PITPNA-AS1 Activated by H3K27ac Sponged miR-98-5p to Regulate Cisplatin Resistance in Gastric Cancer

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Abstract

To evaluate the expression of PITPNA-AS1 and miR-98-5p in gastric cancer tissues as well as their association with progression of gastric cancer, and investigate the role of PITPNA-AS1 and miR-98-5p in developing platinum resistance. RNA sequencing was used to identify candidate IncRNAs and microRNAs related to local recurrence of gastric cancer. qRT-PCR was used to investigate the expression of PITPNA-AS1 and miR-98-5p. CCK-8 and caspase3/7 activity were used to evaluate the cell proliferation and apoptosis rate. Dual luciferase reporter gene assay and RNA pull down were used to evaluate the cross talk between PITPNA-AS1 and miR-98-5p. PITPNA-AS1 and miR-98-5p could regulate cell proliferation and inhibit apoptosis in gastric cancer cell lines. Cisplatin and lobaplatin could significantly suppress the expression of PITPNA-AS1, which interacted with negatively regulated miR-98-5p expression. PITPNA-AS1 overexpression impaired the effect of platinum, which was partially reversed by downregulation of miR-98-5p knock down. In gastric cancer, PITPNA-AS1 and miR-98-5p could regulat cell growth, apoptosis and platinum resistance. They have the potential to be biomarkers and curative therapeutic targets. However, further research on molecular mechanisms are needed.

Introduction

Gastric cancer (GC) is the fourth most commonly diagnosed cancer and the second most common cancer-related mortality globally, with approximately 738,000 people die of GC each year worldwide\(^1\)\(^,\)\(^2\). Despite the remarkable progressive surgical and medical techniques, prognosis of patients with GC remains relatively poor mainly due to recurrence and metastasis\(^3\). Neoadjuvant chemotherapy improves survival in comparison to best supportive care or surgery alone. 5-fluorouracil combinated with cisplatin has been convincingly proved that it is survival benefit for HER-2-positive patients\(^4\)\(^-\)\(^6\). As the main chemotherapy treatment for postoperative GC patients, the efficacy of platinum has been largely limited due to the chemo-resistance\(^7\). Laboratory studies illustrated that resistance to platinum is almost multifactorial, including damaged cellular uptake of platinum\(^8\), reinforced endocellular detoxication by glutathione and metallothionein systems\(^9\), modified patterns of DNA platination, enhanced tolerance to DNA damage\(^10\) and rising restore of DNA damage\(^8\)\(^,\)\(^11\).

Long non-coding RNAs (LncRNAs) are a class of non-coding RNAs longer than 200 nt without protein coding potential. Several LncRNAs were confirmed as biotargets for modulating cisplatin resistance in cancer through the cell cycle, apoptosis and Wnt pathways\(^12\), which acting as a competing endogenous RNA or directly binding to mRNAs or proteins and regulating their expression and functions\(^13\). LncRNA PITPNA antisense RNA 1 (PITPNA-AS1) is located in chromosome 17p13.3, with the function of regulating cell growth and motility of hepatocellular carcinoma via miR-876-5p/WNT5A pathway, which was affirmed by rescue and in vivo experiments\(^14\).

MiRNAs are another type of non-coding RNAs with 19-24 nt in length, which could repress gene expression post-transcriptionally via binding to the 3'-untranslated region(3'-UTR) of multiple target
RNAs\textsuperscript{15}. Wei L, et al. summarized expression levels and potential targets of 53 microRNAs (miRNAs) which participated in platinum resistance of gastric cancer\textsuperscript{16}. It has been reported that several oncogenic miRNAs can promote platinum resistance of gastric cancer, such as miR-20a\textsuperscript{17} and miR-106a\textsuperscript{18} while tumor suppressor miRNAs can reverse resistance to platinum conversely, such as miR-508p\textsuperscript{19} and miR-129-5p\textsuperscript{20}. As a valid tumor suppressor, microRNA-98-5p (miR-98-5p), which is one member of let-7 family, usually downregulate in various cancers, such as nasopharyngeal carcinoma\textsuperscript{21} and endometrial cancer\textsuperscript{22}. But in primary breast cancer swatches, miR-98 expression ascended\textsuperscript{23}. Perhaps miR-98 has completely opposite obligation in different types of cancers. A series assays elucidated that MiR-98-5p expressed significantly low in pancreatic ductal adenocarcinoma tissues and was related to tumor size, TNM stage, lymph node metastasis and survival by reversely regulating MAP4K4 and inhibiting downstream MAPK/ERK signaling\textsuperscript{24}. Until now, few studies are implemented about the function of miR-98-5p in GC.

Although recent advanced studies identify molecular elements of GC, the precise mechanisms of tumourigenesis remain largely unknown\textsuperscript{25}. Therefore, the clarification of new pathogenesis is vital for practical targeted treatment for GC; Many studies verified that IncRNA and miRNA played vital functions in the development and therapeutic resistance of cancers and their aberrant expression emerged as important hallmarks of multiple cancers\textsuperscript{26-28}. However, few studies reported the molecular mechanisms of PITPNA-AS1 and miR-98-5p in GC, especially when comes to their relationship with platinum resistance. Thus, we investigate the role of PITPNA-AS1 and miR-98-5p in GC and their connection with platinum resistance.

Results

RNA sequencing for IncRNA and microRNA in 3 gastric cancer patients

We have performed IncRNA and microRNA sequencing for 3 cisplatin resistant gastric cancer patients’ cancer tissues and 3 cisplatin sensitive gastric tissues; the heat map for top 20 genes were shown in Figure 1a and 1b. We have selected PITPNA-AS1 for further experiments. Based on DIANA, we assumed that miR-98-5p might be the downstream target for PITPNA-AS1.

PITPNA-AS1 expression was correlated with local recurrence in gastric cancer patients

PITPNA-AS1 was highly expressed in 153 gastric cancer tissues compared with para-cancer tissues, and miR-98-5p expression was lower in cancer tissues (Fig. 2a and 2b). In addition, survival analysis demonstrated that PITPNA-AS1 overexpression was combination with worse cancer specific survival (Fig. 2c). On the contrary, miR-98-5p led to reduced risk of death shown in the analysis of cancer specific survival (Fig. 2d). Furthermore, PITPNA-AS1 negatively correlated with miR-98-5p expression (Fig. 2e). Besides, PITPNA-AS1 expressed higher in local recurrent gastric cancer patients than non-recurrent ones, while miR-98-5p was down regulated (Fig. 2f and 2g).
PITPNA-AS1/miR-98-5p regulated cell proliferation and inhibits apoptosis in gastric cancer cell lines

We found that PITPNA-AS1 over-expressed in human gastric cancer cell lines MKN45 and AGS than that in normal gastric mucosal cell line GES-1 (Fig. 3a), besides, miR-98-5p expressed lower in MKN45 and AGS than that in GSE-1 cell line (Fig. 3b). Then we knocked down the expression of PITPNA-AS1 and found that MKN45 and AGS cell proliferation rate was inhibited (Fig. 3c and 3d), however, the apoptosis rate was increased (Fig. 3e and 3f). Moreover, overexpression of miR-98-5p brought decreased cell proliferation rate and enhanced cell apoptosis rate (Fig. 3g-3j);

PITPNA-AS1 negatively regulated the expression of miR-98-5p

We found that knocking down PITPNA-AS1 resulted in overexpression of miR-98-5p (Fig. 4a and 4b). Then we performed dual luciferase reporter gene assay and noticed that PITPNA-AS1 could interact with miR-98-5p (Fig. 4c and 4d). Further RNA pull-down assay indicated that PITPNA-AS1 directly interacted with miR-98-5p (Fig. 4e and 4f).

PITPNA-AS1 expression can be suppressed by cisplatin in gastric cancer cell lines

The half maximal inhibitory concentration $\text{IC}_{50}$ of CDDP in MKN45 is 0.52 ug/mL (Fig. 5a); and the IC50 in AGS is 0.59 ug/mL (Fig. 5b). We used CDDP (0.52 ug/mL) to treat MKN45 and CDDP (0.59 ug/mL) to treat AGS cells for 24 hours and found that PITPNA-AS1 expression can be significantly suppressed (Fig. 5c and 5d).

In the meantime, CDDP induced the expression of miR-98-5p in MKN45 (0.52 ug/mL) and in AGS cells (0.59 ug/mL) (Fig. 5e and 5f). We then noticed that H3K27ac expression was suppressed when treated with CDDP in MKN45 and AGS cells (Fig. 5g).

PITPNA-AS1/miR-98-5p regulated by H3K27ac influenced the effect of platinum

We have developed MKN45-CDDPR cells, the IC50 is 2.60 ug/mL (Fig. 6a). Then we found that PITPNA-AS1 was overexpressed in MKN45-CDDPR compared with parental cells (Fig. 6b); however, miR-98-5p was down regulated (Fig. 6c). Furthermore, we found that H3K27ac was up regulated in MKN45-CDDPR cells (Fig. 6d), which could be significantly suppressed by cisplatin treatment (Fig. 6e). Furthermore, Chip assay showed that H3K27ac enriched more in the promotor region of PITPNA-AS1 in MKN45-CDDPR cells than in parental cells (Fig. 6f and 6g). By treating with C646, the expression of PITPNA-AS1 in MKN45-CDDPR could be significantly suppressed (Fig. 6h). Then we transfected PITPNA-AS1-WT plasmids and found that PITPNA-AS1 knock down could suppress IC50 of MKN45-CDDPR, which could be reversed by miR-98-5p knock down (Fig. 6i).

Discussion

Collectively, in this study, we discovered the role of PITPNA-AS1 and miR-98-5p in gastric cancer through gain and loss-of-function assays and analyzed the mechanism by which PITPNA-AS1 regulates
apoptosis and drug resistance through the miR-98-5p targeting axis.

Gastric cancer is one of the leading public health problems worldwide because of its high incidence, morbidity and mortality rate\textsuperscript{29}. Currently, lacking of screening methods and early symptom, patients are most often diagnosed at advanced stages, with metastatic at distant sites and somber prognosis (median overall survival is 10-12 months)\textsuperscript{30,31}. For locally advanced disease, adjuvant or neoadjuvant therapy which recognized as the optimal therapeutic option is usually introduced with surgery owing to its curability\textsuperscript{30}. Fluoropyrimidine plus oxaliplatin doublet is considered as the preferred first-line regimen due to its comparable survival benefits and lower toxicity\textsuperscript{32}. Overcoming resistance is still a challenge in GC chemotherapy.

LncRNAs are associated with the tumor recurrence and poor prognosis, and abnormal expression has been observed in various tumors\textsuperscript{33}. Mounting evidence elucidated that LncRNAs could act as oncogenes or tumor suppressors by modulating the gene expression and function in tumorigenesis\textsuperscript{34}, which possibly induce significant influence on the alterations of cell proliferation, metastasis, autophagy and apoptosis\textsuperscript{35,36}. Our study indicated that LncRNA PITPNA-AS1 was highly expressed in gastric cancer patients and was associated with poor prognosis. Alteration of gene expression is correlated with the cancer specific survival of patients. PITPNA-AS1 was over-expressed in MKN45 cell line while knocking down PITPNA-AS1 resulted in inhibiting cell proliferation rate and increasing apoptosis rate. We first time inspected the role of PITPNA-AS1 in GC, which founding the basis for further exploration.

Next, we investigated the potential mechanism underlying PITPNA-AS1. Biased on current study, mechanism assays unveiled that PITPNA-AS1 targeted miR-98-5p. Dual luciferase reporter gene assay, RNA pull-down assay and RIP consequence provided powerful evidence that PITPNA-AS1 could interact with miR-98-5p. Moreover, knocking down PITPNA-AS1 resulted in decreased expression of miR-98-5p, which confirmed this discovery again. The anti-tumor function of miR-98-5p has been documented yet. For instances, Fu Y, et al. recognized miR-98-5p underexpression as biomarkers for predicting poor prognosis in pancreatic ductal adenocarcinoma (PDAC) patients because miR-98-5p inhibits proliferation and metastasis via targeting MAP4K4\textsuperscript{24}. Acting as a tumor suppressor, miR-98 could decelerate cancer aggressiveness by inhibiting TWIST expression in non-small cell lung cancers\textsuperscript{37}. In hepatocellular carcinoma (HCC), miR-98-5p could restrain cell proliferation and induce cell apoptosis via inhibition of its target gene IGF2BP1\textsuperscript{38}. As for colon cancer, miR-98 play the role of tumor suppressor gene and inhibits Warburg effect by targeting HK2 (HK2 involves in miR-98-mediated suppression of glucose uptake, lactate production, and cell proliferation, whose expression were negatively correlated with miR-98) in colon cancer cells, which provided promising therapeutic candidate for clinical treatments\textsuperscript{39}.

In our study, miR-98-5p was shown to be downregulated in GC. Overexpression of miR-98-5p led to decreased cell proliferation rate and ascended apoptosis rate. Moreover, inhibition of miR-98-5p partially reversed the inhibitory effects of PITPNA-AS1 on GC cell proliferation and apoptosis. Thus, we draw the conclusion that PITPNA-AS1 exerts its tumor-promotion effect in GC via negatively modulating the
expression of miR-98-5p. Laboratory findings were consistent with literature reports. Guo Q, et al. revealed that IncRNA PITPNA-AS facilitate the cervical cancer progression on the proliferation, cell cycle and apoptosis by targeting the miR-876-5p/c-MET axis\(^\text{40}\).

Aberrant IncRNA expression is certified to strongly implicate in drug resistance in some cancers\(^\text{41,42}\). Our experiments unraveled that cisplatin (CDDP) and lobaplatin (LBP) could suppress PITPNA-AS1 expression and induce expression of miR-98-5p in GC cell lines. Besides, PITPNA-AS1 was overexpressed in MKN45-CDDPR and MKN45-LBPR, which could well resist platinum drugs, compared with their parental cells. However, miR-98-5p gave the opposite results. Furthermore, PITPNA-AS1-WT could reverse the inhibitory effect of platinum while downregulating miR-98-5p have the same phenomenon to some extent. These data demonstrated that PITPNA-AS1/miR-98-5p had a major role in regulating platinum-resistant in GC cells. Consistent with aforementioned findings, Wang Y’s studies have identified that elevated expression of miR-98-5p is associated with promoted resistance to cisplatin treatment through directly targeting Dicer1 and poor clinical outcomes in epithelial ovarian cancer patients\(^\text{43}\). Guo H’s studies have illustrated that cancer-associated fibroblast- derived exosomal who carrying overexpressed miR-98-5p promoted cisplatin resistance in ovarian cancer by downregulating CDKN1A\(^\text{44}\). An existing study has revealed that miR-129 could enhance chemosensitivity to cisplatin by suppressing P-gp protein in GC cells\(^\text{45}\).

Conjointly, these results suggest that miR-98-5p could be used as a novel prognostic factors and critical therapeutic strategy in GC by enhancing chemo-sensitivity for platinum treatment against GC. However, downstream signal molecule and other biological processes requires further investigation.

Materials And Methods

Gastric cancer patients

The GC tissues and local recurrence GC tissues, as well as the corresponding para-cancer tissues were collected from the patients who were diagnosed as GC by surgical resection at the First Affiliated Hospital of Xi’an Jiao Tong University. All of the samples were pathologically diagnosed and stored in liquid nitrogen. All of the patients had signed a written informed consent. The present study gained approval from the Ethics Committee of The First Affiliated Hospital of Xi’an Jiao tong University. And all experiments were conducted in accordance with relevant guidelines and regulations, which consistent with the Declaration of Helsinki regulations.

Cell culture

The human GC cell line MKN45 and AGS were purchased from Shanghai Gaining Biological Technology Co., Ltd (Shanghai, China), and the human gastric epithelial cell line GES-1 was obtained from American Type Culture Collection (Virginia, USA). All the cells were cultured in DMEM medium (HyClone, USA) containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin (HyClone, USA) in a 37°C
and 5% CO2 incubator. The GC cells were then treated with continuous low-dose of cisplatin in a stepwise manner to developed cisplatin resistant GC (MKN45-CDDPR ) cells.

**Cell transfection**

The PITPNA-AS1 knockdown and miR-98-5p overexpression plasmids were purchased from Genechem (Shanghai, China). The above plasmids were delivered into MKN45 and AGS cell lines by using the Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) reagent according to the manufacturer's instruction.

**RNA sequencing**

The RNA sequencing process was guided and supported by Genechem(Shanghai, China). In brief, total RNA was extracted from 3 GC patients’ tissues and corresponding normal tissues by using TRIzol (Invitrogen, Carlsbad, CA). And the RNA purification was performed by using the RNA Clean XP Kit (Beckman Coulter, Kraemer Boulevard Brea, CA) and the RNase-Free DNase Set (QIAGEN, GmbH, Germany). Finally, the Illumina HiSeq 2000/2500 (Illumina Inc, San Diego, CA) was used for RNA sequencing.

**qRT-PCR**

Total RNA was extracted from GC tissues and cell lines by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was generated by using the First-strand cDNA synthesis kit (Tiangen Biotech, Beijing, China). The expression levels of PITPNA-AS1 was tested by conducting qRT-PCR using SYBR® Premix Dimer Eraser kit (Takara Shiga, Japan). And β-actin was used as the inner reference. The miScript microRNA RT PCR kit (Qiagen, Toronto, ON, Canada) was used for cDNA synthesis and qRT-PCR process for miR-98-5p expression. U6 was used as the internal reference. ABI 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) was conducted to perform the qRT-PCR process. The expression level was calculated by 2-ΔΔCt method.

**Western blot**

The total proteins were extracted from GC cells by using RIPA lysis buffer (Sigma-Aldrich, Darmstadt, Germany) and were quantified by BCA Protein Assay Kit (Beyotime, Shanghai, China). Then proteins were diverted onto PVDF membranes (Millipore, USA) after separated by SDS-PAGE. The transferred PVDF membranes were blocked by using 5% skim milk, and then were incubated overnight at 4°C with primary antibodies, which including H3K27ac (1:1000, CST, Shanghai, China) and Histone H3 (1:2000, CST, Shanghai, China). Subsequently, the membranes were incubated with the secondary antibody (1:10000, Beyotime, Shanghai, China). Then the enhanced chemiluminescence (ECL, Beyotime, Shanghai, China) was used to quantify the protein expression levels.

**Cell proliferation**
In order to evaluate the proliferation and cisplatin resistance of GC cells, the cell counting kit-8 (CCK-8) kit (AbMole, USA) was used according to the manufacturer’s protocol. In briefly, GC cells were seeded in 96-well plates with a density of $5 \times 10^3$ cells per well, then 10 μL of CCK-8 reaction solution was supplemented into each well every 24h followed by incubation for 2h. Then the optical density (OD) values of GC cells at 450 nm were detected to assess cell proliferation of GC.

**Cell apoptosis assay**

The Caspase 3/7 Activity Apoptosis Assay Kit (Invitrogen) was used to detect the apoptosis rate of GC cells. According to the manufacturer’s instruction, GC cells were plated into the 96-well plate overnight at 20000 cells per well. Then 50 μL of Caspase 3/7 Substrate (Component A) was added into 10 mL of Assay Buffer (Component B) to make caspase 3/7 assay loading solution. GC cells were incubated in a a 37°C, 5% CO2, incubator for 6 h after treated with camptothecin to induce apoptosis. Then GC cells were added with 100 μL/well of caspase 3/7 assay loading solution, followed by supplemented with the assay loading solution at room temperature under dark conditions for 1 h of incubation. Finally, GC cells were centrifuged at 800 rpm for 2 min, then the fluorescence intensity at Ex/Em=490/525 nm was monitored to evaluate cell apoptosis rate.

**Dual luciferase reporter gene assay**

The plasmids of PITPNA-AS1 wild-type (PITPNA-AS1-WT) and PITPNA-AS1 mutant type (PITPNA-AS1-Mut) were co-transfected with the miR-98-5p-NC mimic into GC cells by using Lipofectamine 2000 (Invitrogen, USA). And then the miR-98-5p-WT and miR-98-5p-Mut vectors were transfected into GC cells as well. Then Dual-luciferase reporter system (Promega, Madison, WI, USA) was conducted to estimate the luciferase activities based on the manufacturer’s instruction.

**RNA pull-down assay**

Biotin-labeled miR-98-5p-WT and miR-98-5p-Mut were synthesized by GeneCreate (Wuhan, China) and were transfected into GC cells which were incubated with lysis buffer (Ambion, Austin, Texas, USA). Then the GC cell lysates were incubated with the streptavidin Dynabeads (Invitgen, USA) precoated with RNase-free bovine serum albumin (BSA) and yeast tRNA (Sigma-Aldrich, USA) overnight at 4 °C. After washed with washing buffer, the bound RNA was purified by using Trizol. Finally, the enrichment of PITPNA-AS1 was identified and estimated by performing qRT-PCR.

**Statistics**

The SPSS 18.0 software and the Graphpad Prism 8.2 software were used to analyze and visualize the data involved in this study. The Limma package were used for RNA sequencing analysis. A paired Student’s t-test was used to evaluate the statistical differences between two groups. And one-way ANOVA was applied for multiple groups comparison. The Kaplan-Meier survival analysis was used to estimate the prognosis of GC patients. Each assay was independently repeated at least three times and all the
statistical results presented in this work were expressed as Mean±Standard Deviation (SD). A p value of <0.05 was indicative of statistically significant difference.

**Declarations**

**Author contributions**

Y.P.L. designed and performed the experiments in this study; and completed the writing of the manuscript. S.X.H. and J.S.W. supervised the progression of the study. X.Z. completed the statistical analyses of the data and contributed to the manuscript editing. Y.N.C. plotted the statistic graphs. G.G. helped to edit the manuscript. All authors gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

**Competing interests**

The authors declare that they have no competing interests.

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