RESEARCH COMMUNICATION

# Neurotrophic Artemin Promotes Motility and Invasiveness of MIA PaCa-2 Pancreatic Cancer Cells

**Ling-Xin Meng[&]*, Yu-Hua Chi[&], Xiang-Xu Wang, Zhao-Jun Ding, Li-Cong Fei, Hong Zhang, Ling Mou, Wen Cui, Ying-Jie Xue**

## Abstract

**Objective: To analyze the capacity of neurotrophic artemin to promote the motility and invasiveness of MIA PaCa-2 pancreatic cancer cells. Methods: MIA PaCa-2 was cultured in vitro and studied using transwell chambers for motility and invasiveness on treatment with different concentrations of aArtemin or its receptor GFRα3 were also determined. Expression of matrix metalloproteinase-2 (MMP-2) and epithelial cadherin (E-cadherin) was quantified using RT-PCR and Western blotting. Results: MIA PaCa-2 pancreatic cancer cell motility and invasiveness was significantly increased with artemin and its receptor GFRα3 with dose dependence (P<0.01). MMP-2 production was also significantly increased (t = 6.35, t = 7.32), while E-cadherin was significantly lowered (t = 4.27, t = 5.61) (P <0.01). Conclusion: Artemin and its receptor GFRα3 can promote pancreatic cancer cell motility and invasiveness and contribute to aggressive behavior. The mechanism may be related to increased expression of MMP-2 molecule and down-regulation of E-cadherin expression.**

**Keywords:** Artemin - invasiveness - motility - pancreatic cancer - MMP-2 - E-cadherin

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## Introduction

Pancreatic cancer is characterized by strong aggressiveness, early metastasis, and poor prognosis. The survival rate for this cancer is among the lowest of all cancer types and 5-year survival rate < 5%. Highly malignant pancreatic cancer has the high incidence of metastasis. Most of the metastasis has occurred when the patient visits. Invasion and metastasis are independent prognostic indicators and have serious adverse effects on prognosis for pancreatic cancer patients (Lomberk, 2008; Jemal et al., 2009).

Proteins belonging to the GDNF family of neurotrophic factors (GDNF, Neurturin, Persephin, and Artemin) and their receptors GFRα1-4/RET have been proposed as therapeutic agents for neurodegenerative diseases on the basis of their ability to promote survival of various neurons including peripheral autonomic, sensory, central motor, and dopaminergic neurons. Artemin and persephin play roles by GFRα3 and GFRα4 in mammals, respectively (Airaksinen et al., 2006). Artemin with GFRα3 receptors tend to form a ligand/receptor complex by glycosyl-phosphatidylinositol (GPI) anchored in the cell membrane, activation of Ret, which pass signals. As glial-derived neurotrophic factor (GDNF) family member, the main physiological function of Artemin and its receptor GFRα1-4/RET is to promote the repair of a variety of neurological diseases, affecting cell growth, differentiation and apoptosis. Several other studies have previously shown that artemin and its receptor GFRα3 can promote the development of glioblastoma multiforme (GBM), tumor invasion and metastasis (Rückert et al., 2011), but the exact mechanism is unknown. Invasion and metastasis of pancreatic cancer prevention study at home and abroad cause increasing attention of scholars.

Our lab once discussed the exogenous nerve growth factor (β-NGF) on MIA PaCa-2 pancreatic cancer cell motility, invasion and the infiltration capacity (Meng et al., 2008). The role of artemin/GFRα3 receptor in the malignant behavior of pancreatic cancer remains has not been reported. We postulated that Artemin may play some role in the motility and invasion of pancreatic cancer. To determine the functional consequences of exogenous Artemin in pancreatic cancer cells, we explored a variety of methods to study the the motility and invasiveness behavior of Artemin and its receptor GFRα3 on pancreatic cancer line cells MIA PaCa-2.

## Materials and Methods

*Cell culture*

Human pancreatic cancer cell line, MIA PaCa-2 was obtained and maintained in Oncology Laboratories of Tianjin Medical University Cancer Hospital & Tianjin Cancer Institute. The MIA PaCa-2 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO,

*Department of Oncology, Affiliated Rizhao People's Hospital, Jining Medical University, Rizhao, Shandong Province, China [&]Equal contributors *For correspondence: menglx001623@163.com*

Paisley, United Kingdom) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. MIA PaCa-2 cells were maintained in a humidified 5% $CO_2$ atmosphere at 37 °C. Adherent cells; grow well, every 72 h to trypsin digestion and passage.

*Matrigel-Based Invasion Assay*

Invasion assay was performed using BD Biocoat Matrigel invasion chambers with 8-μm pore size (BD Biosciences, Heidelberg, Germany). Logarithmic growth phase MIA PaCa-2 cells were treated with 0.25% trypsin digestion, centrifugation, washed and resuspended in serum-free DMEM medium, adjusted the cell concentration of $5 \times 10^5$ cells/ml. 25μl Matrigel gel (1 mg/ml) was applied to the uniform micro-porous membrane of Transwell chamber on each interval of 10 min for three times, and violet lamp for 30 min to gel. According to the manufacturer's instructions, the Matrigel was hydrated with 0.5 mL DMEM (serum-free) and incubated for 2 hours. 100 μl $5 \times 10^5$ MIA PaCa-2 cells were seeded into the upper chamber of the invasion chambers and incubated for 24 hours at 37 °C, 5% $CO_2$ atmosphere. To detect the influence on invasion, 400 μl conditioned medium was added to the lower chamber and incubated for 24 hours, including final concentration of 50 ng/mL, 100 ng/mL, 150 ng/mL and 200 ng/mL of Artemin and GFRα3 DMEM medium, respectively. Blank control group was added equal amount of serum-free DMEM medium. Each group of five samples in parallel. The noninvading cells were removed from the upper surface of the membrane by wiping with a cotton-tipped swab. Cells adhered to the lower surface were fixed in 75% methanol mixed with 25% acetone and then stained with giemsa dye for 15 min. To calculate the total number of invading cells, the membranes were scanned and cell number in every microscopic cutout of the mosaic image of the membrane was counted using the software Zeiss KS300 (Carl Zeiss AG). The assays were performed in duplicate and repeated 3 times.

*Western blotting analysis*

Since the pro-invasive activity of other GDNF-family member is attributed to the activation of metalloproteinases (MMP) (Okada et al., 1999), we also investigated whether Artemin-induced cancer cell invasion was associated with the up-regulation of MMPs and E-cadherin. The MIA PaCa-2 cells stimulated by 150 ng/ml Artemin and GFRα3 were collected, respectively. Cells were rinsed twice with D-Hanks and solubilized with lysis buffer [50 mmol/L Tris (pH 8.0), 1% Nonidet p-40, 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate, 0.5% deoxysodium cholate, 1 × cocktail (Roche, Mannheim, Germany)] for 30 min on ice. The total extract was cleaned by centrifugation at 12 000 r/min for 30 min at 4 °C, and the supernatant was collected. Spectrophotometer was applied to measure the standard protein sample and draw the standard curve of protein determination according to the results. Regression equation was framed by SPSS15.0 curve fitting. The final sample concentration was measured by adjusting the protein concentration in each group. The protein concentration was determined with the Bradford assay (BioRad, CA, United States). A total of 40 $\mu$g of total cell extract was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. 10% polyacrylamide gel electrophoresis was placed for several hours. The separated proteins were transferred onto an Immobilon-PVDF membrane (Millipore, Bedford, MA, United States) and were blocked and incubated with the primary MMP-2 and E-cadherin-specific antibody (1:500) overnight at 4 °C (about 12 h). The membrane with HRP-conjugated secondary antibody was incubated in 37 °C 2 h, colored with DAB, and quantitatively analyzed by applying Kodak gel imaging system (Kodak digital science image station 440). Band position is set according to selection, and the gray-scale mode (Total gray) was used for the amount of protein expression detection of each band.

*Statistical analysis*

Results are expressed as mean ± standard deviation (SEM). For statistical analysis, the Student t test or single factor analysis of variance was used by using SPSS 16.0 statistical package. P value of less than 0.05 was considered significant.

## Results

*Influence of Artemin and GFRα3 on MIA PaCa-2 Pancreatic Cancer Cell Invasion*

To determine a possible contribution of Artemin on the invasive potential of pancreatic cancer cells and on the formation of metastasis, a Matrigel-based invasion assay was used. As Artemin and GFRα3 drug concentration increasing, the number of pancreatic cancer cells MIA PaCa-2 through Matrigel gel were significantly higher than normal group ($P<0.01$). There were statistically significant differences for Artemin, GFRα3 group between 50 ng/ml and 100 ng/ml, 50 ng/ml and 150 ng/ml, 100 ng/ml and 150 ng/ml ($P<0.05$, $P<0.01$, $P<0.05$), and a dose-effect relationship. However, there was no significant difference between 150 ng/ml and 200 ng/ml ($P>0.05$). This suggests that Artemin and GFRα3 had the ability to significantly increase the invasive potential of MIA PaCa-2 cancer cells in vitro, and 150 ng/ml was the optimal concentration to have the best effect for both of them (Table 1, Figure 1).

**Table 1. Artemin and GFRα3 Effects on MIA PaCa-2 Pancreatic Cancer Cell Invasion**

| | Group | Hole number | Cells ( $\bar{x} \pm s$ ) |
|---|---|---|---|
| Control gruop | | 10 | 34.6±7.3 |
| Artemin* (ng/ml) | 50 | 10 | $68.4\pm8.5^{\#}$ |
| | 100 | 10 | $95.1\pm9.7^{\#\Delta}$ |
| | 150 | 10 | $107.4\pm11.4^{\#\Delta}$* |
| | 200 | 10 | $98.4\pm10.2^{\#\Delta}$ |
| GFRα3* (ng/ml) | 50 | 10 | $59.3\pm8.7^{\#}$ |
| | 100 | 10 | $78.3\pm9.9^{\#\Delta\Delta}$ |
| | 150 | 10 | $94.4\pm9.3^{\#\Delta\blacktriangle}$ |
| | 200 | 10 | $86.5\pm9.1^{\#\Delta\blacktriangle}$ |

Note: Compared with control group, t = 4.25, 5.15, 6.26, 5.36, 3.35, 3.48, 4.54, 4.22; $^{\#}P<0.01$; 50 ng/ml compared with the group, t = 2.53, $^{\Delta\Delta}P<0.05$, t = 3.12, 3.86, 3.89, 3.05; $^{\Delta}P<0.01$; 100 ng/ml compared with the group, t = 2.73, 2.18, 2.07; $^{\blacktriangle}P<0.05$

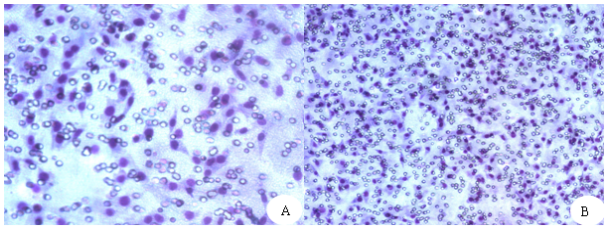


**Figure 1. Cell Invasion Analysis of Artemin and GFRα3 on MIA PaCa-2 Pancreatic Cancer cell lines.** A: Invasion MIA PaCa-2 cancer cells in the control cell line (Invasion assay, Giemsa Magnification is×200); B: Invasion MIA PaCa-2 cancer cells in 150 ng/ml artemin group cell line (Invasion assay, Giemsa Magnification is ×200)

**Table 2. MMP-2 and E-cadherin Protein Expression in Each Group Bands (Total gray, × $10^8$)**

| | control | artemin | GFRα3 |
|---|---|---|---|
| MMP-2 | 1.02±0.02 | 2.17±0.05[a] | 2.02±0.03[a] |
| E-cadherin | 1.36±0.03 | 0.65±0.04[a] | 0.74±0.01[a] |

Note: Compared with control group [a]P <0.01

The numbers of MIA PaCa-2 cancer cells through Matrigel gel in 150 ng/ml artemin group were significantly higher than normal group. Artemin dramatically increased the cell invasive properties of the MIA PaCa-2 cells when compared with control MIA PaCa-2 cells.

*MMP-2 and E-cadherin expression in pancreatic cancer cells by Western blot quantitative detection*

Multiple correlation coefficient of the regression equation was 1, and the coefficient of determination (ie, $r^2$) was 0.9864. F = 3465.349 by analysis of variance, the regression equation was valid. Regression equation was A595 (c) = 0.1431C+0.0083. Take the test, the sample tubes were measured at 595nm absorbance, the results were: 0.617, 0.832. According to the regression equation experiments, the control tube sample concentrations were: 6.4 mg/ml and 7.2 mg/ml. The results were able to meet the enhanced chemiluminescence Western blot test requirements. Application Kodak gel imaging system (Kodak digital science image station 440) quantitative analysis, band position is set according to selection, and the application of gray-scale mode (Total gray) calculated the amount of protein expression of each band. The results showed that after 150 ng / ml artemin, GFRα3 stimulated, the synthesis of MMP-2 pancreatic cancer cells was significantly increased (respectively, t = 6.35, t = 7.32), while E-cadherin was significantly lower (respectively, t = 4.27, t = 5.61), and the difference was statistically significant compared with the control group (P <0.01) Table 2.

## Discussion

Artemin (ART) is the latest to be found in the GDNF family members, consist of the 113 amino acids and seven conserved cysteine residues. Artemin, NTN and PSP is very similar (45% homology), but there are more differences with GDNF (about 30% homology). GDNF, like Artemin, NTN, and PSP have the only biologically activity forming homodimer. Artemin with its receptor complex (GFRα3/RET) tend to form a ligand/receptor complex by glycosyl-phosphatidylinositol (GPI) anchored in the cell membrane, activation of Ret, which pass signals. As shown in the literature, in adult and fetal mice, Artemin expression is present in the nervous system, concentrated in DRG, immature Schwann cells, and weakly in the brain (Baloh et al., 1998). Under physiologic conditions, Artemin is localized in vascular smooth muscle cells, in the adventitia of the dorsal aorta and arteries entering the gut (Baloh et al., 1998; Enomoto et al., 2001; Honma et al., 2002). In the normal pancreas, Artemin was faintly present in smooth muscle cells of arteries but absent in intrapancreatic nerves or ducts. Artemin widely expressed in various tumor tissues, involved in tumor development. Andres et al (Andres et al., 2001) and Baloh et al (Baloh et al., 1998; Andres et al., 2001) demonstrated the increase of neuroblastoma cell proliferation induced by Artemin. Ceyhan et al have found that Artemin and GFRα3/RET receptors were overexpressed in PDAC compared with the normal pancreas, and has a significant role in promoting proliferation and nerve infiltration, however, this effect was not observed in PAC cell lines (Ceyhan et al., 2006). Most intriguingly, however, was the ability of Artemin to increase the invasive potential of pancreatic cancer cells without affecting their proliferation. This finding may imply its contribution to the perineural invasion as well as to the dismal prognosis of pancreatic cancer patients.

Artemin is an important regulator of the induction of neuronal proliferation and regeneration under physiologic conditions (Baloh et al., 2000; Enomoto et al., 2001; Honma et al., 2002) and GFRα3 is upregulated in the distal nerve segment after sciatic transsection (Orozco et al., 2001). On the other hand, mutations of the RET receptor cause several human diseases such as papillary thyroid carcinoma, multiple endocrine neoplasia (types 2A and 2B), and Hirschsprung's disease (Takahashi, 2001). Few reports advocate that Artemin and its receptor control pancreatic cancer cell invasion and metastasis, and their exact roles in the pathogenesis process are poorly understood. The impressive presence of artemin in PDAC tissue and pancreatic cancer cells prompts the question, whether in addition to the presumed chemotactic and attractive activity, a direct effect of artemin on PAC cells.

In this experiment, MIA PaCa-2 cells were studied in vitro to observe the MMP-2, E-cadherin protein expression and the infiltration capacity from the matrix degradation to cell adhesion affected by Artemin and GFRα3 in PAC cells. Tumor cell invasion and metastasis is a complex sequential process including a number of tumor cells and tumor cells, tumor cells and host cells, tumor cells and extracellular matrix (ECM) interactions, in particular, a variety of cytokines involved in the regulation. Ultimately, it can happen that some of the successful transfer of tumor cells. Matrix metalloproteinase (MMP)-2 and MMP-9, regulated by tissue inhibitor of metalloproteinases (TIMP)-2 and TIMP-1, respectively, play important roles in the degradation of the basement membrane during biliary cancer and cholangiocarcinoma invasion, inhibition of this class of enzymes can block the ability of tumor cell invasion and metastasis (Terada et al., 1995; Jo Chae et al., 2004).

Tumor cells co-existence of different subsets, or transfer capability of different subsets of cells mixed with low capacity to transfer cells showed strong ability of invasion and metastasis, the reason may be related to different subsets between the degradation of extracellular matrix components and complementary role of the enzyme, changed the ability to transfer (McLaughlin et al., 1999). Loss and reduce the adhesion of tumor metastasis to obtain the first step. E-cadherin and cell-cell adhesion is closely related to the state to maintain, and plays an important role in the maintenance of cell differentiation, polarity and normal tissue structure (von Burstin et al., 2009). Most cancer cells have the changes of E-cad expression, gene mutation or lack of expression. In vitro experiments showed that, E-cad loss can destroy the cell-cell adhesion, resulting in infiltration capacity. A variety of tumors reduced E-cad were related with invasion and metastasis. Torer N and other findings, some adhesion molecules, including such as E-cad, Ezrin, radixin and moesin, are closely related clinical stage, lymph node invasion and survival in pancreatic cancer patients (Torer et al., 2007). Winter JM found that E-cad protein low expression or absence was related with the occurrence of pancreatic cancer (Winter et al., 2008).

Invasion chamber in vitro detection of tumor cell invasion of highly metastatic cells and screening of the classic methods to be applied to more invasion mechanism (Kao et al., 2008). In this study, the first chambers with Transwell invasion assay were applied to study the PAC cells invasiveness in vitro. The results showed that, as Artemin and GFRα3 increase of drug concentration, pancreatic cancer cells through Matrigel gel significantly increased the number of trends, 150 ng/ml as the best of both the role of concentration. Further testing by Western blot method, Artemin and GFRα3 influence on MIA PaCa-2 ECM degradation enzymes MMP-2 and E-cadherin expression was found after 150ng/ml artemin and GFRα3 stimulated, respectively. The PAC cells significantly increased synthesis of MMP-2, and E-cadherin was significantly decreased. The results indicated that Artemin and GFRα3 can promote MMP-2, reduce the biosynthesis of E-cadherin. Artemin or GFRα3 role in promoting PAC cells the migration and movement by through the increased expression of MMP-2 expression and reduced expression of E-cadherin. With the increased digestive activity of cancer cells toward the ECM, Artemin might promote their invasive behavior and direct them to intrapancreatic/extrapancreatic nerves. Similar results were presented by Okada et al, who showed that GDNF had chemokinetic effects on pancreatic cancer cells and proposed GDNF as a major mediator of celiac ganglionotropic invasion of PAC cells (Okada et al., 1999; Veit et al., 2004). Activation of MMP-9 and integrin expression in tumor cells mediated the pro-invasive activity of GDNF (Okada et al., 1999). Recently, Li et al. (2011) have found that Artemin was a direct target of miR-223 and that miR-223 may have a tumor suppressor function in esophageal carcinoma and could be used in anticancer therapies. Artemin and GFRα3/RET have been proposed as therapeutic agents for pancreatic cancer diseases on the basis of their ability to promote the motility and invasiveness.

In conclusion, We demonstrated for the first time that Artemin and its receptors GFRα3/RET may promote the migration and movement of MIA PaCa-2 cell, and have the potential to increase PAC cell invasion. The mechanism may through increased MMP-2 expression, and reduce E-cadherin expression. Some other pathway might be involved in the downstream signaling. Therefore, we hypothesize that, Artemin and its receptors GFRα3/RETmay have been proposed as novel therapeutic agents for PAC patients in neuropathic pain in malignant disorders.

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Author contributions: Meng LX and Chi YH contributed equally to this work; Meng LX performed the majority of experiments and drafted the manuscript; Chi YH edited and revised the manuscript and was involved in data interpretation; Ding ZJ, Fei LC and Cui W contributed to the literature review; Zhang H and Xue YJ designed and supervised the research and gave funding support.

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