Original Article

Rspo1/LGR5 pathway promotes cervical cancer cell growth and is correlated with the high pathological grade

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Abstract: Objective: To investigate the significance of LGR5 expression on cervical cancer and to explore the role of Rspo1/LGR5 pathway in cervical cancer proliferation. Methods: LGR5 expression was evaluated by immunohistochemistry in 31 pairs of paraffin-embedded cervical carcinoma specimens. The correlation between LGR5 expression and clinicopathological features were statistically analyzed. The potential of Rspo1/LGR5 signal pathway in cervical cancer cell proliferation was determined by cell counting and cell cycle analysis. The activation and blockage of Wnt/β-catenin signaling were analyzed by flow cytometry. Results: LGR5 was expressed at higher levels in the squamous epithelium of tumor tissues compared with the corresponding adjacent tissues. LGR5 expression levels in tumor tissues are correlated with tumor pathological grade and tumor volume. The addition of Rspo1 could promote LGR5 positive SiHa cell proliferation and IL-6 production. C-myc and CD44, the downstream molecules of Wnt/β-catenin signaling, and PCNA, the marker of cell proliferation, were enhanced by Rspo1 stimulation and the enhancement was blocked by DKK, the inhibitor of Wnt/β-catenin pathway. The tumor formation and tumor volume of Rspo1 pretreated group were higher than those of control group. Conclusion: Rspo1/LGR5 pathway could accelerate tumor growth and progression via enhancing Wnt/β-catenin signaling in cervical cancer cells.

Keywords: Cervical cancer, LGR5, Rspo1, β-catenin, CD44, c-myc, IL-6

Introduction

Cervical cancer is the third most common type of gynecological cancer worldwide, accounting for approximately 8% of all female malignancies [1]. Identification of novel molecular markers and mechanisms contributing to the pathogenesis will be meaningful to improve the diagnosis and treatment of the disease. Recent studies highlighted the role of some embryonic stem cell specific regulators as indicator of poorly differentiated cervical cancers [2]. Leucine-rich-repeat-containing G-protein coupled receptor 5 (LGR5) was originally identified as a marker for the intestinal, hair follicle, and stomach stem cells, regulating the embryonic development and even the tumorogenesis of these organs [3-5]. It was suggested that LGR5 is on a higher level of the stem cell hierarchy than CD133, the unanimously agreed stem cell marker [6].

Martens et al demonstrated that the subcolumnar reserve cells of uterine cervix were undifferentiated, omnipotent cervical stem cells which possess the capacity to undergo squamous differentiation. They were also presumed to be the potential target of HPV infection, which was widely assumed to be closely involved in the tumorigenesis of cervical cancer [7]. The uterine cervix shares common features with the intestine, hair follicle and stomach including a constantly and fast renewing epithelium. In the process of normal tissue renewal, cancer stem cells (CSCs) might arise by means of a mutation in normal stem cells...
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and subsequently grow and differentiate to create primary tumors [8]. LGR5/Wnt signaling is closely related to the tumorigenesis of various cancers. Despite the eminent role of LGR5/Wnt signaling in tumorigenesis, the function of LGR5 in uterine cervical cancer remains unclear. Carmon et al demonstrated that R-spondins (Roof plate-specific spondins) functioned as the ligands of LGR5 to regulate the Wnt/β-catenin signaling [9, 10]. Subsequently, Peng et al identified the structure of R-spondin 1 (Rspo1) in complex with the ectodomain of its receptor LGR5 [11]. Hence, we hypothesized that Rspo1/LGR5 pathway might be involved in the malignant behavior of the cervical cancer through Wnt/β-catenin pathway. (Abrami, 2008 #133).

In this study, we found that LGR5 expression on cervical cancer tissues is higher than that on the adjacent tissues, and the expression is correlated with the pathological grade of the cervical cancer. Furthermore, we identified that Rspo1/LGR5 pathway could promote cervical cancer cell proliferation in vitro and tumor growth in vivo via Wnt/β-catenin signaling. In all, we speculated that Rspo1/LGR5 pathway is involved in the malignant biological behavior of cervical cancer cells and is expected to help understand tumor pathology and develop novel and effective therapeutics for cervical cancer.

Materials and methods

Tissue microarrays (TMA) assay for LGR5 expression on cervical cancer tissues

Primary uterine cervical squamous cell carcinoma samples were used for the construction of a tissue microarray. The samples on tissue chip (product number: OD-CT-RpUtr03-003, Shanghai Biochip Co., Ltd. Shanghai, China) were from 31 cervical cancer patients with different TNM stages. Matched pairs of one-millimeter diameter cylinders from two different areas, the center of the tumor tissue and the sample adjacent to the tumor, were included in each case to ensure reproducibility and homogenous staining of the slides. Sections of 4-μm thickness were mounted on poly-L-lysine-coated slides for subsequent staining with an anti-human LGR5 antibody using a two-step protocol as follows. Briefly, the tissue microarrays were incubated at 63°C in a chamber for 1 h, deparaffinized with xylene, and rehydrated with a series of ethanol of different concentrations. The slides were boiled in sodium citrate buffer solution (0.01 M, pH 6.0) for 5 min and EDTA (0.01 M, PH 9.0) for 20 min for antigen retrieval. After immersed in the endogenous peroxidase blocking solution (38.4 ml methanol+12 ml 30% H2O2+9.6 ml distilled water) for 15 min at room temperature and washed with PBS for 3 times, the slides were incubated with rabbit anti-LGR5 antibody (Novus Biologicals, littleton, CO, USA) (1:100 dilution in antibody dilution buffer (DAKO, Glostrup, Denmark)) for 30 min at room temperature. Rinsed three times in PBS, the slides were then incubated for 30 min at room temperature with EnVision™Detection Systems Peroxidase/DAB, Rabbit (DAKO, Glostrup, Denmark) and visualized by 3’3-diaminobenzidine tetra-hydrochloride (DAB) incubation. After stained with hematoxylin and dehydrated with a series of ethanol of different concentrations, the slides were covered for observation.

Evaluation of immunohistochemical variables

Immunohistochemical staining was assessed by Aperio pathological scanning system (Leica, Germany). The staining was scored according to the staining intensity and the distribution of the cells stained. Distribution was evaluated as none (0), < 10% (1), 10% to 30% (2), 30% to 80% (3), > 80% (4). Intensity was evaluated as none (0), faint (1), moderate (2), strong (3), or very strong (4). The final staining scores were calculated as the product of staining intensity multiplied the percentage of stained cells. Each patient was represented by the mean value from the tumor and the adjacent tissue respectively.

Flow cytometry

Surface epitopes of the cells were analyzed by flow cytometry (BD, San Diego, California, USA) using a series of mouse anti-human mAbs (eBioscience, San Diego, CA, USA). For direct immunofluorescence assay, the cells were stained with fluorescein isothiocyanate (FITC)-or phycoerythrin (PE)-conjugated mAbs. For intracellular staining, the cells were fixed with 2% paraformaldehyde, permeabilized with 0.5% saponin, and stained with FITC- or PE-conjugated mouse anti-human mAbs. Negative controls were mouse isotype Ig-PE or Ig-FITC.
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At day 3 and day 5, the cells were digested and resuspended with 100 μl/well trypsin. Then, the number of cells in triplicate wells was counted to obtain the cellular growth curve.

For cell cycle analysis, cells (4×10^4/mL) were seeded in 24 well plate (1 ml/well) and cultured

**Cell proliferation and cell cycle analysis**

Cells (5×10^4/mL) were seeded on 96 well plate (100 μl/well) and cultured for 5 days in RPMI 1640 culture medium (5% FCS) supplemented with Rspo1 (0 ng/mL, 0.2 ng/mL, 1 ng/mL and 5 ng/mL; R&D Systems, Minneapolis, MN, USA).

Figure 1. Immunostaining for LGR5 expression on cervical cancer by tissue microarrays. Standard of immunostaining intensity from score 1 to 3 (A). Representative immunostaining of LGR5 on tumor tissues and tumor adjacent tissues of cervical squamous cell cancer (B). Comparison of LGR5 immunostaining on squamous epithelial of tumor tissue and tumor adjacent tissue (C). LGR5 expression on tumor tissues correlates with tumor grades (D). LGR5 expression in tumor tissues correlates with tumor size (E). LGR5 expression in tumor tissues has no significant correlation with lymph node metastasis (F). *P < 0.05, **P < 0.01, ns, no difference.
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with RPMI 1640 culture medium (5% FCS) stimulated with optimal dose of Rspo1 (0 ng/ml, 0.2 ng/ml, 1 ng/ml and 5 ng/ml). Next day, the cells were collected, washed with PBS, and fixed with 70% ethanol at room temperature overnight. After being washed in cold PBS, the fixed cells were resuspended with 0.5 ml propidium iodide (PI) solution (20 μg/ml; Sigma Chemical Co., saint Louis, Missouri, USA) containing RNAase A (0.1 μg/ml; Sigma Chemical Co., saint Louis, Missouri, USA) at 37°C for 30 min. Then, the samples were subjected to flow cytometric cell cycle analysis for determining DNA contents. The cell histogram was divided into three regions according to cell cycle phase: G0-G1, G2-M, and S phases.

Xenograft tumor assays

Subcutaneous transplantations of SiHa cells into nude mice were performed in accordance with a protocol approved by Animal Care and Use Committee of Soochow University. SiHa cells were pre-treated by Rspo1 (100 ng/ml) or phosphate-buffered saline (PBS) for 30 min at 37°C. Then, SiHa cells (1×10^6/50 μl/site) were injected subcutaneously into right or left flank of the nude mice (8 weeks old). The mice were monitored for every 2 days for tumor growth till the termination of experiments. Then the tumors were harvested, weighed, and examined. Tumor size was assessed by measuring the largest perpendicular diameters with a caliper and recorded as the tumor volume: V = 1/6π (length ×width ×height).

Statistic analysis

Numerical data were expressed as means ± standard deviation. The statistical significance of differences between groups was assessed using the GraphPad Prism6 software, La Jolla, CA, USA. The paired and unpaired two-tailed t-test was used for the comparison of parameters between two groups. Analysis of variance (ANOVA) was performed to determine the differences in the means among the various treatment groups. The SPSS 17.0 software package (SPSS, Inc., Chicago, IL) was used for analysis. P < 0.05 was considered statistically significant. Tumor volume and the Kaplan-Meier survival curve were analyzed by the log-rank test with the Graphpad Prism6 software.

Results

LGR5 is over-expressed on squamous epithelium of cervical cancer tissues

To investigate the clinical significance of LGR5 in cervical carcinogenesis, we first determined LGR5 expression by immunohistochemistry (IHC) staining of cervical cancer tissue array containing 31 cervical squamous cell carcinomas of different stages and the corresponding adjacent tumor tissues. Representative cytoplasmic LGR5 staining is presented in Figure 1A showing the evaluation standard of immunostaining intensity of LGR5 expression. LGR5 was expressed at obviously higher levels in the squamous epithelium of tumor tissues by contrast to detectable but weak expression in adjacent tissues with a statistical significance (Figure 1B, 1C). Moreover, high-grade tumors had more wide-spread and higher intensity LGR5 staining than low-grade tumors. The augmented expression of LGR5 was observed across various grades of cervical cancer with apparent difference between well and poorly differentiated tissues (I versus II P < 0.05, I versus III P < 0.01) (Figure 1B, 1D). However, LGR5 staining scores in tumor adjacent tissues were not correlated with pathological grades of tumor (P > 0.05), indicating that LGR5 might contribute to the differentiation of cervical cancer.

Correlation of LGR5 expression and the clinicopathological features

We also examined whether LGR5 over-expression was associated with other risk factors

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Table 1. Clinical and pathological characteristics of patients of cervical cancer

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such as age, lymph node metastasis and TNM stages. LGR5 expression levels in tumor tissues are correlated with tumor volume ($P < 0.05$) (Figure 1E), but not correlated with patients’ age, TNM grades, or lymph node metastasis (Figure 1F, Table 1). However, LGR5 staining scores in tumor adjacent tissues were not correlated with these clinicopathological features ($P > 0.05$).

Rspo1/LGR5 pathway promotes cervical cancer cell proliferation in vitro

Flow cytometry indicated high level expression of LGR5 molecule on the surface of cervical squamous carcinoma cell line SiHa (Figure 2A). Then, SiHa cells were treated with different doses of Rspo1, the ligand of LGR5, to further investigate the potential mechanism underlying tumor growth promotion by LGR5. Under suboptimal culture conditions (5% serum), the addition of Rspo1 could promote SiHa proliferation in a dose dependant manner (Figure 2B, 2C). Since cell proliferation promotion usually involves modulation of the cell cycle, SiHa cell cycle was analyzed to confirm such effect of LGR5. Flow cytometric cell cycle analysis showed that compared with the control, Rspo1 stimulation could increase the percentage of cells in S phase and decrease the portion of G1/G0 phase (Figure 2D, 2E). The results suggested that Rspo1/LGR5 promote the cervical cancer cell, possibly by accelerating the cell cycle.

Rspo1/LGR5 pathway promotes IL-6 production of SiHa cells in vitro

To determine the effect of Rspo1/LGR5 pathway on cytokine production of SiHa cells, SiHa

Figure 2. Rspo1 promotes SiHa cell proliferation in vitro. LGR5 expression on the surface of cervical squamous carcinoma cell line SiHa was analyzed by flow cytometry (A). The effect of Rspo1 on the proliferation of SiHa cells was evaluated by cell counting (B), microscopic photograph (C). The cell cycle of Rspo1-modulated SiHa cells was analyzed by flow cytometry (D, E). *$P < 0.05$, **$P < 0.01$. 

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Cells were stimulated with different doses of Rspo1 for 24 h, 48 h and 72 h for intracellular staining of IL-6, VEGF and TGF-β. The results indicated ligation of Rspo1 and LGR5 markedly stimulated the production of IL-6 also in a dose dependant and time dependant manner (Figure 3). However, there are no effects on the production of TGF-β (data not shown).

**Rspo1/LGR5 pathway upregulates CD44, c-myc and PCNA expression of SiHa cells**

It was reported that Wnt/β-catenin signaling could be regulated by Rspo1/LGR5 to enhance cell proliferation in intestinal epithelium [12]. However, there is no study identifying the engagement of Rspo1/LGR5 in cervical cancer tumorigenesis by activating Wnt/β-catenin signaling pathway. To determine whether Wnt/β-catenin signaling was involved in the Rspo1/LGR5 pathway, we evaluated the expression of c-myc and CD44, which are the downstream molecules of Wnt/β-catenin signaling. After the Rspo1 administration, the expressions of cell surface CD44 and intracellular c-myc were unregulated in dose dependent manner, and their expression levels were restored by Wnt inhibitor Dickkopf-1 (DKK) (Figure 4A and 4B). Proliferating chain nuclear antigen (PCNA), the marker of cell proliferation, was increased after the Rspo1 stimulation, and the increase could be also blocked by DKK (Figure 4C). All these results demonstrated that Rspo1/LGR5 signaling is closely associated with the activity of some key molecules of the Wnt/β-catenin pathway in cervical cancer cells.

**Rspo1 promotes tumor formation and growth of SiHa cells in vivo**

We then examined the effect of Rspo1/LGR5 pathway on cervical cancer tumorigenesis in vivo by comparing the growth of subcutaneous-ly grafted tumor. Consistent with the increased proliferative capacity in vitro, the LGR5 signal triggered by Rspo1 caused significant differences in tumor formation and tumor growth in vivo. Triggering LGR5 signal induced acceleration in tumor initiation and tumor growth. The tumor formation was observed at day 20 of the control group in contrast today 7 of Rspo1 pre-stimulated group. The tumor free ratio is 60% of the control and 40% of Rspo1 group respectively till the termination of experiments. The xenografted tumors also grew faster than the control (Figure 5). Our data showed that Rspo1/LGR5 pathway drives tumorigenesis by promoting tumor initiation and growth.

**Discussion**

Cervical cancer is the most common of gynecological cancer worldwide. It was unanimously agreed that human papilloma virus (HPV) infection was the main reason leading to malignant transformation of uterine cervix. Further research demonstrated that undifferentiated stem cells of the epithelium in uterine cervix which possess the capacity to undergo squamous differentiation are actually the candidate targets for the HPV infection and therefore the origin of the neoplasia formation and tumor growth [13, 14]. Organista-Nava et al hypothesized that the expression of embryonic stem cell-specific signature might play an important role to promote cell growth, survival,
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Figure 4. Stimulation of LGR5 signal with Rspo1 upregulates CD44, c-myc and PCNA expression of SiHa cells. After the Rspo1 administration, the expressions of intracellular c-myc (upper part of A) cell surface CD44 (upper part B) and were unregulated, and their expression levels were restored by Wnt inhibitor DKK (lower parts of A and B). PCNA, the marker of cell proliferation, was increased after the Rspo1 stimulation, and its increase could be blocked by DKK (C). MFI, mean fluorescence intensity.
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Figure 5. Rspo1 promotes tumor formation and growth of SiHa cells in vivo. SiHa cells were pre-treated by Rspo1 (100 ng/ml) or phosphate-buffered saline (PBS) for 30 min at 37°C. Then, SiHa cells (100×10⁴/50 μl/site) were injected subcutaneously into the flank of the nude mice. Rspo1 could enhance the tumor formation (A) and tumor growth of SiHa cells (B). **P < 0.01.

colony formation, lack of adhesion, as well as cell invasion and migration in cervical cancer [2]. LGR5, a member of the G-protein-coupled receptor family, was originally identified as a novel stem cell marker of the intestinal, stomach epithelium and the hair follicles [15]. In recent years, however, the role of LGR5 in tumorogenesis has been further illustrated in glioma, CRC, liver, ovary and mammary tumors to sustain tumor growth by mutational activation of the Wnt pathway in these tumors [16-18]. LGR5, a stem cell regulator, is now strongly implicated as a novel CSC marker of these tumors. Moreover, the secreted Wnt-enhancer protein Rspo1 was identified as the ligand of the LGR5 and Wnt/β-catenin signaling could be modulated by interaction of Rspo1 and LGR5 [10]. Despite the eminent studies on Wnt signaling in tumorigenesis, the biological function of Rspo1/LGR5 pathway in cervical cancer has not yet been examined. By the tissue microarray analysis, we demonstrated that LGR5 was highly expressed in squamous epithelium of cervical cancer tissues. The LGR5 scores were correlated with the poor pathological grade and tumor volume, suggesting that LGR5 might play important roles in the tumor proliferation, differentiation and the patients’ clinical outcome of cervical cancer.

By the classical Wnt pathway, the secreted Wnt glycoproteins bind to its receptors to inhibit the degradation of β-catenin, allowing its nuclear transportation and gene induction by binding to TCF/LEF transcription factors, and thus regulate the cell survival and proliferation. LGR5 interacts and cointernalizes with Wnt receptors to modulate Wnt signaling [19]. Hence, the ligation of Rspo1 and its receptor LGR5 might link to the biological function of Wnt/β-catenin signaling in cervical cancer cells. To further explore the effects of Rspo1/LGR5 pathway in the malignant behavior of cervical cancer cells, we use Rspo1 to trigger the LGR5 signaling on the squamous cell line of cervical cancer SiHa. Under suboptimal culture conditions (RPMI 1640 medium with 5% FCS), the addition of Rspo1 could promote SiHa proliferation in a dose dependant manner. Furthermore, cell cycle analysis revealed that Rspo1 stimulation could accelerate cell proliferation as a result of significant increase in the percentage of cells in S phase and a concomitant decrease in the percentage of G0/G1 phase cells.

Previous reports demonstrated that many genes regulating tumor initiation, proliferation and differentiation are modulated by Wnt pathway. For example, R-spondins are able to synergize with the Wnt pathway, enhancing Wnt signaling only in the presence of canonical Wnt ligands. To confirm whether the Wnt/β-catenin axis is enhanced by the Rspo1/LGR5 pathway on cervical cancer, we analyzed the downstream molecules of Wnt/β-catenin axis on SiHa cells in the context of Rspo1 trig-
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gering. It was demonstrated that c-myc was a promoter of cervical carcinogenesis and its expression was increased in uterine cervix cancer [20]. CD44, a cell adhesion molecule, can mediate type I receptor function in cervical carcinoma cells. Over-expression of both CD44 and either erbB2 or EGFR may contribute to cervical carcinoma tumor growth and metastasis [21]. It is suggested that, in cooperating with the Wnt/β-catenin axis, Rspo1/LGR5 pathway might promote the cervical cancer cell proliferation and invasiveness by upregulating the expression of c-myc and CD44 protein. Consistent with the previous report that the aberrant activation of the Wnt/β-catenin signaling pathway is common in human cervical cancer our data demonstrated that c-myc and CD44, the Wnt/β-catenin targets, were upregulated on SiHa cells with the stimulation of Rspo1. Furthermore, up-regulation of CD44, c-myc and PCNA could be blocked by DKK-1, an inhibitor of Wnt/β-catenin signaling, suggesting that Rspo1/LGR5 promotes cervical cancer tumorigenesis via activation of Wnt/β-catenin pathway.

To elucidate the clinical relevance of chronic inflammatory and cancer development, we also examined the effects of Rspo1 stimulation on secretion of some key cytokines in tumorigenesis, including IL-6, TGF-β and VEGF. Intracellular staining revealed an up-regulation of IL-6 production. It was consistent with the results of Iris Augustine who reported that silencing of Wnt secretion protein Evi revealed a strong reduction in transcription IL-6 mRNA [22]. The enhanced Wnt signaling stimulates tumor cell proliferation possibly by up-regulation of interleukins and other pro-oncogenic factors, including IL-6. IL-6 has been implicated as a key chronic inflammatory factor in tumorigenesis [23]. In a variety of preclinical models of cervical cancer, high level IL-6 has been shown to be associated with malignancy of cervical cancer through promotion of tumorigenesis, angiogenesis, and metastasis. It has been proved that HPV early protein 6 induces fibroblast senescence to promote cervical tumorigenesis by activation of IL-6/stat3 pathway [24]. Therefore, Rspo1/LGR5 could enhance the IL-6 production and take part in the tumor growth of cervical cancer via Wnt/β-catenin axis.

We next evaluated whether the biological effect of Rspo1/LGR5 pathway in vitro could be translated to in vivo differences in tumor growth by SiHa xenograft model. We demonstrated that LGR5 signal activation by Rspo1 pre-treatment prior to subcutaneous implantation significantly increased tumor formation and tumor growth compared to non-triggering control.

In summary, our data demonstrated that the stem cell marker LGR5 is highly expressed in squamous epithelium of cervical cancer tissues and correlated with the poor tumor pathological grade. Rspo1/LGR5 pathway could accelerate tumor growth and mediate the IL-6 production and c-myc up-regulation via Wnt/β-catenin signaling in cervical cancer cells. Our study co-opts the CSC biomarker LGR5 to illustrate pathogenesis of cervical cancer and provide a potential target for its diagnosis and therapeutic intervention.

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Disclosure of conflict of interest

None.

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