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Original Article

Hypoxia-related miR-210-5p and miR-210-3p regulate hypoxia-induced migration and epithelial-mesenchymal transition in hepatoma cells

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Abstract: MicroRNA-210 (miR-210) has been associated with hypoxia. It is abnormally expressed in many malignant tumors. In a previous study, Illumine Soledad massively parallel signature sequencing of miRNomes was conducted in non-tumor and hepatocellular carcinoma (hepatoma, HCC) tissues. It was found that expression of miR-210 was abnormally high in HCC. However, expression levels and biological activity levels of miR-210-5p in HCC, as well as its relationship with hypoxia, have not been reported. The current study evaluated human HCC cell lines SMMC-7721, Huh-7, HepG2, and MHCC97H and normal human hepatocytes under normoxia and hypoxia conditions. Results showed that both miR-210-5p and miR-210-3p were highly expressed in hepatoma cells. Relative expression of miR-210-5p was much higher than that of miR-210-3p. In addition, both miR-210-5p and miR-210-3p were significantly upregulated under hypoxia, with relative expression of miR-210-5p still much higher than that of miR-210-3p. Hypoxia also promoted the migration of hepatoma cells *in vitro* and expression of epithelial-mesenchymal transition (EMT)-related proteins, including N-cadherin, MMP2, Slug, and Twist1. Inhibition of miR-210-5p and miR-210-3p inhibited the migration and expression of EMT-related proteins but had no effects on the proliferation of hepatoma cells. In conclusion, miR-210-5p and miR-210-3p were highly expressed in hepatoma cells and upregulated under hypoxia. Hypoxia-induced migration and EMT in hepatoma cells were regulated by hypoxia-related miR-210-5p and miR-210-3p. However, proliferation of hepatoma cells was not associated with miR-210-5p and miR-210-3p.

Keywords: Hepatocellular carcinoma, miR-210-5p, miR-210-3p, hypoxia, epithelial-mesenchymal transition, migration

Introduction

Hepatocellular carcinoma (HCC, hepatoma) is a malignant tumor with high morbidity and mortality rates, especially in men. The number of HCC-related deaths is the second highest of all cancer-related deaths. The number of new cases is ranked as the fifth most prevalent [1]. Surgical resections, chemotherapy, radiotherapy, transcatheter arterial chemoembolization (TACE), and targeting therapy are the main treatments for HCC. However, the cancer easily metastasizes during early stages of the disease. Postoperative recurrence and a hypoxic microenvironment result in an unsatisfactory prognosis for patients with HCC. The hypoxic microenvironment plays a critical role in the development and progression of solid tumors, including HCC. Hypoxia is caused by malformation of the tumor microvascular and the special metabolic activity of the tumor [2]. It mediates the progression of malignancy-related biological behaviors, such as tumor proliferation and metastasis, leading to poor prognosis [3]. It has been reported that hypoxia can induce epithelial-mesenchymal transition (EMT) in many epithelium-derived malignant tumors [4]. EMT is a biological process in which epithelial cells are transformed into cells with mesenchymal phenotypes. The loss of epithelial characteristics

and the acquisition of interstitial cell characteristics are characterized by hypoxia, allowing tumor cells to obtain increased migration and invasion abilities.

MicroRNAs (miRNAs) are non-coding single-stranded RNAs of approximately 19-25 nucleotides in length. It has been reported that at least 30% of human genes are regulated by miRNAs [5]. Studies have shown that many kinds of miRNAs are involved in the EMT process of malignant tumors. In addition, miRNAs have been closely related to occurrence and development of HCC. Compared with miRNA expression profiles of normal liver tissues, there is abnormal expression of miRNAs in HCC, such as lower expression of miR-7 [6] and miR-144-3p [7] and higher expression of miR-30d [8] and miR-210 [9]. A previous study used Illumine Soledad massively parallel signature sequencing of miRNomes in non-tumor and hepatocellular carcinoma tissues to show that expression of miR-210 is abnormally high in more than 70% of HCC specimens [10]. Abnormal expression of miR-210 has been detected in many types of tumors. It has been demonstrated that miR-210 is a key regulatory miRNA in hypoxia, targeting hypoxia-inducible factor (HIF)-1α [11-13]. HIF-1α and HIF-1β constitute the HIF-1 complex. Under normoxia, HIF-1α binds to the von Hippel-Lindau E3 ligase complex, then undergoes ubiquitination and proteasomal degradation. On the other hand, hypoxia inhibits the degradation of HIF-1α and leads to its stabilization [14, 15]. HIF-1α can also upregulate expression of miR-210. This has distinct effects on the development of HCC. It has been reported that the formation of a HIF-1α-miR-210-HIF-3α regulatory loop promotes the invasion and metastasis of HCC [16].

Dicer, an enzyme, cleaves pre-miRNAs to generate functional miRNAs. Mature miR-210 can be generated from each strand of the same RNA hairpin structure, as miRNA-5p or miRNA-3p [17], which targets different mRNAs. Whether significantly upregulated miR-210 observed during hypoxia is in the form of miR-210-5p or miR-210-3p has not been reported. Furthermore, respective expression of miR-210-5p and miR-210-3p in HCC has not been reported. The purpose of the current study was to investigate expression levels of miR-210-3p and miR-210-5p in hepatoma cells under normoxia and hypoxia conditions, aiming to explore the effects of hypoxia and hypoxia-related miR-210-5p and miR-210-3p on migration and EMT of hepatoma cells.

Materials and methods

Cell culturing and transfection

Human HCC cell lines (SMMC-7721, Huh-7, HepG2, and MHCC97H) and normal human hepatocytes L02 were obtained from the American Type Culture Collection (ATCC). All cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; Gibco, Grand Island, USA) with 10% fetal bovine serum (FBS; Gibco). They were cultured in a cell incubator under the recommended conditions of 37°C, 20% O_2, 5% CO_2, 75% N_2, and appropriate humidity. The cells were transfected with 100 nM of miR-210-5p inhibitor, miR-210-3p inhibitor, or NC inhibitor using Lipofectamine 2000 Transfection Reagent (Invitrogen, CA, USA), according to manufacturer instructions.

RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from normal hepatocytes, normoxic hepatoma cells, and hypoxia-treated hepatoma cells using the RNAfast2000 kit (Fastgen, Shanghai, China). Complementary DNA (cDNA) was generated by reverse transcription using the extracted RNA as template and a ReverTra Ace® qPCR RT kit (Toyobo, Japan), according to manufacturer protocol. The miRNA was reverse transcribed using a ReverTra Ace-α- First Strand cDNA Synthesis kit (Toyobo) with the stem-loop reverse transcription method. U6 was used as an internal normalized reference. All qRT-PCR reactions were performed using a THUNDERBIRD qPCR kit (Toyobo). Amplification conditions were 95°C for 1 minute; 40 cycles at 95°C for 15 seconds, 60°C for 1 minute; 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds. Relative expression levels of miRNA were calculated by relative quantification using the $2^{-\Delta\Delta Ct}$ method. Each sample was independently analyzed three times. Primer sequences are shown in **Table 1**.

Hypoxia treatment of cells

Two methods were used to simulate hypoxic environments, the $CoCl_2$ induction method [18]

Table 1. Sequence of primers

Gene name	Primer sequence (5'→3')
miR-210-5p	Reverse transcription primer: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGTGT
	Forward primer: AAATGCCCACCGCACACTG
	Reverse primer: GTGCAGGGTCCGAGGT
miR-210-3p	Reverse transcription primer: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC
	Forward primer: CGCTGTGCGTGTGACAGC
	Reverse primer: AGTGCAGGGTCCGAGGTATT
U6	Reverse transcription primer: AACGCTTCACGAATTTGCGT
	Forward primer: CTCGCTTCGGCAGCACA
	Reverse primer: AACGCTTCACGAATTTGCGT
HIF-1α	Forward primer: TCTGGGTTGAAACTCAAGCAACTA
	Reverse primer: CAACCGGTTTAAGGACACATTCTG
β-actin	Forward primer: CATGTACGTTGCTATCCAGGC
	Reverse primer: CTCCTTAATGTCACGCACGAT

and the AnaeroPack method. Regarding the first method, when cells reached approximately 70% confluence in the 6-well plate, SMMC-7721, Huh-7, HepG2, and MHCC97H cells and normal hepatocytes were gently washed with phosphate-buffered saline (PBS). They were then added to the 100 μM $CoCl_2$ solution to simulate the hypoxic environment. The cells were incubated at 37°C with 20% O_2, 5% CO_2, 75% N_2, and suitable humidity for 24 hours. The normoxic group for each cell type was used as a control. Regarding the second method, cells at 70% density were placed in a sealed air-tight container that contained an AnaeroPack (Mitsubishi Gas Company, Tokyo, Japan) [19] and an oxygen indicator. The tank was placed in a cell incubator at 37°C with 20% O_2, 5% CO_2, 75% N_2, and suitable humidity for 24 hours. The normoxic group for each cell type was used as a control.

Western blot analysis

Antibodies against N-cadherin, Twist1, matrix metalloproteinase 2 (MMP2), Slug (Snail2), E-cadherin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (Boston, MA, USA). The antibody against HIF-1α was purchased from Abcam (Cambridge, UK). Cells were harvested and lysed by radioimmunoprecipitation assay (RIPA) buffer (Solarbio, Beijing, China) containing the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF; Solarbio). Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime, China). Aliquots containing 20 μg protein were taken from each sample and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). They were then electro-transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). After blocking in 5% milk in Tris-buffered saline Tween-20 (TBST) for 1 hour at 37°C, the membranes were incubated with primary antibodies at 4°C overnight. They were incubated with secondary antibodies at 37°C for 2 hours. Images were obtained using Image Lab™ software 2.0 (Bio-Rad).

In vitro migration assay

Cell migration was detected using Transwell migration assays in 24-well Cell Migration plates with 8-μm pore polycarbonate membranes (Corning, NY, USA), according to manufacturer instructions. Briefly, 4×10^4 cells were suspended in serum-free medium (100 μl) in the upper chamber. DMEM containing 20% FBS (600 μl) was added to the lower chamber. After incubation at 37°C for 24 hours, the lower surface of the membrane was fixed with 4% paraformaldehyde. It was then stained with Giemsa staining solution. After washing and drying, photos were obtained. The amount of migration was determined based on the average number of cells in five microscope fields.

Cell proliferation assay

SMMC-7721 and Huh-7 cells were seeded into 96-well plates (5×10^3 cells/well) and trans-

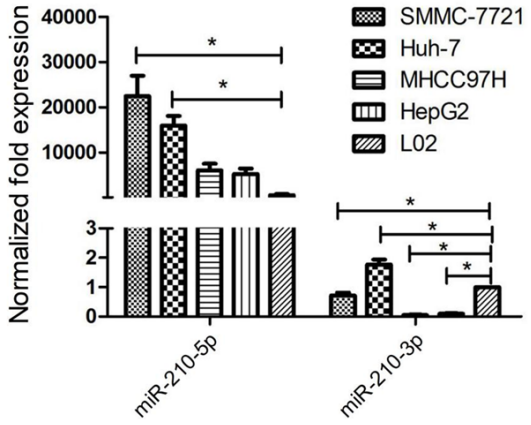

Figure 1. Expression of miR-210-5p and miR-210-3p in hepatocellular carcinoma (HCC) cell lines and normal hepatocytes under normoxia. Relative expression of miR-210-5p and miR-210-3p in human HCC cell lines SMMC-7721, Huh-7, HepG2, and MHCC97H and normal human hepatocytes L02 under normoxia was measured by quantitative reverse transcription polymerase chain reaction. Expression levels were normalized to those of U6 as an internal reference. Each sample was independently analyzed three times and mean values are shown. *, $p < 0.05$.

fected with miR-210-5p inhibitor, miR-210-3p inhibitor, or NC inhibitor. Each group included four duplicate wells. Cell proliferation was detected using Cell Counting Kit-8 (CCK8) assays (Dojinodo, Japan) at 0, 24, 48, and 72 hours, according to manufacturer instructions, under an enzymatic detector at a wavelength of 450 nm.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 software (IBM SPSS, Armonk, NY, USA). Unpaired Student's t-tests were used to determine statistical differences between two groups. Data are presented as the mean ± standard deviation (SD). $P < 0.05$ indicates statistical significance.

miR-210-5p and miR-210-3p overexpressed in hepatoma cells under normoxia

Expression of miR-210-5p in hepatoma cell lines SMMC-7721 and Huh-7 was higher than that in normal hepatocytes (L02). Expression of miR-210-3p was also high in Huh-7 cells. Relative expression of miR-210-5p was significantly higher than that of miR-210-3p in HCC cells ($p < 0.05$; **Figure 1**).

Hypoxia upregulated expression of miR-210-5p, miR-210-3p, and HIF-1α in hepatoma cells

Hepatoma cells SMMC-7721 and Huh-7, with a relatively high expression of miR-210-5p and miR-210-3p, were hypoxia-treated by being cultured with an AnaeroPack for 24 hours or 100 μM $CoCl_2$ for 24 hours. Results from qRT-PCR and Western blotting showed that miR-210-5p and miR-210-3p in HCC cells were significantly upregulated under hypoxia. Relative expression of miR-210-5p in the $CoCl_2$ treated group was significantly higher than that of miR-210-3p (**Figure 2A**, **2B**; $p < 0.05$). Relative mRNA expression and protein expression levels of HIF-1α in the $CoCl_2$ treated group were significantly higher than those in the normoxic group (**Figure 2D**; $p < 0.05$). Relative mRNA expression of HIF-1α in the AnaeroPack group was also increased. However, the difference failed to reach statistical significance, compared to that in the normoxic group (**Figure 2C**; $p > 0.05$). Based on these results, the $CoCl_2$ induction method was selected to simulate hypoxia in all subsequent experiments.

Hypoxia-induced migration and EMT in hepatoma cells in vitro

Compared with the normoxic group, the migration ability of SMMC-7721 and Huh-7 hepatoma cells treated with 100 μM $CoCl_2$ for 24 hours was significantly enhanced (**Figure 3A**, **3B**). To investigate the association between EMT and hypoxia, this study quantitated EMT-related proteins N-cadherin, E-cadherin, MMP2, Slug, and Twist1 in SMMC-7721 and Huh-7 cells. Results showed that expression of N-cadherin, Slug, Twist1, and MMP2 was upregulated, while that of E-cadherin was downregulated (**Figure 3C**). Differences between normoxic and hypoxic groups were statistically significant ($p < 0.05$). Results suggest that hypoxia may induce migration of hepatoma cells and EMT *in vitro*.

Hypoxia-related miR-210-5p and miR-210-3p regulated hypoxia-induced migration and EMT in hepatoma cells

The number of migrated transmembrane cells transfected with 100 nM miR-210-5p inhibitor and 100 nM miR-210-3p inhibitor was significantly lower than that of those transfected with NC inhibitor at the same concentration, as well as that of the untreated groups ($p < 0.05$;

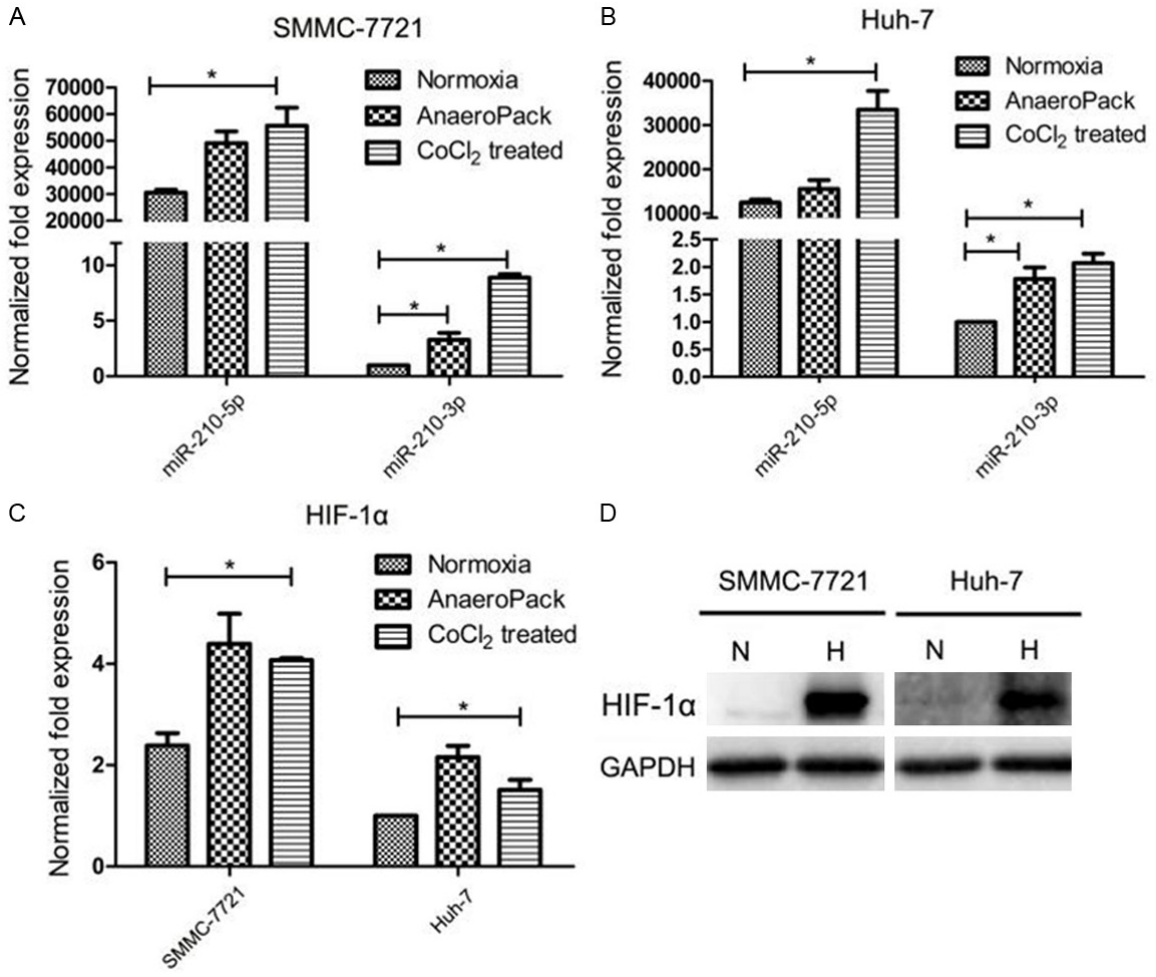

Figure 2. Relative expression of miR-210-5p, miR-210-3p, and HIF-1α in hepatocellular carcinoma cells under normoxia and hypoxia. (A and B) Relative expression of miR-210-5p and miR-210-3p in SMMC-7721 and Huh-7 under normoxia and hypoxia (AnaeroPack and $CoCl_2$). (C) mRNA and (D) Protein expression of HIF-1α in SMMC-7721 and Huh-7 under normoxia and hypoxia. (N: normoxia; H: hypoxia. *, $p < 0.05$).

Figure 4A, **4B**). Results suggest that inhibiting miR-210-5p and miR-210-3p inhibited the migration of hepatoma cells *in vitro*. At the same time, to further explore the relationship between EMT and hypoxia-induced upregulation of miR-210-5p and miR-210-3p, this study quantitated the expression of EMT-related proteins. Compared to levels in the NC group, N-cadherin, Slug, Twist1, and MMP2 were downregulated and E-cadherin was upregulated in the miR-210-5p inhibitor group and miR-210-3p inhibitor group (**Figure 4C**). Inhibition of miR-210-5p and miR-210-3p also inhibited hypoxia-induced EMT in hepatoma cells. Results suggest that migration and EMT induced by hypoxia in hepatoma cells were both inhibited by hypoxia-induced suppression of miR-210-5p and miR-210-3p induced by hypoxia.

Inhibition of miR-210-5p and miR-210-3p shows no effects on proliferation of HCC cells

The effects of inhibition of miR-210-5p and miR-210-3p on proliferation of SMMC-7721 and Huh-7 cells were analyzed using CCK8 assays. Compared with that in NC inhibitor and untreated groups, inhibition of miR-210-5p and miR-210-3p produced no significant effects on the proliferation of hepatoma cells (**Figure 5**; $p > 0.05$).

Discussion

Abnormal expression of miR-210 in some tumor tissues and cells is related to the malignant biological behavior of tumors. However, respective expression levels of miR-210-5p and miR-210-

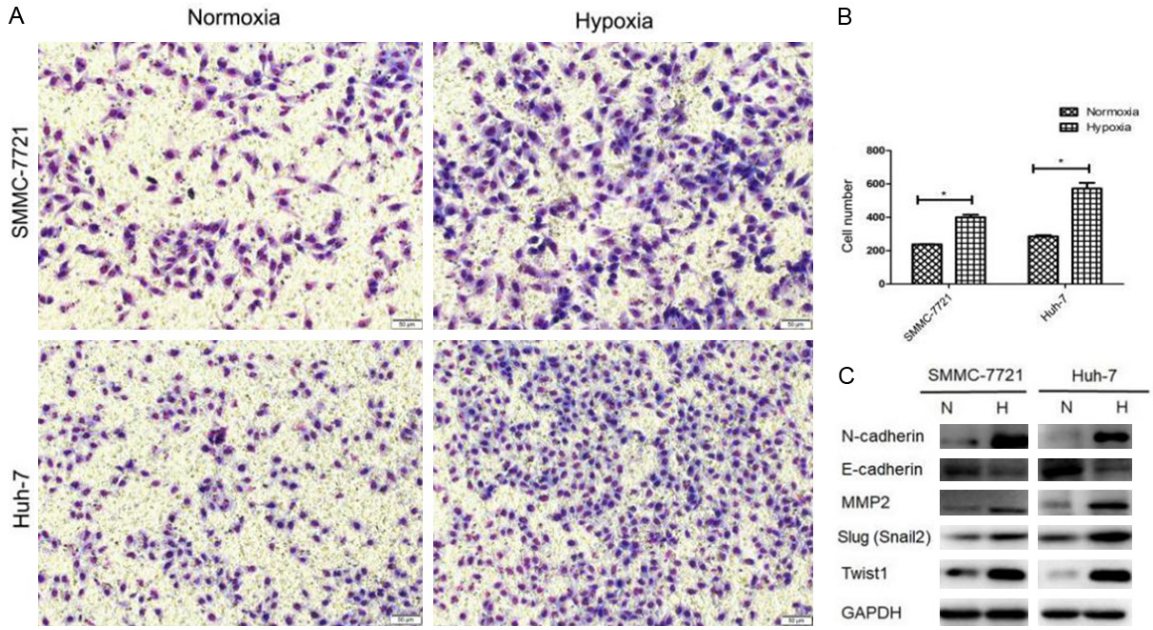

Figure 3. Migration and expression of epithelial-mesenchymal transition (EMT)- related molecules in hepatocellular carcinoma cell lines under normoxia and hypoxia. A. Evaluation of the migration ability of SMMC-7721 and Huh-7 cells under normoxia and hypoxia using Transwell migration assays with Giemsa staining and 400 × magnification. B. The number of cells was calculated using Giemsa staining. C. Representative Western blot images of EMT-related molecules N-cadherin, E-cadherin, Slug, Twist1, and MMP2 in SMMC-7721 and Huh-7 cells after inhibition of miR-210-5p and miR-210-3p. GAPDH was used and an internal control (N: normoxia; H: hypoxia. *, $p < 0.05$).

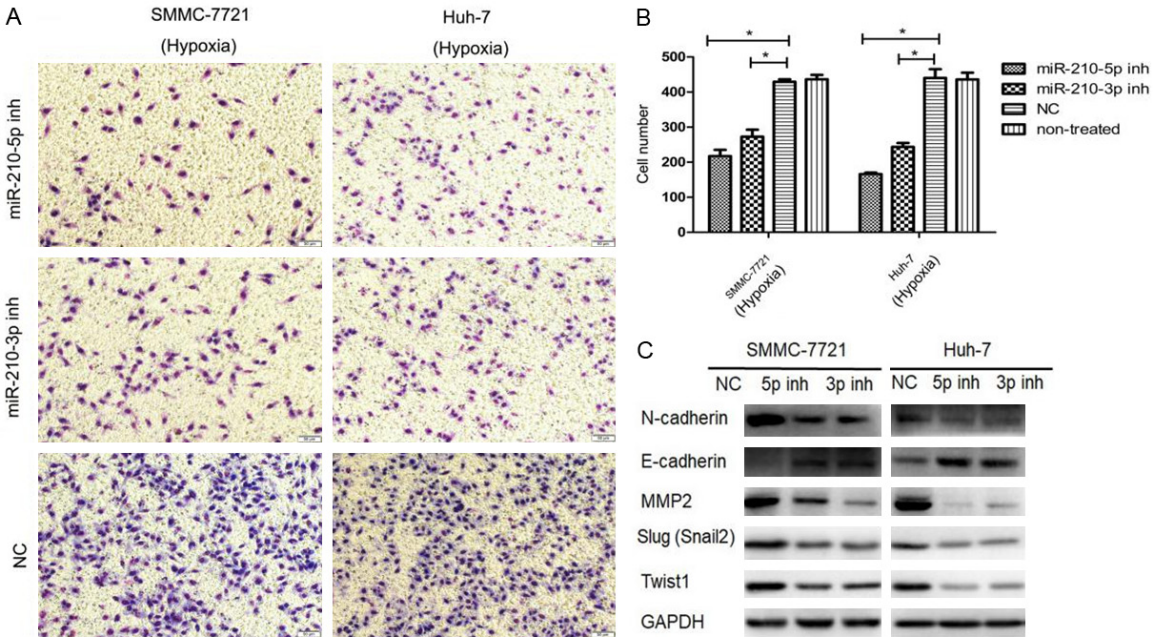

Figure 4. Inhibition of miR-210-5p and miR-210-3p inhibited hypoxia-induced migration and epithelial-mesenchymal transition (EMT) in hepatoma cells. A. Evaluation of the migration ability of SMMC-7721 and Huh-7 cells using Transwell migration assays with Giemsa staining and 400 × magnification after transfection with 100 nM miR-210-5p inhibitor and miR-210-3p inhibitor under hypoxia. The NC inhibitor and non-treated cells were used as controls. B. The number of cells was determined using Giemsa staining. C. Representative Western blot images of EMT-related molecules in hepatocellular carcinoma cell lines after inhibition of miR-210-5p and miR-210-3p under hypoxia. (miR-210-5p inh: miR-210-5p inhibitor group; miR-210-3p inh: miR-210-3p inhibitor group; NC: negative inhibitor group. *, $p < 0.05$).

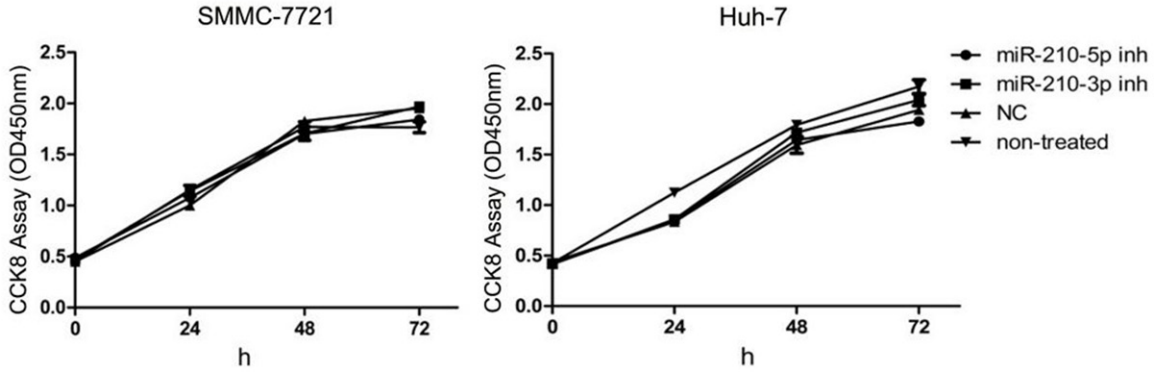

Figure 5. Evaluation of thes effect of inhibition of miR-210-5p and miR-210-3p on cell proliferation in SMMC-7721 and Huh-7 cells using CCK8 assays. The NC inhibitor and non-treated cells were used as controls.

3p in hepatoma cells have not been reported. The current study found that both miR-210-5p and miR-210-3p were overexpressed in hepatoma cells, compared to levels in normal hepatocytes. The most prominent and robust miRNA that is induced in response to the hypoxia microenvironment is miR-210. It is stably upregulated under hypoxia. However, the classical hypoxia-induced upregulated miR-210 reported by previous studies is primarily miR-210-3p [20]. The relationship between miR-210-5p and hypoxia has not been previously reported. The current study chose the $CoCl_2$ induction method to simulate the hypoxic environment, in part because of the instability of the AnaeroPack method. Using the $CoCl_2$ method, Fe^{2+} is replaced by Co^{2+} in heme [21] and the iron chelate enzyme is inactivated by intracellular ion replacement, leading to inhibition of the oxidative reaction of cells [22]. Co^{2+} is able to bind oxygen at a high concentration and forces the cells into a hypoxic state, even while in a normoxic environment. Using the model, it was found that both miR-210-5p and miR-210-3p were significantly upregulated under hypoxia. This result supports the previous report, suggesting that miR-210 induced by HIF-1α could be regarded as a tumor hypoxia marker [23] and demonstrating that both miR-210-5p and miR-210-3p are hypoxia-related miRNAs. At the same time, expression of HIF-1α proteins in the $CoCl_2$-induced hypoxia group was significantly upregulated. This was consistent with the stable expression of HIF-1α protein often being regarded as an important marker for successful hypoxia induction [24]. In addition, hypoxia can promote the migration of hepatoma cells [25], as found in current results. Hypoxia stimulation is one of the most significant factors for the acceleration of EMT in the microenvironment of hepatoma cells [26]. EMT enhances the motility and invasiveness of tumor cells [27, 28] and plays a critical role in the metastasis of malignant tumors. Activation of EMT transcription factors, such as snail or slug, and the reduction of epithelial proteins, including E-cadherin, have been defined as EMT hallmarks [29]. In addition, the acquisition of another EMT transcription factor Twist1 and the mesenchymal marker N-cadherin promotes the EMT process [30, 31]. Increased levels of matrix metalloproteinases, such as MMP2, are also related to EMT [32, 33]. Present findings indicating that N-cadherin, Slug, Twist1, and MMP2 were significantly upregulated and that E-cadherin was downregulated are consistent with previously reported theories suggesting that hypoxia is able to induce the migration and EMT of hepatoma cells *in vitro*.

Therefore, this study further explored whether hypoxia-induced migration and EMT might be affected by hypoxia-induced miR-210-5p and miR-210-3p. Results demonstrated that inhibition of miR-210-5p and miR-210-3p could inhibit the migration of hepatoma cells *in vitro*. Inhibition of miR-210-5p and miR-210-3p also inhibited EMT, as N-cadherin, Slug, Twist1, and MMP2 were significantly downregulated and E-cadherin was upregulated. Based on these results, hypoxia-related miR-210-5p and miR-210-3p can regulate hypoxia-induced migration and EMT in hepatoma cells.

The significance of the current study is the finding that miR-210-5p and miR-210-3p were abnormally high expressed in hepatoma cells

and upregulated under hypoxia. Expression of miR-210-5p was much higher than miR-210-3p in nomorxia and hypoxia in hepatoma cells. Hypoxia-induced migration and EMT in hepatoma cells was regulated by hypoxia-related miR-210-5p and miR-210-3p. However, the proliferation of hepatoma cells was not associated with miR-210-5p and miR-210-3p.

The current study did not explore the targets of miR-210-5p or miR-210-3p affecting hypoxia-induced migration and EMT in HCC. Therefore, this should be addressed in future studies, aiming to improve the understanding of the mechanisms underlying this network. Current results, however, lay a firm foundation for further studies on the roles of miRNAs in occurrence, development, and metastasis of HCC.

Acknowledgements

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Disclosure of conflict of interest

None.

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