ISLR affects colon cancer progression by regulating the epithelial–mesenchymal transition signaling pathway
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This study aims to determine the mechanism of ISLR on the progression of colon cancer. TCGA database was used to analyze ISLR expression in colon cancer tumor tissues. qRT-PCR and western blotting were used to detect ISLR expression in colon cancer cells. CCK-8, colony formation, EDU, wound healing and transwell assays were used to measure cell viability, proliferation, migration and invasion of colon cancer cells, respectively. The signaling pathway enrichment analysis of ISLR was analyzed on the basis of the KEGG database. The protein expression of genes related to signaling pathway was measured by western blotting. Results of TCGA analysis, qRT-PCR and western blotting showed that ISLR was upregulated in colon cancer tumor tissues and cells. High level of ISLR was related to low overall survival of patients with colon cancer. ISLR silence significantly inhibited cell viability, proliferation, migration and invasion of colon cancer cells. ISLR overexpression markedly enhanced the cell viability, proliferation, migration and invasion of colon cancer cells. KEGG database analyzed expression induced a significant promotion of tumor growth of colon cancer [13]. PEG10 is differentially expressed in colon cancer and increased in PEG10 expression correlates with decreased overall survival of patients with colon cancer [14]. CBX8 regulates the expression of miR-378a-3p and thus affects the progression of colon cancer [15]. DSG2 is low expressed in colon cancer and the overall survival of colon cancer patients was associated with DSG2 expression [16]. Immunoglobulin superfamily containing leucine-rich repeat (ISLR) is a leucine-rich repeat sequence in the human immunoglobulin superfamily with a molecular mass of 46 kDa [17]. Studies have shown that ISLR is expressed in various human tissues including skeletal muscle, small intestine, thyroid and others [18,19]. The loss of ISLR delayed the regeneration of skeletal muscle in adult mice [20]. The absence of ISLR in mouse stromal cells significantly impaired intestinal regeneration and inhibited the tumorigenesis of colon [19]. Besides, the findings of Li et al. have shown that the ISLR gene is involved in gastric carcinogenesis [21]. However, the mechanism of ISLR in colon cancer remains to be further studied.

Keywords: colon cancer, epithelial–mesenchymal transition, ISLR, migration

Introduction
Colon cancer is one of the most common cancers worldwide and the second leading cause of cancer-related death [1,2]. The most significant feature of colon cancer is metastasis [3]. More than 20% of patients with colon cancer have metastasis at the time of initial diagnosis [4,5]. At present, the most effective treatment method for colon cancer is immunotherapy combined with surgical resection, as well as chemotherapy and radiotherapy [6,7]. Researches have indicated that the targeted therapy revealed better effect on colon cancer [8,9]. Therefore, finding the new targets will be of great significance for the treatment of colon cancer.

At present, many genes with abnormal expression have been identified in colon cancer [10,11]. The abnormal expression of genes is associated with the development of colon cancer and the survival of patients with colon cancer [12]. ILF3 is reported to be overexpressed in colon cancer and correlated with a poor survival rate [11]. Hong et al. have reported that highly FGFR4 expression induced a significant promotion of tumor growth of colon cancer [13]. PEG10 is differentially expressed in colon cancer and increased in PEG10 expression correlates with decreased overall survival of patients with colon cancer [14]. CBX8 regulates the expression of miR-378a-3p and thus affects the progression of colon cancer [15]. DSG2 is low expressed in colon cancer and the overall survival of colon cancer patients was associated with DSG2 expression [16]. Immunoglobulin superfamily containing leucine-rich repeat (ISLR) is a leucine-rich repeat sequence in the human immunoglobulin superfamily with a molecular mass of 46 kDa [17]. Studies have shown that ISLR is expressed in various human tissues including skeletal muscle, small intestine, thyroid and others [18,19]. The loss of ISLR delayed the regeneration of skeletal muscle in adult mice [20]. The absence of ISLR in mouse stromal cells significantly impaired intestinal regeneration and inhibited the tumorigenesis of colon [19]. Besides, the findings of Li et al. have shown that the ISLR gene is involved in gastric carcinogenesis [21]. However, the mechanism of ISLR in colon cancer remains to be further studied.

Epithelial–mesenchymal transition (EMT) is a critical factor for regulating invasion and migration of cancer...
EMT-induced by TGF-β markedly increased migration, invasion, and tumor development in mice [25]. Pan et al. have found that circFNDC3B-218aa plays a tumor-suppressive role in colon cancer by inhibiting the EMT progression [26]. However, whether ISLR could regulate the EMT progression in colon cancer remains unclear. Thus, we investigated the effect of ISLR on the EMT signaling pathway in colon cancer cells in this study.

Materials and methods

Cell culture

Human normal colonic epithelial cell line NCM460 and human colon cancer cell lines CX-1, SW480, HCT-116, HT29 and LoVo were purchased from Procell Life Science&Technology Co., Ltd. (Wuhan, China). All cells are cultured in DMEM containing 10% FBS and placed under a constant temperature containing 5% CO₂ in a sterile incubator.

Cell transfection

Si-RNAs targeting ISLR (si- ISLR #1, si- ISLR #2 and si- ISLR #3) and negative control (si-NC), and ISLR overexpressed plasmid pc-ISLR, and negative control (pc-NC) were purchased from GenePharma (Shanghai, China). The transfection was conducted by Lipofectamine 3000 (Thermo Fisher Scientific, Shanghai, China) in light of the instructions. Cells were collected after the transfection for 48 h.

Cell counting kit-8 assay

The cell viability of colon cancer cells was detected by cell counting kit-8 (CCK-8) (Sigma-Aldrich, St. Louis, Missouri, USA). Cells were inoculated into 96-well plates and cultured in a constant temperature incubator. 10 µl of CCK-8 reagent was added into the well, following by another 4 h culture. Finally, the absorbance (450 nm) was read under a microplate reader (Molecular Devices, Shanghai, China).

Colony formation assay

The proliferation ability of colon cancer cells was detected by colony formation assay. Cells were seeded into 6-well plates (2×10³ cells/well) and cultured for 7 days. Subsequently, the cells were fixed with paraformaldehyde at room temperature for 30 min and then stained with crystal violet for 10 min. Finally, the colony numbers were counted under the microscope.

EDU assay

The proliferation ability of colon cancer cells was detected by EDU assay. Colon cancer cells were seeded into 24-well plates with 48 h culture. Then, the EDU detection kit (Beyotime, Shanghai, China) was used to treat cells for 2 h at 37°C. Subsequently, cells were fixed with formaldehyde for 30 min and then washed with PBS. Apollo reagent was added and incubated in the dark for 30 min, and then the cells were stained with Hoechst 33342 (Sigma-Aldrich) for 30 min in the dark. Cells labeled and unlabeled by EDU were counted under a microscope, and pictures were taken.

Wound healing assay

The migration ability of colon cancer cells was detected by wound healing assay. Cells were seeded into six-well plates and cultured for 24 h. A pipette tip was used to scratch the cell monolayer, followed by 48 h culture in a serum-free medium. The pictures were taken at different time points (0 and 48 h). The migration rate was calculated by using ImageJ software (V1.8.0.112; National Institutes of Health).

Transwell test to detect cell migration and invasion

The migration and invasion ability of colon cancer cells was detected by transwell assay. Colon cancer cells with serum-free medium were seeded into the upper chamber precoated with or without Matrigel (BD Biosciences, California, USA). Concurrently, the complete medium contained with 10% FBS was added into the lower chamber. After culture for 48 h, these nonmigrated or non-invaded cells were removed off the upper chamber by using a cotton swab. The cells on the lower side of the membrane were fixed with paraformaldehyde and stained with crystal violet. Finally, the migrated or invaded cells were counted under a microscope and calculated the relative migration or invasion rates.

qRT-PCR

Total RNA of colon cancer cells were extracted by using TRizol reagent (Invitrogen, Carlsbad, California, USA). The cDNA was synthesized from RNA by using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The amplification system is 50 µl including 5 µl cDNA, 1 µl upstream and downstream primers, 10 µl SYBR Green I dye, 33 µl ddH₂O. Amplification temperature and time are: 95°C - 5 min; 94°C - 15 s, 55°C - 30 s, 72°C - 20 s, 4°C - 60 min, 45 cycles are set. GAPDH is used as the internal reference gene, and the primers of all genes are designed and synthesized by the company. All PCR analyses were repeated three times.

Subcellular fractionation

Cytoplasmic and nuclear extracts were extracted from HCT-116 or CX-1 cells by using NE-PER kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Western blotting was used to detect the protein expression of YAP and TAZ in the cytoplasm or nuclear. GAPDH was used as the cytoplasmic control and LaminB1 was used as the nuclear control.

Western blotting

Total protein of cells was extracted by using RIPA lysis buffer (Solarbio, Beijing, China) containing protease inhibitors.
inhibitors and centrifuged at 12 000g for 20 min at 4°C. The protein concentration was determined by BCA protein assay (Beyotime, Shanghai, China). Subsequently, protein (50 μg) was separated by SDS-PAGE and then transferred onto PVDF membranes. Then, the membranes were blocked and incubated with primary antibodies (ISLR, MMP2, Vimentin, PDLIM4, E-cadherin, N-cadherin, Snai2 and GAPDH were purchased from Sigma-Aldrich; YAP, p-YAP, TAZ, p-TAZ and LaminB1 were purchased from Cell Signaling Technology, Danvers, Massachusetts, USA) overnight at 4°C and incubated with the second antibody for 1 h at 37°C. After washing with TBST, ECL enhanced chemiluminescence detection system (Thermo Scientific, Rockford, Illinois, USA) was used to detect the protein bands.

Statistical analysis
Statistical analysis was performed with GraphPad Prism 7.0. The two-tailed t test was used for comparison between two groups, and one-way ANOVA was used for comparison among multiple groups. Data were shown as means ± SD and repeated in triplicate in this study. Statistical significance was considered when $P < 0.05$.

Results
ISLR is highly expressed in colon cancer tissues and cells
As shown in Fig. 1a, the analysis on the basis of the TCGA database suggested that the ISLR expression was markedly increased in colon cancer tissues. High level of ISLR is related to the low overall survival of patients with colon cancer (Fig. 1b, $P < 0.05$). Then, we detected the ISLR expression in human normal colonic epithelial cells NCM460 and colon cancer cells (CX-1, SW480, HCT-116, HT29 and LoVo) by qRT-PCR and western blotting. As shown in Fig. 1c,d, the expression of ISLR was significantly upregulated in colon cancer cells compared with human normal colonic epithelial cells NCM460 ($P < 0.05$).

ISLR promotes proliferation and inhibits apoptosis of colon cancer cells
To investigate the role of ISLR in the development of colon cancer, three ISLR interference fragments (si-ISLR#1, #2 and #3) and one ISLR-overexpressing plasmid (pc-ISLR) were used in this study. The data in Fig. 2a showed that the protein expression of ISLR was...
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significantly decreased by si-RNAs, especially si-ISLR#1 and si-ISLR#3 ($P < 0.05$). Meanwhile, the ISLR protein expression was markedly enhanced by pc-ISLR (Fig. 2b, $P < 0.05$). Then, we measured cell viability of colon cancer cells by CCK-8 assay. The data showed that silencing of ISLR significantly inhibited the cell viability of HCT-116 cells, while ISLR overexpression increased cell viability of CX-1 cells (Fig. 2c,d, $P < 0.05$). Besides, we detected the proliferation of colon cancer cells by performing colony formation and EDU assays. The results showed that the proliferation ability of colon cancer cells was significantly reduced by ISLR silencing and enhanced by ISLR overexpression (Fig. 2e–h, $P < 0.05$).

**ISLR promotes migration and invasion of colon cancer cells**

We investigated the effect of ISLR in the migration and invasion of colon cancer cells. The results of the transwell assay showed that the invasion ability of colon cancer cells was significantly decreased by si-ISLRs and increased by pc-ISLR (Fig. 3a,b, $P < 0.05$). As shown in Fig. 3c,d, low expression level of ISLR markedly reduced the migration ability, while high level of ISLR increased the migration ability of colon cancer cells ($P < 0.05$). The results of wound healing assay were consistent with those of transwell assay. The migration rate of colon cancer cells was significantly reduced by si-ISLRs and enhanced by pc-ISLR (Fig. 3e,f, $P < 0.05$).
ISLR activates the epithelial–mesenchymal transition signaling pathway in colon cancer cells

The enriched KEGG pathway analysis was performed to explore the underlying mechanism of ISLR in colon cancer. The results suggested that ISLR activated the EMT signaling pathway (Fig. 4a). Then, we analyzed the genes enriched in the EMT signaling pathway (Fig. 4b). The protein expression of those genes (EMT signaling pathway enriched genes MMP2, Vimentin and PDLIM4, and EMT related proteins E-cadherin and N-cadherin) was measured by western blotting. As shown in Fig. 5a, the silence of ISLR remarkably decreased the protein expression of MMP2, Vimentin and N-cadherin, and increased the protein expression of PDLIM4 and E-cadherin (P < 0.05). Conversely, overexpression of ISLR significantly increased the protein expression of MMP2, Vimentin, and N-cadherin, and decreased the protein expression of PDLIM4 and E-cadherin (Fig. 5b, P < 0.05). Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) are the downstream effectors of the Hippo pathway [27]. Studies have demonstrated the regulatory roles of YAP and TAZ in the EMT process [28,29]. The results showed that the levels of p-YAP and p-TAZ were markedly increased by ISLR silence while decreased by ISLR overexpression (Fig. 5c,d, P < 0.05). ISLR silence inhibited YAP/TAZ nuclear translocation, while ISLR overexpression promoted YAP/TAZ nuclear translocation (Fig. 5c,f, P < 0.05). Additionally, we found Snai2 (Slug) was decreased by ISLR silence and increased by ISLR overexpression (Fig. 5c,d, P < 0.05).

ISLR promotes the progression of colon cancer cells by activating the epithelial–mesenchymal transition signaling pathway

Snai2, one of the three members in the SNAIL family transcription factors, is a key factor in the development of EMT [30]. Snai2 can directly bind to the promoter region of E-cadherin gene to inhibit its transcription, thereby promoting cancer cell migration [31,32]. In order to confirm whether ISLR affects the development of colon cancer by regulating the EMT signaling pathway, we transfected Snai2 si-RNAs to block the EMT signaling pathway in colon cancer cells. The western blotting results showed...
ISLR regulates the development of colon cancer. ISLR activates the EMT signaling pathway. (a) The Enriched KEGG pathway analysis showed that ISLR activates the EMT signaling pathway; (b) The genes enriched in the EMT signaling pathway were analyzed by Enriched KEGG pathway analysis.
that Snai2 protein expression was markedly decreased by Snai2 si-RNAs, specifically si-Snai2#1 (Fig. 6a, \( P < 0.05 \)). As shown in Fig. 6b, Snai2 silence significantly reduced protein expression of MMP2, Vimentin and N-cadherin, and enhanced protein expression of PDLIM4 and E-cadherin (Fig. 6b, \( P < 0.05 \)). The data of colony formation assay showed that Snai2 silence significantly inhibited cell proliferation of colon cancer (Fig. 6c, \( P < 0.05 \)). Then, we detected the effect of Snai2 silence on the ability of invasion and migration in colon cancer cells by transwell and wound healing assay, respectively. The results showed that Snai2 silence markedly decreased the ability of invasion and migration in colon cancer cells (Fig. 6d,e, \( P < 0.05 \)). In addition, Snai2 silence eliminated the effect of ISLR overexpression on protein expression of EMT signaling pathway and EMT related proteins, cell proliferation, invasion, and migration in colon cancer cells (\( P < 0.05 \)).

Discussion
ISLR was a new member of the Ig superfamily that containing a LRR with conserved flanking sequences and a C2-type Ig-like domain [18]. ISLR was highly expressed in the gastric cancer tissues and suggested poor overall survival of patients with gastric cancer [21]. At the same time, it has been reported in the literature that ISLR can affect tumorigenesis in the colon [19]. In this study, analysis of the TCGA database found that ISLR was highly expressed in colon cancer tissues, and the expression level of ISLR was directly related to the prognosis and survival rate of patients with colon cancer. The data of qRT-PCR and western blotting showed that the expression of ISLR was markedly higher in colon cancer cells than that in normal colonic epithelial cells. These data suggested that ISLR may play an important role in the progression of colon cancer.
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To confirm whether ISLR could affect cell development, we measured the cell viability and proliferation of colon cancer cells after transfection with si-ISLR or pc-ISLR. The results of the CCK-8 assay showed that the cell viability of colon cancer cells was suppressed by si-ISLRs and enhanced by pc-ISLR. ISLR silence inhibited cell proliferation of colon cancer. These results indicated that ISLR can affect cell growth of colon cancer.

The ability of cancer cells to migrate and invade promotes the development and metastasis of cancer [33]. Therefore, controlling the migration and invasion of cancer cells is also important for controlling the development of tumors [33]. In this study, transwell combined with cell...
scratch experiment was used to verify the influence of ISLR on the migration and invasion of colon cancer cells. The results showed that when the silence of ISLR markedly decreased the migration and invasion ability of both HCT-116 and CT-26 colon cancer cells, whereas ISLR overexpression significantly enhanced the migration and invasion ability of colon cancer cells. These results indicated that ISLR can affect the migration and invasion of colon cancer cells.

In previous studies, it has been known that tumor migration and invasion are closely related to EMT [34]. The occurrence of EMT can enable static epithelial cells to acquire the ability to migrate and invade [35]. The enriched KEGG pathway analysis shown that ISLR can activate the EMT signaling pathway, and was enriched in matrix MMP2, Vimentin and tumor suppressor gene PDLIM4. We transfected si-ISLR and pc-ISLR to detect the activity of the EMT signaling pathway, and the results showed that the expression of MMP2, Vimentin and N-cadherin were inhibited by si-ISLR while the tumor suppressor gene PDLIM4 and tumor metastasis suppressor gene E-cadherin were increased by si-ISLR. On the contrary, overexpression of ISLR can promote the expression of MMP2, Vimentin and N-cadherin, and inhibit the expression of PDLIM4 and E-cadherin. These results showed that ISLR can promote the invasion and migration of colon cancer cells by activating the EMT signaling pathway.

From the above results, we know that ISLR can affect the proliferation, migration and invasion of colon cancer cells, and ISLR can activate the EMT signaling pathway, so guessed whether ISLR affects the development of colon cancer by regulating the EMT signaling pathway? For this reason, Snai2 siRNA was used to block EMT progress to further prove this hypothesis. The results indicated that Snai2 siRNA suppressed the expression of MMP2, Vimentin and N-cadherin, and enhanced the expression of PDLIM4 and E-cadherin. Additionally, Snai2 siRNA weakened the proliferation, migration and invasion of colon cancer cells. These results indicated that inhibiting the EMT signaling pathway can alleviate the promoted effect of ISLR overexpression on colon cancer and slow down the development of colon cancer cells.

In conclusion, ISLR was upregulated in colon cancer tumor tissues and cells. Overexpression of ISLR markedly increased cell viability, proliferation, migration, and invasion of colon cancer cells. Besides, ISLR activated the EMT signaling pathway. Inhibition of the EMT signaling pathway can eliminate the promoted effect of ISLR on colon cancer cells.

Acknowledgements
C.C. and T.L. designed the study; S.Y. and B.W. performed the research; W.H. and J.L. analyzed data; C.C. wrote the paper.

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

Conflicts of interest
There are no conflicts of interest.

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