Downregulation of Lgr6 inhibits proliferation and invasion and increases apoptosis in human colorectal cancer

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Abstract. The aim of the present study was to analyze the role of leucine-rich repeat-containing G-protein coupled receptor 6 (Lgr6) in the proliferation and invasion of colorectal cancer (CRC) cells, and to investigate its possible mechanisms. The expression of Lgr6 in CRC tissues was observed by real-time quantitative polymerase chain reaction and western blotting. Then cell viability, apoptosis and cell invasion were measured by MTT, flow cytometry or Matrigel-Transwell system, respectively in CRC cells after transfected with Lgr6 siRNA or Lgr6 vector. Furthermore, the expression of apoptosis-associated protein and PI3K/AKT signaling targets and predictive biomarkers are urgently required.

Introduction

Colorectal cancer (CRC) is one of several types of malignant tumor with the highest cure rate (1,2). In previous years, the morbidity and mortality rates of CRC have significantly increased as a result of an aging population, and changes in eating habits and lifestyles (3). The development of distant metastasis is a main cause of cancer-associated mortality in patients with CRC (4). Biomarkers, including CEA and CA19-9, have failed to detect CRC in its early stages (5), and there is a less sensitive marker for the prognosis of CRC; therefore, treatment targets and predictive biomarkers are urgently required.

The proteins coded by the leucine-rich repeat-containing G-protein coupled receptor (Lgr)4/5/6 family are G-protein-coupled receptors, which comprise leucine-rich repeats and have homology with glycoprotein hormone receptor (6-8). As stem cell markers, these genes have been examined in multiple organs, including the skin, liver and intestines, for several years (8). Lgr ligands are R-spondin (RSPO) ligands, and previous reports have demonstrated that Lgr receptors can activate Wnt/β-catenin signaling by binding to RSPO ligands (9-12). It is known that the Wnt/β-catenin pathway is important in CRC tumorigenesis, and that aberrant activation of the Wnt pathway is associated with ~90% of cases of CRC in humans (13,14); therefore, we hypothesized that the abnormal expression of Lgr is associated with CRC tumorigenesis.

Several previous studies have suggested that Lgr5 negatively regulates the Wnt pathway, which was observed in CRC tumors (15,16). The expression level of Lgr5 was also increased in gastric cancer (GC), which was associated with growth and node metastasis (17), and patients with Lgr5+ GC had a poorer prognosis, compared with those with Lgr5- GC (18). The overexpression of Lgr5 was shown to promote the development of CRC, and the silencing of Lgr5 reduced the proliferation, migration and colony formation in SW480 and HT-29 cells (19). Although the expression pattern of Lgr5 in CRC has been investigated, the role of Lgr6 in CRC remains unresolved. However, on analyzing the structures of Lgr5 and Lgr6, it was identified that they share ~50% sequence homology (7), and a previous study suggested that Lgr6 is a potential tumor-associated gene (20). Lgr6 has been reported as a tumor suppressor gene (11) and a susceptibility gene (21). The exact roles of Lgr receptors vary due to the different expression patterns and/or interactions of the gene and RSPO family members in a given cellular context (16). As a stem-cell marker, Lgr6 has been reported in multiple studies in the taste buds (22), lungs (23) and skin (24), however, its role in CRC...
remains to be fully elucidated. Therefore, the present study aimed to obtain this information by further examining the putative biological significance of Lgr6 in CRC.

Materials and methods

Patients and tissue samples. The present study was approved and supervised by the Research Ethics Committee of the Affiliated Hospital of Nantong University (Nantong, China). Written informed consent was obtained from all patients. Paired CRC and adjacent normal colon tissue samples were obtained between October 2015 and December 2017 from 45 patients who underwent primary surgical CRC resection at the hospital. The patients included 20 women and 25 men, aged between 37 and 83 years. A total of 13 patients had World Health Organization (WHO) grade (25) T1 disease, 14 patients had WHO grade T2 disease, and 14 patients had WHO grade T3, 4 patients had WHO grade T4 disease. All tissue samples were immediately frozen in liquid nitrogen until use.

Cell lines, in vitro culture, plasmids and transfection. HCT-116 and SW480 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in RPMI-1640 (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and incubated at 37˚C in a 5% CO2 atmosphere. The pcDNA3.1 vector containing a wild-type Lgr6 insert was provided by General Biosystems (Anhui, China). The sequences of small interfering RNAs (siRNAs) for Lgr6 were purchased from GenePharma Co., Ltd. (Suzhou, China). The siRNA sequences were as follows: Lgr6 siRNA1, sense, 5'-CCCUGGAUCUA GCUGGAGATT-3' and antisense, 5'-UUCAGCAGUACAGUGGGTTT-3'; Lgr6 siRNA2, sense, 5'-GCAUCCAGUGACCC CUATT-3' and antisense, 5'-UAGGCCGAGUAUGGAG TCTT-3'; Lgr6 siRNA3, sense, 5'-CCUGGAAAGUGUCUCA AA UTT-3' and antisense, 5'-AUUGUGACAGUACUGGATTT-3'; negative control, sense, 5'-UUUCUGCAAGCUGACUG GCTT-3' and antisense, 5'-ACUGUGACGUGUCGAGATT T-3'. The cells were transfected with Lgr6 siRNA, negative control (siCtrl), or pcDNA3.1-Lgr6 cDNA at ~50% density using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Reverse-transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from the cells or tumor tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and 2 µg total RNA from each sample was used for cDNA synthesis using a cDNA synthesis kit (Tiangen, Beijing, China). The qPCR analysis was performed using SYBR-Green (Invitrogen) according to the manufacturer's protocol, the reaction system contained 2 µl cDNA, 0.5 µl forward primer (10 nM), 0.5 µl reverse primer (10 nM), 10 µl SYBR-Green buffer and 7 µl distilled water. Data collection was conducted using an ABI 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling conditions were as follows, 50˚C for 2 min and 95˚C for 2 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. The conditions for melt curve analysis were 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 min. The primer sequences were as follows: B-cell lymphoma-2 (Bcl-2), sense, 5'-CAGGAAACGCGCCGG AT-3' and antisense, 5'-CTGGGGGGCTTTCGCTTC CCC-3'; Bcl-2-associated X protein (Bax), sense, 5'-GGGTGTGCTG CCCCCCTTCTAC-3' and antisense, 5'-CTGGAGACAGGG ACATCGT-3'; caspase-3, sense, 5'-TGCTATGTGAGCG GTGTAG-3' and antisense, 5'-GGCACCCCCACCGAA AAC-3'; Lgr6, sense, 5'-TGACGGCTTACCCTGGACCTCA-3' and antisense, 5'-AGAGAATGCTGTCCTGAGGTG-3'; GAPDH, sense, 5'-GAAGGTGTAAGTCGGAGTC-3' and antisense, 5'-GAAGATGGTGTGAGGATTCTC-3'. The relative expression of each gene was quantified using the 2^ΔΔCt method (26), and GAPDH was used as the internal control.

Western blot analysis. Whole-cell extracts from the cultured cells or tissues were prepared and subjected to western blot analysis using RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Total protein (50 µg) was resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk powder for 1 h at room temperature. The membranes were then incubated with primary antibodies overnight at 4˚C. All primary antibodies were purchased from Abcam (Cambridge, MA, USA) as follows: Rabbit anti-Lgr6 (cat. no. ab205760; dilution 1:2,000), mouse anti-β-actin (cat. no. ab8226; dilution 1:10,000), rabbit anti-pan-AKT (cat. no. ab8805; dilution 1:500), Rb anti-pan-AKT (phospho-T308; cat. no. ab38449; dilution 1:800), rabbit anti-Bcl-2 (cat. no. ab32124; dilution 1:1,000), rabbit anti-Bax (cat. no. ab32503; dilution 1:1,000), and rabbit anti-active-caspase-3 (cat. no. ab2302; dilution 1:200). The membranes were washed three times with PBS-Tween-20 for 10 min and then incubated with the goat anti-mouse (cat. no. ab6789; dilution 1:5,000) or goat anti-rabbit (cat. no. ab6721; dilution 1:10,000) secondary antibody for 1 h at room temperature. The blots were developed using an enhanced chemiluminescence Western Blotting Detection System (Invitrogen) and X-ray film. The band density was quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell invasion assay. The invasive capability of the cells was determined using Matrigel-coated invasion chambers with a pore size of 0.8-µm (BD Biosciences, San Jose, CA, USA). The cells were resuspended with 2% FBS, and ~1x105 cells were transfected with Lgr6 siRNA (siLgr6), negative control (sictrl), or pcDNA3.1-Lgr6 cDNA added to the inner chamber; the outer chamber contained 20% FBS. Following incubation at 37˚C in 5% CO2 for 24 h, the cells on the upper surface of the inner chamber were removed with cotton swabs. The invaded cells that adhered to the lower surface of the membrane were fixed, stained with crystal violet, and observed through an optical microscope (TS100F; Nikon Corporation, Tokyo, Japan).

Cell proliferation assay. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to measure cell proliferation. Briefly, ~5,000 cells per well were cultured in 96-well plates for 24 h and transfected with Lgr6 siRNA, negative control, or pcDNA3.1-Lgr6 cDNA. Cell proliferation was determined 48 h later. MTT solution (5 mg/ml
in PBS) was added to each well. The solution was incubated at 37°C for 4 h, the medium was removed, and 150 µl of dimethyl sulfoxide was added to each well. The absorbance of each well was measured at 490 nm using the Multiskan FC automated microplate reader (Thermo Fisher Scientific, Inc.).

**Cell apoptosis assay.** Cell apoptosis was evaluated by flow cytometry using an Annexin V-PE/7-AAD Apoptosis Detection kit (BD Biosciences) according to the manufacturer's protocol. Briefly, the transfected cells were harvested, washed twice in cold PBS, and resuspended in 500 µl binding buffer. For staining, 5 µl of Annexin V-PE and 5 µl of 7-AAD were combined and gently mixed, and the cells were stained in the dark for 15 min at room temperature. The cells were immediately analyzed by flow cytometry.

**Statistical analysis.** The statistical analysis was performed using SPSS 17.0 (SPS, Inc., Chicago, IL, USA). The survival rate of patients with CRC was calculated using the Kaplan-Meier survival analysis method. Statistically significant differences were determined using one-way analysis of variance. The results are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

### Results

**Lgr6 is upregulated in human CRC tissues.** To reveal the function of Lgr6 in CRC, its levels in CRC tissues and adjacent normal tissues were detected by western blotting or RT-qPCR analysis. The results indicated that the expression of Lgr6 in CRC tissues was significantly higher, compared with that in adjacent normal tissues (Fig. 1A-c). In addition, a high expression of Lgr6 was associated with poor differentiation, lymph node metastasis, and advanced clinical stage, but not with age, sex or tumor diameter. Lgr6, leucine-rich repeat-containing G-protein coupled receptor 6.

<table>
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<th>High expression n (%)</th>
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<td>44 (56.41)</td>
<td>34 (43.59)</td>
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High expression of Lgr6 was associated with poor differentiation, lymph node metastasis, and advanced clinical stage, but not with age, sex or tumor diameter. Lgr6, leucine-rich repeat-containing G-protein coupled receptor 6.

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Downregulation of Lgr6 inhibits the proliferation of CRC cells in vitro. The HCT-116 and SW480 cells were transfected with Lgr6 siRNA with a different target sequence or negative control to determine the role of Lgr6 in CRC cells in vitro. As shown in Fig. 3A and B, the levels of Lgr6 were successfully reduced in the HCT-116 and SW480 cells when transfected with Lgr6 siRNA1 or Lgr6 siRNA3, respectively. Lgr6
Figure 1. Expression of Lgr6 is increased in CRC. (A) Representative western blot analysis of Lgr6 protein in CRC (T1-T4) and paired normal tissues (N1-N4) from patients. β-actin was used as a loading control to normalize the protein levels of Lgr6 in each sample (n=45). (B) Determination of mRNA levels of Lgr6 in CRC tissues and paired normal tissues by reverse transcription-quantitative polymerase chain reaction analysis (n=45). (C) Quantification of protein levels of Lgr6. **P<0.01 vs. normal tissues. CRC, colorectal cancer; Lgr6, leucine-rich repeat-containing G-protein coupled receptor 6.

Figure 2. Kaplan-Meier survival curves for high vs. low expression of Lgr6 in 117 patients with colorectal cancer. Patients with a high expression of Lgr6 had significantly shorter overall survival rates, compared with those with a low expression (P=0.001). Lgr6, leucine-rich repeat-containing G-protein coupled receptor 6.

Figure 3. Silencing Lgr6 inhibits cell proliferation in colorectal cancer cell lines. (A) Expression of Lgr6 was determined in HCT-116 cells and (B) SW480 cells transfected with siCtrl or one of three Lgr6 siRNAs (siRNA1, siRNA2 and siRNA3). MTT analysis of (C) HCT-116 cells and (D) of SW480 cells. Flow cytometric analysis of (E) HCT-116 cells and (F) SW480 cells. **P<0.01 vs. siCtrl group; ##P<0.01 vs. siLgr6 group. Lgr6, leucine-rich repeat-containing G-protein coupled receptor 6; siRNA, small interfering RNA; siCtrl, negative control siRNA; siLgr6, cells transfected with Lgr6 siRNA3; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Rescue, cells with Lgr6-knockdown transfected with pcDNA3.1-Lgr6 cDNA.
siRNA3 showed the optimal interference efficiency; therefore, this sequence was used for subsequent experiments. The MTT assay revealed that the knockdown of Lgr6 in HCT-116 and SW480 cells significantly decreased cell growth, and that the overexpression of Lgr6 in these knockdown cells rescued cell proliferation (Fig. 3c and d). Flow cytometric analysis was used to further identify the growth-inhibitory effects of Lgr6 siRNA. The results showed that Lgr6 siRNA induced the apoptosis of HCT-116 and SW480 cells following 48 h of transfection (Fig. 3E and F). In addition, western blot analysis was used to examine the expression of several proteins associated with apoptosis. As shown in Fig. 4, inhibition via Lgr6 siRNA in the HCT116 and SW480 cells elevated the expression of caspase-3 and Bax at the (Fig. 4A-D) mRNA and (Fig. 4E-I) levels, whereas the expression of Bcl-2 was markedly downregulated. Collectively, these data suggested that the decreased cell number induced by Lgr6 siRNA was partly caused by enhanced cell apoptosis.

Downregulation of Lgr6 inhibits the invasion of CRC cells in vitro. Matrigel invasion assays were used to determine the invasive capabilities of Lgr6 in CRC cells. It was found that silencing Lgr6 in the HCT-116 and SW480 cells decreased the number of invasive cells, compared with that in the control cells (Fig. 5A-D). The overexpression of Lgr6 in these knockdown cells rescued cell invasion. These data indicated that Lgr6 was also involved in the invasion of CRC.

Discussion

The upregulation of Lgr6 has been confirmed in several types of cancer, including basaloid skin tumors (31) and gastric cancer (17). In the present study, it was demonstrated that Lgr6
was upregulated in CRC tissues, and that high levels of Lgr6 in CRC were associated with tumor differentiation. Lgr6 is also reported to be involved in several important cell processes, including proliferation, invasion and metastasis (11). Lgr6 mutation has been found in CRC, which has been suggested as one of the candidate cancer genes (32). Mokarram et al (33) found that the methylation of Lgr6 was stage-dependent and is thus associated with cell proliferation (7). In accordance with data in Table I and Fig. 2, the levels of Lgr6 were associated with tumor stage and the 5-year survival rate; however, the actual biological functions of Lgr6 and the underlying mechanisms in CRC remained to be fully elucidated. To address this problem, two CRS cell lines (HCT-116 and SW480), which express high levels of Lgr6, were used in the present study.
The results of the present study showed that siRNA targeting Lgr6 in the HCT-116 and SW480 cells led to the inhibition of endogenous mRNA and protein levels of Lgr6 in vitro, in an efficient and specific manner. It was found that the downregulation of Lgr6 in HCT-116 and SW480 cells contributed to decreased cell invasion. These data suggested that Lgr6 is vital in the metastasis of CRC, which is consistent with previous findings in other cancer cells, including GC and lung cancer. In addition to a marker of adult stem cells, Lgr6 has been reported as a potential marker for cancer stem cells (8). The accumulation of Lgr6 in later stages retains cancer stem cell features, which are involved in self-renewal and poor differentiation of lung adenocarcinoma (34). Additional experiments demonstrated that the knockdown of Lgr6 in HCT-116 and SW480 cells significantly inhibited cell proliferation in vitro. These results were also found in CRC (19) when Lgr5 was silenced, which shares ~50% sequence homology with Lgr6. Rho GTPases are regulated by Lgr5 (35) and have been shown to be involved in cell cycle entry and progression (36). As targeting Rho GTPases provides a therapeutic opportunity for patients with cancer (36), further investigations are required to identify whether Rho GTPases are regulated by Lgr6 in CRC. In the present study, flow cytometric analysis demonstrated that Lgr6-silencing led to the apoptosis of HCT116 and SW480 cells. In accordance with these results, transfection with Lgr6 siRNA resulted in changes in apoptosis-associated proteins, including increased expression levels of caspase-3 and Bax, and decreased expression of Bcl-2 in HcT-116 and SW480 cell lines. Taken together, these results indicated that Lgr6 was important in regulating the proliferation and invasion of CRC cells.

Previous studies have revealed that Lgr6 activates multiple signaling pathways, including the Wnt/β-catenin pathway (34,37); however, no studies have focused on its role in activating the PI3K/AKT pathway in CRC. In the present study, AKT/PI3K was inactivated when Lgr6 was inhibited by siRNA, indicating that the role of Lgr6 in CRC may partly depend on the PI3K/AKT pathway. PI3K/AKT is an important pathway in multiple types of cancer, including CRC, and has been shown to directly regulate cell growth, migration or invasion (28,29,38); therefore, it is reasonable to suggest that the decreased cell proliferation and invasion observed in Lgr6 siRNA-transfected CRC cells were the result of decreased PI3K/AKT activity.

In conclusion, data from the present study demonstrated that Lgr6 was significantly upregulated in CRC. Lgr6 promoted CRC cell proliferation and migration in vitro and activated the PI3K/AKT signaling pathway. These results suggested that Lgr6 may be a predictive biomarker and a novel therapeutic target for patients with advanced CRC, and may improve the prognosis of patients.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

FQ was a major contributor in writing the manuscript and analyzed the data. C-QD and L-RZ performed the histological examination of CRC tissues. CB and FW performed the experiments of CRC cells in vitro. JQ and Y-FL analyzed the patient data, and supervised the work of the research group.

Ethics approval and consent to participate

The present study was approved and supervised by the Research Ethics Committee of the Affiliated Hospital of Nantong University. Written informed consent was obtained from all patients.

Consent for publication

The patients have provided written informed consent for the publication of any associated data excluding identifying information, including names, initials, date of birth or hospital numbers, images.

Competing interests

The authors declare that they have no competing interests.

References


