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Methylation-induced silencing of SPG20 facilitates gastric cancer cell proliferation by activating the EGFR/MAPK pathway

Zhangjian Zhou [a, 1], Wei Wang [b, 1], Xin Xie [a], Yongchun Song [a], Chengxue Dang [a], Hao Zhang [a, *]

[a] Department of Surgical Oncology, The First Affiliated Hospital of Xi'an Jiaotong University, 227W, Yanta Road, Xi'an, Shaanxi, China
[b] Department of Obstetrics and Gynecology, The First Affiliated Hospital of Xi'an Jiaotong University, 227W, Yanta Road, Xi'an, Shaanxi, China

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ABSTRACT

Spastic paraplegia 20 methylation was characterized in gastric cancer in our previous study. However, its mechanism remains unknown. Cell proliferation, colony formation, flow cytometry, wound healing, *in vitro* Transwell assays and *in vivo* xenografts were performed. A nomogram model was established to make a more accurate prognostic prediction for gastric cancer patients. Knockout of Spastic paraplegia 20 promoted gastric cancer cell proliferation, G2/M arrest *in vitro* and tumor growth *in vivo*. The EGFR/MAPK pathway was activated as a consequence of Spastic paraplegia 20 deletion. EGFR kinase or ERK1/2 inhibitors impaired Spastic paraplegia 20 knockout-induced cancer cell growth. Gastric cancer patients with poor spartin expression (72/161, 44.7%) exhibited a worse prognosis compared with the high expression group with median survival times of 16 and 54 months, respectively. The nomogram model stratified gastric cancer patients into 3 distinct prognostic groups with 3-year survival rates of 100%, 77%, and 35%. Furthermore, it had a better discrimination than the TNM staging system (C index: 0.785, AIC: 752.8708 VS. C index: 0.712; AIC: 775.1223). Methylation-induced Spastic paraplegia 20 silencing facilitates gastric cancer cell proliferation by activating the EGFR/MAPK signaling pathway. The nomogram based on spartin expression provided significantly better discrimination compared with the traditional AJCC TNM staging system and provided an individualized prediction of the survival for gastric cancer patient survival.

1. Introduction

Always considered as a "genetic" disease, cancer has recently been recognized to involve frequent "epigenetic" abnormalities [1]. Aberrant DNA methylation, the most widely studied epigenetic alteration in human cancer diseases, typically results in stable transcriptional silencing and reduced gene expression [2]. Over the past decade, we have learned that during the initiation and tumorigenesis of human cancers, a large number of critical tumor suppressor genes undergo methylation-induced transcriptional silence, which leads to altered cellular signaling that manifests in carcinogenesis [1,3].

Spastic paraplegia-20 (SPG20) encodes spartin, a multifunctional protein that is involved in intracellular epidermal growth factor receptor trafficking [4]. Additionally, down-regulation of spartin results in cytokinesis arrest, which may cause carcinogenesis [5]. We evaluated the methylation status of the SPG20 gene in gastric cancer in our previous study [6]. We demonstrated that hypermethylation of SPG20 can be used as a biomarker for gastric cancer screening and that spartin expression can be used as a prognostic factor for gastric cancer patients. However, the mechanism of SPG20 methylation in gastric carcinogenesis remains unclear. In the present study, we explored the role of SPG20 in gastric cancer through a series of experiments to assess the effect of methylation-induced SPG20 silencing on tumorigenesis. In addition, we validated these results in an enlarged cohort and constructed a new prognostic nomogram model to predict the survival of gastric cancer.

2. Materials and methods

2.1. Cell lines and clinical specimens

Human gastric cancer cell lines were cultured as previous

* Corresponding author. 277 W. Yanta Road, Xi'an, 710061, Shaanxi, China.
E-mail address: hao.zhang@mail.xjtu.edu.cn (H. Zhang).
[1] These authors contributed equally to this work.

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described [6]. Clinical tissues and matched preoperative peripheral blood samples from 67 gastric cancer patients were obtained from the First Affiliated Hospital of Xi'an Jiaotong University. Additionally, peripheral blood samples from 35 healthy individuals were obtained as control. For the survival analysis and nomogram modeling, we adopted formalin-fixed and paraffin-embedded tissues from 161 gastric cancer patients who underwent radical gastrectomy at the First Affiliated Hospital of Xi'an Jiaotong University between 2005 and 2013. Inclusion criteria for eligible patients were also described in the previous study [6]. This study was approved by the ethical committee of the First Affiliated Hospital of Xi'an Jiaotong University. Written informed consent was obtained from all patients for participation and inclusion of their data in this study.

2.2. Bisulfite modification and methylation-specific PCR and Western blotting analysis

Details were described in the previous study [6].

2.3. RNA extraction and quantitative real-time PCR

Total cellular RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). For RT-PCR analysis, the reverse transcription procedure was completed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manual. The following primers for SPG20 were utilized: forward, 5′-TGGGTGGGAATCTGCTAGACA-3'; reverse, 5′—CCCTGCACTTGGAGTTGAG G-3'. The amplification product size was 247. The two-step SYBR Green I (Takara, Tokyo, Japan) real-time PCR conditions were as follows: step 1: 95 °C for 30 s; step 2: 95 °C for 5 s and 60 °C for 30 s for 40 cycles. The relative mRNA level was expressed as the fold change relative to that of the GAPDH gene and normalized to the control group.

2.4. Knockout of SPG20 using the lenti-CRISPR/Cas9 system

The SPG20 knockout gastric cancer cells were established by CRISPR/Cas9 (clustered regularly interspaced short palindrome repeats)-guided genome editing. The procedure was performed according to previous papers from the Feng Zhang Laboratory [7–9]. We designed the sgRNAs for target coding sequence using a CRISPR design tool (http://crispr.mit.edu/) [9]. Target coding sequence was ATGGAGCAAGAGCCACAAAATGGA-GAACCTGCTGAAATTAAGATCATCAGAGAAGCATATAA-GAAGGCCTTTTTATTTGTTAACAAAGGTCTGAATACA-GATGAATTAGGTCAGAAGGAAGAAGCAAAGAACTACTA-TAAGCAAGGAATAGGACACCTGCTCA-GAGGGATCAGCATTTCATCAAAAGAGTCTGAACACACAGGTCCTGGG. The sgRNAs for three target sites were as follows: 5′-TACA-GATGAATTAGGTCAGA-3'; 5′-GAATAGGACACCTGCTCAGA-3'; 5′-TCAAAAGAGTCTGAACACAC-3'. To express the sgRNA in the lentivirus vector, two complementary oligonucleotides were created (http://genome-engineering.org/gecko/) [7]. For the production of each lentivirus, 6 μg of these transfer plasmids were co-transfected with 3 μg and 6 μg of packaging plasmids pMD2.G (addgene #12259, Cambridge, MA, USA) and psPAX2 (addgene #12260, Cambridge, MA, USA), respectively, in 80% confluent HEK293T cells cultured in 60-mm plates using 50 μl Plus Reagent™ (Thermo Fisher Scientific, Waltham, MA, USA) and 30 μl Lipofectamine 2000™ (Thermo Fisher Scientific, Waltham, MA, USA). Empty transfer plasmid "lentiCRISPRv2" (addgene # 52961, Cambridge, MA, USA) was used as a control to produce the corresponding virus. SGC7901 and MKN28 cells grown in 60-mm dishes were transduced directly with 200 μl of lentivirus containing supernatant. After 24 h, 2 μg/ml puromycin was added to select successfully infected cells. To assess the cleavage efficiency of sgRNA-guided Cas9 for target 2, PCR amplification of the specific region including the Cas9 excision site was performed (Forward primer: TCCTCTTAAAATATATTCAGGTAGG; reverse primer: GACCT-TAGGGCTGGATGCAGATGAA). Detection of genomic insertions or deletions was performed using T7 Endonuclease I (New England Biolab, Beverly, MA, USA) according to the manual. Amplification products were further confirmed by sequencing. Alteration of SPG20 expression was evaluated by Western blotting.

2.5. In vivo xenograft

BALB/C nude mice were obtained from Animal Experimental Center of Xi'an Jiaotong University. All animal experiments were approved by the Animal Ethics Committee of Xi'an Jiaotong University. MKN28-SPG20-KO cells and corresponding LenticrisprV2 control (1×10^7 cells suspended in 100 μl of PBS) were subcutaneously injected into the right flank of mice from two groups (five mice per group). The tumor size was monitored by caliper every 5 days for 30 days. The neoplasm volume was calculated using the following formula: V (mm^3) = ½ length (mm) × width $(mm)^2$ [7].

2.6. Statistical analysis

Continuous data were presented as the mean ± standard deviation and compared using the student's *t*-test. Categorical variables were compared using the χ^2 test or Fisher's exact test. Univariate analysis was performed to explore the associations of clinicopathological factors with overall survival. All parameters that were statistically significant in the univariate analysis were included in the nomogram model. Overall survival was estimated using the Kaplan-Meier method and log-rank test. All statistical tests were two-sided, and *P*-values <0.05 were considered to be statistically significant. Statistical analyses were performed using SPSS 13.0 and R software version 3.1.0 (http://www.r-project.org) with the "rms" package. Figures were created using GraphPad Prism 5 and R software.

3. Results

3.1. 5-Aza-2′-deoxycytidine treatment of gastric cancer cell lines

Previous results indicated that SPG20 was hypermethylated in BGC823 and NCL-N87 cells but not in MKN28 or SGC7901 cells [6]. Here, we examined the biological effect of 5-aza-dC on BGC823 and NCL-N87 cells. In the context of pharmacological treatment, BGC823 and NCL-N87 cells were cultured with5 μM 5-aza-dC, whereas medium without 5-aza-dC was used in the control groups. After incubated with 5-aza-Dc for 3 days, the growth rates of BGC823 and NCL-N87 cells were both significantly reduced ($P < 0.05$) (Supplement 1). In the scratch assay, 5-aza-dC inhibited BGC823 and NCL-N87 cell migration compared with the controls ($P < 0.05$) (Supplement 1). Invasion ability was measured by Transwell invasion assay. Cell invasion was significantly inhibited by 5-aza-dC treatment both in BGC823 and NCL-N87 cell lines ($P < 0.05$, Supplement 1). In flow cytometry experiments, compared with control cells, the percentage of BGC823 and NCL-N87 cells undergoing apoptotic cell death significantly increased ($P < 0.05$, Supplement 1). As shown in Supplement 1, a significant reduction was indicated in colony formation in 5-aza-dC treated gastric cells compared with control cells ($P < 0.05$).

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