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Overexpressed MALAT1 promotes invasion and metastasis of gastric cancer cells via increasing EGFL7 expression

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ABSTRACT

Objective: Long non-coding RNAs (lncRNAs) have been demonstrated to participate in various cancers. Here, the role and its potential mechanism of MALAT1 in invasion and migration of gastric cancer (GC) were investigated.
Methods: Gastric adenocarcinoma tissues and matched normal adjacent tissues were isolated from 25 patients with GC. The expression of epidermal growth factor-like domain-containing protein 7 (EGFL7) was detected in the normal gastric mucosa epithelial GES-1 cell line and three different differentiation GC cell lines, including MKN28 (well-differentiated adenocarcinoma), SGC7901 (moderately differentiated adenocarcinoma) and BGC823 (poorly differentiated adenocarcinoma). Tumor xenotransplant mouse model was established with the injection of cell line pretreated with lentiviral vectors for si-MALAT1 or si-control.
Results: The expression of MALAT1 was up-regulated in GC tissues and three cell lines. Si-MALAT1/pcDNA-MALAT1 induced the decrease of cell invasion and migration, while the effects were reversed by the transfection of pcDNA-EGFL7/si-EGFL7. ChIP assay showed that MALAT1 regulated EGFL7 expression by altering the level of H3 histone acetylation in EGFL7 promoter. In tumor xenotransplant mice, down-regulated MALAT1 contributed to the inhibition of tumor metastasis.
Conclusions: Up-regulated MALAT1 promoted the invasion and metastasis of GC, and the increase of EGFL7 expression was a potential mechanism via altering its H3 histone acetylation level.

1. Introduction

Gastric cancer (GC) is one of the leading causes of death from cancer worldwide. There are 989,600 subjects per year suffering from GC, and approximately half of all GC cases occur in Eastern Asia [1]. Although the incidence rates have reduced substantially in most parts of the world, the mortality rates of GC are still high in the less developed regions [2]. Multi-factors contribute to the pathogenesis of GC, including the alterations of environment and genetics [3]. Due to the limitation of biomarkers specific for GC, patients are usually diagnosed after metastasis, leading to the high rates of recurrence, poor prognoses and even death. There are many mechanisms involving tumor suppressors, oncogenes and cell adhesion molecules have been proposed to explain the GC metastasis [4]. However, it has yet to be comprehensively elucidated.

In recent years, the role of long non-coding RNAs (lncRNAs) has been demonstrated to function as a regulator of gene expression by epigenetic modification and transcriptional as well as post-transcriptional regulation in various diseases, especially in cancers [5,6]. Metastasis associated lung adenocarcinoma transcript 1 (MALAT1), a highly conserved non-coding RNA amongst mammals, has firstly been shown a critical regulator in the expression of metastasis-associated genes in lung cancer cells [7,8]. In the past decade, accumulating data has demonstrated that MALAT1 is linked to other cancer types, including renal cell carcinoma [9], liver cancer [10], cervical cancer [11] and digestive system cancer [12–14]. The recent studies revealed that MALAT1 participated in GC by promoting cell proliferation and peritoneal metastasis [10,15]. However, the knowledge of the role and its mechanisms of MALAT1 in GC remains limited.

Epidermal growth factor-like domain-containing protein 7 (EGFL7), also known as vascular endothelial statin, is encoded by the *EGFL7* gene and involved in regulating vascular tube formation [16]. Up-regulated EGFL7 expression has been observed in several cancers such as hepatocellular carcinomas [17], colorectal cancer [18], breast cancer [19] and brain tumors [20]. Furthermore, the elevated EGFL7 is also closely

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correlated to the invasion and metastasis of GC [21]. The non-coding RNA miR-126 was reported to contribute to the epigenetic modification of EGFL7 via histone deacetylation in cancer cells [22]. In the present study, we aimed to explore the role of MALAT1 in EGFL7 expression, gaining the knowledge in the molecular mechanisms of GC.

2. Materials and methods

2.1. Specimen collection

The human study was approved by the Ethics Committee of the Affiliated Hospital of Logistics University of Chinese People's Armed Police Forces, and the written informed consents were obtained from all participants. Gastric adenocarcinoma tissues and matched normal adjacent tissues were isolated from 25 patients with GC who were hospitalized and underwent surgical excision in Affiliated Hospital of Logistics University of Chinese People's Armed Police Forces. The tissues were stored at −80 °C immediately after removing.

2.2. Cell culture

The normal gastric mucosa epithelial GES-1 cell line and three different differentiated GC cell lines, including MKN28 (well-differentiated adenocarcinoma), SGC7901 (moderately differentiated adenocarcinoma) and BGC823 (poorly differentiated adenocarcinoma), were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured in RPMI 1640 medium (Gibco, USA) containing with 10% fetal bovine serum (FBS) (Gibco, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C.

2.3. Cell transfection

When the BGC823 or MKN28 cell lines grew to 70% to 80% confluence, the small interfering RNA-MALAT1 (si-MALAT1), si-EGFL7, pcDNA-MALAT1 or pcDNA-EGFL7 were transfected into cells using Lipofectamine® 2000 Reagent (Thermo Fisher Scientific, USA) in a 6-well cell culture plate following to the manufacturer's instructions. Transfected a si-control or empty pcDNA and served as negative control. Twenty-four h after incubating, the cells were harvested and performed the following experiments. Real-time PCR was used to detect the expression of MALAT1 and EGFL7 and confirm the transfection efficiency. SiRNAs were synthesized by Guangzhou RiboBio Co. Ltd. (Guangzhou, China), and pcDNA plasmids were prepared by the Life Technologies (Beijing, China).

2.4. Real-time PCR

Total RNA was abstracted from GC tumor tissue and cell lines with Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The complementary DNA (cDNA) was produced by RNA using a PrimeScript™ Reverse Transcription Kit (TakaRa, Japan). The primers specific for MALAT1, EGFL7, GAPDH and β-actin were designed and synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). GAPDH and β-actin acted as the reference genes to normalize the expression of MALAT1 and EGFL7, respectively. Real-time PCR was explored to detect the quantities of MALAT1 and EGFL7 expression. The procedures were as follows: activation of enzymes at 95 °C for 5 min, 45 cycles of denaturation at 95 °C for 20 s, annealing at 62 °C (or 60 °C) for 30 s, and extension at 72 °C for 20 s. The relative expression levels of MALAT1 and EGFL7 were calculated with the $2^{-\Delta\Delta CT}$ method.

2.5. Western blot

The protein expression of EGFL7 in GC tissues and cell lines was determined with western blot. The tissue specimen was prepared and homogenized on ice in PBS. An RIPA lysis buffer (Beyotime, China) was used to harvest the protein from tissue homogenate and GC cells. The protein concentrations of supernatants were measured by a BCA Protein Assay Kit (Beyotime, China). Thirty micrograms of protein from each sample were loaded on 10% SDS denatured polyacrylamide gel and then transferred to nitrocellulose membrane and blocked in 5% nonfat milk. The primary anti-EGFL7 antibody (Abcam, USA) and anti-β-actin antibody (Abcam, USA) were diluted to 1: 500, and incubated with membrane overnight at 4 °C. After being washed, horseradish peroxidase-conjugated secondary antibody was then incubated for 1 h at room temperature. β-Actin served as a control protein to normalize the EGFL7 expression. The signals were visualized with a BeyoECL Plus ECL Kit (Beyotime, China).

2.6. Cell migration and invasion assay

The migration and invasion of GC cell lines were measured with Transwell chambers (Corning, USA). The method was described previously [23]. In the upper chamber with (for invasion) or without (for migration) the pretreatment of Matrigel (BD Biosciences, USA), a cell suspension of 0.2 ml in RPMI-1640 medium with 2% FBS was seeded. In the lower chamber, 0.8 ml RPMI 1640 containing 10% FBS was supplemented and acted as attractant. Then, the cells were cultured in the chamber for 6 h (for migration) or 24 h (for invasion) at 37 °C. Removed the upper chamber, and the cells in lower chambers were fixed with 4% paraformaldehyde and stained with 2% crystal violet for 10 min. The migrating cells in six fields on each chamber were observed to calculate the average counts.

2.7. Cell viability assay

The viability of BGC823 cell line was evaluated using MTT assay (Beyotime, China) following to the manufacturer's instructions. In brief, after the transfection of siRNAs or pcDNA, 40 μl MTT stock solution was added into medium for 4 h. After discarding the supernatant, 200 μl dimethyl sulfoxide (DMSO) was supplemented. The absorbance at 570 nm was detected and recorded to calculate the cell viability.

2.8. Chromatin immunoprecipitation (ChIP) assay

The histone acetylation of EGFL7 promoter was evaluated using the ChIP Assay Kit (Upstate Biotechnology, USA) according to the manufacturer's instructions. In brief, BGC823 cells were fixed with formaldehyde at room temperature for 10 min to cross-link the protein-DNA complexes, and re-suspended into the ChIP lysis buffer containing 1% SDS on ice for another 10 min. The DNA fragments with the lengths of 200 to 1000 bp were obtained, and the chromatin was immunoprecipitated with the equal amount of antibodies against histone H3 and histone H4 overnight at 4 °C, with anti-IgG acting as control. Protein-DNA-antibody complexes were precipitated with protein A-agarose beads for 2 h at 4 °C. Input or DNA in the complex was subjected to real-time PCR.

2.9. Establishment of tumor xenotransplant nude mice

The animal related experiments were approved and conducted in accordance with the Animal Care and Use Committee of the Affiliated Hospital of Logistics University of Chinese People's Armed Police Forces. Tumor xenotransplant nude mouse model was established with subcutaneous injection of BGC823 cells. Before injection, the BGC823 cell line was pre-treated with stable transfection of lentiviral vector for si-MALAT1 to down-regulate the expression of MALAT1, and the cells with stable transfection of lentiviral vector for si-control acted as the control. A total of 16 BALB/c nude mice aged eight-week old were randomly divided into two groups. Half of mice received the injection of BGC823 cells with si-MALAT1 transfection, and the other half of mice received the injection of BGC823 cells with si-control transfection. Seven