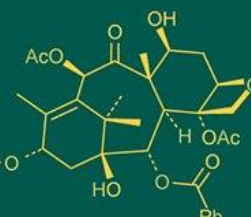
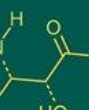
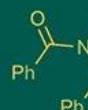


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Targeting of LGR5 as colorectal cancer stem cell marker in colorectal cancer patients

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Abstract

Background: Colorectal cancer (CRC) is a major public health problem worldwide, and it represents the third most frequent neoplasm among diagnosed cancers, as well as ranking second in the number of deceased patients for this type of tumor.

Objective: This study investigates the Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), which is a key component in the tumor stage-specific molecular network of CRC. Initially identified as a marker of normal intestinal stem cells, LGR5 has garnered significant attention for its potential roles in CRC tumorigenesis and chemotherapy resistance. This is attributed to its function as a WNT target gene.

Methods: A total of 50 formalin-fixed paraffin-embedded tissue blocks of new diagnosis and recurrence colorectal cancer were collected from the histology teaching lab at The Medical City Hospitals, Baghdad, Iraq. LGR5 expression was assessed through immunohistochemical staining of a tissue.

Results: The evaluation showed that LGR5 was used as a marker to target cancer stem cells in colorectal cancer.

Conclusion: LGR5 was over-expressed in the recurrence sample compared with low expression in the newly diagnosed sample.

Keywords: Cancer stem cells, colorectal cancer stem cells, LGR5, IHC

1. Introduction

Colorectal cancer (CRC) is one of the major public health problems globally, being ranked as the third most common neoplasm and second leading cause of cancer-related deaths worldwide [1]. Colorectal cancer is likely to progress slowly over two to 10 years before metastasis occurs. The development of CRC is a complex and multistep process that involves the integration of multiple genetic, epigenetic, and environmental determinants. Colorectal cancer stem cells possess the dual characteristics of self-renewal and the ability to initiate tumorigenesis; this represents a distinct subpopulation within tumors. However, their stem cell-like features and resistance to conventional therapies could cause these cancer-initiating cells to be responsible for carcinogenesis, which leads to tumor growth, metastasis, and relapse [2]. Cancer stem cells (CSCs) are self-renewable stem cells that generate more of the same type or differentiate into multiple tumor cell types. In colorectal cancer, the primary method for identifying CSCs involves the use of specific markers, including CD133, CD44, and LGR5, that aid in the purification and analysis of CSCs. CSCs contribute to cancer recurrence and metastasis as they resist conventional chemotherapy and/or radiotherapy treatments. These cells are very resilient because of their highly expressed drug efflux pumps and efficient DNA repair processes. They are likely pivotal in tumor initiation and development due to the higher frequency of tumor formation by these cells. This unique concept has resulted from the close interaction between CSCs and their microenvironment, including stromal cells, immune cells, and extracellular matrix components that impact CSCs' behavior [3]. LGR5 was remarked as an optimum marker to identify ordinary stem cells of the intestines; its presence in the actively proliferating cells at the base of intestinal crypts indicates its probable role in cancer stem cells. Studies revealed that LGR5-positive cells in colorectal cancers exhibit characteristics commonly associated with cancer stem cells, such as self-renewal, tumorigenicity, and chemoresistance [4].

These properties are further enhanced by increased invasiveness and metastatic potential^[5]. The recurrence of tumors is associated with the persistence and re-initiation of oncogenic progression by LGR5-positive cells following therapeutic interventions^[6]. In order to maintain the stem cell niche and control cell proliferation and differentiation, the Wnt signaling pathway becomes important. R-spondins are signaling molecules that stimulate the Wnt pathway, and LGR5 acts as a receptor for them. This activation leads to an increase in Wnt signaling and an improvement in stem cell functioning. Additionally, the signaling cascade contributes to the development of colorectal cancer and is critical for intestinal homeostasis maintenance^[7]. Here, LGR5-positive CSCs have demonstrated a capacity to initiate tumorigenesis in xenograft models. Therefore, their critical role in the pathogenesis of CRC is underscored. Moreover, the expression of LGR5 is associated with aggressive tumor phenotypes and adverse clinical outcomes. These cells contribute to the heterogeneity of tumors and are implicated in the tumors' resilience against conventional therapeutic modalities, including chemotherapy and radiotherapy^[6].

2. Materials and Methods

2.1 Ethics approval

Ethics approval statement: The project was approved by the Higher Institute of Forensic Sciences Ethics Committee number T.H. 3345 on 23/12/2024.

2.2 Samples collection and clinicopathological study

The study included a sample of 50 patients consisting of males and females aged 30-55 years. Samples were collected from the Histology Teaching Lab at The Medical City Hospitals, Baghdad, Iraq. Colorectal samples were validated by histopathological assay and separated into two groups: Group I included twenty samples obtained from patients who were recently diagnosed with colorectal cancer^[21], and Group II included thirty samples obtained from patients who had undergone chemotherapy and had tumor relapses.

2.3 Histopathological staining protocol

Paraffin-embedded sections of colorectal cancer tissue were sectioned at a thickness of 4 µm using an electronic microtome. These sections were subsequently mounted on positively charged slides and allowed to dry overnight at room temperature. The slides underwent treatment with PolyExcel H₂O₂ for 5 min to inhibit endogenous peroxidase activity. Subsequently, PolyExcel was administered to the sections for 10 min at ambient temperature to facilitate the target antigen binding. The next step included the slides being subjected to incubation with PolyHRP for 10 min at ambient temperature to promote the detection of the antigen-antibody complex. Then, the sections were incubated with StunnDAB solution for 5 min at ambient temperature to enable visualization of the antigen-antibody complex. Ultimately, a peroxidase substrate was incorporated to augment the staining intensity, where a fixing reagent was utilized to stabilize the resultant staining.

2.4 Staining with Haematoxylin and Eosin

Haematoxylin and Eosin (H & E) staining is a conventional tissue staining method; it is the most routine technique for tissue slides when working in histopathology. First, the slides were washed twice with 1X PBS for 3 min to terminate the remaining medium. Later, the slides were

treated with xylene for 2 min, followed by sequential immersion for 30 sec in graded alcohol of concentrations 100% to 70%. Then, the slides were immersed in haematoxylin solution for 30 sec and rinsed in water for one minute. Next, the slides were stained with eosin for 5 sec, rinsed in water, dehydrated through a graded ethanol series (70% to 100%) for 15 sec each, and finally cleared in xylene for 1 min. It is worth noting that a drop of DPX mounting medium was added to the slides to preserve the stained tissue.

2.5 Tissue Processing and Immunohistochemistry Examination

A. Tissue Processing

Tissue samples were deparaffinized using standard protocols. Rehydration was achieved by immersing slides in a graded ethanol series (100% to 70%) for 2 min each, followed by a 3 min rinse in distilled water. Slides were then buffered to prevent non-specific antibody binding and background staining.

B. Antigen Retrieval

Heat-induced epitope retrieval (HIER) was employed to unmask antigens. Slides were immersed in Tris-EDTA buffer (pH 9.0) and heated in a water bath at 96°C for 30 min. After cooling to room temperature, they were rinsed in a wash buffer for 10 min.

C. Immunohistochemistry

Slides were incubated with hydrogen peroxide for 5-10 min to block endogenous peroxidase activity, followed by a 5-min wash. The primary antibody, anti-LGR5, was diluted at 1:50 and applied to the slides for 30 min at room temperature in a humidity-controlled chamber. After a 5-minute wash, a secondary antibody conjugated to horseradish peroxidase (HRP) was applied and incubated for 30 min. Following another 5-minute wash, the DAB chromogen substrate was added for 5 min to visualize the antigen-antibody complex. Slides were then washed, dehydrated, and mounted with DPX.

2.6 Microscopic Examination and Scoring

Stained slides were examined under a light microscope at 10X, 20X, and 40X magnifications. The presence of brown-stained cell membranes assessed positive staining. The intensity of staining was scored semi-quantitatively as follows:

Score 0: No positive cells

Score 1: 10% or fewer positive cells

Score 2: 11-30% positive cells

Score 3: 31-50% positive cells

Score 4: More than 50% positive cells

3. Results

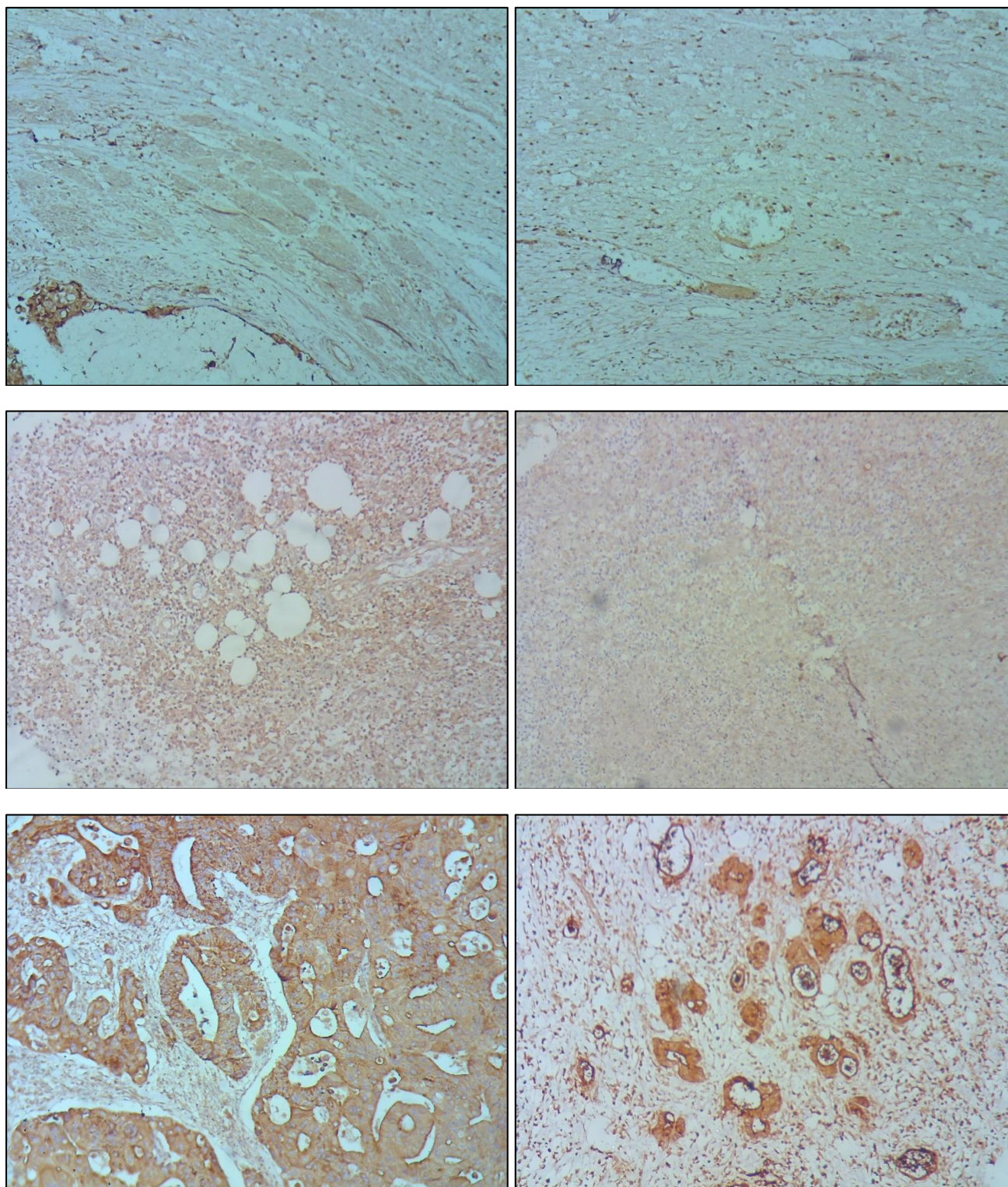
3.1 Microscopic Examination and Scoring

Table 1 shows the number and percentage of patients in each group with different LGR5 scores (a protein involved in cell growth and differentiation) from 0 to +4. Group I refers to patients newly diagnosed, while Group II refers to patients experiencing a recurrence after previous treatment. The higher proportion of patients with higher LGR5 scores in the "Recurrence" group compared to the "New diagnosis" group, where the number of samples with +3 and +4 scores is 9 and 13, respectively, for the recurrence group.

Table 1: Frequency of LGR5 cell scoring in colorectal cancer patients' groups.

LGR5 Score	Group I New Diagnosis		Group II Recurrence		Total	
	No.	%	No.	%	No.	%
Score 0	8	40	0	0	8	16
Score +1	7	35	2	6.6	9	18
Score +2	3	15	6	20	9	18
Score +3	2	10	9	30	11	22
Score +4	0	0	13	43.4	13	26
Total	20	100	30	100	50	100

Immunohistochemistry (IHC) staining for LGR5 was performed on a colorectal cancer tissue sample. Figures 1 and 2 present the immunohistochemistry staining revealed LGR5 expression in both new diagnosis and recurrence samples of colorectal cancer, respectively, observed at 100X magnification. Figure 1 shows that the positive LGR5 expression was indicated by brown coloration. Intense LGR5 staining, as observed in Figure 2, suggests robust and widespread LGR5 immunoreactivity within the tumor cells. The CSCs, characterized by their ability to self-renew and differentiate, are implicated in tumor initiation, progression, metastasis, and therapeutic resistance.

**Fig 1:** Immunohistochemistry staining for new diagnosis sample and LGR5 expression in colorectal cancer (100X).

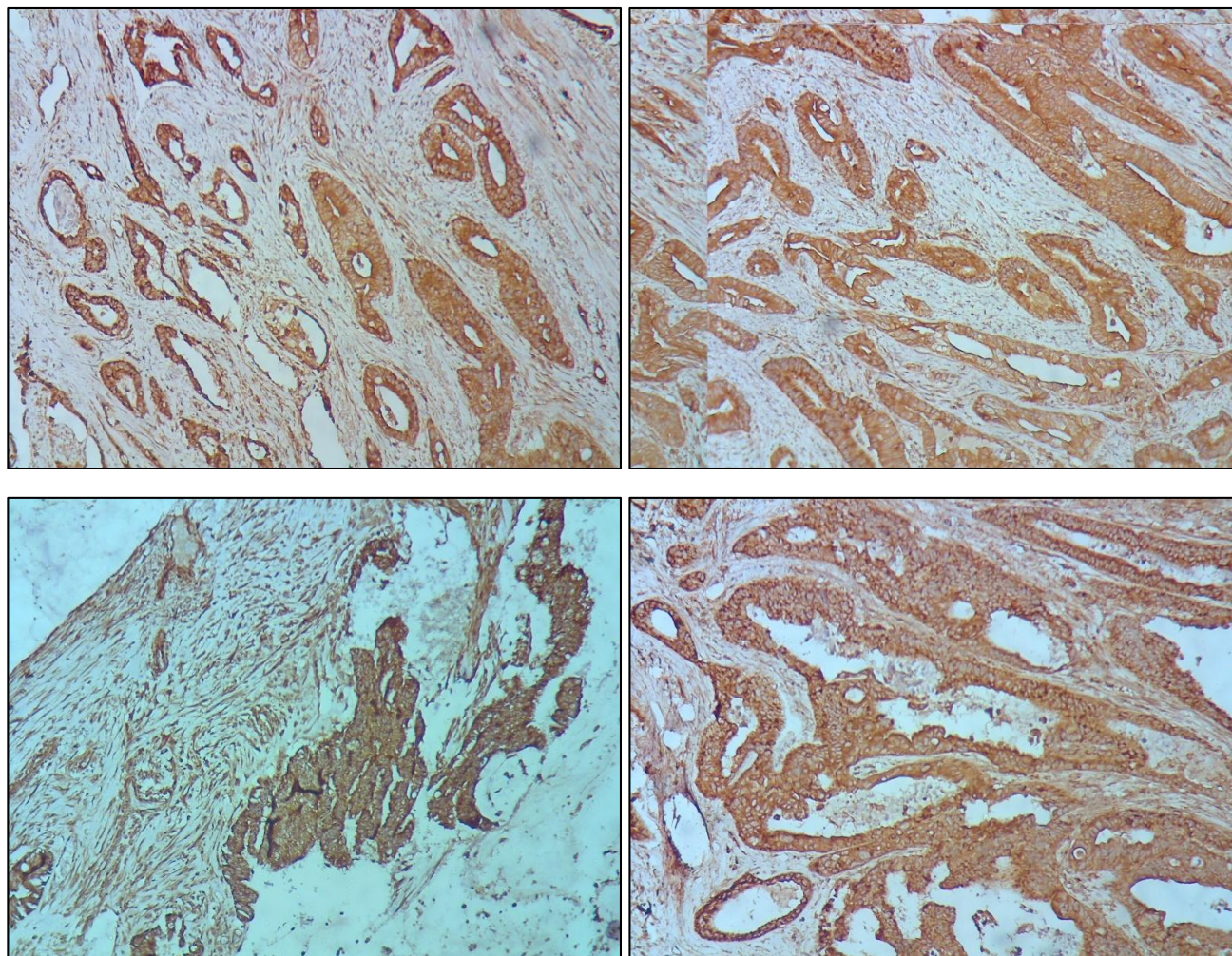


Fig 2: The immunohistochemistry staining for recurrence sample and LGR5 expression in colorectal cancer (100X).

Tumor cells with LGR5 appear brown, while those without appear blue or violet. We found that the percentage of LGR5-positive cells differed between groups. Patients newly diagnosed with the disease (Group I) had lower

LGR5 expression (around 33%) compared to patients experiencing a recurrence (Group II), who showed high LGR5 expression (around 82%), as illustrated in Figure 3.

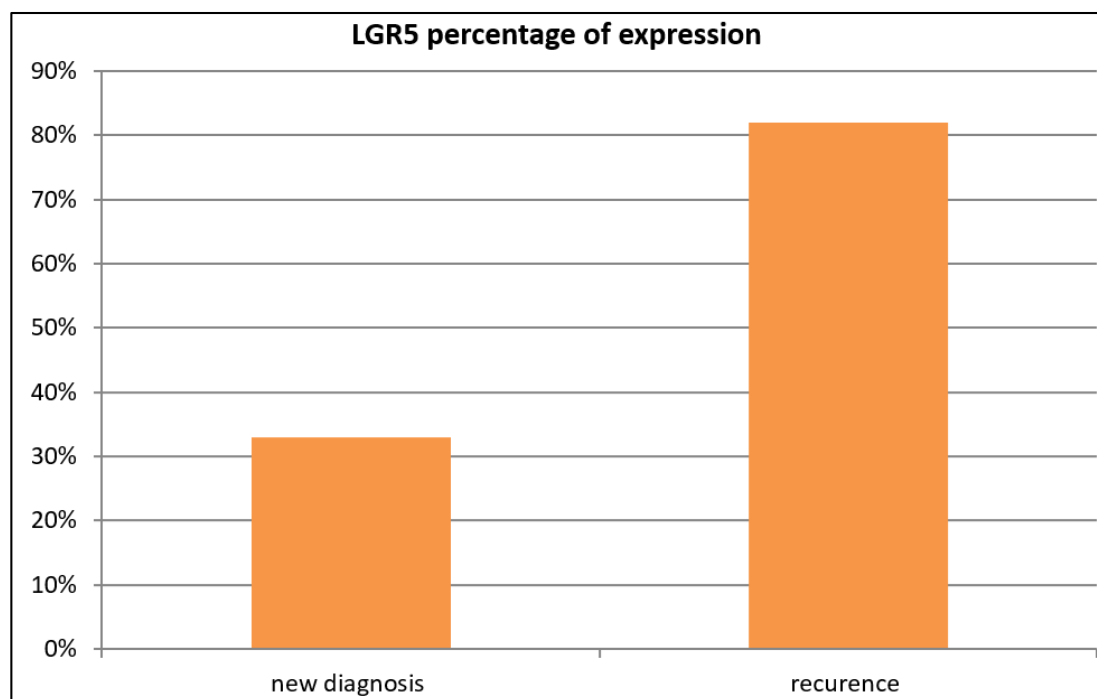


Fig 3: A comparison of the LGR5 percentage of expression between new diagnosis and recurrence

4. Discussion

Our findings demonstrate the potential for targeting CSCs in CRC by exploiting LGR5 as a marker. LGR5 is a well-established marker for intestinal stem cells that is critical in maintaining and self-renewing CSCs in CRC. This suggests that therapeutic strategies aimed at inhibiting LGR5 function or disrupting its downstream signaling pathways may offer a promising approach for selectively eliminating CSCs and improving treatment outcomes for CRC patients. Colon cancer is the second leading cause of cancer-related deaths, with a subset of particularly aggressive cancer cells known as colon cancer stem cells (CCSCs) driving tumor growth and resistance to therapy. These CCSCs possess self-renewal and differentiation capabilities, which enable them to perpetuate the disease and evade treatment^[8, 9].

Previous studies revealed the critical role of LGR5 in CRC pathogenesis^[10, 11]. Elevated LGR5 expression levels were observed in recurrent CRC, for which a potential association with disease recurrence risk is posed^[12]. Also, our finding aligns with previous studies demonstrating that the Wnt/ β -catenin signaling pathway, which is activated by LGR5, becomes dysregulated during the transition of colorectal cancer (CRC) from adenoma to carcinoma^[13]. Further results showed that LGR5 is a marker for cancer stem cells in CRC. Additionally, the association between elevated LGR5 expression and more aggressive tumor characteristics further supports this perspective^[14]. Additionally, the importance of LGR5 and β -catenin expression levels in adenomatous polyps was previously established, where their potential as valuable prognostic markers for CRC development and progression is noted. Mechanisms underlying the role of LGR5 in CRC recurrence and its potential as a therapeutic target^[15]. Differential expression of stem cell markers, including LGR5 and CD133, was present across different types of colorectal cancer. In contrast to normal mucosa and adenomas, these markers showed increased expression in advanced stages of colorectal cancer. Here, their potential involvement in colorectal tumorigenesis and progression is highlighted^[16]. This observation is further supported by another study that reported significant LGR5 overexpression in CRC tissues and established its independent prognostic value for improved overall survival^[17].

In our study, IHC was employed to assess LGR5 protein expression within tumor cells. This technique utilizes a brown chromogen to visualize LGR5 protein within tumor cells, allowing for qualitative assessment of LGR5 abundance based on staining intensity. Furthermore, LGR5-positive tumor cells were compared to the total number of tumor cells within the observed field, enabling the estimation of the percentage of cancer stem cells within the tumor, as previously described in^[18]. It is good to mention that CSCs are likely responsible for tumor initiation, growth, metastasis, progression, recurrence, and resistance to conventional therapies^[19, 20]. This highlights the limitations of current treatment approaches that primarily target proliferating cells, as these therapies may fail to eliminate the CSC population and lead to disease relapse. Our results emphasize the need for novel therapeutic strategies that specifically target CSCs. These strategies could focus on inducing CSC differentiation, thereby preventing their self-renewal and tumorigenic potential or inhibiting the signalling pathways that maintain the stem-cell state^[22].

5. Conclusion

In our investigation, LGR5, a protein posited to serve as a marker for colorectal cancer stem cells, was observed to exhibit significant activity in recurrent neoplasms. This finding underscores the potential significance of cancer stem cells in the recurrence of the pathology. Concentrating on LGR5 may yield improved therapeutic outcomes, particularly in instances that present challenges to current treatment modalities.

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7. Funding

None.

8. Conflict of Interest

There is no known conflict for this work.

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