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Original Article

HIF-1α regulates miR-210 to affect biological behavior of breast cancer cells

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Abstract: Background: Rapidly growing tumors outstrip their vascular supply and become hypoxic, and hypoxia is the characteristic of most solid tumors. Hypoxia related to tumor invasion, angiogenesis and apoptosis, it increased the risk of metastasis and affected the prognosis. As a hypoxia inducible product, HIF-1α and miR-210 expressions significantly up-regulated in the breast cancer tissues, and were closely related to the occurrence, development and the prognosis of breast cancer. This study explored the regulatory effect of hypoxia inducible factor-1α (HIF-1α) on microRNA-210 (miR-210) and its impact on proliferation, invasion, and apoptosis of breast cancer cells. Methods: Plasmid DNA expressing short hairpin RNA (shRNA) targeting HIF-1α was constructed and transfected into MCF-7 cells by Lipofectamine 2000. The expression level of gene and protein HIF-1α and miR-210 were detected by quantitative Real-time PCR technology or Western blot, respectively. Effect of Effect of HIF-1α gene knockdown on MCF-7 cells was also investigated. Results: Knockdown of HIF-1α gene decreased expression level of miR-210, weakened the ability of cell proliferative and invasion, and increased apoptosis of MCF-7 cells. Conclusion: HIF-1α may affect growth and invasion of breast cancer through positive regulation of miR-210 expression.

Keywords: Hypoxia inducible factor-1α, microRNA-210, breast cancer, regulation, biological behavior

Introduction

Breast cancer is a major cause of cancer-related deaths in women worldwide, and local recurrence and distant metastasis are major factors affecting poor patient prognosis [1-3]. Hypoxia, or inadequate tissue oxygenation, within tumors has negative implications for survival of breast cancer patients independently of other prognostic parameters, such as clinical tumor stage, tumor histology grade, or lymph node status. Hypoxic tumors are associated with a more aggressive cancer phenotype, increased risk of metastasis, increased tumor resistance to radiotherapy and chemotherapy, and cancer immune suppression [4-6].

In response to hypoxic stress, cells can alter gene transcription through oxygen-monitoring machinery such as hypoxia-inducible factors (HIFs), which are a major component of hypoxia signaling pathways. HIF-1 is a heterodimer composed of one oxygen-sensitive subunit, HIF-1α, and one oxygen-insensitive subunit, HIF-1β. Under normoxia or normal oxygen levels, HIF-1β is active, and prolyl hydroxylases hydroxylate specific proline residues (Pro402 and Pro564 in HIF-1α) that recruit proteasomes to degrade HIF-1α [7, 8]. In hypoxic tumor microenvironments, oxygen-dependent HIF-1α degradation is halted, allowing HIF-1α to accumulate.

Recent studies suggest that HIF-1α could specifically regulate cellular adaptation to hypoxia through recruitment of microRNAs (miRs) [9, 10]. miRs are small, single-stranded noncoding RNAs that regulate protein expression by either inhibiting messenger RNA (mRNA) translation or promoting mRNA degradation. A single miR can affect the translation and activity of more than 100 mRNAs, suggesting that miRs have a wide influence over cellular processes [11].

miR-210 is a hypoxia-regulated miR that has been investigated extensively in cancer. miR-210, like most miRs, has a 6-8-nucleotide-long seed sequence that recognizes and binds com-

Table 1. qPCR primer sequences

Primer	Sequence
Specific stem-loop	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGCC-3'
miR-210	Forward: 5'-CGCCTGTGCGTGTGA CAGCG-3'
	Reverse: 5'-GTGCAGGGTCCGAGGT-3'
U6	Forward: 5'-GCTTCGGCA GCACATATACTAAAAT-3'
	Reverse: 5'-CGCTTCACGAATTTGCGTGTCAT-3'

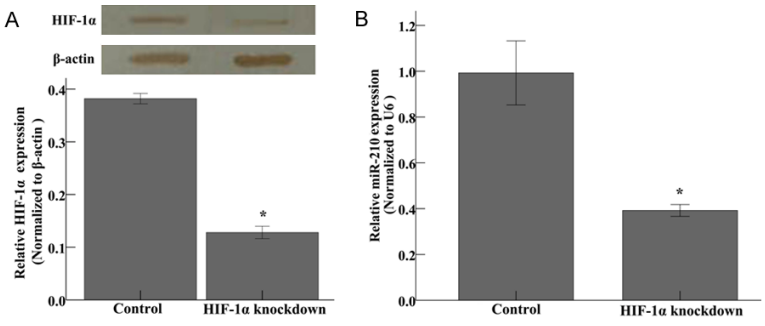

Figure 1. A: Representative western blot and corresponding quantification of relative HIF-1α expression in protein extracts from negative control oligos MCF-7 cells or cells transfected with HIF-1α shRNA. β-actin was used as a loading control. B: Relative miR-210 expression from qPCR of reverse-transcribed RNA extracts from negative control oligos MCF-7 cells or cells transfected with HIF-1α shRNA. $^{*}P < 0.05$.

plementary pairing sites on target 3'UTRs. Limited genes have been confirmed as bona fide targets of miR-210 thus far. Therefore, previous studies have used prediction software, such as TargetScan and PicTar, to search for potential miR-210 target mRNAs in silico by matching 3'UTRs to the seed region of miR-210. In addition to the 3'UTR, studies examining 5'UTRs and mRNA coding regions also have revealed target pairing sites complementary to the seed region of miR-210 [12].

Hypoxia induces miR-210 overexpression, and its expression levels in breast cancer are an independent prognostic factor for patient survival [13]. However, it is unclear whether HIF-1α binds to miR-210 seed regions to regulate miR-210 expression and further affect breast cancer cell behavior. Therefore, we inhibited HIF-1α expression and examined the effects on miR-210 expression and proliferation, invasion, and apoptosis of breast cancer cells to analyze biological effects of miR-210 regulation by HIF-1α.

Materials and methods

Cell culture

Human breast cancer cell line MCF-7 was obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and was maintained in DMEM (Solarbio, Beijing, China) supplemented with 10% fetal bovine serum (FBS, Sijiqing, Hangzhou, China) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Cell transfection

4×10^5 cells/ml MCF-7 cells were seeded and grown overnight in six-well plates. The next day, cells at a confluence of 80%~90% were transfected with 5 μg/ml shRNA targeting HIF-1α (Genesil, Wuhan, China) or negative control oligonucleotides using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions.

Western blot analysis

Seventy-two hours after transfection, the cells were washed twice with cold PBS and total cellular protein was extracted using Epiquik whole cell extraction kit (Epigentek, Farmingdale, NY, USA). Protein extracts (20 μg) were resolved on SDS-PAGE and transferred to PVDF membranes. The membranes were then blocked with 5% nonfat milk powder, and following overnight incubation of membranes with primary antibodies against HIF-1α (1:500, Beyotime, Shanghai, China), specific reactive bands were detected using an anti-mouse IgG secondary antibody (1:1000, Beyotime, Shanghai, China) conjugated to horseradish peroxidase. Immune-reactive bands were visualized with a DAB detection kit (Beyotime, Shanghai, China). Equal loading was verified using an anti-β-actin antibody (Beyotime, Shanghai, China); all assays were measured in triplicate from experiments performed 3 times.

Quantitative real-time PCR

Expression of miR-210 and reference gene *U6* was measured by quantitative real-time PCR

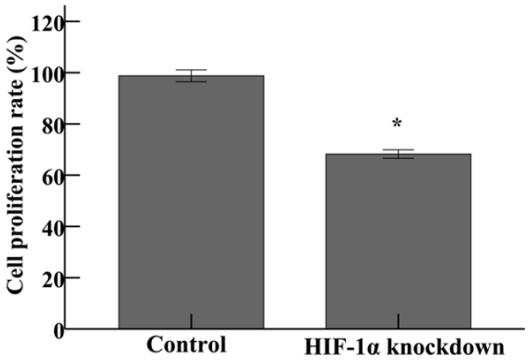

Figure 2. Cell proliferation rate of negative control oligos MCF-7 cells or cells transfected with HIF-1α shRNA as determined by MTT assays. $^{*}P < 0.05$.

(qPCR). Primer sequences are shown in **Table 1**. Total RNA was isolated from MCF-7 cells using TriZol reagent (Tiangen, Beijing, China), and complementary DNA was synthesized by reverse transcription. In brief, reverse transcription was carried out in 20 μL total reaction volumes containing 1 μL of template RNA, 2 μL of 10× RT mix, 2 uμL of Super Pure dNTP (2.5 nM each), 2 μL of specific primers, and 1 μL of reverse transcriptase (Beyotime, Shanghai, China). Reactions were incubated at 37°C for 60 min.

qPCR reactions were performed in 20 μL total volumes containing 1 μL of RT product, 10 μL of 2× SuperReal PreMix Plus (with SYBR Green I), 0.6 μL of forward primer, 0.6 μL of reverse primer, and 0.4 μL of 50× ROX reference dye. Reactions were incubated at 95°C for 15 min, followed by 45 cycles of 95°C for 10 s and 62°C for 31 s. All reactions were repeated three times. Relative expression of miR-210 and *U6* was analyze by using the Stratagene Mx3000P software (Agilent Technologies, Inc., Santa Clara, CA, USA) and fold changes were calculated using the $2^{-\Delta\Delta Ct}$ normalization method.

Cell proliferation assay

MTT cell proliferation assays were performed according to manufacturer's instructions (Beyotime, Shanghai, China). Briefly, 1×10^4 cells/well was plated in 96-well plates. Plates were incubated for 24 h in a humidified atmosphere containing 5% CO_2 at 37°C. Then, 50 μL of MTT (5 mg/mL) in PBS were added and incubated for 4 h in a humidified atmosphere containing 5% CO_2 at 37°C. Next, 150 μL of DMSO were added after removal of the supernatant. A microplate

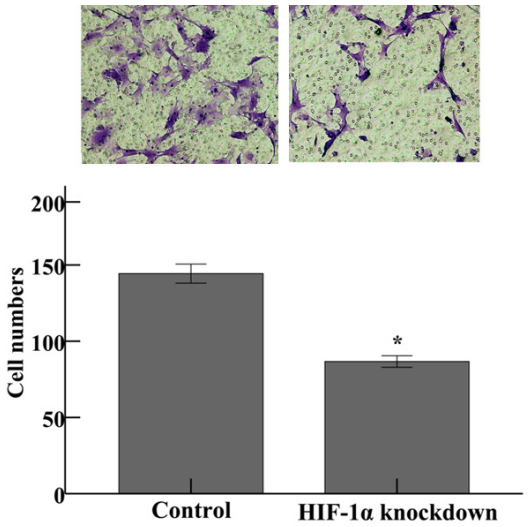

Figure 3. Representative microscopy field images and overall cell quantification from transwell cell invasion assays with negative control oligos MCF-7 cells or cells transfected with HIF-1α shRNA. $^{*}P < 0.05$.

reader (Bio-Rad, Hercules, CA, USA) was used to determine absorbance at 570 nm. Each assay was performed in triplicate wells and repeated three times.

Cell invasion assay

We used a transwell insert (24-well insert with 8-μm pore size; Corning Inc., Corning, NY, USA) coated with Matrigel (BD Biosciences, San Jose, CA, USA) to assess breast cancer cell invasion in vitro. Briefly, MCF-7 cells after 48 h from the end of transfection were starved in media without FBS overnight, and then 1×10^6 cells were resuspended in FBS-free media and placed in insert top chambers in triplicate. Lower chambers were filled with 10% FBS as the chemoattractant and incubated for 24 h. After 24 h, cells on upper surfaces of the membranes were removed using cotton buds, and cells on lower surfaces of the inserts were fixed and stained with 0.1% crystal violet. Five visual fields of each insert were randomly chosen and photographed under a light microscope at 100× magnification. Cells in the photographs were counted, and data were summarized as mean ± standard deviation (SD).

Flow cytometry analysis

Cells were collected after trypsinization, centrifuged at 200 × g for 5 minutes, and washed with PBS. Cells were incubated with 100 μL

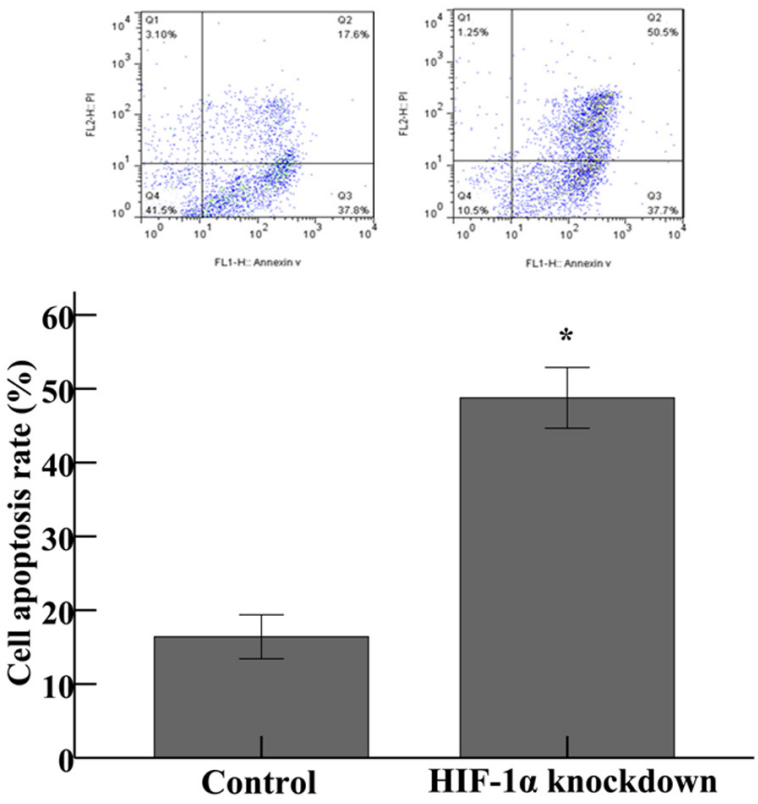

Figure 4. Representative flow cytometry cell scatter plots and overall quantification of cell apoptosis rate (% of cells stained for annexin-V and propidium iodide) of negative control oligos MCF-7 cells or cells transfected with HIF-1α shRNA. $^{*}P < 0.05$.

Annexin-V-FLUOS staining reagent containing 2% propidium iodide (Roche, Basel, Switzerland) at room temperature for 15 min. Flow cytometry was used to obtain apoptotic index from the percentage of cells double-stained by annexin-V and propidium iodide.

Statistical analysis

All data were analyzed by SPSS17.0 (IBM, Armonk, NY, USA) and expressed as mean ± SD. Data were analyzed by independent samples t-test, and $P < 0.05$ was considered statistically significant.

Results

Expression of HIF-1α and miR-210

Western blots showed that Seventy-two hours after transfection, HIF-1α expression of HIF-1α knock down MCF-7 cells (0.382 ± 0.004) significantly reduced in comparison to negative control oligoes cells (0.128 ± 0.005) (**Figure 1**).

Further, qPCR revealed that HIF-1α knockdown resulted in a corresponding significant decrease (about 60%) in miR-210 expression (0.993 ± 0.056) in comparison to negative control oligoes cells (0.392 ± 0.010).

Proliferation rate post-transfection

MTT assays showed that HIF-1α knockdown significantly reduced MCF-7 cell proliferation in comparison to negative control oligo cells forty-eight hours after transfection (**Figure 2**).

Cell invasion post-transfection

Transwell invasion assays showed that HIF-1α knockdown significantly reduced the number of cells that invaded into the lower side of transwell membranes in comparison to negative control oligoes cells (**Figure 3**).

Apoptosis rate post-transfection

Flow cytometry of annexin-V/propidium iodide-stained cells showed that HIF-1α knockdown significantly increased apoptosis rate of MCF-7 cells in comparison to negative control oligoes cells (**Figure 4**).

Discussion

Recent studies show that HIF-1α and miR-210 are frequently upregulated in breast cancer cell lines and tissues and play important roles in breast cancer growth and metastasis [14, 15]. In this study, we found that HIF-1α knockdown by shRNA could also remarkably decrease miR-210 expression in MCF-7 cells, suggesting HIF-1α may regulate miR-210 expression.

miR-210 has a hypoxia responsive element (HRE) on its proximal promoter [16], and HIF-1α can combine with HRE to regulate miR-210 expression [17]. In oxygen-dependent regulatory systems, prolyl hydroxylases normally can

induce degradation of HIF-1α but can also be inhibited by miR-210, resulting in tissue accumulation of HIF-1α [9]. Moreover, recent studies showed that a miR-210 feedback loop also downregulates succinate dehydrogenase complex subunit D (SDHD), another inhibitor of HIF-1α. In this way, miR-210-mediated repression of SDHD promotes HIF-1α stabilization and thereby further promotes miR-210 production to drive the positive feedback loop [18]. HIF-1α promotes increased expression of miR-210, and miR-210 promotes stabilization of HIF-1α, suggesting that a positive feedback loop is indeed at work [14, 15].

Metastasis is a main reason for breast cancer-related deaths. Our results show that HIF-1α knockdown in breast cancer cells significantly inhibits cell proliferation and cell migration and significantly increases apoptosis rate in vitro; indicating that HIF-1α-induced miR-210 expression aberrantly regulates proliferation, migration, and apoptosis of breast cancer cells. These results suggest a new potential target for breast cancer treatment, and methodologies for therapeutic delivery are currently in development and testing to selectively inhibit inappropriately upregulated HIF-1α-induced miR-210 expression in breast cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

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