GFRα2 prompts cell growth and chemoresistance through down-regulating tumor suppressor gene PTEN via Mir-17-5p in pancreatic cancer

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ABSTRACT

Nerve growth factors and their receptors have received an increasing attention in certain cancers since they play an important role in regulating tumorigenesis, biological process and metastasis. Here we aimed at characterizing a new function of one of the subtypes of growth factor receptors (GFR), GFRα2, in pancreatic cancer. In this study, we showed that GFRα2 was up-regulated in pancreatic adenocarcinoma and was positively correlated with tumor size and perineural invasion, which indicated that it may be associated with cell growth and apoptosis. Mechanically, we discovered that high GFRα2 expression level leads to PTEN inactivation via enhancing Mir-17-5p level.

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Introduction

Currently, pancreatic adenocarcinoma (PDAC) is one of the most aggressive human malignancies. Due to the lack of early symptoms, most of the patients are diagnosed at late stage; thus, the average survival time is usually less than 6 months and the 5-year survival rate hovers around 5% [1,2]. Even for those patients who received radical resection, they still suffer from the problem of recurrence [3]. Chemoresistance is a major barrier for treating PDAC, which can arise de novo or requires refractoriness. Gemcitabine has been recognized as the first-line drug to treat PDAC for a long time; however, insensitivity due to singleness of therapeutic target is the major concern during its clinical application [4]. Hence, investigating the molecular mechanism of chemoresistance is becoming a real challenge.

Growth factor receptor α (GFRα) contains four members – GFRα1, GFRα2, GFRα3 and GFRα4 – cooperating with transmembrane receptor tyrosine kinase RET, which receives signal from glial cell line-derived factor (GDNF) family [5]. These structures are crucial for normal development of kidney and nerves [6,7]. GFRα2 is a common receptor for both GDNF and neurturin. Besides the role of maintaining endocrine homeostasis in pituitary [8], it has been reported to be correlated with neuropathic pain [9] and neuronal plasticity [10]. However, its specific function in cancer is still not clear.

Mir-17-5p belongs to Mir-17-92 cluster [11], which is involved in a wide range of biological functions and processes such as development, energy metabolism, immune response, cell differentiation and carcinogenesis [12,13]. Previous studies indicated that Mir-17-5p was often up-regulated in various cancers and functions as an oncogenic MirRNA [14–16]. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) gene is a tumor suppressor gene that acts a pivotal role in cell growth, development, signal transduction and apoptosis [17,18]. PTEN inactivation leads to many types of cancer including PDAC [18]. Mass spectrometry analysis showed that GFRα2 interacts with PTEN [19], which may offer a new way to clarify the downstream mechanism of GFRα2 to influence biological process of cancer. The Mir-17-5p/PTEN axis has been reported to be highly

http://dx.doi.org/10.1016/j.canlet.2016.06.016
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related to chemoresistance in certain cancer such as ovarian cancer and prostate cancer [16,20]. However, the specific correlation between GFRα2 and PTEN and whether the chemoresistant axis of Mir-17-5p/PTEN exists in PDAC are still unknown.

This study shows that GFRα2 may enhance cell survival by repressing the expression level of PTEN via recruiting Mir-17-5p, and GFRα2/Mir-17-5p/PTEN could enhance cell survival and chemoresistance in PDAC. Thus, inhibiting this pathway may be a potential target in PDAC therapy.

Materials and methods

Patients and tissue collection

58 pairs of PDAC samples and two normal pancreases were collected from pa-
tients who underwent surgery resection at the Department of General Surgery of Ruijin Hospital between 2008 and 2010 and were well-documented with clinical information. Surgical specimens were confirmed by pathological examination and all of the patients did not receive any anti-tumor treatment like chemotherapy or radiotherapy before surgery. Tumor staging was determined by the latest edition of the TNM system of the American Joint Committee. The project was approved by the Ethics Committee of Ruijin Hospital affiliated to Shanghai Jiao Tong University and all subjects signed an informed consent form. Details about the patients’ information will not be disclosed.

Immunohistochemistry analysis

All samples were fixed in 4% paraformaldehyde (pH 7.4) overnight at 4°C and then dehydrated in different concentrations of ethanol. Immunohistochemistry staining and analytical methods were performed according to our group’s previous methods [21]. Anti-GFRα2 (Proteintech, #21973-1-AP) and anti-PTEN (Cell Signaling Technology, #9188) were used in the experiments.

Cell culture and reagents

PanC-1, Mia Paca-2 and HEK-293T were purchased from ATCC and were au-

Plasmid construction

 Lentivirus vector pGO-LV-PDI (without fluorescence) was digested by restric-
tion endonuclease (NEB) between BamHI and EcoRI; full-length cDNA of GFRα2 and PTEN were cloned from PanC-1 and then ligated by ClonExpress II One Step Cloning Kit (Vazyme Biotech Co., Ltd, #C112-02). The shRNA targeted GFRα2, purchased from Sigma, was inserted into pLKO.1 vector. Empty vectors were used as negative control.

Virus packaging and infection

 Lentivirus plasmids were transfected into HEK-293T with virus packing plas-
mids by Lipo2000 (Invitrogen) to produce lentivirus. The culture supernatants were collected at 48 h and 72 h and added to pancreatic cancer cell lines to construct stable cell lines via puromycin selection.

Luciferase report assay

 Plasmids containing wild-type Luc-PTEN (WT) and mutant Luc-PTEN 3′UTR were specially synthesized. Mir-17-5p mimics were synthesized by Biotrend (Shanghai, China). Luciferase activity of the indicated cells were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) 60 h after transfection according to the manufacturer’s instruction. Activities were normalized to Renilla luciferase.

Western blot analysis

 Cells were lysed with pre-chilled lysis buffer (Beyotime, #P0013) and total pro-
teins were qualified by BCA assay (Beyotime, #P0012). Then western blot assay was performed as described previously [21]. Antibodies were the same as in IHC staining. GAPDH was used as internal control (Abcam). The western blot images were cap-
tured by a Tanon-5200 Chemiluminescent Imaging System (Tanon, Co., Shanghai).

RNA isolation and qPCR analysis

 Total RNA were isolated by TRIzol (Invitrogen) according to the manufacturer’s protocol. The quality and concentration of RNA were evaluated by a spectropho-
tometer. 1 μg of total RNA was used for first-strand synthesis (TIANGEN Biotech (Beijing) Co., Ltd). Real-time PCR was performed by the SYBR Green PCR method using the All-in-One miRNA qPCR Detection Kit (GeneCopoeia, Rock, MD, USA) in triplic-
ate. The following forward primers were purchased from GeneCopoeia: has-Mir-

Colonization formation assay and cell growth counting

 For colonization formation assay, 500 cells were plated into a 6-well plate. After 2 weeks of regular culture, cells were washed with PBS and then fixed with methanol for 15 min before staining with 1% Giemsa for 15 min and washed 3 times with PBS. For cell counting assay, cells were plated in a 96-well plate at a density of 2×103 cells. Every two days one set of cultures was tested followed by TransDetect cell count-
ing kit (CKK-8, TransGen Biotech) at 450 nm.

Coimmunoprecipitation (Co-IP) assay

 Co-IP assay was performed according to our previous protocol [22]. Briefly, cells were lysed in pre-chilled lysis buffer (Beyotime, #P0013) with protease inhibitor cock-
tail (Roche). Protein A beads were incubated with anti-PTEN antibody (Cell Signaling Technology, #9188) for 4 h and then incubated with total protein lysis overnight. Then the precipitated protein was used for western blot analysis as the previous method.

Study approval

 This study was approved by the Ethics and Research Committees of Ruijin Hos-
pital, Shanghai Jiao Tong University, School of Medicine and conducted in accordance with the Declaration of Helsinki Principles. The procedures for pancreatic tumor re-
section were described in detail to all patients before admission, and informed consent was obtained for all participating patients.

Results

GFRα2 is up-regulated in PDAC and positively correlated with tumor size and poorer prognosis

The protein expression level of GFRα2 was measured in 58 tumor samples and their corresponding peritumor tissues by IHC. GFRα2 expression was nearly negative in the two normal pancreatic tissues (Fig. 1A), while significant differences were found between tumor and peritumor tissues (Fig. 1B and C). The association between gene expression and other clinical parameters was shown in Table 1, indicat-
ing that patients with higher GFRα2 expression had a larger tumor size and higher rate of perineural invasion compared with those with lower GFRα2 level. In addition, the GFRα2 expression level was correlated with poorer prognosis (Fig. 1D). These results implicated that GFRα2 may play a role in cell survival rate, espe-
cially growth and apoptotic ability.

GFRα2 could enhance progression of PDAC cells and down-regulate PTEN expression

To further investigate the function of GFRα2 in pancreatic cancer, we constructed stable GFRα2 over-expression (pGMLV-PDI) and knock-down (pLKO.1) cell lines using two pancreatic cancer cell lines: PanC-1 and Mia Paca-2. Colony formation assay and cell prolifera-
tion rate assay were carried out in control and over-expression groups. It was clearly shown that both the colony formation and the proliferation abilities were significantly enhanced in GFRα2 over-
expression (GFRα2 OE) group (Fig. 2A–C). We also injected the two group cell lines into nude mice to study subcutaneous tumor for-
mation. As expected, over-expression of GFRα2 resulted in increased tumor growth in vivo (Fig. 2D). At the same time, we used shRNA to
knock-down the expression level of GFRα2 (Fig. 2E). We found that knock-down of GFRα2 in pancreatic cancer cells attenuated cell proliferation and colony formation (Fig. 2F and G). Further, we checked the expression level of PTEN in GFRα2 OE and control cells of PanC-1 and MiaPaca-2. The results showed that PTEN presented lower level in the GFRα2 OE group (Figs. 2H and 3G). These findings suggested that GFRα2 could prompt tumor growth and repress PTEN expression level. In order to validate the capacity of GFRα2 in promoting cell growth in vivo, we also detected the Ki-67 index in the xenografts (Supplementary Fig. S1A and B) and the result showed that the index was much higher in the GFRα2 OE group.

**Mir-17-5p was up-regulated by GFRα2 and directly targeted to PTEN**

According to one study published in 2015 [23], we asked whether the correlation between Mir-17-5p and GFRα2 also existed in PDAC. We checked the Mir-17-5p level in MiaPaca-2 cells, which express low levels of endogenous Mir-17-5p, before and after over-expressing GFRα2. The results showed that Mir-15-5p was significantly up-regulated in the GFRα2 OE group (Fig. 3A). Since MirRNAs are often involved in regulatory feedback pathways, we wanted to know whether Mir-17-5p was also the case. Indeed, by using the MirRNA database TargetScan, we identified PTEN mRNA as one of the putative Mir-17-5p targets. To validate this prediction, we treated MiaPaca-2 cells with Mir-17-5p mimics and control MirRNA, and detected the PTEN expression level with qRT-PCR (data not shown) and western blot assay (Fig. 3B). In line with these results, we identified the binding sites of Mir-17-5p in PTEN 3′UTR by luciferase report assay (Fig. 3C and D). Taken together, we indicated that PTEN was directly regulated by Mir-17-5p and this MicroRNA was the bridge between GFRα2 and PTEN. In order to validate the regulation relationship clearly, we first used Co-IP assay to detect whether GFRα2 could interact with PTEN directly as the previous mass spectrometry indicate [19]; however, this direct interaction seemed not to exist in pancreatic cancer cell line (Fig. 3E). Next, we compared PTEN level and cell proliferation rate by CCK-8 after over-expressing GFRα2 with or without Mir-17-5p mimics and inhibitor (Fig. 3F). These results demonstrated that GFRα2 could not interact with PTEN directly and Mir-17-5p could further decrease the PTEN level and promote cell proliferation, inhibiting it could have the opposite effects (Fig. 3G and Supplementary Fig. S1C, D).

**Mir-17-5p expression level was up-regulated in PDAC and positively correlated with GFRα2 expression level in PDAC samples**

Based on the previous findings, we assessed whether Mir-17-5p was up-regulated in PDAC. MicroRNAs were extracted from the previous tissues and qRT-PCR was used to detect Mir-17-5p expression level. Scatter diagram was shown to indicate that expression of Mir-17-5p in tumor tissues was much higher than that in

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Table 1

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* Significant difference (p<0.05).
peri-tumor tissues (Fig. 4A). Higher expression of Mir-17-5p also showed a positive correlation with the expression of GFRα2 and is associated with poorer prognosis as GFRα2 did (Fig. 4B, C and Table 2).

GFRα2/mir-17-5p/PTEN was correlated with chemoresistance in PDAC

In order to study the connection between GFRα2 and chemoresistance, we used gemcitabine (GEM, Selleckchem), the first-line chemotherapeutics in PDAC therapy, as an apoptotic inducer. 50 μM GEM was added in the culture medium for 48 h and flow cytometry (Annexin-V/PI) was used to detect the apoptotic and dead rate. Compared with the control group, GFRα2 could significantly inhibit the apoptotic rate (p < 0.05). Next, we used Mir-17-5p mimics and inhibitor to treat the GFRα2 OE group; we found that Mir-17-5p could enhance the chemoresistance to GEM, while the cancer cells could become sensitive to GEM after inhibiting the Mir-17-5p (compared with the control group, p < 0.05). If we over-expressed the level of PTEN (adenovirus, Ad) before treating with Mir-17-5p mimics, the apoptotic rate will become higher (p < 0.05) (Fig. 5A–C). These results demonstrated that GFRα2 could enhance the chemoresistance to GEM through inhibiting PTEN via up-regulating the Mir-17-5p level. The mechanism can be described in Fig. 5D.

Discussion

Chemoresistance and fast growth rate are the main reasons for patients of PDAC suffering from short overall survival time [24], especially for those with advanced carcinoma. This is a multistep process that involves complicated factors, and the activation of oncogenes and loss of tumor suppressors are thought to act key steps...
in the progression of PDAC [25–27]. Therefore, it is necessary to study the underlying mechanisms of tumor growth and chemoresistance. This study presented a new role of GFRα2 in tumorigenesis and chemoresistance in pancreatic ductal adenocarcinoma (PDAC). First, we found that GFRα2 was up-regulated in pancreatic cancer tissues and was related to prognosis, tumor size and chemoresistance. Next, over-expression of GFRα2 did promote tumor growth and down-regulate PTEN expression level at the same time. Third, we found that Mir-17-5p was up-regulated after GFRα2 over-expression in pancreatic cancer cell lines and its expression pattern also correlated with chemoresistance. This correlation could also be validated in patients’ tissues. With the rescued experiments, we found that Mir-17-5p played an important role in connecting GFRA2 and PTEN.

Lastly, we used gemcitabine (GEM) to treat pancreatic cancer cells before and after ectopic expression or knock-down of GFRα2 and Mir-17-5p. It is highly consistent with our hypothesis that GFRα2 and Mir-17-5p could enhance chemoresistance. Taken together, our results indicated that GFRα2/Mir-17-5p/PTEN axis played an important role in tumorigenesis and chemoresistance.

Previous studies about GFRα2 mainly focused on its function in the field of neuroscience. GFRα2-RET signaling provided necessary trophic signals for proper soma size and epidermal innervation in the IB4-binding nonpeptidergic neurons and development cutaneous sensory neurons [28,29]. GFRα2 also correlated with nerve injury or neurotrophic pain in some certain diseases. Although a few studies have pointed that GFRα2 acted as an oncogene in cancer,
and one article reported it was highly related to perineural invasion and abdominal pain in PDAC [10]. However, the underlying mechanism in PDAC was still largely unknown.

Mature MirRNAs are non-coding RNA molecules with 21–25 conserved nucleotides. The small cellular RNAs can regulate the biological process of target mRNAs by degrading the mRNAs or inhibiting their translation [30]. Thus, up-/down-regulation of MirRNAs has been linked to the progression of many cancers [31,32]. Lines of evidence have shown that Mir-17-5p, which belongs to the Mir-17-92 cluster located in 13q31.3, was up-regulated in various types of cancer and correlated with cell cycle, apoptosis and proliferation. Although some studies have already indicated that it acted a role in chemoresistance in ovarian cancer and targeted PTEN, the specific way of Mir-17-5p activation and its role in PDAC are still unknown [16,20]. Recently, a study based on bioinformatic analysis with hepatitis C virus (HCV) showed that Mir-17-3p (a mature type of Mir-17) could bind to the 5′UTR region of GFRα2, promoting translational enhancement instead of repression [23]. Although we could not verify this finding in pancreatic cancer cells (data not shown), it inspired us to detect whether there was a regulatory correlation between GFRα2 and Mir-17 (including its two mature types: Mir-17-5p/3p). Although the correlation and the function between Mir-17-5p and PTEN have been reported in various cancers, its regulation and activation pathway have not been studied, especially in PDAC.

In our study, we found a new way of GFRα2 to regulate PTEN expression and it is effective to inhibit the GFRα2/Mir-17-5p/PTEN axis in terms of tumor growth and chemoresistance. However, since most of the findings were obtained from cells and animal tests, it is still unknown whether inhibiting GFRα2 and Mir-17-5p could influence other normal functions in vivo, and how to induce the Mir-17-5p inhibitor into the living body is also a problem that needs further investigation in the future.

Table 2

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* Significant difference (p<0.05).

Funding

This work was supported by the National Science Foundation of China (No. 81472237), National Basic Research Program of China (973 Program, 2015CB964800) and Innovation Training Programs for Undergraduates to Jiangning Gu, Chinese Academy of Sciences (CAS).
Acknowledgments

We are grateful to the experimental support of the Uli Schwarz public laboratory platform in PICB; Dr. Yujie Chen offered great help to our research work.

Conflict of interest

The authors declare no conflict of interest.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2016.06.016.

References


Fig. 5. GFRα2/Mir-17-5p/PTEN was correlated with chemoresistance in vitro. (A) Basic information for therapeutic approaches. (B) Flow cytometry analysis of dead and apoptotic rate of these cells (3 replicates). Notice that the cell dead rate accounted for more than 10% in the control group, which was much higher than any other group. (C) Statistical results of dead and apoptotic rate in these cells. (D) Model for the GFRα2/Mir-17-5p/PTEN axis. GFRα2 activation could repress PTEN through improving Mir-17-5p to enhance cell survival and this axis can help cancer cells resist chemotherapeutics (gemcitabine).


R. Song, Q. Liu, T. Liu, J. Li, Connecting rules from paired miRNA and mRNA expression data sets of HCV patients to detect both inverse and positive regulatory relationships, BMC Genomics 16 (Suppl. 2) (2015) S11.


