IncRNA-GPAND Promotes Gastric Cancer Progression via RUNX2 and MMP13

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Research

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Abstract

Background: Gastric cancer (GC) is still one major reason for cancer-related death worldwide and in China, which ranks the second highest common cancer death rate. It is of great importance to study the molecular mechanisms by which gastric cancer develops.

Methods: In this study, in situ hybridization histochemistry (ISHH) was used to examine the lncRNA-GPAND expression levels using gastric cancer tissue array. The real-time live-cell imaging system was used to investigate the effect of GPAND on cell proliferation and apoptosis of GC cell lines. Cell cycle of AGS cell line was examined after GPAND was suppressed using the flow cytometry (FCM). Transwell method was used to study the effect of GPAND on the invasion characteristics of GC cell line. Then the next generation sequencing (NGS) was used to study the potential molecular mechanism and the pathway, and the RT-qPCR was performed to verify the potential targets found by NGS method.

Results: It was shown that GPAND was significantly over-expressed in the gastric cancer (GC) tissues (n=215) compared with the paired non-cancerous tissues (n=215), the expression levels of GPAND of GC tissues of TNM stage I-II (n=45) were significantly higher than that of stage III-IV (n=147). It has shown that knockdown of GPAND inhibited the AGS and N87 cell proliferation and promoted the cell apoptosis of AGS and N87 cell lines significantly, and the G1 phase percentage was remarkably increased in GPAND knockdown group of AGS cell line compared with control group. Moreover, suppression of GPAND inhibited the AGS cell invasion significantly. It was found via the NGS method that RUNX2 and MMP13 were significantly up-regulated when the GPAND was over-expressed. **Conclusions:** These observations suggest the lncRNA-GPAND/RUNX2/MMP13 axis to be a viable therapeutic target for gastric cancer.

Introduction

As it has been reported, there were about 27,600 newly diagnosed cases and 11,010 cancer-related deaths for gastric cancer in the United States in 2020 [1]. Although the incidence and mortality of gastric cancer has steadily declined since 2000, it remains one of main important cause of cancer mortality in China, e.g. 15.1% for Chinese males, and 11.1% among females[2]. The current therapy, especially the target therapy for gastric cancer is rather unsatisfactory. Hence, to investigate the molecular mechanism promoting the tumorigenesis, progression, invasion and metastasis of gastric cancer is of great significance.

Long non-coding RNAs (lncRNAs) has been the research highlights for these years[3, 4]. Previous studies have showed that lncRNAs are able to play important roles in gastric cancer[5] development via transcriptional and translational regulation of the oncogenes and cancer suppressor genes, which usually mediated by other non-coding RNAs such as microRNAs and circRNAs[6, 7].

The runt-related transcription factor 2 (RUNX2) is an important transcription factor for embryogenesis[8], plays a major role at the late stage of chondrocyte differentiation[9], and impacts skeletal development via regulating the differentiation of chondrocytes and osteoblasts and the expression of many

extracellular matrix protein genes including those of Col1a1, Col1a2, Spp1, Bglap, and Mmp13 during chondrocyte and osteoblast differentiation[10]. RUNX2 is abnormally up-regulated in prostate cancer[11, 12] and breast cancer cell lines[13, 14] and tissues[15]. High RUNX2 expression correlates with the triple-negative breast cancer subtype and with lower patient survival rates compared with samples displaying low RUNX2 expression[16]. Furthermore, it was also correlated with the drug-sensitivity of pancreatic cancer[17]. The high expression of Runx2 indicates a trend toward a poor survival rate of osteosarcoma[18] partly due to its oncogenesis role of osteosarcoma[19].

The matrix metalloproteinases (MMPs) are a family of structurally related enzymes playing a role in multiple physiological processes, i.e., embryonic, adult tissue development and remodeling, evaluation, uterine implantation, wound healing, etc, and MMPs may have the function in some pathological conditions, such as arthritis, atherosclerosis and cancer invasion and metastasis. Human collagenase-3 (MMP-13) is a matrix metalloproteinase involved in a variety of malignant tumors including gastric cancer[20, 21], cervical cancer[22, 23], skin melanoma, breast cancer[24], colon cancer[25], head and neck carcinomas[26], chondrosarcomas[27] and basal cell carcinomas of the skin[28]. The increased expression of MMP13 usually predicts metastasis and poorer outcome of malignant tumors.

GPAND, i.e., the abbreviation for “gastric progression associated non-coding RNA”, refers to the long non-coding RNA, AK125207. Here, we found that GPAND was significantly over-expressed in the gastric cancer tissues compared with the paired non-cancerous tissues. The depletion of GPAND inhibited the cell proliferation and invasion, and increased the cell apoptosis and G1 phase percentage of gastric cancer cells significantly. RUNX2 and MMP13 were significantly up-regulated in GPAND overexpression groups compared with control group via NGS technique and western blotting experiment. Our findings suggest that GPAND/RUNX2/MMP13 might be the potential therapeutic targets of the gastric cancer.

Materials And Methods

Ethics.

The procedures performed in this study were approved by the Ethics Committee of the Shanghai Outdo Biotech Company, the member of National Human Genetic Resources Sharing Service Platform (Shanghai, China). The present study was performed in accordance with the ethical standards of The Institutional and National Research Committee and with The Declaration of Helsinki.

Tissue arrays and interpretation criteria for In Situ Hybridization (ISH) results

HStmA180Su15, HStmA180Su17 and HStmAde180Sur-03 tissue arrays (Outdo Biotech Co. Ltd., Shanghai, China) are used in this study to examine the GPAND expression levels. The hematoxylin-eosin staining and GPAND in situ hybridization of these tissue arrays can be seen in the S. file-1. The ISH results were interpreted by senior pathologists. The rating criteria for staining intensity was as follows: no staining was negative (- or 0), weak positive was light yellow (+ or 1), moderate positive was brown (++ or 2), and strong positive was brown (+++ or 3). (Note: If there are both moderate and strong positive

tissues, we usually record 2-3). If located in the nucleus, the positive rate of staining was calculated by recording 100 cells randomly in each field of vision, and then the percentage of positive cells in 100 cells was recorded as $\times 1\%$. According to the same principle, the percentage of positive cells in the other two visual fields was $\times 2\%$ and $\times 3\%$, and the average of positive staining rates in the tissues was $\times 1\%$, $\times 2\%$ and $\times 3\%$. If located in the cytoplasm or membrane, we also chose three different visual fields of staining intensity to calculate the positive rate and then take the average value. The standardization scheme of original experimental data is as follows: 1) Staining intensity score: 0 (negative), 1 (1+), 2 (2+), 3 (3+); 2) Positive staining percentage score: 0 (negative), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%); 3) Total score and grouping: grouping with the product of "staining intensity score" and "staining positive rate score" as the total score.

Cell lines and cell culture

The cell lines used in this study were human gastric cancer cell lines BGC823, MGC803, MKN28, SV-HUC-1, NCI-N87, AGS, MNK45, OACP4C, SGC7901, SK-GT2 and normal gastric mucosal cell line, GES-1, were used. BGC823, MGC803, MKN28, SV-HUC-1, MNK45, SGC7901 were kind gifts from Professor Xiaodi Zhao (Xijing Digestive Disease Hospital, Air Force Medical University). OACP4C was purchased from the Baili Biotech (Shanghai) and SK-GT2 was purchased from the Huiying Biotech (Shanghai). AGS and NCI-N87 were purchased from ATCC and all cells were maintained in RPMI-1640 medium (Hyclone Cat.No.SH30809.01B, USA) supplemented with 10% fetal bovine serum (Hyclone Cat.No.SH30087.01, USA) in the humidified sterile incubator at 37°C, 5%CO₂.

Constructs and transfection

The PCR product of GPAND was amplified using the GeneAmp PCR System 2400 thermal cycler firstly 94 °C for 1 min, then 98 °C for 15sec, 58 °C for 15 sec, 68 °C for 4 min for 30 cycles, and 68 °C for 5 min finally. 15 μ L GPAND PCR product and pcDNA3.1+ vector were used for digestion via EcoRI/XhoI which was followed by digestion product recycling, linkage of the target segment to the vector, conversion of ligation products, and the identification positive clones. Passage the cell one day before transfection to make it 70%-80% confluence, the Lipofectamine 2000 (invitrogen, Cat. No. 11668019) transfection reagent and the Opti-MEM (invitrogen, Cat. No. 31985070) medium were used for transfection according to the manufacturer's instruction.

Cell transfection

The human gastric cancer cells in the logarithmic growth phase were used to prepare the single cell suspension by trypsin digestion and were counted. The cell concentration was adjusted to 8×10^4 cell/ml, 100ul cell suspension were inoculated per well in a 96-well plate, and were cultured with 10% fetal cattle serum (Hyclone Cat.No.SH30087.01 USA) medium at 37°C for 24 h. 96-well plate was used for transfection and the amount of medium is 50ul. Pipette 0.125ul siRNA liquor into 25ul Opti-MEM medium (Gibco Cat.No.31985-070 USA) as liquid A, 0.25ul Lipofectamine 2000 (Invitrogen Cat.No. 11668019 USA) was dissolved in 25ul Opti-MEM medium as liquid B and then it was mixed for 5 minutes. Finally,

the liquid A and liquid B were mixed, and added to the cell culture plate after standing for 20 minutes. After 4 hours of incubation, the transfection medium was replaced by the growth medium containing APO-488 probe, 100ul per well. The primers siRNA and miRNA sequences used were listed in the table S1 and table S2 respectively.

Cell proliferation assays

To examine cell viability, Cell Counting kit-8 (CCK-8; Nanjing KeyGen Biotech Co., Ltd.China) was used according to the manufacturer's protocols. AGS and N87 cells were seeded onto 96-well plates (1×10^4 cells/well), and then the cell viability was measured every 24 h for 5 days. The number of the viable cells was counted by evaluation of absorbance values at 450 nm by the Multiscan MK3 microplate reader (Thermo Fisher Scientific, Inc.USA). For monitoring of cell confluence, the AGS and N87 cells transfected were seeded at 8,000 cells per well in 96-well plates in the media, respectively. Five wells were used under each condition. Cells were cultured at 37°C and 5% CO₂ and monitored using the Real-Time Live-Cell Imaging System (Incucyte Zoom, Essen BioScience, USA). Cells were cultured with medium supplemented with siRNAs at the indicated doses for 4 hours and then the culture medium was replaced with the culture medium. Cell confluence was monitored and measured using the IncuCyte Zoom system (Essen Bioscience, Ann Arbor, MI, USA). The dynamic pictures were taken once per 2h to capture 3-4 fields of view/well/time for recording and was analyzed by IncuCyte ZOOM automatic software (Version: IncuCyte ZOOM 2016B, USA) and database.

Cell apoptosis analysis

The cell apoptosis was analyzed using the Real-Time Live-Cell Imaging System (Incucyte Zoom, Essen BioScience, USA) as mentioned above. In this section, CellEvent® Caspase-3/7 Green ReadyProbes® Reagent (ThermoFisher/MP, Cat.No.R37111, USA), e.g., Apo-488, a fluorogenic, no-wash indicator of activated caspase-3/7 for live- and fixed-cell applications, is used. Activation of caspase-3 is an early indicator of apoptosis and CellEvent® Caspase-3/7 Green reagent allows rapid and sensitive detection of cells destined for cell death. CellEvent® Caspase-3/7 Green reagent is a four amino acid peptide (DEVD) conjugated to a nucleic acid-binding dye that is non-fluorescent when not bound to DNA. The CellEvent® Caspase-3/7 Green reagent is intrinsically non-fluorescent, as the DEVD peptide inhibits binding of the dye to DNA. Upon activation of caspase-3/7 in apoptotic cells, the DEVD peptide is cleaved and the free dye can bind DNA, generating a bright green fluorescence. The fluorescence emission of the dye when bound to DNA is 530 nm and can be observed using a standard FITC filter set.

Cell cycle analysis

Here, the Cell cycle detection kit (KeyGEN BioTECH Cat.No.KGA511 China) was used. 1×10^6 cells were collected, centrifugated, discarded the supernatant, washed with pre-cooled PBS, added with pre-cooled 70% ethanol, and fixed overnight at 4°C, for each sample 48 hours after cell transfection. Then the cells were collected by centrifugation, washed once with 1mL PBS, added 500uLPBS containing 50ug/mL propidium bromide (PI), 100ug/mL RNase A, 0.2% Triton X-100, and were incubated at 4°C in the dark for

30 minutes. Finally, the cell cycle was detected with a flow cytometer (BD calibur, USA) according to standard procedures and the results were analyzed with the cell cycle fitting ModFitLT™ software (version 3.0; Verity Software House, Inc.).

Cell invasion analysis

The Matrigel (Cat.No.356234, BD Co.Ltd, USA) was dissolved overnight at 4°C, diluted with a pre-cooled serum-free medium at a volume ratio of 1:3, and then 40ul matrigel was added into the pre-cooled transwell chamber (Cat#REF353097, Co.LtdUSA), and was incubated at 37°C for 2 hours to be solidified. The excess liquid in the chamber was aspirated, 100ul and 600ul serum-free medium was added to the upper and lower chambers respectively, and was equilibrated overnight at 37°C. On the second day of cell transfection with siRNA, 1×10⁵ cells were counted, resuspended with 100ul serum-free DMEM (Hyclone Cat.No.SH30019.01, USA) medium, added to the upper chamber of Transwell chamber, and add 600ul complete medium to the lower chamber. After incubating at 37°C and 5% CO₂ for 24 or 48 hours, the cell was taken out, the cells in the upper chamber were wiped with a cotton swab, and then was fixed with 4% paraformaldehyde for 15 minutes, washed once with PBS, stained with crystal violet for 10 minutes, and was washed once with PBS. In the end, we checked whether the cells pass through the small holes and took photos for statistics using the Micro plate spectrophotometer (Cat.No.Multiscan MK3. Thermo Fisher Scientific, USA)

RNA-sequencing and data analysis

Total RNA was isolated from AGS cells transfected GPAND siRNAs for RNA-sequencing analysis. RNA extraction was performed using the RNAiso Plus Total RNA extraction reagent (TAKARA, Cat.No.9109, China) following the manufacturer's instructions and checked for a RIN number to inspect RNA integrity by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, USA). Qualified total RNA was further purified by RNA Clean XP Kit (Beckman Coulter, Inc. Kraemer Boulevard Brea, CA, Cat. No. A63987, USA) and RNase-Free DNase Set (QIAGEN, Cat.No.79254, GmbH, Germany). Part of RNA was sequenced by The Shanghai Biotechnology Corporation with Illumina HiSeq X10 for transcription expression analysis, and the other part of it was used to perform qRT-PCR examination to verify the results. The VAHTS mRNA-seq v2 Library Prep Kit for Illumina (Vazyme, Cat.No.NR601-02, China) and VAHTS Universal V6 RNA-seq Library Prep Kit for Illumina® (Vazyme, Cat.No.NR604-02, China) were used for library construction. The Qubit® 2.0 Fluorometer was used to detect the concentration of the library, and the Agilent 4200 was used to detect the size of the library. The quality control standard of sequencing data is as follows: the amount of data is 6G/sample, and the ratio of the quality of each base greater than 20 (Q20) is not less than 85%. RNA-seq reads quality was evaluated using FastQC and mapped to the human genome reference assembly (hg38) using Bowtie 2. Significant differential expressed genes (DEGs) were identified as those with a False Discovery Rate (FDR) value above the threshold ($q\text{-value} \leq 0.05$), fold-change ≥ 2 and FPKM > 5 using edgeR software. For functional analysis, the selected DEGs were mapped to each term of the GO and KEGG databases, the number of genes of each item was calculated, and then a hypergeometric test was applied to screen the GO or KEGG pathway terms with

significant enrichment. In addition, the data were analyzed using Ingenuity Pathway Analysis® (IPA, www.ingenuity.com) to further identify biological processes enriched for the DEGs. For pathway analysis, MapMan package was used, and Volcano plots Ggrepel packages was used to make volcano plot.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted from the gastric and normal gastric cell lines using the TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and examined on a NanoDrop machine according to the manufacturer's protocols (Eppendorf). Electrophoresis and spectrophotometric methods were used to examine the concentrations and purity of the RNA samples. cDNA was prepared using ImProm-II™ Reverse Transcription System according to manufacturer's protocols (cat. no. A3800; Promega Corporation). The temperature protocol of reverse transcription reactions was as follows: i) mRNA, 85°C for 5 min, 30°C for 10 min, 42°C for 60 min and 85 °C for 10 min; and ii) 85°C for 5 min, 42°C for 60 min and 85°C 10 min for miRNA. The Taq plus Master mix (Vazyme P212-01 China) was used for qPCR and each reaction was performed in triplicate. The thermocycling conditions used for RT-qPCR were as follows: Initial denaturation at 95°C for 5 min, followed by 40 cycles of 95 °C for 15 sec and 60°C for 34 sec. The $2^{-\Delta\Delta Cq}$ method[29] was utilized to calculate the relative expression levels of mRNA and miRNA (primers used can be seen in Table S3), using GAPDH and U6 as reference genes for mRNA and miRNA, respectively (Primers used were listed in Table S7-S8).

miRNA target prediction

In order to predict potential miRNA target genes, microRNA.org website (<http://www.microrna.org/microrna/home.do>) was used, the binding free energy was calculated and binding sites were analyzed using <http://bibiserv.techfak.uni-bielefeld.de/rnahybrid> website.

Statistics

Data were expressed as Mean±Sd or Mean±SEM (n=3) (According to the statistics method used, see in the legends). SPSS statistical software package, version 17.0 (SPSS, Chicago, IL, USA) was used. The Wilcoxon signed-rank test was used to compare the immunohistochemistry scores of cancer and matched adjacent non-cancerous tissues. The one-way ANOVA followed by Tukey's post hoc test were performed for cell proliferation and apoptosis experiments. P values < 0.05 were considered statistically significant.

Results

Up-regulation of GPAND in GC tissues and cell lines

In Situ Hybridization (ISH) method was used to evaluate the expression levels of GPAND of gastric cancer (GC) tissues using GC tissue arrays (Fig.1, the hematoxylin-eosin staining and GAPDH in situ hybridization of tissue arrays can be seen in the S. Fig-1). It was shown that GPAND was expressed in the nucleus, membrane and cytoplasm of gastric cancer cell and was significantly up-regulated in the gastric

cancer tissues (n=215) compared with the paired non-cancerous tissues (n=215) (Fig.2A, $p < 0.0001$). Further analysis revealed that the expression levels of GPAND of GC tissues of TNM stage I-II (n=45) were significantly higher than that of stage III-IV (n=147) (Fig.2B, $p < 0.001$). Tumor volume subgroup analysis showed that GPAND was remarkably up-regulated in the tissues of tumor volume $\geq 100\text{cm}^3$ (n=46) compared with that of $\leq 15\text{cm}^3$ (n=73) (Fig.2C, $p < 0.05$). However, it was not showed that GPAND expression levels of patients aged $\leq 55\text{y}$ (n=44) was significantly different than that aged $\geq 75\text{y}$ (n=50) (Fig.2D). Interestingly, when compared with the survival of patients with GPAND staining score of 10-12 (n=23) (Fig.2E), the survival of those scored 0 (n=15) was not remarkably different with them. Furthermore, there was not significant difference of the GPAND staining score between male (n=161) and female (n=75) (Fig.2F). We then examined the expression levels of GPAND using several GC cell lines and found that it was significantly up-regulated in the MGC803, NCI-N87, AGS, OACP4C, and SK-GT2 cell lines compared with normal gastric mucosa cell line, GES-1 (Fig.2G). All the staining score of Fig.2 can be seen in the Table S1-S6, respectively.

Depletion of GPAND inhibited the cell proliferation and promoted apoptosis of GC cell lines

In this section, to assess the role played by GPAND on the proliferation of GC cell lines, two methods i.e., the real-time live-cell imaging system analysis and the cell counting kit-8 were used. Here, AGS and SK-GT2 cell line were analyzed after GPAND was knocked down. To evaluate the proliferation difference, cells transfected with negative control (NC) siRNA and the cells cultured using culture medium supplemented with the same amount of transfection media, i.e., the blank control, were used as the control groups. We found that depletion of GPAND inhibited significantly the cell proliferation of AGS (One-way ANOVA, Fig.3A, $p < 0.0001$) and SK-GT2 (One-way ANOVA, Fig.4A, $p < 0.0001$) compared with NC groups using the real-time live-cell imaging method. Meanwhile knockdown of GPAND promoted apoptosis of AGS (One-way ANOVA, Fig.3B, $p < 0.05$) and SK-GT2 (One-way ANOVA, Fig.4B, $p < 0.01$) cell line compared with NC groups. Moreover, CCK-8 experiment was also performed using SK-GT2 cell line and it showed that down-regulation of GPAND led to the inhibition of growth of SK-GT2 cell line from the third day after transfection of siRNAs (One-way ANOVA, Fig.4A, $p < 0.01$).

GPAND promoted cell cycle of AGS cell line

According to the finding that depletion of GPAND could inhibit the growth and promote the apoptosis of GC cell lines, we speculated that maybe GPAND could affect the cell cycle of these cell lines. Hence, we performed the flow cytometry experiment to study this speculation. We found that after GPAND was knockdown using two siRNAs, i.e., si-1 and si-2, which showed effective depletion of GPAND in the AGS cell line (Fig.2). Fig-2, G1 phase percentage was remarkably increased in AGS cell line (si-1 vs NC: 26.62 ± 0.380 vs 23.05 ± 0.191 , $p < 0.001$; si-2 vs NC: 25.76 ± 0.383 vs 23.05 ± 0.191 , $p < 0.001$) compared with the control group at 48h (Fig.5C-D). Furthermore, G1 phase percentage was also significantly increased in GPAND knockdown group (si-1: 31.24 ± 0.599 vs 16.99 ± 0.554 , $p < 0.001$; si-2: 32.36 ± 0.320 vs 16.99 ± 0.554 , $p < 0.001$) compared with control group at 24h (Fig.5A-B).

Suppression of GPAND inhibited the cell invasion of AGS cell line

In addition to study the roles played by the GPAND on the proliferation, apoptosis and cell cycle of GC cell lines. It is of interesting that if the GPAND could affect the invasion of GC cell line. We then investigated the effect of suppression of GPAND using both siRNA-1 and siRNA-2 on the invasion characteristic of AGS cell line at 48h and 72h. It was found that the suppression of GPAND inhibited the AGS cell invasion significantly compared with the negative control group at 48h (si-1 vs NC: 130.250 ± 8.498 vs 195.375 ± 20.311 , $p < 0.001$; si-2 vs NC: 105.125 ± 6.334 vs 195.375 ± 20.311 , $p < 0.001$) (Fig.6A-B) and 72h (si-1 vs NC: 111.375 ± 8.911 vs 199.750 ± 21.816 , $p < 0.001$; si-2 vs NC: 90.125 ± 11.154 vs 199.750 ± 21.816 , $p < 0.001$) (Fig.6C-D).

Downstream molecular change induced by depletion of GPAND

Given that the roles played by GPAND in the proliferation, apoptosis, cell cycle and invasion of AGS cell line, in order to explore the potential molecular mechanism by which GPAND, the next generation sequencing method was used to analyze the relative gene profile change after the cDNA of GPAND was transfected compared with the negative control. Here, we found that MMP13 and RUNX2 gene were up-regulated in the GPAND up-regulated group compared with the control group (Table 1 & 2, Fig.7A-B). The representative graph of the potential mechanism by which GPAND-RUNX2-MMP13 axis promoted the invasion and metastasis of gastric cancer is shown in the figure 8.

Discussion

AK125207 was screened by us using the commercial microarray (Arraystar, USA) 7 years ago according to the coding-potential predicting tool "<http://lilab.research.bcm.edu/cpat/>", it was predicted as non-coding RNA. Furthermore, the source by which AK125207 was acquired is <http://nred.matticklab.com/cgi-bin/ncrnadb.pl> and the annotation of the AK125207 was non-coding RNA. In this study, to explore the expression levels of AK125207 (named as GPAND) in the gastric cancer tissues, we used the commercial tissue arrays via in-situ hybridization histochemistry (ISHH) method other than using the gastric cancer tissues via the real-time quantitative PCR examination was firstly because of the difficulty for us to acquire the enough gastric cancer samples with the survival follow-up outcome at the beginning of this study. In order to investigate the clinical meaning of GPAND, it is of great importance to use the samples with the survival data. Hence, we bought the commercial tissue arrays to meet our study needs. Secondly, ISHH technique makes it possible for us to further know the localization of GPAND on the cancer cells, however, RT-qPCR method only provide the quantitative analysis results. Thirdly, in our study positive staining score was evaluated by assessing the relative intensity and scope of positive staining (see in the method section) in the cancer cells area which combined both the staining intensity and the scope factors, providing more meaning and data than RT-qPCR method in our view. Of course, the shortcoming of tissue arrays is obvious for the random and uncertain sampling, hence, to investigate enough amount samples is very important and we here analyzed 215 samples and we were lucky to some extent that the expression level score of GPAND of gastric cancer tissues were found to be significantly up-regulated

compared with the normal gastric tissues. However, we did not conclude that the survival was correlated with the expression levels of GPAND. Hence, it is of interesting that if more samples were used for analysis, could the remarkable survival difference be observed? We expect to answer this question in the following study.

After observing the difference of expression levels of GPAND in the gastric cancer tissues and normal tissues, we then investigate the roles played by the GPAND of the proliferation, apoptosis, cell cycle and invasion characteristics in the gastric cancer cells. To our surprise, the knock down of GPAND led to the inhibition of proliferation, promotion of apoptosis, increase of G1 phase, and stronger invasive capability. Especially, to make it sure that the depletion of GPAND may affect the proliferation of gastric cancer cells, two methods, i.e., CCK-8 and real-time live-cell imaging system experiments were performed for at least 3 times using two siRNAs targeted the GPAND, for this step is very critical for the further investigation of the roles of the GPAND. Although only SK-GT2 cell line was used to perform CCK-8 experiment, it also could support the view of the function of GPAND on the proliferation of gastric cancer cells to some extent in order to verify the results obtained by the live-cell imaging system.

Due to the observation that the GPAND maybe exert its oncogenic role in the development of gastric cancer, it is of great interest to explore its molecule mechanism by which the GPAND may function as the oncogene. Hence, the next generation sequencing was performed to study the potential mechanism. In this section, both siRNA-1 and siRNA-2 were used to knock down the GPAND, and it is of great meaning that MMP13 and RUNX2 were overexpressed compared with the negative control group. As far as we know, MMP13 functions as the oncogene of gastric cancer, colon cancer, breast cancer and etc. RUNX2 promotes the development of multiple tumors such as prostate cancer and breast cancer and is a negative prognostic indicator of the survival of osteosarcoma.

RUNX2, a mesenchymal stem marker[30], was reported by Chao Han et al that the high expression of RUNX2 and low expression of FN1 analyzed using the GEO2R tool could be the poor predictive marker of long survival in diffuse, poorly differentiated, and lymph node-positive GC[31]. Moreover, [ZJ Guo](#) et al also found that the levels of RUNX2 expression in gastric cancer tissues were correlated with the differentiation degrees, invasion depth and lymph node metastasis. COX regression analysis indicated that RUNX2 was an independent prognostic indicator for GC patients[32]. It was also found that the RUNX2/OPN axis regulated the ability of osteosarcoma cells to attach to pulmonary endothelial cells as a key step in metastasis of osteosarcoma cells to the lung[33]. These findings supported to some extent our research that maybe the long non-coding RNA GPAND exerted its oncogene role via RUNX2 and MMP13.

More interestingly, we also examined the mRNA expression levels of MMP13 and RUNX2 in the gastric cancer cell line and found that both of them were significantly up-regulated in the gastric cancer cell line compared with the gastric mucosal cell line, GES-1. Furthermore, we also examined the roles played by the MMP13 and RUNX2 in the gastric cancer cell line (data not shown), the potential mechanism by

which the GPAND affect the MMP13 and RUNX2 expression are being studied and these findings will be reported in the next article.

In conclusion, in this study, we preliminarily reported that lncRNA GPAND may function as the oncogene in the development of gastric cancer via MMP13 and RUNX2. The GPAND might be a potential therapeutic target of gastric cancer if the regulatory mechanism by which it affected the MMP13 and RUNX2 were more elucidated.

Abbreviations

GC:Gastric cancer, GPAND : Gastric cancer progression associated non-coding RNA, RT-qPCR : Quantitative reverse transcription PCR, FCM; flow cytometry, CCK8: cell counting kit-8, NGS: next generation sequencing, ISHH: in situ hybridization histochemistry, Runx2: Runt-related transcription factor 2, MMP13: matrix metalloproteinases 13

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree to publish this paper.

Competing interests

No potential conflict of interest was reported by the authors.

Authors' contributions

Y W initiated the project, designed the experiments, wrote the manuscript and interpreted the data. P X finished the data analysis. CB W, and ZB T performed the experiments. All authors read and approved the final version of the manuscript.

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Availability of data and materials

The data and materials are available.

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Tables

Table 1. Gene profile of GPAND Up-regulation group-1			
Gene name	Qvalue	Updown	log2FC
URGCP-MRPS24	0.009752	UP	6.272947
TICAM2	3.62E-13	UP	5.759538
RP11-140H17.1	9.97E-27	UP	5.50768
RP11-66B24.7	1.56E-33	UP	5.070263
ISY1-RAB43	6.28E-05	UP	4.944514
LEFTY2	7.36E-06	UP	4.431153
AP000640.10	0.039624	UP	4.113889
NTSR2	0.02531	UP	4.067092
ACTC1	5.80E-05	UP	3.911762
ADAM1A	0.009991	UP	3.502547
RUNX2	0.007422	UP	3.479325
MMP13	0.000243	UP	3.461601
RP11-495P10.5	5.77E-05	UP	3.434059
EEF1DP3	0.001474	UP	3.420137
ERVMER61-1	0.007982	UP	3.390571

Table 2. Gene profile of GPAND Up-regulation group-2			
Gene name	Qvalue	updown	log2FC
RP11-66B24.7	7.30E-29	UP	5.197578
CTD-3222D19.2	0.000541	UP	5.184666
AP003419.11	4.40E-08	UP	5.123315
LEFTY2	1.04E-07	UP	4.837296
AMIGO3	2.18E-17	UP	4.65509
RP11-94I2.4	0.040335	UP	4.288095
RP11-495P10.5	8.76E-09	UP	4.261071
ACTC1	3.58E-06	UP	4.254367
CTD-2349B8.1	0.0096	UP	3.804667
RUNX2	0.000861	UP	3.702207
DBIL5P	0.0096	UP	3.614764
MMP13	5.57E-05	UP	3.56879
ADAM1A	0.003647	UP	3.498786
TUBBP10	0.003685	UP	3.407775
ERVMER61-1	0.037378	UP	3.397506

Figures

Figure 1

A

B

Figure 1

Representative graphs of GPAND of In Situ Hybridization. A. Representative graph of in-situ hybridization (ISH) of GPAND of gastric cancer tissue array. a. Hematoxylin-eosin staining of the cancer tissue; b. ISH graph (×5) ; c. ISH graph (×20); d. ISH graph (×40). B. Representative graph of in-situ hybridization (ISH) of GPAND of normal gastric tissue array. a. Hematoxylin-eosin staining of the gastric tissue; b. ISH graph

(×5) ; c. ISH graph (×20); d. ISH graph (×40). The red arrow: nuclear staining; the white arrow: cytoplasm staining; the yellow arrow: cell membrane staining. Bar: 500 μm

Figure 2

Figure 2

Analysis of GPAND staining score and its expression levels of gastric cancer cell lines. A-F. Data were expressed as Mean $\pm$ SEM (n=3) and unpaired t-test was performed to analyze the GPAND staining score. G. Data were expressed as Mean $\pm$ SD (n=3) and unpaired t-test was performed. The relative expression

levels measured using RT-qPCR method of GPAND of gastric cancer cell lines and normal gastric mucosal cell line, GES-1.

Figure 3

Figure 3

Knockdown of GPAND inhibited the cell proliferation and promoted apoptosis of GC cell lines, AGS. One-way ANOVA followed by Tukey's post hoc test was performed in this section to compare the effect of GPAND knockdown on the proliferation and apoptosis of AGS cell line. In this section, siRNA-1 (100 nM)

and siRNA-2 (100 nM) (Table.S1) were transfected to knockdown the expression of GPAND. A. The phase object confluence of AGS cells, measured by the real-time live-cell imaging system proliferation, showed the significant proliferation difference between GPAND siRNA-1 transfected groups and the control groups. There was not remarkable difference between GPAND siRNA-1 and 100 nM GPAND siRNA-2 transfection groups. B. The green object count, measured by the real-time live-cell imaging system proliferation, showed the significant apoptosis (the green fluorescence indicated cell apoptosis) difference between GPAND siRNA-1 transfected groups and the control groups. C. Representative graphs of cell proliferation taken by the real-time live-cell imaging system (Bar: 300 μ m). D. Representative graphs of apoptosis taken by the real-time live-cell imaging system (Bar: 300 μ m). NC: siRNA negative control; Blank ctrl: Blank control; GPAND si: GPAND siRNA; ***: $P < 0.001$; *: $P < 0.05$.

Figure 4

Figure 4

Knockdown of GPAND inhibited the cell proliferation and promoted apoptosis of GC cell lines, SK-GT2. One-way ANOVA followed by Tukey's post hoc test was performed in this section to compare the effect of GPAND knockdown on the proliferation and apoptosis of SK-GT2 cell line. In this section 100 nM and 50 nM siRNA-2 (Table.S1) was used to suppress the GPAND expression. A. The phase object confluence of SK-GT2 cells, measured by the real-time live-cell imaging system proliferation, showed the significant

proliferation difference between GPAND siRNA transfected groups and the control groups. There was not remarkable difference between 50 nM and 100 nM GPAND-siRNA transfection groups. B. The green object count, measured by the real-time live-cell imaging system Proliferation, showed the significant apoptosis (the green fluorescence indicated cell apoptosis) difference between GPAND siRNA transfected groups and the control groups. There was not significant difference between 50 nM and 100 nM GPAND-siRNA transfection groups. C. Representative graphs of cell proliferation and apoptosis taken by the real-time live-cell imaging system (Bar: 300 μ m). D. The CCK-8 experiment showed that the GPAND suppression inhibited the growth of SK-GT2 cell line from the third day after siRNA transfection was performed. NC: siRNA negative control; Blank ctrl: Blank control; GPAND si: GPAND siRNA; CDDP: cisplatin ***: $P < 0.001$; **: $P < 0.01$.

Figure 5

GPAND promoted cell cycle of the AGS cell line Here, AGS cell line was used to examine the roles played by the GPAND on the cell cycle using the flow cytometer method. A & C. Representative graphs of the flow cytometer. G1 phase percentage was remarkably increased in GPAND depletion cell compared with the control group at 24h (B) and 48h (D).

Figure 6

Figure 6

Suppression of GPAND inhibited the cell invasion of the AGS cell line A & C. Representative graphs of the invasion cells of the transwell experiment. The suppression of GPAND inhibited the AGS cell invasion significantly compared with the negative control group at 48h (B) and 72h (D).

Figure 7

Figure 7

Molecular change induced by cDNA transfection of GPAND A.Heat map of the next generation sequencing of GPAND up-regulation after cDNA was transfected for 36h and negative control groups. B. Heat map of the next generation sequencing of GPAND depletion after cDNA was transfected for 48h and negative control groups.

Figure 8

Figure 8

The representative graph showing the potential mechanism by which GPAND-RUNX2-MMP13 axis promoted the invasion and metastasis of gastric cancer.

Supplementary Files

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