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Role and Regulatory Mechanism of LGR5 in the Occurrence and Development of Breast Cancer

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ABSTRACT

Breast cancer is a phenomenon in which mammary epithelial cells proliferate out of control under the action of various carcinogenic factors. This study aimed to explore the role and regulatory mechanism of LGR5 in the occurrence and development of breast cancer. Fresh breast cancer case samples and normal breast cell samples were collected. Western blot was used to analyze the expression of LGR5 and (phosphorylated IQGAP1)/(IQGAP1). Construct metastatic human breast cancer MDA-MB-231 cell line knocking down LGR5. The relative expression levels of LGR5 and phosphorylated IQGAP1 were detected by Western blot. The relative activity of the above three types of cells after 24 h of culture was detected by CCK8 assay. The migration ability of the cells was analyzed by the cell scratch test, and the invasive ability of the cells was analyzed by the transwell detection. Construct LGR5-knockdown, LGR5-overexpressing and LGR5-ΔC-overexpressing (lack of C-terminal tail) human colon cancer LoVo cell lines. The mechanism of the reaction between LGR5 and IQGAP1 was examined by immunoprecipitation. Expression of LGR5 and phosphorylation ratio of IQGAP1 showed opposite trends in breast cancer and normal body. Viability, migration and invasion ability of breast cancer cells were all affected by LGR5 expression and IQGAP1 phosphorylation ratio. LGR5 affected IQGAP1 phosphorylation process by linking to IQGAP1 through its own C-terminus. This study proved that LGR5, which is highly expressed in breast cancer cells, interacts with IQGAP1 through its C-terminal end, reducing the phosphorylation ratio of IQGAP1, thereby improving the viability, migration and invasion ability of breast cancer cells, and ultimately increasing the malignant transformation rate of breast cancer.

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YZ and DG conceived and designed experiments. YZ, QM, HT, QZ and DG performed experiments, data collection and analysis and provided technical support. DG wrote the manuscript. All authors provided final approval for submitted and published version.

Key words
Breast cancer, LGR5, IQGAP1

INTRODUCTION

Breast cancer is a condition in which mammary epithelial cells multiply uncontrollably as a result of a variety of carcinogenic causes (Akram *et al*., 2017; Katsura *et al*., 2022). Symptoms such as breast lumps, nipple discharge, and axillary lymph node enlargement are common in the early stages of the disease (Ahmad, 2019). Cancer cells may metastasis far away at the late stage, resulting in multi-organ tumors that directly threaten the patient's life (Momenimovahed and Salehiniya, 2019). Breast cancer is known as the pink killer because it is the most common malignant tumor in women, while male breast cancer is relatively uncommon (Anastasiadi and Georgios, 2017). Breast cancer has become one of the solid tumors with the best curative effect as medical technology has advanced. Common markers for breast cancer tumor examination include serum cancer antigen 15-3 (CA15-3), serum carcinoembryonic antigen (CEA), serum cancer antigen 125 (CA125) and so on, which provide supplementary evidence for the diagnosis of breast cancer, and monitor postoperative recurrence and metastasis (Khatcheressian *et al*., 2013; Mlika-Cabanne *et al*., 1998; Leonard *et al*., 2004).

Leucine-rich repeat G-protein-coupled receptor 5 (LGR5) is a G-protein-coupled receptor located on the cell membrane with an R-sponge protein ligand and is a well-established stem cell marker (Xu *et al*., 2019; Barker and Clevers, 2010). LGR5 can promote tumor growth in gastrointestinal, liver, lung, and ovarian cancers, mainly by increasing the activation of the Wnt/β-catenin signaling pathway (Morgan *et al*., 2018). LGR5 is not only considered to be a stem cell marker for breast cancer, but several recent studies have also demonstrated its cancer-promoting role in breast cancer. By analyzing the KEGG signaling pathway data, the researchers proposed the

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possibility that LGR5 enhances signaling downstream of HER-2 and VEGF receptors by increasing the expression of β-catenin (Chen *et al*., 2018; Kumar *et al*., 2014). It has also been revealed that LGR5 is overexpressed in breast cancer tissues compared with non-cancerous tissues, and its expression level is negatively correlated with the survival rate of breast cancer patients (Yang *et al*., 2015). This was further confirmed by follow-up studies, who found that the expression level of β-catenin correlated with the expression level of LGR5, which was further increased in triple-negative breast cancer tissues compared to other types of breast cancer (Hou *et al*., 2018).

IQGAP1, also known as p195, is a widely expressed Ras GTPase-activating-like protein encoded by the IQGAP1 gene in humans (Tanos *et al*., 2018). IQGAP1 is a scaffold protein that governs actin cytoskeleton organization, transcription, cellular adhesion, and cell cycle regulation, among other physiological functions (Briggs and Sacks, 2003; Wu and Chen, 2014). According to an investigation of IQGAP1 expression in human tissues, the scaffold is more or less ubiquitously expressed in human tissues (Peng *et al*., 2021). The nucleus, plasma membrane, and cytoplasm are the most common places where it can be found (Smith *et al*., 2015). In other words, it can be found in every cell and in all sorts of tissues. According to expression analyses, IQGAP1 is overexpressed in many cancers, and in more aggressive colorectal and ovarian cancers, IQGAP1 is localized in the invasive front of the neoplasm, indicating a role in cell motility (Wei and Lambert, 2021; Tanos *et al*., 2018). Importantly, IQGAP1 binding partners account for about 10% of genes with elevated expression in metastatic cells (Johnson *et al*., 2009).

The most common Wnt pathway mediated by LGR5 has been reported in many literatures, and it has been applied in breast cancer (Park *et al*., 2021; Neiheisel *et al*., 2022). In contrast, the literature shows that LGR5 reduces phosphorylation levels at Ser-1441/1443 of IQGAP1, resulting in increased binding of Rac1 to IQGAP1, thereby increasing cortical actin levels and enhancing normal cell-cancer cell adhesion. This mechanism has not been validated in breast cancer (Carmon *et al*., 2017).

This study aimed to explore the role and regulatory mechanism of LGR5 in the occurrence and development of breast cancer. Appropriate clinical, cellular and molecular-level experiments were designed for the study. Western blot analysis was used to detect the expression of LGR5 and phosphorylated/ unphosphorylated IQGAP1 in human breast cancer. Immunohistochemical staining was used to analyze the expression of LGR5 and phosphorylated IQGAP1 in human breast cancer. CCK8 assay detected the activity of breast cancer cells. Cell scratch test detected the migratory ability of cells. Transwell test detected the invasive ability of cells. Immunoprecipitation was used to detect the molecular mechanism of the interaction between LGR5 and IQGAP1.

MATERIALS AND METHODS

Experiment design

Clinical experiments: 30 pairs of fresh breast cancer case samples and normal breast cell samples were collected, and Western blot was used to analyze the expression of LGR5 and (phosphorylated IQGAP1)/ (IQGAP1) in human breast cancer.

Cell experiments: (1) Construct metastatic human breast cancer MDA-MB-231 cell line knocking down LGR5. The relative expression levels of LGR5 and phosphorylated IQGAP1 were detected by Western blot in human normal breast cell line, MDA-MB-231 cell line and breast cancer cell line with LGR5 knockdown. The relative activity of the above three types of cells after 24 h of culture was detected by CCK8 assay. The migration ability of the cells was analyzed by the cell scratch test, and the invasive ability of the cells was analyzed by the transwell detection. (2) Construct LGR5-knockdown, LGR5-overexpressing and LGR5-ΔC-overexpressing (lack of C-terminal tail) human colon cancer LoVo cell lines. The mechanism of the reaction between LGR5 and IQGAP1 was examined by immunoprecipitation.

Western blot analysis

Collect cells from each group and fill each six-well plate with 200 μl of cell lysate. The cells were sonicated and then lysed for 1 h on cold. At 4 °C, the lysed cell sample was centrifuged for 15 min at 12,500 rpm. Then, in a clean centrifuge tube, transfer the supernatant from the centrifuge tube. Protein concentration was measured using a -actin or -tubulin protein measurement kit. The protein samples that were analyzed were kept at -80 °C. The protein loading concentration in Western blot electrophoresis was 50 g per well. After SDS-PAGE electrophoresis, the membrane was transferred and blocked. To utilize concentration, the proteins LGR5, IQGAP1, and phosphorylated IQGAP1 were diluted using a primary antibody (1: 500, anti-human, Thermo-Fisher, USA). The samples were shaken overnight at 4 °C in an incubator. After washing with PBS, the samples were incubated for 30 minutes at room temperature in the dark with the secondary antibody (1: 1000, anti-human, Thermo-Fisher, USA). Finally, the developer was utilized for photography and development.

Immunohistochemistry analysis

Each group's breast cancer tissues were seeded

in a six-well plate and fixed for 24 h in 4 percent paraformaldehyde at room temperature. PBS solution was used to wash these samples three times. After that, the samples were sealed for 30 min with 5% BSA. FBP17 and PPRRD (1: 500, anti-human, Thermo-Fisher, USA) were used as primary antibodies. The samples were incubated with secondary antibody (1: 1000, Thermo-Fisher, USA) for 30 min in the dark at room temperature after being shaken overnight at 4 °C and washed three times in PBS. Finally, the samples were examined under an inverted microscope while photographs were taken. Positive area analysis was performed using Image J software.

Cell scratch test

After the cells of each group were digested and counted, 8×10^5 cells were divided into 35mm^2 culture dishes. Use a marker to draw a line on the bottom of the dish as a mark, aspirate the culture medium, and use a 10 μl pipette tip to mark the cells in the dish perpendicularly to the marker. Rinse with PBS to remove the marked cells, add serum-free culture medium to continue culturing. Take pictures at 24h, and select the intersection of the line drawn by the marker and the cell scratch as the observation point, and then observe at a fixed point.

Transwell detection

Twenty-four h before the experiment, the cells of different groups were replaced with serum-free medium, and the culture was continued. Before inoculation, soak the 24-well plate and transwell chamber with 1×PBS for 5 min to moisten the chamber. Digest the cells, wash the cells with serum-free medium, resuspend the cells in serum-free medium, count the cells and dilute to adjust the cell density to 5×10^5/ml. Inoculate 0.2 ml cell suspension (5×10^4 cells) into the transwell chamber, and then add 0.7 ml of RPMI-1640 medium containing 10% FBS to the lower 24-well plate, 3 replicate holes per group, and place them in a 37 °C incubator for 24 h to terminate the culture. Add 1 ml of 4% formaldehyde solution to each well of the above cells, and fix them at room temperature for 10 min. Aspirate the fixative solution, wash once with 1× PBS. Add 1ml 0.5% crystal violet solution to each well, wash with 1×PBS three times after dyeing for 30 min. Use a cotton swab to carefully wipe off the cells that have not migrated in the transwell, place them under a 200× microscope for observation.

Immunoprecipitation analysis

The cells in each group were given an adequate amount of cell IP lysis solution (including protease inhibitors), and they were lysed at 4°C for 30 min, then centrifuged at 12,000g for 30 minutes, and the supernatant was obtained for use. In the supernatant, add 1 μg of the matching antibody and 50 μl of protein A/G beads, then incubate overnight at 4°C with gentle shaking. Centrifuge protein A/G beads to the bottom of the tube at 3000g for 5 minutes at 4°C after immunoprecipitation. Wash the pellet three times with 1 ml of lysis buffer after removing the supernatant. Finally, 15 μl of 2× SDS buffer was added and incubated for 10 minutes in a boiling water bath.

Statistical analysis

The paired T test was employed as the statistical tool in this project. The mean ± standard deviation is used to express the experimental data. SPSS 23.0 was used to conduct the statistical analysis. Origin 2022 and Adobe Illustrator 2021 were used to create the figures.

RESULTS AND DISCUSSION

Expression of LGR5 and phosphorylation ratio of IQGAP1 showed opposite trends in breast cancer and normal body

Figure 1A shows the findings of a Western blot examination of the relative expression levels of the proteins LGR5 and IQGAP1 (phosphorylated/non-phosphorylated) in breast cancer and normal human breast tissue. The figure shows that, when compared to normal breast tissue, the expression of LGR5 in breast cancer tissue is higher, but the phosphorylation ratio of IQGAP1 is lower. This meant that LGR5 expression was inversely linked with the IQGAP1 phosphorylation ratio.

Figure 1B shows the results of immunohistochemical staining for LGR5 and phosphorylated IQGAP1 expression in breast cancer tissue and normal human breast tissue. The expression of LCR5 in breast cancer tissue was higher than in normal breast tissue, whereas the expression of phosphorylated IQGAP1 was lower, as shown by the staining results, which was compatible with the results of Western blot analysis. The results of Western blot analysis and immunohistochemical staining corroborated each other, proving that LGR5 expression is inversely linked with IQGAP1 phosphorylation ratio. The effect of LGR5 on IQGAP1 phosphorylation could have implications for the development and progression of breast cancer, which would need to be validated in follow-up studies.

Viability, migration and invasion ability of breast cancer cells were all affected by LGR5 expression and IQGAP1 phosphorylation ratio

The results of Western blot analysis of the expression of LGR5 and phosphorylated IQGAP1 (pSer(IP)) in human normal breast cell line, metastatic human breast cancer cell MDA-MB-231 line and LGR5-knockdowned MDA-MB-231 cell line are shown in Figure 2A. It can

be seen from the figure that compared with normal breast cells, the expression of LGR5 in MDA-MB-231 cells was significantly increased, while the expression of phosphorylated IQGAP1 was correspondingly decreased. By knocking out the gene expressing LGR5 in MDA-MB-231 cells, the expression of LGR5 was decreased, while the expression of phosphorylated IQGAP1 was increased. These results were consistent with the results in clinical experiments, and further illustrated the causal relationship between LGR5 and IQGAP1 - the expression of LGR5 negatively regulated the phosphorylation level of IQGAP1.

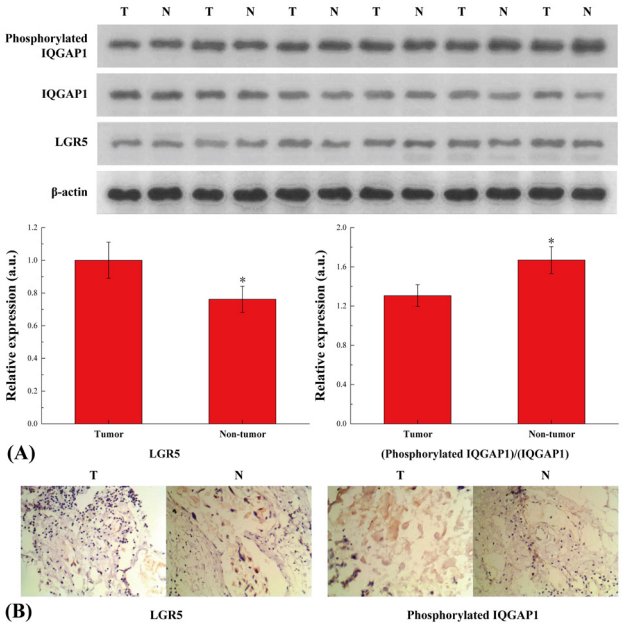

Fig. 1. (A) The results of Western blot analysis of the expression of LGR5 and IQGAP1 in human breast cancer, including the original gel image and the relative value of LGR5 and IQGAP1 (phosphorylated/non-phosphorylated). The symbol * means $p < 0.05$ compared to Tumor group. (B) The results of immunohistochemical analysis of LGR5 and phosphorylated IQGAP1 expression in breast cancer.

The findings of the CCK8 assay assessing the relative cell viability of the three cell lines after 24 h of culture are shown in Figure 2B. The viability of breast cancer cells was much higher than that of normal breast cells, as shown in the picture, and this was directly associated to the malignant proliferation of cancer cells. The viability of MDA-MB-231 cells was considerably reduced when the gene encoding LGR5 was knocked out, which was statistically different from that of normal MDA-MB-231 cells. This showed that a decrease in LGR5 expression has an effect on the viability and proliferation of breast cancer cells. Simultaneously, based on prior experimental findings, it may be deduced that LGR5 influenced breast cancer cells through changing IQGAP1 phosphorylation.

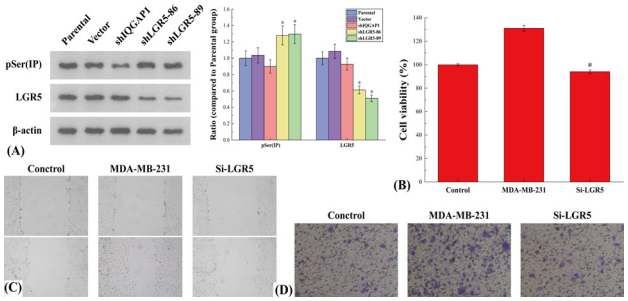

Fig. 2. (A) The results of Western blot analysis of the expression of LGR5 and phosphorylated IQGAP1 (pSer(IP)) in human normal breast cell line, metastatic human breast cancer cell line and LGR5-knockdowned MDA-MB-231 cell line, including the original gel image and the relative values of LGR5 and pSer(IP). The symbol * means $p < 0.05$ compared to Parental and Vector groups. (B) The results of CCK8 assay analyzing the relative cell viability of the three cell lines after 24 h of culture. The symbol * means $p < 0.05$ compared to MDA-MB-231 group. (C) The results of cell scratch test to analyze the migratory ability of cells in the three cell lines. (D) The results of transwell detection to analyze the invasive ability of cells in the three cell lines.

Figure 2C shows the results of a cell scratch test to determine the migratory ability of cells in the three cell lines. The ability of breast cancer cells to migrate was much higher than that of normal breast cells, as seen in the picture. The capacity of breast cancer cells to migrate was considerably reduced when the gene encoding LGR5 was knocked out. This matched the experimental results of the CCK8 cell viability assay.

The results of transwell detection to analyze the invasive ability of cells in the three cell lines are shown in Figure 2D. Through the analysis of the images, it can be concluded that the trend of invasive ability of normal breast cells and breast cancer cells before and after knocking out the gene expressing LGR5 was completely consistent with the trend of cell activity and migration ability shown by the aforementioned experimental results.

Analysis of the cell viability, cell migration and invasion ability of the three cell lines by CCK8 assay, cell scratch test and transwell detection showed that high level expression of LGR5, i.e., decreased phosphorylation level of IQGAP1, significantly increased the rate of malignant transformation of breast cancer. Therefore, if the expression of LGR5 is inhibited in any way, the development of breast cancer can be delayed to a certain extent.

LGR5 affected IQGAP1 phosphorylation process by linking to IQGAP1 through its own c-terminus

The results of immunoprecipitation to detect the reaction mode between LGR5 and IQGAP1 are shown in Figure 3. Figure 3A showed the results of the repeated experiments of knockout of LGR5-expressing genes in LoVo cells, once again demonstrating that the expression of LGR5 was negatively correlated with the phosphorylation ratio of IQGAP1. Figure 3B was the focus of this experiment. When LCR5 (wild type) was overexpressed in LoVo cells, the phosphorylation ratio of IQGAP1 decreased accordingly. When the overexpressed LCR5 lacked the C-terminal end, the phosphorylation ratio of IQGAP1 returned to the normal level. This fully demonstrated that LGR5 connected with IQGAP1 through its C-terminal end, thereby affecting the phosphorylation process of IQGAP1. By exploring the mechanism of the interaction between LGR5 and IQGAP1, it further provides new possibilities for the treatment of breast cancer.

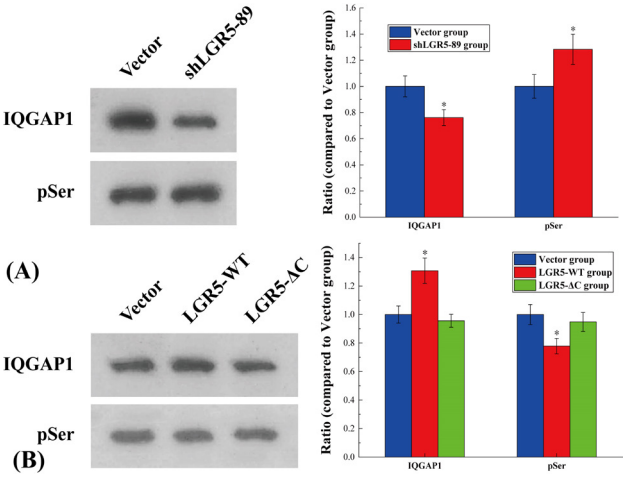

Fig. 3. The results of immunoprecipitation to detect the reaction between LGR5 and IQGAP1. (A) Relative expression levels of IQGAP1 and phosphorylated IQGAP1 in LoVo cell lines and cell lines after LGR5 knockout. The symbol * means $p < 0.05$ compared to Vector group. (B) Relative expression of IQGAP1 and phosphorylated IQGAP1 in LoVo cell line, LGR5 overexpressing cell line and LGR5-ΔC (lack of C-terminal tail) overexpressing cell line. The symbol * means $p < 0.05$ compared to Vector group.

CONCLUSION

In this study, through clinical experiments, in vitro cell experiments and molecular-level experiments, it was successfully proved that LGR5, which is highly expressed in breast cancer cells, interacts with IQGAP1 through its C-terminal end, reducing the phosphorylation ratio of IQGAP1, thereby improving the viability, migration and invasion ability of breast cancer cells, and ultimately increasing the malignant transformation rate of breast cancer. This provides a new potential therapeutic strategy targeting LGR5 for the treatment of breast cancer. Nevertheless, more in-depth studies are still needed to improve our conclusions and to better understand the role and regulatory mechanism of LGR5 in the occurrence and development of breast cancer.

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IRB approval

The research protocol has been reviewed and approved by the Ethical Committee and Institutional Review Board of Zhejiang Provincial People's Hospital, Hangzhou Medical College, Hangzhou 310012, China.

Ethics Statement

All research activities complied with all relevant ethical regulations and were performed in accordance with relevant guidelines and regulations of Zhejiang Provincial People's Hospital, Hangzhou Medical College, Hangzhou 310012, China.

Statement of conflict of interest

The authors have declared no conflict of interests.

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