MiR-142 modulates human pancreatic cancer proliferation and invasion by targeting hypoxia-inducible factor 1 (HIF-1α) in the tumor microenvironments

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ABSTRACT

MicroRNAs regulate most protein-coding genes, including genes important in cancer and other diseases. In this study, we demonstrated that the expression of miR-142 could be significantly suppressed in pancreatic cancer specimens and cell lines compared to their adjacent tissues and normal pancreatic cells. Growth and invasion of PANC-1 and SW1990 cells were attenuated by overexpression of miR-142 (Lai et al., 2015; Pavlakis et al., 2013). Various microRNAs, such as miR-200 (Yu et al., 2010), miR-146a (Ma et al., 2014), miR-196a (Mees et al., 2009) and the let-7 family (Li et al., 2009b) have been confirmed to be tumor suppressors; meanwhile, pancreatic cancer malignancy was found positively associated with miR-196a (Huang et al., 2014), miR-212 (Ma et al., 2014) and miR-31 (Laurila et al., 2012). We chose to focus on miR-142 because it is a potential anticancer target of concern; however, as far as we know, its potential functional mechanisms remain uncertain and need further exploration in pancreatic cancer.

Hypoxia-inducible factor 1α (HIF-1α), the oxygen-regulated subunit of heterodimeric basic-helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) transcription factor hypoxia-inducible factor 1 (HIF-1), could regulate the transcription of genes involved in oxygen homeostasis in response to hypoxia (Tan et al., 2016; Tang et al., 2011). Hypoxia is a common feature of solid tumors such as osteosarcoma (Zheng et al., 2015), colorectal carcinoma (Ghanbari et al., 2015) and liver cancer (Zhang et al., 2014). These findings suggest that miR-142 could play a tumor-inhibitive role in the above cancers; however, as far as we know, its potential functional mechanisms remain uncertain and need further exploration in pancreatic cancer.

In almost all malignancy studies, microRNA (miRNAs) are found especially critical because they can be the results of chromosome lesions, modulated by classic cell signaling, and they themselves can act as both oncogenes and tumor suppressors (Bartel, 2004; Lan et al., 2015). Recently, a close association between miRNAs and pancreatic cancer tumorigenesis has been elucidated (Lai et al., 2015; Pavlakis et al., 2013). Various microRNAs, such as miR-200 (Yu et al., 2010), miR-146a (Ma et al., 2014), miR-486 (Mees et al., 2009) and the let-7 family (Li et al., 2009b) have been confirmed to be tumor suppressors; meanwhile, pancreatic cancer malignancy was found positively associated with miR-196a (Huang et al., 2014), miR-212 (Ma et al., 2014) and miR-31 (Laurila et al., 2012). We chose to focus on miR-142 because it is closely related to many different malignancies, such as osteosarcoma (Zheng et al., 2015), colorectal carcinoma (Ghanbari et al., 2015) and liver cancer (Zhang et al., 2014). These findings suggest that miR-142 could play a tumor-inhibitive role in the above cancers; however, as far as we know, its potential functional mechanisms remain uncertain and need further exploration in pancreatic cancer.

INTRODUCTION

Pancreatic cancer is regarded as highly malignant, known for its progressive growth, early-stage metastasis, and poor response to both radiotherapy and chemotherapy. In the past 10 years, despite newly-approved therapeutic methods and large evolutions regarding nursing care, no revolutionary progress has been seen in the survival distribution and reproduction in any medium provided that the original work is properly attributed.

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In this research, it was confirmed that miR-142, known as tumor-suppressive miRNA, had a regulatory relationship with an oncogene, HIF-1α. We confirmed that miR-142 inhibited pancreatic cancer cell proliferation and invasion, partly by choosing HIF-1α as its binding site. The HIF-1α level in cells was downregulated by manual overexpression of miR-142, which also brought down the protein expression of vimentin and VEGF-C while promoting the protein expression of E-cad under hypoxic microenvironments, suggesting that miR-142 could inhibit the hypoxic microenvironment-induced epithelial-mesenchymal transition (EMT). These findings bring about novel knowledge of miR-142’s character and functional mechanism regarding the pathobiology of pancreatic cancer. Besides, a potential therapeutic blueprint for treating pancreatic cancer might be developed accordingly in the future.
RESULTS
miR-142 is largely downregulated in both pancreatic cancer tissues and cell lines and hinders the proliferation and invasion of pancreatic cancer cells

To quantify the expression level of miR-142 in pancreatic cancer tissues and cell lines, SYBR green quantitative PCR analysis was performed. Among a large group of 42 case pairs of primary pancreatic cancer tissues and their adjacent normal tissues, a significantly lower level of miR-142 was observed in pancreatic cancer tissues compared with the paired adjacent normal tissues (Fig. 1A). Besides, compared with normal cell line HPC-Y5, the miR-142 expression level was significantly lower in all four human pancreatic cancer cell lines, PANC-1, SW1990, Hup and CFPAC-1 (Fig. 1B).

Since the expression was lower in PANC-1 and SW1990 cells, the two cell lines were chosen for further experiments. The function of miR-142 in pancreatic cancer cell lines was then investigated. PANC-1 and SW1990 cell lines were transfected with miR-142 mimics or miR-142 inhibitor to achieve miR-142 overexpression or inhibition, respectively, and the transfection efficiency was verified using real-time PCR (Fig. 1C). MTT and Transwell assays were performed to monitor the cancer cell proliferation and invasion. Results from MTT assays revealed that over-expression of miR-142 inhibited the replication of the PANC-1 and SW1990 cell lines while down-regulation of miR-142 promoted the proliferation of the PANC-1 and SW1990 cell lines (Fig. 1D,E). Moreover, Transwell assays (Fig. 1F,G) showed that cell invasion ability was significantly inhibited by ectopic expression of miR-142 in PANC-1 and SW1990 cells. By comparison, when the miR-142 inhibitor silenced endogenous miR-142, the cell invasion (Fig. 1F,G) was enhanced. These results indicate that miR-142 appeared to be tumor-suppressive in pancreatic cancer cell lines in vitro.

miR-142 inhibits the expression of HIF-1α via directly binding to HIF-1α 3’ UTR

To clarify the molecular mechanism of how miR-142 inhibits the growth of pancreatic cancer cell, the TargetScan (http://www.targetscan.org/vert_71/), miRanda (http://www.microrna.org/microrna/home.do) and miRWalk (http://zmf.umm.uni-
The TargetScan, miRanda and miRWalk online tools were used to predict potential targets of miR-142. The expression levels of five candidate targets, ASH-1L, HIF-1α, HMGA1, HMG1B, TCF7, were determined in response to miR-142 overexpression in PANC-1 and SW1990 cells (Fig. 2A). Among the monitored gene candidates, HIF-1α was chosen for further experiments because the expression of HIF-1α was reduced the most significantly (Fig. 2A). The protein expression of HIF-1α was then determined in response to miR-142 overexpression or inhibition. As exhibited by western blot assays, increased miR-142 level in PANC-1 and SW1990 cell lines significantly repressed HIF-1α protein expression, and knockdown of miR-142 increased the protein level of HIF-1α as compared with negative control (NC) (Fig. 2B). These data suggested that miR-142 inhibits the expression of HIF-1α in pancreatic cancer cells.

To further investigate the underlying mechanism, a wild-type HIF-1α 3′ UTR luciferase reporter vector (wt-HIF-1α) was created to confirm this prediction, accompanied by a mutated HIF-1α 3′ UTR luciferase reporter vector (mut-HIF-1α) by manually mutating the sequence of the putative 5 base pair miR-142 binding site in the 3′ UTR region of HIF-1α (Fig. 2C). The miR-142 mimic/ inhibitor and wt-HIF-1α vector were co-transfected into the SW1990 cell line. Compared to control cells, in miR-142 mimic transfected cells the HIF-1α 3′ UTR’s luciferase activity was significantly reduced, while it was increased in miR-142 inhibitor transfected cells (Fig. 2C). Besides, the miR-142-mediated repression of HIF-1α 3′ UTR luciferase reporter activity could be abolished by mutating the putative miR-142 binding sequence in the HIF-1α 3′ UTR (Fig. 2C).

**miR-142 expression is negatively correlated with HIF-1α expression in pancreatic cancer tissues**

Next, SYBR green quantitative PCR analysis was performed to quantify the mRNA expressional level of HIF-1α in pancreatic cancer tissues. Results showed that HIF-1α expression increased in pancreatic cancer tissues compared with the adjacent normal tissues.
The expression of HIF-1α was significantly up-regulated in hypoxia (1% O₂) treatment compared to that in the normoxia (20% O₂) condition, as exhibited by real-time PCR analysis. The data are showed as mean±s.d. of three independent experiments.

**Fig. 3. MiR-142 expression is negatively correlated with HIF-1α expression in pancreatic cancer tissues.** (A) The expression of HIF-1α was determined using SYBR green quantitative PCR in pancreatic cancer tissues compared to the adjacent normal tissues in a panel of matched tissues from 42 pancreatic cancer patients. (B) Western blot analysis and (C) immunohistochemical analysis were used to measure the protein levels of HIF-1α in pancreatic cancer tissues compared to the adjacent normal tissues. (D) An inverse correlation between miR-142 and HIF-1α expression levels were observed. **P<0.01; one-way ANOVA and Spearman’s rank correlation analysis. The data are showed as mean±s.d. of three independent experiments.

**miR-142/ HIF-1α axis regulates hypoxia-induced cell proliferation and invasion**

According to previous studies, hypoxia has a close association with cancer cell invasion (Chouaib et al., 2012; Raja et al., 2014; Tan et al., 2015; Tsujinaka et al., 2011). Given the key role of HIF-1α in the regulation of genes associated with angiogenesis, tumor resistance, invasion and metastasis under hypoxia conditions (Kim et al., 2015; Li et al., 2009a; Park et al., 2010), further experiments were arranged to investigate how miR-142/HIF-1α axis regulates tumor biological function in hypoxic microenvironment in pancreatic cancer. The protein expression of HIF-1α in SW1990 cells was significantly up-regulated in hypoxia (1% O₂) treatment compared to that in the normoxia (20% O₂) condition, as exhibited by western blot assays (Fig. 4A). Inversely, the expression of miR-142 was repressed in hypoxia (1% O₂) treatment compared to that in the normoxia (20% O₂) condition, as exhibited by real-time PCR assays (Fig. 4B). si-HIF-1α was transfected into SW1990 cells to achieve HIF-1α knockdown, and the inhibitory efficiency was verified using western blot assays (Fig. 4C). The proliferation and invasion of SW1990 cells in response to miR-142 overexpression or HIF-1α knockdown in hypoxia (1% O₂) treatment or normoxia (20% O₂) condition was then monitored using MTT and Transwell assays (Fig. 4D,E). Results showed that hypoxia could significantly promote SW1990 cell proliferation and invasion compared with that of the normoxia condition, while the promotive effect of hypoxia on SW1990 cell proliferation and invasion could be partly restored by miR-142 overexpression and HIF-1α knockdown (Fig. 4D,E). As exhibited by the western blot assays, the protein expression of HIF-1α, vimentin and VEGF-C was up-regulated, while the protein expression of E-cad was down-regulated by hypoxia, suggesting that EMT transformation was induced in the hypoxic microenvironment (Fig. 4F). After either HIF-1α knockdown or miR-142 overexpression, the effect of hypoxia on the above factors could be partly restored, thus regulating the cell proliferation and invasion (Fig. 4F). Taken together, miR-142/HIF-1α axis regulates hypoxia-induced cell proliferation and invasion of pancreatic cancer.

The correlations between miR-142/HIF-1α axis and the pathological stage of pancreatic cancer

To validate the correlation between miR-142/HIF-1α axis and the pathological stage in pancreatic cancer, 42 pancreatic cancer cases were analyzed, and the relative expression levels of HIF-1α and miR-142 according to clinicopathological parameters were exhibited in Table 1. As exhibited, the expression of HIF-1α (P=0.016) and miR-142 (P=0.038) was positively or negatively correlated with the stage of pancreatic cancer, respectively, and miR-142 was negatively correlated with lymphatic metastasis (P=0.043). Taken together, miR-142/HIF-1α axis was correlated with the pathological stage of pancreatic cancer, and miR-142 was correlated with lymphatic metastasis of pancreatic cancer.

**DISCUSSION**

Abnormalities in miRNAs’ expression are observed almost in all fields of malignant biology, such as cell replication, apoptosis, invasion and/or metastasis, and they may serve as either tumor inhibitors or promoters (Hwang and Mendell, 2007). In this study, we chose miR-142 as the research aim because of its potential suppressive function in human malignancies. Song et al. (2009) indicated that cell proliferation in both colorectal carcinoma and osteogenic sarcoma cell lines was inhibited by overexpression of miR-142. Nevertheless, so far, miR-142’s role in pancreatic cancer tumorigenesis and the detailed functional mechanisms of how miR-142 performs remain uncertain. In our
study, the real-time PCR results indicated that miR-142’s expression level was obviously down-regulated both in pancreatic cancer tissues and cell lines. Manual enhanced expression of miR-142 significantly inhibited the proliferation and invasion of pancreatic cancer cells. Conversely, knocking down of miR-142 might accelerate cell growth and invasion. The above results expressed that miR-142 could potentially be tumor-suppressive in pancreatic cancer.

Since the effect of certain miRNAs on carcinogenesis depends on their downstream targets (Chen et al., 2015; Profumo et al., 2015), TargetScan, miRanda and miRWalk online tools were used to predict the candidate targets of miR-142. Among five candidates, HIF-1α was shown to be the most significantly down-regulated by miR-142 overexpression. Overexpression of miR-142 could down-regulate the protein level of HIF-1α, while knockdown of miR-142 could up-regulate the expression of HIF-1α. The luciferase reporter

Fig. 4. miR-142/ HIF-1α axis regulates hypoxia-induced cell proliferation and invasion. (A) The protein expression of HIF-1α was determined in hypoxia (1% O₂) treatment compared to that in the normoxia (20% O₂) condition as exhibited by western blot assays. (B) The expression of HIF-1α was determined in hypoxia (1% O₂) treatment compared to that in the normoxia (20% O₂) condition as exhibited by real-time PCR assays. (C) si-HIF-1α was transfected into SW1990 cells to achieve HIF-1α knockdown. The transfection efficiency was verified using western blot assays. (D) The SW1990 cells’ proliferation in response to miR-142 overexpression or HIF-1α knockdown in hypoxia (1% O₂) treatment compared to that in the normoxia (20% O₂) condition was monitored using MTT assays. (E) The SW1990 cells’ invasion in response to miR-142 overexpression or HIF-1α knockdown in hypoxia (1% O₂) treatment compared to that in the normoxia (20% O₂) condition was monitored using Transwell assays. (F) The protein expression of HIF-1α, E-cad, vimentin and VEGF-C was monitored in response to miR-142 overexpression or HIF-1α knockdown in hypoxia (1% O₂) treatment compared to that in the normoxia (20% O₂) condition using western blot assays. **P<0.01; one-way ANOVA. The data are showed as mean±s.d. of three independent experiments.
Table 1. Relative expression levels of HIF-1α and miR-142 according to clinicopathological parameters

| Parameter            | Total | High exp | Low exp | P-value | High exp | Low exp | P-value |
|----------------------|-------|----------|---------|---------|----------|---------|---------|
| Gender               |       |          |         |         |          |         |         |
| Male                 | 23    | 7        | 16      | 0.432   | 15       | 8       | 0.826   |
| Female               | 19    | 8        | 11      | 0.747   | 13       | 6       |         |
| Age                  |       |          |         |         |          |         |         |
| <50                  | 21    | 7        | 14      | 0.747   | 13       | 8       | 0.513   |
| ≥50                  | 21    | 8        | 13      | 0.038   | 9        | 10      | 0.016   |
| TNM staging          |       |          |         |         |          |         |         |
| I                    | 19    | 10       | 9       | 19      | 9        | 10      | 0.138   |
| II                   | 10    | 2        | 6       | 6       | 3        | 5       |         |
| III–IV               | 23    | 5        | 18      | 21      | 16       | 5       |         |
| Differentiation      |       |          |         |         |          |         |         |
| High                 | 8     | 2        | 6       | 0.781   | 3        | 5       |         |
| Median               | 21    | 8        | 13      | 0.394   | 17       | 9       | 0.822   |
| Low                  | 13    | 5        | 8       | 0.043   | 11       | 9       | 0.126   |
| Pathological type    |       |          |         |         |          |         |         |
| Tubular              | 26    | 8        | 18      | 17      | 9        | 0       |         |
| Papillary            | 16    | 7        | 9       | 11      | 11       | 5       |         |
| Lymphatic metastasis |       |          |         |         |          |         |         |
| Positive             | 20    | 4        | 16      | 0.043   | 11       | 9       | 0.126   |
| Negative             | 22    | 11       | 11      | 17      | 9        | 4       |         |

In summary, it was identified that through targeting HIF-1α, pancreatic cancer cell proliferation and invasion are inhibited by miR-142 expression. miR-142/HIF-1α axis was correlated with the stage of pancreatic cancer, and miR-142 was correlated with lymphatic metastasis.

In conclusion, the results of our study suggest that miR-142 is a promising therapeutic target for pancreatic cancer treatment in the future.
After 4 h of incubation, the supernatant was removed so that the reaction could be stopped. Then, to dissolve the formazan product, 100 μl DMSO could be added. Then, to dissolve the formazan product, 100 μl DMSO could be added.

**Transwell assay**

Evaluation of the invasive and migratory potential of cells was performed with Transwell inserts with 8 μm pores (Corning, New York, USA). For invasion assay, each upper insert was seeded with 3×10^4 cells suspended in serum-free medium at 24 h after transfection. The upper inserts were pre-coated with matrigel matrix (BD, New York, USA). The lower chambers were each filled with 500 μl medium containing 10% FBS. 48 h later, we used a cotton swab to remove non-invaded cells from the upper surface of the Transwell membrane. Then, on the lower membrane surface, the invaded cells were fixed with methanol, stained with 0.1% crystal violet, photographed and counted under a microscope. For migration assay, similar procedures were conducted, except that 2×10^4 cells were applied and the inserts were not pre-coated by matrigel. Six random fields were counted at 100× magnification for each insert. This experiment was conducted three times separately.

**Western blot analysis**

The protein expression of HIF-1α, E-cadherin, vimentin and VEGF-C in pancreatic cancer cells was detected by immunoblotting. RIPA buffer containing 1% PMSF was used to lyse the cells. Proteins were then loaded onto a 15% SDS-PAGE minigel and transferred onto PVDF membrane under an electric field. Then the blots were probed with 1:1000 diluted rabbit polyclonal antibodies against HIF-1α, E-cadherin, vimentin and VEGF-C (Abcam, USA) at 4°C overnight. After that, we incubated the blots with HRP-conjugated secondary antibody (1:5000). ECL substrates were used to visualize signals (Millipore, Billerica, USA). For normalization, the endogenous ß-actin was also tested.

**Luciferase reporter assay**

After seeding into a 24-well plate, SW1990 cells were cultured overnight, then co-transfected with the wild-type/mutated HIF-1α 3′ UTR luciferase reporter vector and pRL-TK plasmids, or transfected with miR-142 mimics/antisense/miR-142 inhibitor. 48 h after transfection, the luciferase assays were performed with the help of the Dual Luciferase Reporter Assay System (Promega, Madison, USA).

**Hypoxia treatment**

SW1990 cells or miR-142 mimics or si-HIF1-α transfected cells were seeded into 6-well plates or Transwell upper inserts, cultured in hypoxia chamber in a humidified atmosphere with 94% N₂, 5% CO₂ and 1% O₂ for 24 h then change to normoxia for additional 24 h for real-time PCR, western blot analysis and Transwell assay. For MTT assay, cells seeded in 96-well plates and cultured in hypoxia condition for 24 h then change to normoxia for additional 24, 48, 72 and 96 h.

**Statistical analysis**

Data were processed using SPSS 17.0 statistical software (SPSS, Chicago, IL, USA), and displayed as mean±s.d. of three independent experiments. By guest on January 10, 2021http://bio.biologists.org/Downloaded from

**Author contributions**

Conceptualization: W.W. and W.S.; Formal analysis and investigation: Y.L. and X.G.; Writing-original draft preparation: Y.L.; Writing-review and editing: W.W. and N.J.; Funding acquisition: W.S. and W.W.; Resource: X.W. and N.J.
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