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Hypoxia induces the overexpression of MicroRNA-21 in pancreatic cancer cells

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Abstract

Background—Pancreatic cancer cells exist in a hypoxic microenvironment containing numerous factors which impact tumor survival, proliferation and metastasis. MicroRNAs (miRs) are differentially expressed in cancer but also altered by hypoxia. We hypothesized that hypoxia could induce miR-21 expression, an oncomir in pancreatic cancer cells.

Materials and Methods—We examined how hypoxia regulates miR-21 expression in pancreatic cancer cell lines (BxPC-3, AsPC-1) by stem-loop RT-PCR. Chromatin immunoprecipitation (ChIP) assays were used to study how hypoxia alters HIF-1 binding to the hypoxia response element of miR-21. BxPC-3 and AsPC-1 cells were transfected with a constitutively stable HIF-1 subunit or vector control (pcDNA3.1) to determine the influence of miR-21 in normoxia. The effect of mature miR-21 sense and antisense oligonucleotides on proliferation and apoptosis in hypoxic and normoxic conditions was assessed via WST-1 assay and flow cytometry.

Results—MiR-21 levels increased in all cell lines grown in hypoxic conditions versus normoxia while siRNA targeting HIF-1 reduced miR-21 expression. Hypoxic conditions resulted in direct binding of HIF-1 to the predicted binding site in miR-21. Transfection with a constitutively stable HIF-1 expression plasmid in normoxia resulted in upregulated miR-21, similar to that seen in hypoxia. Cells transfected with antisense constructs targeting miR-21 had reduced proliferation and increased apoptosis in normoxia whereas miR-21 overexpression abrogated hypoxia-associated reductions in proliferation.

Conclusions—MiR-21 is induced by hypoxia in pancreatic cancer cells via HIF-1 upregulation. MiR-21 overexpression allows cells to avoid apoptosis in a hypoxic

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microenvironment. Inhibition of miR-21 expression may increase cellular susceptibility to hypoxia in pancreatic cancer.

Keywords

Pancreatic cancer; hypoxia; micro-RNA; HIF-1alpha

INTRODUCTION

Pancreatic cancer is the fourth most common cause of cancer-related death in the United States.^{1, 2} In the best of circumstances, when disease is confined to the pancreas without lymphatic or hematogenous spread, five-year overall survival remains less than 30% with a median survival of approximately 18 - 24 months.³ Unfortunately, the majority of patients are not amenable to resection due to locally advanced or metastatic disease. This dismal prognosis owes to the aggressive nature of the disease resulting in advanced stage at the time of presentation and its inherent resistance to chemotherapy and radiation. Much research has focused on identifying gene expression patterns that might explain its pathogenesis and aggressive nature. Thus far, results have been disappointing.

Hypoxia is an essential component of the neoplastic microenvironment often allowing a selective advantage for tumor cells over otherwise non-invasive cells more sensitive to a low oxygen state. The evidence for hypoxia in pancreatic cancer is in their characteristic avascular appearance on computed tomography⁴ and from intratumoral oxygen tension measurements.⁵ Hypoxic conditions in solid malignancies may confer resistance to conventional radiation and chemotherapy.^{6, 7} A functional link between hypoxia and microRNA expression in colon and breast cancer cell lines was shown by Kulshreshtha *et al.*⁸ and in several other cancers.⁹⁻¹¹ Interestingly, the majority of hypoxia-associated microRNAs predicted *in silico* are differentially overexpressed in pancreatic cancer.

MicroRNA research holds the promise of providing molecular insight into carcinogenesis and disease progression. These small non-coding RNAs, when in mature form, are incorporated into the RNA-induced silencing complex (RISC) where they act by binding to a conserved sequence of the 3' untranslated region of protein-coding target genes, leading to mRNA degradation or translational inhibition depending on perfect or imperfect complementarity, respectively.¹² Target genes involved in apoptosis or cell cycle progression allow microRNAs to act as oncogenes and tumor suppressor genes.¹³⁻¹⁵ These *oncomirs* have been described in both hematologic and solid tumors. One such miR, miR-21, is overexpressed in multiple malignancies including pancreatic, esophageal, lung, breast and colon cancers.¹⁶⁻²⁰ This oncogenic miR has been linked to tumor aggression and carcinogenesis, in part, by preventing apoptosis.^{21, 22} MiR-21 is significantly overexpressed in pancreatic cancer as detected by in situ hybridization and its strong expression predicts limited survival in patients with node-negative disease.²³ Little is known, however, of the mechanism by which upregulation of miR-21 occurs.

In this study we explored the role of hypoxia in pancreatic cancer. Of particular interest was the hypoxic regulation of miR-21 and its relevance in tumor pathogenesis. We show that miR-21 is upregulated in pancreatic cancer cells in response to hypoxia through expression of hypoxia inducible factor (HIF)-1, and that miR-21 expression allows for cell survival in the hypoxic environment.

MATERIALS AND METHODS

Cell lines and culture conditions

Human pancreatic cell lines (AsPC-1 and BxPC-3) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS) in 21% O₂ and 5% CO₂ at 37°C. Hypoxia was induced in a hypoxic chamber (Billups-Rothenberg, Inc. Del Mar, CA) with 1% oxygen in parallel with cells maintained in normoxia. O₂ and CO₂ were measured and controlled using dual flow meters in order to monitor, measure, and keep concentrations consistent between experiments.

Real-time reverse transcription PCR (qRT-PCR)

RNA was extracted from cells using TRIzol (Invitrogen) according to manufacturer's recommendations. RNA quality and quantity were determined by the Nanodrop ND-1000 Spectrophotometer (Nanodrop Products, Wilmington, DE). After treatment with DNase (Invitrogen, CA, USA), 20ng of RNA was reverse transcribed into cDNA with Taqman Reverse Transcriptase Kit (Applied Biosystems, Inc, CA, USA). Real time RT-PCR was performed using microRNA-21-specific primers from the Taqman MicroRNA Assay using the Ct method and normalized with snoRNA U48.

Chromatin immunoprecipitation (ChIP) assay

Samples were cross-linked using 1% formaldehyde for 10 minutes and reactions stopped with 125mM glycine for 5 minutes. Cells were washed twice with 5ml of ice-cold phosphate-buffered saline (PBS) followed by centrifugation at 1500rpm 4°C for 10 minutes. Lysis buffer was supplemented with protease inhibitors (CalBiochem, CA, USA) and added to pellets followed by 10 minutes incubation on ice. Sonication was performed for DNA shearing to fragments between 200 bp and 1 kb. Supernatant was recovered and diluted in ChIP dilution buffer (Millipore Corp, MA, USA), followed by immunoprecipitation overnight at 4°C with polyclonal anti-HIF-1 antiserum (Abcam, Inc, MA, USA). Negative control beads (MOCK) were prepared in the absence of anti-HIF-1 antiserum. Immunocomplexes were recovered by addition of 50% slurry of salmon sperm DNA-protein A-agarose to samples and washed. The immunoprecipitated DNA was retrieved from the beads by incubation in elution buffer at 65°C for 3h. Cross-linking was reversed using 200mM NaCl at 65°C overnight followed by proteinase K digestion at 45°C for 2h. DNA was then purified using a PCR purification kit (Qiagen, Inc, CA, USA), and PCR was performed with primers designed to span the region of hypoxia response elements (HRE) predicted for HIF-1 in the 5' direction of miR-21.⁸ CTGAGGCAAAGGGAAATG (forward); GAGACCAGCCTGGCTAACAC (reverse). PCR products were amplified from 1µg, resolved on an 1.5% agarose gel and visualized by ethidium bromide staining.

Transient Transfection

Cells were transfected with the precursor of miRNA-21 or negative Control#1 (Ambion, TX, USA) and antisense oligo of miR-21 (Exiqon, Denmark) at final concentrations of 100 nM using LipofectAMINE 2000 reagent (Invitrogen, CA, USA). The transfection mixture was dissolved in Opti-MEM serum free media (Invitrogen) and at the time of transfection cells were seeded in medium with 10% FBS and no antibiotics. Six hours after transfection, medium was exchanged for full growth medium.

Cell proliferation

For cell proliferation, cells were seeded in a 96-well plate (5000 cells/well). After transfection cell viability was determined at 48h using WST1 reagent (Roche, CA, USA)

according to the manufacturer's protocol. Absorbance at 490 nm was measured via kinetic microplate reader (Spectra Max 190; Molecular Devices, CA, USA). Experiments were done in triplicate and repeated three times.

Plasmids

Mutant versions of HIF-1 with double proline-to-alanine substitutions making it constitutively active have been described.²⁴ The three-HRE-thymidine kinase (tk)-luciferase reporter is a HIF-responsive construct containing a tandem of hypoxia-responsive elements from the erythropoietin promoter. Plasmid was provided as a kind gift from Dr. Mircea Ivan (Indiana University).

Western blot analysis

Whole-cell extracts were prepared in 300µl of ice-cold lysis buffer (10mM TRIS-HCL, 150mM NaCl, 1% TRITON X-100, 2mM EDTA, 0.5% NP40) with protease inhibitors Cocktail II (Calbiochem, Germany), 48 hours after transfection. Cells were washed with ice cold PBS and lysed. 60 µg of protein was separated on 4-20% polyacrylamide gels (BioRad, CA, USA). Immunoblot analysis was performed using the following antibodies: anti-HIF-1 dilution 1:200 (BD Transduction Laboratories, CA, USA), and anti-vinculin – dilution 1:500 (Santa Cruz, CA, USA). Secondary horseradish peroxidase (HRP) antibody was detected using ECL Western Analysis System (Amersham Bioscience, NJ, USA).

Apoptosis Analysis

At 48h after transfection with anti miR-21, cells were collected, washed with cold PBS and stained with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI). The BD Pharmingen Detection Kit for Annexin V was used according to manufacturer's protocol. Cells that were FITC-/PI- were considered viable, FITC+/PI- cells were considered early apoptotic, and FITC+/PI+ cells were considered nonviable.

Statistics

All experiments were completed in triplicate and repeated. Continuous data were compared by Student's T test. Data are presented as mean ± standard deviation unless otherwise stated. $p < 0.05$ was considered significant.

RESULTS

MiR-21 expression is upregulated by hypoxia in vitro

Pancreatic cancer cell lines, BxPC-3 and AsPC-1, were cultured for 48 hours in normoxic or hypoxic conditions and miR-21 expression was assessed by RT-PCR. Cells cultured in hypoxic conditions for 48 hours resulted in significantly increased expression of HIF-1 (Fig. 1A, $p = 0.0015$). Given our previous report of differential miR-21 overexpression in resected human pancreatic cancers,¹⁹ we first sought to define the impact of hypoxia on miR-21 expression by qRT-PCR in human pancreatic cancer cell lines. AsPC-1 and BxPC3 cultured in hypoxic conditions for 48 hours showed increased miR-21 expression (Fig. 1B). Chromatin immunoprecipitation (ChIP) assay were used to assess recruitment of HIF-1 to the predicted hypoxia response element (HRE) region for miR-21 under normoxic and hypoxic conditions. The HIF-1 binding site within the predicted region of miR-21 was enriched, indicating direct binding of miR-21 by HIF-1 (Fig. 1C). The anti-HIF-1 antiserum (but not MOCK control) immunoprecipitated the miR-21 fragment in hypoxic AsPC-1 cells but very little in the normoxic cells.

MiR-21 induction in hypoxia is dependent on HIF-1 α

The role of HIF-1 in hypoxia-regulated miR-21 expression was first tested by RNA interference. Under hypoxic conditions, silencing of HIF-1 in the BxPC-3 cell line attenuated the miR-21 overexpression (Fig. 2). A similar trend was observed in AsPC-1, but did not reach statistical significance. Next, we transduced BxPC-3 and AsPC-1 cell lines with a constitutively stable HIF-1 subunit or vector-only control (pcDNA3.1) under normoxic conditions. HIF-1 stabilization was achieved by altering the two alpha subunits that are subjected to oxygen-dependent hydroxylation and proteasomal degradation via VHL-dependent ubiquitylation.²⁴ The presence of exogenous HIF-1 after transfection was confirmed via immunoblot (Fig. 3A). Constitutively stable HIF-1 resulted in increased miR-21 expression in both pancreatic cancer cell lines (Fig 3B).

miR-21 increases proliferation and overrides hypoxia-induced cell cycle arrest

Hypoxia treatment induces cell cycle arrest at the G1/S transition.²⁵ In normoxia, when HIF-1 is destabilized and not functional, miR-21 overexpression did not significantly increase cell proliferation whereas inhibition of miR-21 did decrease proliferation. Conversely in hypoxia, miR-21 inhibition did not further reduce proliferation but increasing miR-21 expression resulted in increased proliferation (Fig. 4).

miR-21 enhances pancreatic cancer cell survival

Transfection of cells with anti-miR-21 resulted in a modest decrease in cell proliferation. Thus we determined the role of miR-21 on cell survival. BxPC-3 (and AsPC-1 data not shown) transfected with anti-miR-21 had greater apoptosis as compared to controls either in normoxic or hypoxic conditions (Fig. 5).

DISCUSSION

MicroRNA are often dysregulated in tumors and have therefore been classified as oncomirs, which modulate cell proliferation and survival.^{26, 27} miR-21 was among the first oncomirs identified, and is overexpressed in multiple histologic subtypes of cancer including glioblastoma, lung cancer and leukemia.^{28, 29} Increased miR-21 levels in pancreatic cyst fluid have been shown to be a predictive marker for ductal adenocarcinoma.³⁰ Other recent studies also provide evidence that miR-21 can upregulate Bcl-2, leading to chemoresistance in pancreatic cancer cells.^{31, 32} Prior studies from our group have identified a microRNA expression profile unique for pancreatic cancer and identified that miR-21 expression inversely correlated with overall survival¹⁹ and was also expressed in human pancreatic cancer cell lines.^{23, 32} In the present study we demonstrate that miR-21 is regulated by hypoxia-induced HIF-1 expression in human pancreatic cancer cell lines. We found that hypoxia-induced regulation of miR-21 through expression of HIF-1 enhanced survival and decreased apoptosis in human pancreatic cancer cell lines. It is known that the majority of tumors from pancreatic cancer patients express mutated KRAS.^{19, 33} In the present study we found that even cell lines with wild-type KRAS (BxPC-3) showed an increase in miR-21 expression under hypoxic conditions and relied on miR-21 for survival. Future studies in both wild-type and KRAS mutant pancreatic tumors will be important to further characterize the relationship between miR expression and individual molecular subtypes of pancreatic cancer. Importantly these data uncover a novel role for miR-21 in pancreatic cancer and suggest it may be a relevant therapeutic target for this disease.

The tumor microenvironment is characterized by dysregulated blood flow, angiogenesis, and a lack of oxygen, which can modulate tumor gene expression and function.^{34, 35} Other groups have shown that oncomir expression in tumor cells can be modulated by expression of HIF-1.^{36, 37} Norman *et al.* provides evidence that hypoxia increases miR-210 in lung and

melanoma cells resulting in silencing of genes that regulate immune recognition by cytotoxic T cells.³⁸ Another study showed that hypoxic induction of miR-210 mediates the metastasis of hepatocellular carcinoma.³⁹ miR-155 is another oncomir regulated by hypoxia in lung cancer cells and its increased expression protects cells from radiation damage.⁴⁰ Evidence of hypoxia regulating oncomirs in other cancers has been well documented. Studies in cell lines from other solid tumors such as breast, colon, and renal have observed a role for miR-21 in regulating cell growth and survival.^{10, 41, 42} However, the role of hypoxic conditions in regulating oncomir expression in pancreatic cancer is poorly understood.

This study primarily focused on how hypoxia regulates miR-21 since this oncomir has a significant role in pancreatic cancer. Indeed, pancreatic cancer cell lines incubated in hypoxic conditions increased miR-21 expression. Also, attenuating HIF-1 expression via siRNA under hypoxic conditions abrogated induction of miR-21 expression. Finally, pancreatic cancer cells transfected with miR-21 siRNA cultured under both normoxic and hypoxic conditions had decreased proliferation and greater apoptosis compared to mock-transfected cells. These data support the role for basal expression of miR-21 as an essential regulator of pancreatic cancer cell survival under both normoxic and hypoxic conditions. Our data suggests that hypoxic conditions in the pancreatic tumor microenvironment can increase the HIF-1 expression, leading to increased miR-21 and tumor cell survival. This work has elucidated a novel and important role for miR-21 as an oncomir that is regulated by the hypoxic environment. The present study focused only on one important oncomir, miR-21, which has documented importance for pancreatic cancer cell survival. However we are aware that numerous additional oncomirs may be regulated by hypoxia. Indeed, using an array approach, a recent study in retinoblastoma demonstrated that hypoxia upregulates several miRs including miR-181, miR-125, and miR-491.⁴³ Importantly, further studies will enhance our understanding of the redundancy across individual miRs that are regulated by hypoxia in pancreatic cancer.

MiR-21 is becoming recognized as an important oncomir that modulates various cellular functions including cell proliferation and survival when expressed in pancreatic cancer cells. This study showed that miR-21 expression is regulated by HIF-1 and provides rationale for treatment modalities that reduce hypoxia in the tumor microenvironment. This approach could reduce miR-21 expression, and potentially improve of targeted therapies for pancreatic cancer.

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Figure 1. Hypoxia induces MiR-21 and recruits HIF-1 to MiR-21 promoter in pancreatic cancer cell lines

A) Cells were grown in 21% (normoxia) or 1% oxygen (hypoxia) for 48 hours. MiR-21 was assayed by qRT-PCR and normalized to snoRNA U48. B) Immunoblot of HIF-1 from lysates of cells cultured at normoxic or hypoxic conditions. C) ChIP analyses of recruitment of HIF-1 to the predicted hypoxia response element (HRE) region of miR-21 following treatment of AsPC-1 cells grown at normoxic or hypoxic conditions for 72 hours.

Figure 2. MiR-21 upregulation by hypoxia is attenuated by inhibition of HIF-1 with siRNA
BxPC-3 and AsPC-1 cells were transfected with HIF-1 siRNA and cultured in normoxic or hypoxic conditions for 48 hours and miRNA expression was assessed by RT-PCR.

Figure 3. HIF-1 overexpression in normoxia

A) Western blot of HIF-1 expression 48 hours after transfection with constitutively stable pcDNA HIF-1 ; B) MiR-21 overexpression through transfection of the cells with pcDNA HIF-1 by qRT-PCR in normoxia

A)

Figure 4. miR-21 increases proliferation and overrides hypoxia-induced cell cycle arrest
AsPC-1 cells were transfected with mature miR-21, antisense to miR-21 and NC-non coding and were cultured for 48 hours in normoxic or hypoxic conditions. Proliferation was measured by WST-1 assay.

Figure 5. Pancreatic cancer cell survival is attenuated by anti-miR21 siRNA

AsPC-1 cells were treated with 100nm anti-miR21 siRNA were harvested after 48 hours of incubation and using Annexin V-FITC was stained to assess apoptosis.