Original Paper

Lgr5-Positive Cells are Cancer-Stem-Cell-Like Cells in Gastric Cancer

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Key Words

Lgr5 • Gastric cancer (GC) • Cancer stem cells (CSCs) • Diphtheria toxin fragment A (DTA) • Tumorigenesis

Abstract

Background/Aims: Effective treatment of gastric cancer (GC) requires better understanding of the molecular regulation of its carcinogenesis. Identification of cancer stem cells (CSCs) in GC appears to be a critical question. **Methods:** We analyzed Lgr5 expression in GC specimen. We used an adeno-associated virus (AAV) that carries diphtheria toxin fragment A (DTA) under the control of Lgr5 promoter (AAV-pLgr5-DTA) to transduce human GC cells. The growth of GC cells with/without depletion of Lgr5-positive cells was studied *in vitro* in an MTT assay, and *in vivo* by analyzing bioluminescence levels. **Results:** A portion of GC cells in the resected specimen expressed Lgr5. GC cells that formed tumor spheres expressed high Lgr5. Selective depletion of Lgr5-positive GC cells resulted in significant growth inhibition of GC cells *in vitro* and *in vivo*. **Conclusion:** Lgr5-positive cells may be CSCs-like cells in GC and may play a pivotal role in the tumorigenesis of GC. Treating Lgr5-positive GC cells may substantially improve the therapeutic outcome.

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Introduction

Gastric carcinoma (GC) is one of the leading causes of cancer-related death in China [1-3]. So far, surgical removal of the stomach baring the primary tumor remains the most effective treatment. However, less than half of GC patients are diagnosed at early stages and amenable to potentially curative surgical therapy [1-3]. Moreover, patients who have undergone radical tumor resection have a high rate of relapse, resulting in a pretty low 5-year survival rate [1-7].

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Cancer stem cells (CSCs) are cancer cells with characteristics of stem cells. CSCs are tumorigenic, and are responsible for cancer relapse and metastasis [8-15]. Identification of CSCs is critical for developing efficient cancer treatment, and is expected to improve the current therapy on rapidly growing cancers and highly metastatic cancers [8-15].

The Wnt target gene *Lgr5* has been recently identified as a novel stem cell marker of the intestinal epithelium [16, 17] and the hair follicle [18, 19]. In the stem cell niche of the intestine crypt and hair follicle, *Lgr5* is specifically expressed in actively cycling cells. Transplantation and lineage-tracing experiments have demonstrated that *Lgr5*-positive cells are responsible for maintaining all cell lineages of the intestine and the hair follicle throughout long periods of time and can build entire new intestine tissue and hair follicles [16, 17, 20], respectively. Moreover, *Lgr5*-positive follicle stem cells have been shown to contribute to the formation of papillomavirus-induced tumor in epidermis [21]. Further, *Lgr5* has been shown as a marker for CSCs in some types of cancer [22-27]. In stomach, *Lgr5*⁺ stem cells drive self-renewal and can build long-lived gastric units *in vitro* [28]. *Helicobacter pylori* infection has been associated with the increases in *Lgr5*-positive cells in the stomach of patients with gastric cancer, and been hypothesized to relate to gastric carcinogenesis, based on clinical data [29-32]. However, the role of *Lgr5*-positive cells in GC has not been extensively tested for their CSC characteristics *in vitro* using molecular biological methods.

Here we reported expression of *Lgr5* in a portion of the GC cells in the resected specimen. We used an adeno-associated virus (AAV) that carries diphtheria toxin fragment A (DTA) under the control of *Lgr5* promoter (AAV-p*Lgr5*-DTA) to transduce human GC cells. The growth of GC cells with/without elimination of *Lgr5*-positive cells was examined *in vitro* in an MTT assay, and *in vivo* by analyzing bioluminescence levels. GC cells that formed tumor spheres expressed high *Lgr5*. Selective depletion of *Lgr5*-positive GC cells resulted in significant inhibition of the GC cell growth *in vitro* and *in vivo*.

Materials and Methods

Specimens from patients

Fifty resected GC specimens (paired GC and the adjacent normal tissue (NT)) in this study were histologically and clinically diagnosed at the First Affiliated Hospital of Liaoning Medical University from 2008 to 2013. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

*Cell culture and transduction with AAV-p*Lgr5*-DTA*

Human GC cell line SNU-5 was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA). Primary GC cells were cultured in the same media. All cells were incubated in a humidified chamber with 5% CO₂ at 37°C.

AAV (serotype 8)-p*Lgr5*-DTA was prepared as has been previously described [33-35]. Briefly, we used a pAAV-CMV-GFP plasmid (Clontech, Mountain View, CA, USA), a packaging plasmid carrying the serotype 8 rep and cap genes, and a helper plasmid carrying the adenovirus helper functions (Applied Viromics, LLC, Fremont, CA, USA) in this study. The transgene was DTA or null (as a control) under the control of a human *Lgr5* promoter. The AAV-p*Lgr5*-DTA construct was prepared from a full-length human *Lgr5* promoter, amplified by PCR with EcoRI-restriction-endonuclease-forward and NheI-restriction-endonuclease-reverse primers, using the human genomic DNA as a template. The construct was then subcloned into the 50-EcoRI and 30-NheI sites of the pAAV-CMV-GFP vector to replace the CMV promoter (pAAV-*Lgr5*-GFP). DTA fragment was cut out from a DTA containing plasmid pFB-CMV-DTA with EcoRIII and BamHI restriction endonucleases, and then ligated to the EcoRIII and BamHI sites of plasmid pAAV-*Lgr5*-GFP after same enzymes' cutting to remove GFP. 2A peptide-linked multicistronic vectors that are used to express multiple proteins from a single open reading frame (ORF) and that the small 2A peptide sequences, when cloned between genes, allow for efficient, stoichiometric production of discrete protein products within a single vector through a novel "cleavage" event within the 2A peptide sequence. Sequencing was performed

to confirm the correct orientation of the prepared pAAV-Lgr5-DTA. AAV was prepared by triple transfection of the pAAV-Lgr5-DTA or control pAAV-Lgr5-null plasmid, R2C8 (containing AAV2 Rep and AAV8 capsid genes) and pAd5 (containing adenovirus helper genes) into HEK293 cells by Lipofectamine 2000 reagent (Invitrogen). The viruses were purified using CsCl density centrifugation and then titered by a quantitative densitometric dot-blot assay.

For cell transduction *in vitro*, the GCs cells were incubated with AAV at a MOI of 100 for 12 hours. For cell transduction *in vivo*, 10^9 AAV were injected directly into the implanted tumor (10^6 cells at time of injection). For preparation of luciferase-carrying SNU-5 cells, the cells were transduced with an AAV-pCMV-luciferase, which was similarly prepared as AAV-pLgr5-DTA and AAV-pLgr5-null.

Primary Tumor Sphere Culture

Purified tumor cells by flow cytometry were washed, acutely dissociated in oxygenated artificial cerebrospinal fluid and subjected to enzymatic dissociation. Tumor cells were then resuspended in tumor sphere media (TSM) consisting of a serum-free DMEM, human recombinant EGF (20ng/ml; Sigma-Aldrich), bFGF (20ng/ml; Sigma-Aldrich), leukemia inhibitory factor (10ng/ml; Sigma-Aldrich) and N-acetylcysteine (60μg/ml; Sigma-Aldrich), and then plated at a density of 2×10^6 cells/60mm plate.

In vivo tumor model and imaging of the implanted tumor by bioluminescence

All mouse experiments were approved by the Institutional Animal Care and Use Committee at the First Affiliated Hospital of Liaoning Medical University. Surgeries were performed under ketamine/xylazine anaesthesia, according to the Principles of Laboratory Care, supervised by a qualified veterinarian. NOD/SCID mice were purchased from Jackson Labs (Bar Harbor, ME, USA).

Luciferase-carrying SNU-5 cells of 10^6 were subcutaneously implanted into 12-week-old NOD/SCID mice. The tumor growth in the living animals was monitored and quantified by luminescence levels. Bioluminescence was measured with the IVIS imaging system (Xenogen Corp., Alameda, CA, USA). All of the images were taken 10 minutes after intraperitoneal injection of luciferin (Sigma-Aldrich, St. Louis, MO, USA) of 150 mg/kg body weight, as a 60-second acquisition and 10 of binning. During image acquisition, mice were sedated continuously via inhalation of 3% isoflurane. Image analysis and bioluminescent quantification were performed with living image software (Xenogen Corp).

Apoptosis assay

Apoptosis was evaluated by Double Stain Apoptosis Detection Kit (Genscript, Piscataway, NJ, USA), based on Hoechst 33342 (HO; stain all cells) and Propidium Iodide (PI; stain dead cells) staining, according to the instruction of the manufacturer. Quantification was performed with 5 repeats per condition.

Cell viability assay

For assay of cell growth, cells were seeded into 24 well-plate at 1×10^4 cells per well in the conditioned media and subjected to a Cell Viability Kit (MTT, Roche, Indianapolis, IN, USA), according to the instruction of the manufacturer.

RT-qPCR

RNA was extracted from tissues from patients, or from cultured cells, with RNeasy kit (Qiagen, Hilden, Germany). cDNA was then synthesized by reverse transcription (Qiagen). Quantitative real-time PCR (RT-qPCR) was performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were analyzed using $2^{-\Delta\Delta Ct}$ method for quantification of the relative mRNA expression levels. Values of genes were first normalized against GAPDH, and then compared to controls.

Immunohistochemistry

Specimens were fixed with 4% paraformaldehyde for 4 hours, and then cyro-protected in 30% sucrose overnight. Tissue samples were sectioned in 6μM. A primary antibody used in immunohistochemistry is rabbit polyclonal anti-Lgr5 (Abcam, Cambridge, MA, USA). ABC method was applied, followed by counterstaining with hematoxylin (Abcam).

Western blot

The protein was extracted from the resected specimens or adjacent normal tissue (NT), or cultured cells. Primary antibodies were anti-Lgr5 (Abcam) and anti-GAPDH (Cell Signaling, San Jose, CA, USA). GAPDH was used as a protein loading control. The secondary antibody was HRP-conjugated anti-rabbit from Jackson ImmunoResearch Labs (West Grove, PA, USA). The protein levels were first normalized to GAPDH, and then normalized to control, quantified by NIH Image J software (Bethesda, MA, USA).

Statistical analysis

All statistical analyses were carried out using the SPSS 18.0 statistical software package. All values are depicted as mean $\pm$ standard deviation from 5 individuals and are considered significant if $p < 0.05$. All data were statistically analyzed using one-way ANOVA with a Bonferoni correction, followed by Fisher's Exact Test to compare two groups.

Results

GCs express higher Lgr5 in some cancer cells

Here, we aimed to find out the role of Lgr5-positive cells in the GCs. We examined Lgr5 levels in 50 resected GC specimens, and compared to the paired adjacent normal tissue (NT) from the same patient. We found significantly higher levels for Lgr5 in GCs, compared to NT, by mRNA (Fig. 1A: increased by 5.6 ± 0.9 fold) and by Western blot (Fig. 1B: increased by 7.9 ± 0.7 fold). Moreover, in the sections from the resected GC specimens, we found that some, but all GC cells expressed Lgr5 (Fig. 1C). Then we put the dissociated GC cells in a sphere-

Fig. 1. CRCs express high Lgr5 in some cancer cells. (A-B) Lgr5 levels were examined in 50 resected GC specimens, compared with the paired adjacent normal tissue (NT) from the same patient, by RT-qPCR (A), and by Western blot (B). (C) Representative images for Lgr5 in GC and NT. (D) Tumor sphere formation. (E) A representative image for Lgr5 in tumor-sphere-forming GC cells. $*p < 0.05$, $n = 50$. Statistics: one-way ANOVA with a Bonferoni correction, followed by Fisher's Exact Test to compare two groups. Scale bar is $50 \mu\text{m}$.

Fig. 2. AAV-pLgr5-DTA specifically ablates Lgr5-positive GC cells. (A) Schematic of an AAV-pLgr5-DTA. The transduction with this virus specifically kills Lgr5-positive cells. AAV-pLgr5-null was also prepared, and used as a control for viral transduction. (B-C) Twelve hours after GC cells were transduced with viruses, the apoptosis of the cells was evaluated, showing by representative images (B), and by quantification (C). (D) Forty-eight hours after viral transduction, the GC cells were harvested for RT-qPCR for Lgr5. * $p < 0.05$. $n = 5$. Statistics: one-way ANOVA with a Bonferoni correction, followed by Fisher's Exact Test to compare two groups. Scale bar is 20 μm .

forming culture (Fig. 1D). We isolated sphere-forming GC cells and found that nearly all these cell expressed Lgr5 (Fig. 1E). These data suggest that Lgr5-positive cells in GC are likely CSC-like cells, which prompted us to analyze the role of Lgr5-positive cells in GCs.

Ablation of Lgr5-positive cells inhibits growth of GCs in vitro

We then used human GC cell line SNU-5 in our study. To determine the role of Lgr5-positive cells in the tumorigenesis of GC, we prepared an AAV-pLgr5-DTA. The transduction using AAV-pLgr5-DTA virus specifically killed Lgr5-positive cells. AAV-pLgr5-null was also prepared a control for viral transduction (Fig. 2A). Twelve hours after GC cells were transduced with the viruses, the apoptosis of the cells was evaluated, showing significant increases in apoptotic cells by AAV-pLgr5-DTA, compared to those by AAV-pLgr5-null, by representative images (Fig. 2B), and by quantification (Fig. 2C). Forty-eight hours after viral transduction, the GC cells in culture were harvested for RT-qPCR for Lgr5. We found that the Lgr5 transcripts in AAV-pLgr5-DTA-treated cells decreased by more than 90%, suggesting that most of the Lgr5-positive cells had been ablated by AAV-pLgr5-DTA (Fig. 2D).

Moreover, selective elimination of Lgr5-positive cells almost completely inhibited the growth of GCs *in vitro*, in a MTT assay (Fig. 3).

Fig. 3. Ablation of Lgr5-positive cells inhibits growth of GCs *in vitro*. Cell growth was quantified in a MTT assay. * $p < 0.05$. $n = 5$. Statistics: one-way ANOVA with a Bonferoni correction, followed by Fisher's Exact Test to compare two groups.

Fig. 4. Ablation of Lgr5-positive cells inhibits growth of GCs *in vivo*. (A) Schematic of the model. Luciferase-carrying GC cells were subcutaneously transplanted into NOD/SCID mice to form tumors. Thirty days later, the mice received direct injection of either AAV-pLgr5-DTA, or AAV-pLgr5-null as a control, into the tumors. The growth of the tumors was then evaluated by bioluminescence after another 30 days. (B-C) Bioluminescence was evaluated 30 days after viral injection, shown by representative images (B), and by quantification (C). * $p < 0.05$. $n = 5$. Statistics: one-way ANOVA with a Bonferoni correction, followed by Fisher's Exact Test to compare two groups.

Ablation of Lgr5-positive cells inhibits growth of GCs *in vivo*

We then examined the effects of ablation of Lgr5-positive cells on the growth of GCs *in vivo*. For this purpose, GC cells were transduced with AAV-pCMV-Luciferase to allow tracing of the cells in living animals. Then the cells were subcutaneously transplanted into NOD/SCID mice to form tumors. Thirty days later, either AAV-pLgr5-DTA, or AAV-pLgr5-null as a control, was directly injected into the tumors of the mice. The growth of the tumors was then evaluated by bioluminescence after another 30 days (Fig. 4A). We found that injection with

AAV-pLgr5-DTA reduced the tumors by $89.5 \pm 12.7\%$, shown by representative images (Fig. 4B), and by quantification (Fig. 4C). These data suggest that ablation of Lgr5-positive cells inhibited growth of GCs *in vivo*.

Discussion

Comprehension of the molecular mechanisms underlying the tumorigenesis of GCs is extremely important for effective therapies. Recently, the importance of cancer stem cells (CSCs) to cancer growth and metastasis has been acknowledged [8, 9], since therapeutic treatments targeting CSCs may efficiently inhibit the growth and pathogenesis of cancer.

Indeed, although in the previous studies, identification of CSCs has been largely relied on flow-cytometry-based examination of CD133, side population and high aldehyde dehydrogenase (ALDH) activity, increasing evidence has shown the limitation of these methods. For example, not all CD133-positive cells are CSCs [36], and increased ALDH activity can be also detected in non-stem/progenitor and non-cancer cells [37, 38]. These reports suggest that isolation of CSCs with these generalized methods lack of cancer specificity is not ideal.

There is clinical evidence for a role of Lgr5+ cells in the carcinogenesis of GC [29-32]. However, most of these evidence come from the analyses on the clinical samples. A direct evidence for Lgr5-positive cells as CSC-like cells in GC is lacking. In our current work, we specifically killed Lgr5-positive cells in GCs, which nearly completely inhibited growth of GCs both *in vitro* and *in vivo*. The *in vivo* prolonged inhibition of tumor growth may result from the long-term expression of the transgene by AAV infection, compared to the transient expression of the transgene by adenoviral infection [33-35]. In case that new Lgr5-positive cells may regenerate from Lgr5-negative cells to acquire CSC properties, the presence of the transgene in these cells should induce expression of DTA after activation of Lgr5 promoter, resulting in elimination of newly formed Lgr5-positive cells for a long period.

Since AAV has been used in clinic and has been proven of safety in humans, our approach here may be readily translated into clinical application [33-35, 39-43]. However, Lgr5 is not a specific CSC marker and it is also expressed by normal intestinal stem cells and hair follicle. As a result, targeting Lgr5-positive cells by gene therapy should be applied specifically to the tumor, rather than systematically.

Besides our studies on GC cell line, we also achieved strong data on human GC specimens. Significant higher levels of Lgr5 in GC specimens, as well as unique Lgr5 expression in tumor sphere from human GCs, supports our conclusion that Lgr5-positive cells play a critical role in tumorigenesis of CRCs, and possibly act as CSCs. Our results thus highlight Lgr5 as a promising target for GC therapy.

Disclosure Statement

The authors have declared that no competing interests exist.

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