Silencing of RegIV by shRNA causes the loss of stemness properties of cancer stem cells in MKN45 gastric cancer cells

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Abstract. Regenerating islet-derived family member 4 (RegIV) is overexpressed in several types of tumours, including pancreatic and gastric cancer (GC). However, the role it plays in gastric cancer stem cells (GCSCs) remains unknown. The present study tested the hypothesis that the silencing of RegIV by shRNA in GC cells may cause the loss of their stemness properties, indicating the inhibition of growth, proliferation and increased sensitivity to chemoradia-tion-induced cell death. MKN45 poorly differentiated human GC cells were propagated as mammospheres in stem cell culture conditions. Mammospheres were identified as CSCs using generally acknowledged CSC markers such as CD44. A panel of 21-nucleotide shRNAs were designed to target RegIV gene expression. Several shRNA constructs were identified that led to significant reduction in RegIV mRNA expression. Furthermore, the stemness properties of control mammospheres and RegIV knockdown mammospheres were compared by tumourigenicity assay in vivo and plate colony formation assay in vitro. Finally, we evaluated the treatment response in both mammospheres which underwent chemoradiation. The knockdown expression of RegIV by shRNA deprived CSCs of their stemness properties and increased the effectiveness of cell killing following chemoradiation. Inhibition of endogenous RegIV expression may be a new therapeutic strategy for human GC.

Introduction

Gastric cancer (GC) is a world health burden and is the second leading cause of cancer-related mortality worldwide, despite improvements in prognosis as a result of early diagnosis (1). Only slight progress has been made in the treatment strategies for GC during the past 30 years (2). Accumulating evidence in recent years strongly indicates the existence of cancer stem cells (CSCs) in solid tumours of a wide variety of organs, including GC (3). Therefore, we searched for new effective treatment methods for GC.

RegIV, a member of the regenerating gene family, is involved in digestive tract malignancies, including the pancreas, colorectum and stomach, as well as in benign diseases such as ulcerative colitis (4–6). RegIV overexpression in tumour cells has been associated with cell growth, survival, adhesion and resistance to apoptosis and can even predict the intrinsic chemoresistance in advanced GC (7). A few research groups recently investigated its possible applications for cancer biomarkers and acquired marked results. RegIV was found in the serum of patients with GC which could predict the peritoneal dissemination in gastric adenocarcinoma (8-10). However, the relationship between CSCs and RegIV in human GC has yet to be reported. Thus, a better understanding of the relationship between CSC and RegIV may help to improve early diagnosis and may aid in identifying a new molecular therapeutic target for GC.

Materials and methods

Cells and animals. The human poorly differentiated GC cell line MKN45 was from the Cell Bank of the Chinese Academy of Sciences. Cells were cultured in log growth phase in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum and 0.01 mg/ml bovine insulin at 37°C in a humidified atmosphere of 5% CO2. We used 30 4-week-old Balb/cA nu/nu female mice from the Shanghai Experimental Animal Centre of the Chinese Academy of Sciences (Shanghai, China), they were maintained in plastic cages (5 mice/cage) in a room with constant temperature (22±1°C) with a dark-light cycle (12 h/12 h). Animal experiments were performed in accordance with the ethics code by the Ethics Committee of the Chongqing Medical University.

Cancer stem cell culture. To obtain CSCs and to propagate them as mammospheres, cells floating in the supernatant
of 4-day-old cultures were collected by centrifugation for 5 min at 500 g, washed in Hank's buffered salt solution, and resuspended in phenol red-free DMEM supplemented with 0.4% bovine serum albumin (BSA, Sigma), 5 µg/ml bovine insulin, 20 ng/ml basic fibroblast growth factor (bFGF, Sigma), 10 ng/ml epidermal growth factor (EGF, Sigma) at a density of 1,000 cells/ml. Growth factors were added to the mammosphere cultures every 3 days.

Flow cytometry of CD44, CD24 and CD133 expression. Expression of CD44, CD24 and CD133 was analysed in cells derived from monolayer cultures or in 15-day-old mammospheres following incubation in trypsin-EDTA dissociation with a Pasteur pipette and passage through a 40-µm sieve. At least 10⁵ cells were pelleted by centrifugation at 500 g for 5 min at 4˚C, resuspended in 10 µl of monoclonal mouse anti-human CD44-phycocerythrin (PE) antibody, monoclonal mouse anti-human CD24-PE antibody and a monoclonal mouse anti-human CD133-PE antibody, respectively, and incubated for 20 min at 4˚C.

RegIV knockdown by lentivirus-mediated short hairpin RNA. RegIV knockdown in mammospheres was performed by infection with a lentivirus that expresses human RegIV-specific short hairpin RNA (shRNA) and contains a green fluorescent protein gene under a separate promoter for tracking the transfection efficiency. Briefly, the lentivirus vector plasmid encoding human RegIV-specific shRNA (Sigma-Aldrich) was transfected with capsule and packaging plasmids using SuperFect (Qiagen, Valencia, CA, USA) into HEK293T cells, and after 48 h, supernatant was collected and used as infection solution without enrichment. The three predesigned target sequences for human RegIV (GeneBank accession: NM_001159352) are, 5'-TGGATTGTTTTTCTCAAATAATA-3', 5'-ATGGATTGTTTTTCTCAAATAAT-3', and 5'-TGATGATGGATTGTTTTTCTCAAAT-3'. The following sequence was used in this experiment: 5'-TGGATTGTTTTTCTCAAATAATA-3'. The scramble shRNA obtained from Addgene (Cambridge, MA, USA) was used as control. Forty-eight hours after viral infection, RegIV knockdown was confirmed by real-time-PCR analysis.

In vivo xenograft assay. Cells were derived from RegIV-KD or control mammospheres by incubation in trypsin-EDTA dissociation with a Pasteur pipette. We adjusted the concentration of the cell suspension to be inoculated to 5x10⁵/ml in PBS. Then, 0.2 ml of the cell suspension was subcutaneously injected in the right hind limb of the mice, respectively. Fifteen mice were injected in each group. Mice were observed daily and inspected for tumour growth each week for 8 weeks.

Plate clone formation assays. RegIV-KD and control mammospheres were incubated in trypsin-EDTA dissociation with a Pasteur pipette. They were seeded at 1,000 cells in each 6-well plate and cultured in DMEM medium containing 10% FCS for ~14 days. When most cell clones reached >50 cells, they were fixed with 4% paraformaldehyde for 15 min and stained with 1% crystal violet at room temperature. Each experiment was repeated three times.
Results

Growth factors induce MKN45 suspension cells to propagate as mammospheres. Non-CSCs failed to proliferate under the growth factors rich, low attachment and serum-free culture conditions. Only CSCs could clonally proliferate to form suspension mammospheres which was considered to be one of the most important characteristic of CSCs (12). Mammospheres formed in significant numbers in cells with growth factor treatment for 15 days, while a few MKN45 cells formed sphere bodies without growth factor treatment. Notably, those sphere bodies could be gradually detached into monolayer culture cells in 2 days after RegIV knockdown, which indicated the loss of the stemness capacity (Fig. 1).

Surface marker expression profile in mammospheres of MKN45. To further verify whether those mammospheres were CSCs, we analysed the expression patterns of cell surface markers for CSCs by using FACS for the mammospheres of human GC cell line MKN45. Based on previous published reports regarding CSCs in solid tumours, the following markers were studied: CD44, CD24 and CD133. The results of the FACS studies for CD44, CD24 and CD133 are shown in Fig. 2. Mammospheres of MKN45 showed a high level of expression of CD44 with up to 87% of cells expressing CD44, while they showed as little as 5% expression of CD24 and CD133, which is consistent with other verified reports (13). Thus, we interpreted these data by the fact that those mammospheres are suitable candidates for CSCs of MKN45 cells.

Validation of lentiviral shRNA constructs for RegIV knockdown. Lentiviral constructs shRNA-RegIV (nos. 1-3) and controls (scrambled sequence) were first examined in submerged MKN45 mammosphere cultures. The vector also contains a human EF1-α promoter driving the GFP marker gene for tracking transduced cells (Fig. 3). Therefore, cells that receive silencing constructs can be detected by fluorescence of GFP at the single cell level. We confirmed the detection of GFP in the mammospheres of MKN45 in 2 days of post-transfection under laser confocal microscopy (Fig. 4A). The real-time quantitative PCR analysis revealed a strong reduction of RegIV at the mRNA level for designed shRNA (no. 1) when compared to the scrambled control. The other two shRNA-RegIV constructs (nos. 2-3) showed no differences in mRNA expression levels when compared to the scrambled control (Fig. 4B). These data indicate that shRNA-RegIV1 plasmid is the most effective construct in knockdown RegIV expression. Hence, the stable transfected clone was used for further studies.

RegIV knockdown reduces tumourigenicity and clone formation of MKN45 mammospheres in vivo and in vitro. With serum stimulation, cells from control mammospheres showed higher clone formation ability than RegIV-KD cells and significantly increased rate of clone efficiency (Fig. 5A).

To verify RegIV-KD in MKN45 CSCs may have a significant role in supporting tumourigenicity in vivo, we injected these RegIV-KD cells subcutaneously in nude mice. We...
observed that RegIV-KD cells produced fewer (4/15) and much smaller tumours than those from control mammospheres (13/15) (Fig. 5B). The tumour diameter was monitored every week up to 8 weeks. In the experiment, control mammospheres generated tumours of greater volume, formed measurable tumour masses in animals in the first week of post-injections (Fig. 5B).

RegIV knockdown enhances chemoradiosensitivity and apoptosis in MKN45 mammospheres. The ability of chemo-reagent
to inhibit the growth of RegIV-KD and control mammospheres was assessed by cell viability and apoptosis assay. It was observed that RegIV-KD mammospheres significantly decreased cell viability to 33% (5-FU) and 44% (cisplatin) as compared to control mammospheres. Similar results were obtained in the Annexin V cell death assay wherein RegIV-KD results in significantly higher cell death as compared to control (Fig. 6A).

To assess the radiosensitivity of the RegIV-KD cells, the comet assay was performed on the irradiated tumour cells. RegIV-KD cells showed a higher percentage of DNA in the tail when compared to the control, suggesting that the DNA damage was higher in radiosensitive RegIV knockdown MKN45 cells (Fig. 6B).

Discussion

Although the biological function of RegIV is poorly understood, it has been reported that RegIV may function as a growth and antiapoptotic factor in colon and gastric cancer (6,14,15). According to previous literature, RegIV expression in different cell types is associated with regeneration, survival and migration (6,16,17). RegIV is systematically overexpressed in colon, pancreatic and gastric cancer (GC) and in diseases that predispose to colon cancer such as ulcerative colitis (5,18,19). However, the role RegIV plays in CSCs has not been fully elucidated. We demonstrated for the first time that the knockdown expression of RegIV deprived CSCs of their stemness properties by a series of experiments, including chemoradioreistance in vitro and xenograft assay in vivo. Other studies confirmed RegIV expression in gastric, colorectal and pancreatic carcinoma, and that RegIV has a potential role in diagnosing digestive tract neuroendocrine tumours (20-22). Gastric cancer stem cells with overexpressing RegIV protein grew more rapidly and were more resistant to 5-FU and cisplatin treatment. Furthermore, previous studies had shown that RegIV overexpression was thought to be chemoresistant in GC patients (7,23). Furthermore, RegIV was recently reported to be an important target gene of GLI1 (24). Thus, we concluded that the RegIV plays a key role for maintaining the stemness properties of CSCs and the SHH-GLI1-RegIV signal cascade may be involved.

CSCs use multiple signalling pathways to control self-renewal and differentiation (25,26). Misregulation of these pathways may lead to the loss of CSC properties. Numerous signalling pathways have been implicated in this process including Wnt, Notch, EGF, PTEN and SHH (27-31). Furthermore, frequent misregulation of crucial embryonic signalling pathways (i.e., the Hedgehog signalling pathway) contribute to the process of gastric carcinogenesis (32). The
Hedgehog signal is transmitted by transmembrane protein Smoothened (SMO) through binding to a second receptor Patched (Ptc) in extracellular and terminated in intracellular Hedgehog signal transduction via Hedgehog transcription factors that are glioblastoma factors GLI1, GLI2 and GLI3.

In conclusion, this is the first report to demonstrate RegIV was able to manipulate the stemness properties of CSCs in GC cells. Our study contributes to the body of research on gastric carcinogenesis and provides insight into the possible network of signalling pathways through the GLI1/RegIV axis. It may also help to provide new insight into treatment strategies for GC. Further studies are required to determine whether the biological behaviour of GC patients may be achieved by regulating RegIV.

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References