth of invasion, lymph node metastasis, and distant metastasis. These results suggest that high expression levels of Lgr5 receptors are usually correlated with poor tumor prognosis and bad patients' outcome. Furthermore, Lgr5 was seen more frequently in advanced colorectal cancer, as 11.1% of stage II cancer show score 3 Lgr5 expression, 50% of stage III were score 3 and 73.7% of stage IV were score 3 Lgr5 expression. This significant positive correlation between Lgr5 expression and stage of CRC (p<0.05) suggesting that Lgr5 may play an important role not only in tumor initiation but also in progression of the tumor. Uchida, et al., [21]. Merlos, et al., [31] and Takahashi, et al., [25], also found increased Lgr5 expression in advanced stages of CRC cases. Simon, et al., [30], found significant correlation between score of Lgr5 expression and advanced tumor stage in gastric adenocarcinoma. In contrast to our results Takeda, et al., [24] and Gerger, et al., [32] found that in adenocarcinoma Lgr5 expression was negatively associated with advanced tumor stage. McClanahan, et al., [17] found no significant correlation between score of Lgr5 expression and stage of ovarian tumors where Lgr5 score was high in stage I and II ovarian tumors and appears to decrease in stage III and IV tumors suggesting that overexpression of Lgr5 may be an early event in tumorigenesis and not in tumor progression. Sun, et al., [33] found no significant correlation between score of Lgr5 expression and the stage of endometrial carcinoma as Lgr5 is highly expressed in the endometrium during the initial stages of tumorigenesis, but is remarkably down-regulated in advanced tumors. Becker, et al., [10] stated that high Lgr5 expression score were independent of stage in esophageal adenocarcinoma.

From the current study, we found that Lgr5 as stem cell marker of cells with intestinal differentiation was expressed and presented with different expression patterns in normal colonic mucosa,
expression was positively correlated with stage of CRC.

Conclusion:

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

References


