

ORIGINAL ARTICLE

Correlation between LGR5 stem cells and location of tumor as well as age, sex and metastasis in colon adenocarcinoma

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Summary

Purpose: G-protein receptors belong to a large family of receptors which includes more than 800 kinds. An interest for these receptors arose upon noticing their expression on skin, intestine and breast stem cells.

Methods: We examined the tissues of 53 patients who had been diagnosed with adenocarcinoma of the colon. There were no exclusion criteria. We measured the expression levels of LGR5 by immunohistochemistry and we correlated those and the location of the colon primary tumor with the age, sex and the metastatic potential.

Results: The median values of the two groups- the orthosigmoid and the other colon location- were 1 and 3.5 respectively with no statistical significance and with $p=0.132$. Spearman Rho indicated no statistical significance between the expression of LGR5 and age with $p=0.219$. Moreover, the Mann-

Whitney U test showed no statistical significance between LGR5 and sex with $p=0.778$. Fisher's test, however, showed a statistically significant result between metastasis and expression of LGR5 with $p=0.025$.

Conclusions: It becomes apparent that patients with increased expression of these two markers are characterized by a more aggressive form of the disease with an increased rate of metastasis. Also, interactions on both paths lead to a simultaneous overexpression, thus proving the diversity of carcinogenic pathways. Racial and other factors are not affected, and ultimately the need to target these indicators in treatment protocols is imperative.

Key words: LGR5, receptor, adenocarcinoma, colon, stem cells, metastasis

Introduction

The G-protein receptors belong to a large and multifactorial membrane protein family with over 800 genes that regulate their expression [1]. They participate in signal transductions essential to cell immune response, signal transduction within neural pathways, muscular or cardiac contraction, excretion of important enzymes, regulation of blood pressure and so on. This family

of receptors responds to a large scale of stimuli from photons to ions, minor organic molecules, peptides and proteins [2] (Figure 1). With regard to stimuli, the receptor undergoes changes to cause the proper response and activity inside the cytosol.

The LGR5 gene acts synergistically with the gene of the WNT receptor, expressing a family of

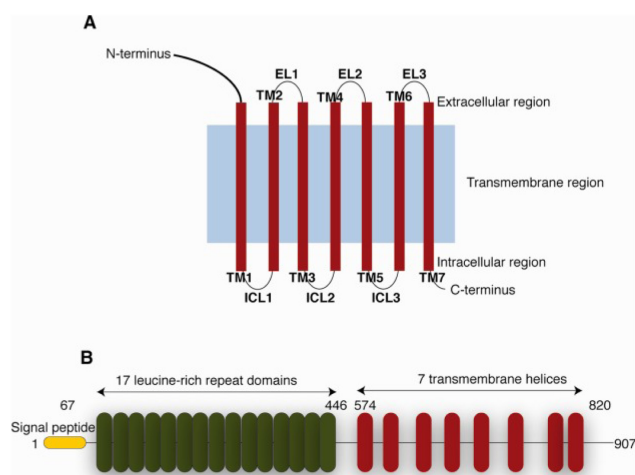


Figure 1. Schematic illustration of GPCR and LGR5. **A:** general architecture GPCR. **B:** LGR5 contains a peptide followed by repeated leucine peptides. It also contains a connection between the last peptide and the first transmembrane region with 7 similar following regions.

agonists of the WNT molecular pathway called R-spondins. This indicator has been found in stem cells of the stomach and the small intestine of mice [3], while it has also been identified in stem cells of the human intestine [4]. In studies with *in situ* hybridization, the mRNA of the gene for LGR5 was found at the base of the crypt which is the same pattern as the one found in mice [3-5].

A high percentage of LGR5⁺ stem cells were isolated in adenomas of the large intestine. Although it is known that the number of stem cells is substantially high, only a part of them are active every time and thus the percentage of the expression of the indicator changes every minute according to the circumstances [6]. Therefore, the fact that a small part of stem cells is active at any given time probably explains the low rate of the development of an adenoma.

Becker et al suggested that LGR5 may be a better marker of cancer stem cells (CSCs) in colorectal carcinoma (CRC) [7]. Elevated LGR5 expression has been observed in several types of cancers, including hepatocellular carcinoma [8], CRC [9], ovarian cancer [10], and basal cell carcinoma [11]. In particular, many studies have suggested that LGR5 plays a key role in CRC carcinogenesis and is associated with poor outcome of CRC patients [12-14]. Therefore, it was expected that LGR5 expression in CRC would be an ideal prognostic marker that is correlated with low survival.

The purpose of the study was to show how the expression of LGR5 affects the aggressiveness of the disease and whether it is related to other individual characteristics, thus outlining a personalized disease profile.

Methods

In our study, using immunohistochemistry, we measured the levels of LGR5 in patients' tissues who were operated for adenocarcinoma of the colon. We included 53 tissues and later we correlated LGR5 levels and other factors such as the location of the primary tumor in the colon, the age, sex and metastasis. There were no exclusion criteria and the study was conducted at the National and Kapodistrian University of Athens in the Pathology Department of Laiko General Hospital, Athens.

Statistics

The statistical analysis of the data was made with the use of Jamovi (vers 0.9.5.16) and R (vers 3.2.2) software. For the study of the correlation between the location of the carcinoma and the expression of the LGR5 marker the Mann-Whitney U test was used. The Mann-Whitney U test examines whether 2 average values between independent samples are equal. Since it is a non-parametric test it aids in investigating the correlation in populations where normality cannot be satisfied. Moreover, the same test was applied in order to investigate the existence of a correlation between sex and LGR5 expression. In both cases a value of $p < 0.05$ for significance level 95% was used as a criterion of statistical significance. The Spearman Rho test was utilized for the statistical evaluation of the relation between age and LGR5 expression, while for the relation with the presence of metastasis the Fisher's exact test was used. The Fisher's exact test is used to investigate the correlation between categorical variables when the number of the observations is small (< 1000).

Results

Correlation between the location of the tumor and LGR5 level

The location of the cancer was used as an independent variable since the number of cancer cases in the descending colon was small ($n=7$). Two groups were created; one with the cases where the tumor was located in the orthosigmoid and the other with the tumor located elsewhere in the colon.

In each case the expression of the marker was used as a dependent variable. Four grades were created in relation to the signal intensity of the cancer stem cells.

The median values of the groups of the orthosigmoid and the other colon locations were 1 and 3.5 respectively. The distributions of the two groups did not have statistical differences (Mann-Whitney $U=255$, $n_1=21$, $n_2=32$, $p=0.132$).

Correlation between age and LGR5 expression

The Spearman Rho test did not result in a statistically important correlation between age and

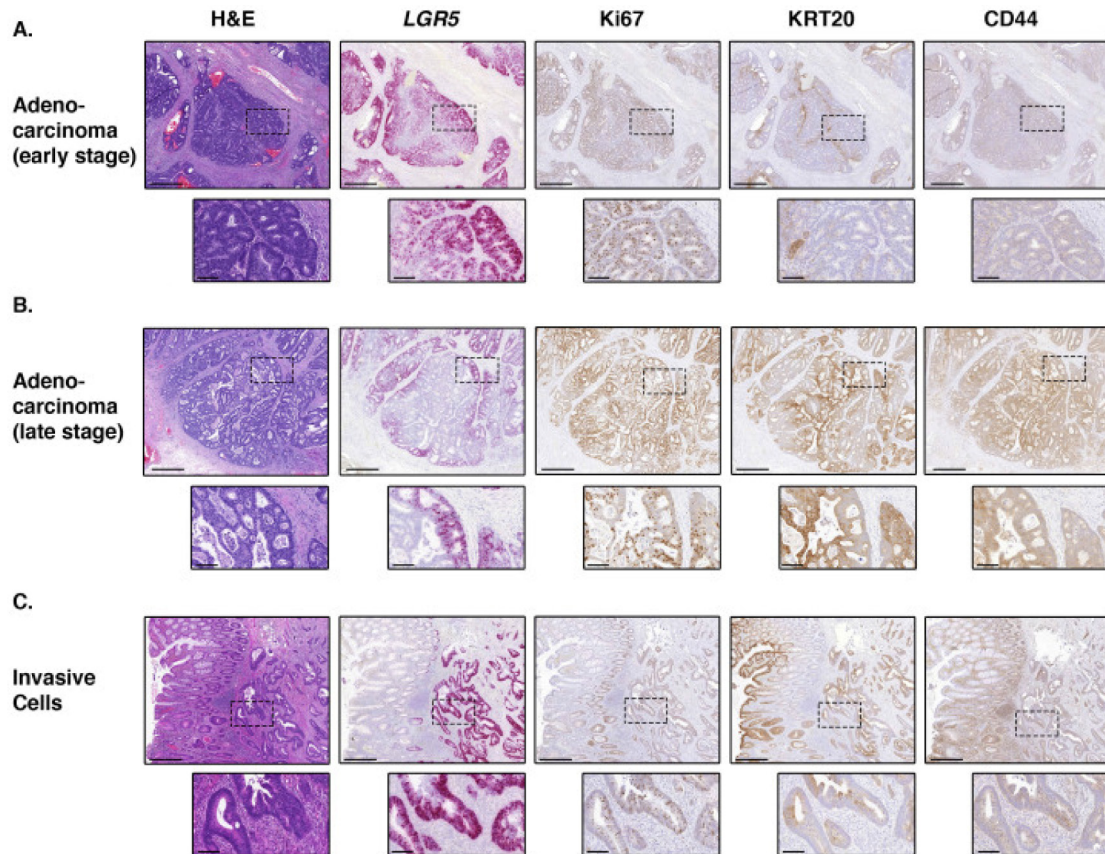


Figure 2. The expression of LRG5 in the different stages of adenocarcinoma (A, B, C). The expression of LRG5 in the stage of the insertion of cancer cells is particularly high.

the expression of LGR5. Specifically, the statistical inducers were $ps=-0.172$, $p=0.219$ (Figure 2).

Correlation between sex and LGR5 level

The Mann-Whitney U test did not lead to a statistically important correlation between sex and the expression of LGR5. The median LGR5 value for both male and female groups was 3. As for Mann-Whitney, the values were $U=334$, $n1=28$, $n2=25$ and $p=0.778$.

Correlation between the presence of metastasis and the LGR5 expression

The appearance of the metastases was statistically significant in relation to the increased expression of the LGR5 indicator ((Figure 3, $p=0.025$). This is the key point of the study, emphasizing the aggressiveness that characterizes the tumors, rich in these LGR5⁺CSC.

Discussion

Many studies have shown that the expression of LGR5 is positively associated with poor prognosis in CRC [11,13,15]. The prognostic value of LGR5 in CRC patients is controversial, and an insufficient

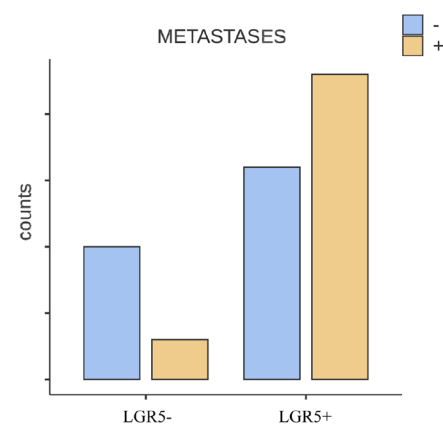


Figure 3. The presence of metastases is more common in LGR5+ colon cancer, contrary to the LGR5- colon cancer ($p=0.025$).

sample size and several other factors likely resulted in opposite results of different clinical studies, but in general it is acceptable that LGR5 CSC have a negative role in carcinogenesis.

This is supported by several studies which have shown that LGR5 is closely associated with tumorigenesis and tumor invasion in CRC and is likely to be a relevant marker of CSCs in CRC [11,16]. CSCs are thought to be responsible for the

local invasion, metastasis and recurrence of malignant tumors because of their self-renewal and multi-differentiation potential and thus are also a major obstacle to improving overall cancer survival. This is not a thought now, because we have some results which follow this statement. Studies have demonstrated that elevated LGR5 expression significantly correlates with lymphatic invasion, vascular invasion, tumor depth, lymph node metastasis, and tumor recurrence [17,18].

Selective ablation of LGR5⁺ CSCs in LGR5⁺ caspase9 knock-in organoids leads to tumour regression, followed by tumour regrowth driven by re-emerging LGR5⁺ CSCs, as Mariko Shimokawa et al showed in their study [19], something very important, because makes the need for a targeted therapy necessary.

A meta-analysis of 7 studies comprising 1833 CRC patients, including 6 studies comprising 1781 patients for overall survival (OS) and 3 studies comprising 528 patients for disease-free survival (DFS) reported that the results showed that high LGR5 expression was associated with poor prognosis in terms of OS (HR: 1.87, 95% CI: 1.23–2.84; $p=0.003$) and DFS (HR: 2.44, 95% CI: 1.49–3.98; $p<0.001$), supporting the theory that high levels of LGR5 lead to a more aggressive disease [20].

However, another study showed that LGR5 CSCs identifies intestinal CSCs in mouse tumours engineered to recapitulate the clinical progression of human CRC. They demonstrated that selective LGR5⁺ cell ablation restricted primary tumour growth, but did not result in tumour regression, because tumours are maintained by LGR5⁻ cells that continuously attempt to replenish the LGR5⁺ CSC pool. These data highlight distinct CSCs dependencies for primary vs metastatic tumour growth, and suggest that targeting CSCs may represent a therapeutic opportunity for managing metastatic disease [21].

In our study the results show that the location of the growing tumour does not seem to relate to the degree of the expression of the LGR5 marker from the cancer stem cells. This is probably due to the fact that the stem cells of the colon and the rectum, where intestinal tumors originate from the same population of the embryonic cells of stomach, the small intestine, the colon and the organs related to them, are all developed from the endoderm. This process takes place through the activation of a complex network of transcription factors and through the interaction between the endoderm

and the mesoderm. Studies in drosophila showed that stem cells in the intestine come from the endoderm as well [22]. A recent study on embryonic stem cells revealed that every LGR5 intestine stem cell has the same transcriptional profile regardless of its location [23]. The statistical analysis of the results seems to confirm the above statement.

There is no indication that the expression of LGR5 depends on the sex. The gene that expresses the LGR5 protein is located in the chromosome 12. A study of the LGR5 and endometrial cancer cells in mouse models showed an increase in the expression of the LGR5 after the effect of progesterone [24]. Nonetheless, a corresponding correlation effect between sex hormones and intestinal cells has not been proved yet. The relation between age and the effect of CRC has been studied thoroughly [36–38]. Age is considered the most important risk factor for the appearance of CRC. Nonetheless, the effect of age on cellular and molecular levels in the intestinal tissues has not yet been clarified. A recent study [25] came to the conclusion that the percentage of the LGR5 intestinal stem cells does not decrease as age progresses. Instead, the decrease of the regenerative potential of the intestinal epithelium that is observed as age increases is due to lower activation of the WNT molecular path. After a statistical analysis of the observations of the present study it is concluded that there is no correlation between age and the percentage of expression of the LGR5 in cancer stem cells. According to current theories about intestine carcinogenesis these cancer stem cells come from the intestinal stem cells [26–28].

Finally, according to our prior discussion and summary our results are compatible with the results of other studies about the metastatic potential and its dependence on the expression of the aforementioned marker. The strong expression of LGR5 in the intestinal cancer stem cells plays an important role in the metastatic potential of the tumour and it seems that the tissues that express the marker have a much higher probability to develop an isolated metastasis. Therefore, at an initial removal of the primary tumour LGR5 levels could successfully lead to further supervision of the patient in order to find a possible metastasis promptly.

Conflict of interests

The authors declare no conflict of interests.

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