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# MiR-210 regulates cell cycle in nasopharyngeal carcinoma cell line (CNE-1) under hypoxic condition by reducing the expression of cyclin D1*

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**Abstract** ***Objective:*** The aim of our study was to determine the underlying mechanism of miR-210 on regulation of the cell cycle in nasopharyngeal carcinoma cell line CNE-1, particularly through regulation of cyclin D1, under hypoxic conditions. ***Methods:*** The CNE-1 cell line was induced with hypoxia, and the expression levels of endogenic miR-210 and cyclin D1 were detected by real-time PCR and Western blotting. Next, the luciferase assay was used to confirm that cyclin D1 is a target gene for miR-210. Cell cycle and cell proliferation were detected in CNE-1 cells that were cultured under hypoxic conditions with either overexpression or knockout of miR-210 using flow cytometry and MTT assay, respectively. ***Results:*** Hypoxia induced the expression of miR-210, resulting in reduced mRNA and protein levels of cyclin D1 and repression of cyclin D1 in CNE-1 cells. Further analysis indicated that miR-210 directly binded to the 3'UTR of the cyclin D1 gene, thus regulated the expression of cyclin D1. The flow cytometry assay showed that, under hypoxic conditions, miR-210 blocked CNE-1 cells in the G1 phase, and miR-210 also inhibited the proliferation of CNE-1 cells. ***Conclusion:*** Under hypoxic conditions, miR-210 directly reduced the expression of cyclin D1, leading to CNE-1 cells blocked in G1 phase.

**Key words** nasopharyngeal carcinoma; miR-210; cyclin D1; cell cycle

MicroRNAs (miRNAs) are a class of endogenous non-coding RNA of 19 to 24 nucleotides in length, which are differentially expressed in a variety of tumors. MicroRNAs are closely related to tumor proliferation, differentiation, invasion, metastasis, and response to therapy, thus they have important implications in the early diagnosis and therapy of tumors [1–3]. A high incidence of nasopharyngeal carcinoma occurs in southern China, particularly in Guangdong. Currently the primary treatment for nasopharyngeal carcinoma is radiotherapy, but it has ineffecient therapeutic effects and nearly a half of the treated patients end up with recurrence and metastasis five years after radiotherapy. Hypoxia is a common phenomenon in the center of solid tumors. miR-210 is a type of miRNA regulated by hypoxia-inducible factor 1 (HIF-1). Under hypoxic conditions, HIF-1 initiates the expression of miR-210. In this study, we aimed to determine the underlying mechanism of miR-210 regulation of the cell cycle under hypoxic conditions by establishing a hypoxic culture model of nasopharyngeal carcinoma cell line, CNE-1. Determining the underlying mechanism of miR-210 regulation will enable us to further reveal the regulatory network of cell cycle molecules in nasopharyngeal carcinoma cells challenged with hypoxia.

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## Materials and methods

### Reagents

The nasopharyngeal carcinoma cell line CNE-1 was purchased from the Chinese Academy of Medical Sciences (Shanghai, China). The hsa-miR-210, hsa-miR-210 inhibitors, and negative control (NC, a random RNA sequence of 22 nucleotides with no binding sites on human mRNA) were purchased from Genepharma (Shanghai, China). The polyclonal rabbit anti-human cyclin D1 antibody was purchased from Abcam (ab16663, US).

### Experimental methods

*Cell culture and hypoxia induction*

CNE-1 cells were cultured with 10% FBS-DMEM at 37 ℃ with 5% $CO_2$. And then, the CNE-1 cells were cultured in a hypoxic system (Biospherix) in 10% FBS-DMEM, at 37 ℃ with 5% $CO_2$, and 1% oxygen. After culturing for 36 h, cells were removed from the hypoxia incubator and

immediately processed to avoid prolonged exposure of cells to the air which may affect the results.

*MiRNA transfection*

The transfection of miR-210, miR-210 inhibitors, and NC were performed using Lipofectamine 2000 (Invitrogen Corp., USA), according to the manufacturer's instructions. Twenty pmol miRNA/well was used in a 6-well culture plate.

*Real-time PCR*

Total RNA was extracted from CNE-1 cells cultured under hypoxic and normoxic conditions, and those cells hypoxia-transfected with miR-210, miR-210 inhibitors, and NC. A total of 1 μg RNA from each sample was used for reverse transcrption (M-MLV, Takara). Using the resulting cDNA from reverse transcription as templates, cyclin D1 gene (CCND-1) expression levels were analyzed in each sample according to the manufacture's instruction for SYBR® Green Realtime PCR Master Mix (Toyobo, Japan). The primers for CCND1 were as follows: Forward 5'-ACACGGACTACAGGGGAGTTT-3', Reverse 5'-GGT TTCCACTTCGCAGCACA-3'; and the primers for the internal control of GAPDH were as follows: Forward 5'-TCCATGACAACTTTGGTATCG-3', Reverse 5'-TG TAGCCAAATTCGTTGTCA-3'.

*Western blotting*

CNE-1 cells cultured under hypoxia and normoxia conditions, and those hypoxia-transfected with miR-210, miR-210 inhibitors, and NC were lysed. Pyrolysis products were transfered with 10% SDS-PAGE at the constant current of 300 mA for 1.5 h, and blocked with 3% milk-PBST at room temperature for 1 h. After overnight incubation with a primary antibody (rabbit anti-human cyclin D1 antibody, 1:5000 dilution) at 4 ℃, the product was incubated with rabbit anti-mouse secondary antibody (1:5000 dilution) for 2 h. Chemiluminescence was detected using an ECL Western Blotting Detection Kit.

*Luciferase assay*

Construct 3'UTR-PGL3 vector for cyclin D1 comprising miR-210 target and the corresponding mutant reporter vectors. Under hypoxic culture conditions, reporting vectors were co-transfected with NC and miR-210, respectively, and the amount of vectors was 200 ng/well, with the amount of miRNA was 4 pmol/well. Thirty-six h after transfection, cells were harvested, lysed, and placed on ice. A total of 10 μL of each sample was added to 100 μL fluorescent substrates, and the enzyme activity was detected on a plate reader after mixing.

### Flow cytometry for cell cycle analysis

CNE-1 cells cultured under hypoxic conditions were harvested 24 h after transfection with miR-210 and NC, miR-210 inhibitor and an inhibitor NC. Samples were made into single cell suspension, and then centrifuged at 2000 rpm for 5 min. The supernatant was discarded. The samples were fixed with 70% cold ethanol at 4 ℃ for at least 18 h. Cell suspension (1 mL) with a cell density of $10^6$ cells/mL were washed three times with PBS, resuspended in 1 mL PI staining solution and incubated for 30 min at 37 ℃. Following incubation, the cells were analyzed by flow cytometry. Final concentration of PI dye was 50 μg/mL and final concentration of RNase A was 20 g/mL.

### MTT assay

CNE-1 cells were planted into 96-well plates under hypoxic conditions at a density of 3000 cells/well, and transfected with miR-210 and NC, with a group of untreated blank controls. At 24, 48, and 72 h after transfection, cells were incubated with 10 μL MTT (5 mg/mL) solution for 4 h at 37 ℃. After carefully discarding the supernatant, 100 μL of DMSO was used to dissolve the precipitate. The absorbance values were measured at 570 nm using an enzyme-linked immunosorbent assay plate reader. CNE-1 cells were planted into 96-well culture plates under hypoxic conditions at a density of 3000 cells/well and transfected with miR-210 inhibitor and an inhibitor NC. After 72 h, the cells were incubated with 10 μL MTT (5 mg/mL) solution for 4 h at 37 ℃. After carefully discarding the supernatant, 100 μL of DMSO was used to dissolve the precipitate. The absorbance values were measured at 570 nm using an enzyme-linked immunosorbent assay plate reader.

### Statistical analysis

All data were expressed as $\overline{\chi} \pm$ SD from 4 replications of the experiment. Statistical analysis was conducted using independent-sample $t$ test (SPSS 19.0), $P < 0.05$ was considered to be statistically significant.

## Results

### MiR-210 reduced the expression of cyclin D1 under hypoxic conditions

The transcription levels of CNE-1 miR-210 and cyclinD1 were detected under normoxic and hypoxic conditions using real time PCR. The results showed that the expression of miR-210 was elevated by 3.2-fold under hypoxic conditions compared to normoxic conditions, whereas the cyclin D1 mRNA level dropped to 0.21 times lower than that under normoxic condition (Fig. 1a, Normoxia 1.00 ± 0.07 vs. Hypoxia 3.21 ± 0.12, $P < 0.01$). Meanwhile, the protein level of cyclin D1 also dropped after hypoxic induction (Fig. 1b, Normoxia 1.00 ± 0.17 vs. Hypoxia 0.21 ± 0.05, $P < 0.01$).

Under hypoxic conditions, the mRNA of cyclin D1 was detected using real time PCR after transfection of miR-210 in vitro. As shown in Fig. 1c, compared with NC, cyclin D1 was reduced by about 30% in CNE-1 cells after transfection of miR-210. However, the protein level of

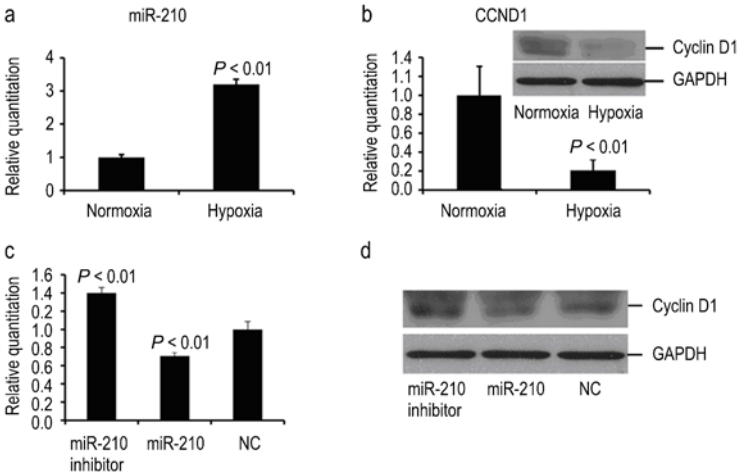


**Fig. 1** Expression levels of miR-210 and cyclin D1. (a) The expression levels of miR-210 under hypoxic conditions and normoxic conditions; (b) CCND1 levels under hypoxic conditions and normoxic conditions; (c) CCND1 levels of CNE-1 cell transfected with miR-210 inhibitor, miR-210, and NC transfected; (d) Levels of cyclin D1 after transfected with miR-210 inhibitor miR-210, and NC

cyclin D1 was reversed to 1.4 times that of NC in cells transfected with miR-210 inhibitor (Fig. 1c, miR-210 0.68 ± 0.08 vs. NC 1.00 ± 0.17, $P < 0.01$; miR-210 inhibitors 1.40 ± 0.12 vs. NC 1.00 ± 0.17, $P < 0.05$). Under the same treatment, the protein and mRNA level of cyclin D1 had consistent change, namely, the protein levels of cyclin D1 after transfection of miR-210 were lower than those in NC, and in cells transfected with miR-210 inhibitor, the protein level of cyclin D1 was reversed to the level of the NC (Fig. 1d).

## Cyclin D1 is a target gene of miR-210

Through online prediction software Findtar 3, we have predicted three binding sites of miR-210 on the 3'UTR (Table 1) of cyclin D1 mRNA, implying that miR-210 can directly bind to the 3'UTR (Table 1) of cyclin D1 mRNA, thereby regulating the expression of cyclin D1 protein. To verify the hypothesis, the miR-210 binding sites with the highest Loop Score were chosen, and the 100 bp upstream and 100 bp downstream fragments were amplified, inserted into the pGL3 vector, construct a luciferase reporter vector for cyclin D1 3'UTR, and co-transfected with miR-210 or NC into CNE-1 cells. Enzymatic activity was detected by luciferase activity microplate following transfection. The results showed that the transfection activity of miR-210 was only 64% of that in NC, thus proving that cyclin D1 is directly regulated by miR-210 (Fig. 2a, miR-210 0.64 ± 0.06 vs. NC 1.00 ± 0.09, $P < 0.01$). In addition, after replacement of the AC with TG in the core region of the binding sites (GGCACG) to destroy the conserved sequence of miR-210 recognition, results of luciferase activity showed that cells transfected with miR-210 and NC had similar enzymatic activity, indicating that mutation eliminated miR-210 binding sites in this sequence. These results further proved that the cyclin D1 gene was a target gene of miR-210 and was directly regulated by miR-210 (Fig. 2b).

## MiR-210 blocked the cell cycle of CNE-1 cells at G1

Cyclin D1 is a key protein for regulation of cell cycles from the G1 to S phase. Under hypoxic conditions, CNE-1 cells were transfected with miR-210 and NC, followed by PI staining and flow cytometry analysis. The results showed that G1 phase cells accounted for 64.2% of the miR-210-transfected cells and 53.5% of NC-transfected

**Table 1** Prediction of binding sites miR-210 on cyclin D1 3'UTR using Findtar 3

| miRNA | mRNA | Position | Structure | Loop score | △G | Recommendation |
|---|---|---|---|---|---|---|
| hsa-miR-210 | NM-053056 | 1965-1987 | 3' AGUCGGCGAC --- AGUGUGCGUGUC 5'<br>5' GCT ACCGTTG A CT TC - CAGGCA CGG 3' | 18.75 | 24.40 | Good |
| hsa-miR-210 | NM-053056 | 2812-2833 | 3' AGUCGGCGACAGUGUGCGUGUC 5'<br>5' TCGGAGGCCATCTCGGGCACAG 3' | 15.00 | 24.30 | Good |
| has-miR-210 | NM-053056 | 2992-3012 | 3' AGUCGGCGACAGUGUGCGUGUC 5'<br>5' AAGGCTGGTGGCAAGTGCACGG 5' | 15.00 | 26.00 | Good |

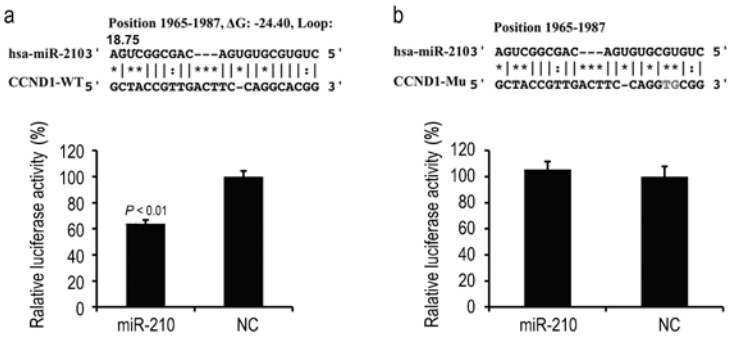


**Fig. 2** Luciferase assay. (a) Enzymatic activity of CNE-1 cells after transfected with miR-210 and NC; (b) After the conserved sequence of miR-210 recognition destroyed, enzymatic activity of CNE-1 cells after transfected with miR-210 and NC

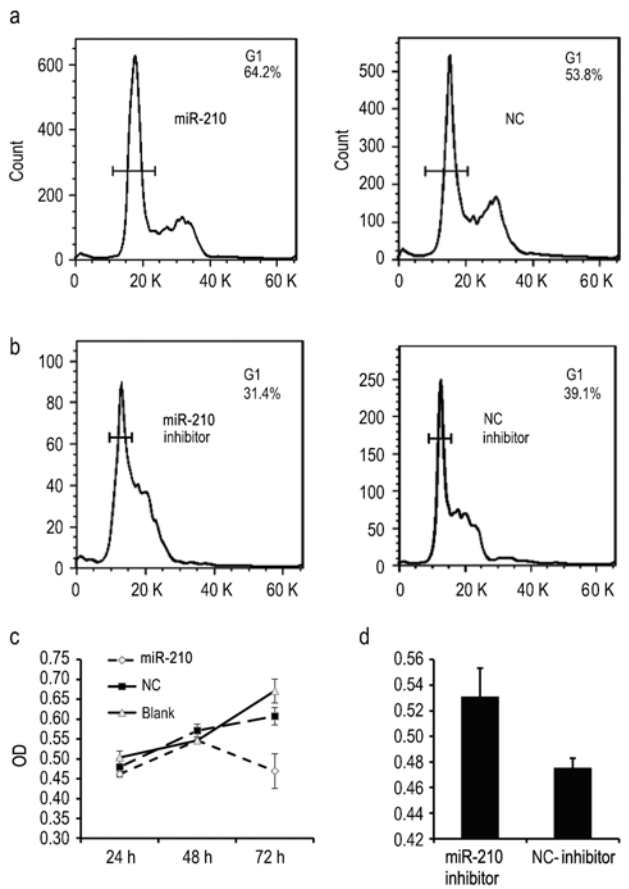


**Fig. 3** Flow cytometry assay. (a) G1 phase cells of CNE-1 cells transfected with miR-210 and NC; (b) G1 phase cells of CNE-1 cells transfected with miR-210 inhibitor and NC inhibitor; (c) Proliferation levels of CNE-1 cells transfected with miR-210, NC, and blank at 24 h, 48 h, and 72 h; (d) Proliferation levels of CNE-1 cells transfected with miR-210 inhibitor and NC inhibitor at 72 h

cells, respectively (miR-210 0.642 ± 0.04 vs. NC 0.535 ± 0.03, $P < 0.05$; Fig. 3a).

### MiR-210 inhibited the proliferation of CNE-1 cells

MiR-210 blocked the cell cycles of CNE-1 in G1 phase by inhibiting the expression of cyclin D1, which was bound to inhibit cell proliferation. Under hypoxic conditions, transfection of miR-210 inhibitor relieved the hypoxia-induced G1 phase blocking in CNE cells. As shown in Fig. 3b, the proportion of G1 phase cells decreased by 7.7% after transfection with miR-210 inhibitor, compared to 39.1% for those transfected with NC. Fig. 3c showed the difference in CNE-1 cell proliferation with transfection of miR-210, NC, and the blank control during 72 h under hypoxic conditions. After 72 h, the number of cells transfected with miR-210 was decreased compared with those transfected with NC or blank, indicating that miR-210 can inhibit cell proliferation (Fig. 3c). Under hypoxic conditions, CNE-1 cells were transfected with miR-210 inhibitor and NC inhibitors so as for inhibition of endogenous miR-210, followed by an MTT assay after 72 h. The results showed that miR-210 inhibitors restored CNE-1 cell proliferation (Fig. 3d, miR-210 inhibitors 0.537 ± 0.02 vs. NC inhibitors 0.497 ± 0.01, $P < 0.05$). These results indicate that, under hypoxic conditions, miR-210 overexpression can block cell cycles at the G1 phase and inhibit tumor cell proliferation. The suppression of endogenous miR-210 relieved the G1 phase blocking caused by hypoxia-induced endogenous miR-210, restoring the proliferation of tumor cells.

## Discussion

MiR-210 is an important miRNA regulated by HIF-1. Under hypoxic conditions, HIF-1 initiates the expression of miR-210 [4]. This study verified this phenomenon in a nasopharyngeal carcinoma cell line CNE-1. MiR-210 has been shown to have a direct or indirect role in regulating cell differentiation, development, migration, X chromosome inactivation, and DNA binding processes under hypoxia [5], and these functions are adaptive changes to a hypoxic environment. This is consistent with the fact that, in induced hypoxia, miR-210 expression is increased to promote the expression of VEGF, so as to stimulate angiogenesis in hypoxic tissues that need to increase the blood supply and relieve the pressure of hypoxia [6].

MiR-210 performs a diverse number of functions and is capable of simultaneously regulating a plethora of genes,

partially due to the multi-target effects of miRNA. However, miRNA has limited ability to regulate an individual gene, which is integral to "fine tuning" [7]. Jie *et al* has shown that, by simultaneous regulation of a set of related genes with specific functions, miRNA can trim the cumulative impact of each regulated gene, and ultimately alter cell function [8]. The results in this study showed that hypoxia-induced expression of miR-210 can inhibit the expression of cyclin D1, thereby resulting in cell cycle blocking in the G1 phase and inhibition of CNE-1 cell proliferation; whereas the opposite effect can be obtained by relieving the function of miR-210 using an inhibitor. Although the proportion of G1 phase cells was only 10.7% more in CNE-1 cells transfected with miR-210 than that in the NC group [5, 9], we propose that miR-210 simultaneously inhibits a key transcription factor E2F3 in cell cycles and related protein cyclin D1 and G2/M phase. Considering the impact of superimposed upstream regulatory genes and G2/M phases, hypoxia-inducible expression of miR-210 slows down the entire cell cycle, inhibits cell proliferation, and enables the cells to adapt to changes in a hypoxic environment.

Furthermore, in the present study, blocking in G1 phase of CNE-1 cells could be detected after 36 h of hypoxia. In comparison to He's study where the blocking of G2/M phases by miR-210 needed to be verified by the process of synchronized release, the cell cycle arrest in this study had stronger inhibition of the cell's G1 phase than the G2/M phase. Therefore, this study discovered key mechanisms of miR-210 on cell cycle regulation, further improving the networks of miR-210 regulation on cell cycles.

Rothe [10] previously found that the prognosis of patients with high expression of miR-210 in breast cancer was often poor, and miR-210 could increase the resistance of tumors to radiotherapy, thus inhibiting cell proliferation and promoting tumor invasion and metastasis. These phenomena may be related with inhibition of the cell cycle. Radiation-sensitive cells are usually fast proliferating cells, whereas the slow proliferating cells are less sensitive to radiation. For example, poorly differentiated nasopharyngeal carcinoma cells are more sensitive to radiotherapy than highly differentiated. The inevitable result of miR-210 inhibition of cell cycle is the inhibition of cell proliferation, which is likely to reduce the radiosensitivity of nasopharyngeal carcinoma cells. The migration and invasion of tumor cells has a close relationship with epithelial-mesenchymal transformation (EMT), and cell cycle is often inhibited under EMT. Thus, the blocking regulation of cell cycle by miR-210 has important academic and clinical significance for tumor development and treatment.

## References


1. Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet, 2010, 11: 597–610.
2. Ambros V. The functions of animal microRNAs. Nature, 2004, 431: 350–355.
3. Ventura A, Jacks T. MicroRNAs and cancer: short RNAs go a long way. Cell, 2009, 136: 586–591.
4. Corn PG. Hypoxic regulation of miR-210: shrinking targets expand HIF-1's influence. Cancer Biol Ther, 2008, 7: 265–267.
5. Fasanaro P, Greco S, Lorenzi M, *et al*. An integrated approach for experimental target identification of hypoxia-induced miR-210. J Biol Chem, 2009, 284: 35134–35143.
6. Hua Z, Lv Q, Ye W, *et al*. MiRNA-directed regulation of VEGF and other angiogenic factors under hypoxia. PLoS One, 2006, 1: e116.
7. Bartel DP, Chen CZ. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nat Rev Genet, 2004, 5: 396–400.
8. He J, Zhang JF, Yi C, *et al*. miRNA-mediated functional changes through co-regulating function related genes. PLoS One, 2010, 5: e13558.
9. He J, Wu J, Xu N, *et al*. MiR-210 disturbs mitotic progression through regulating a group of mitosis-related genes. Nucleic Acids Res, 2013, 41: 498–508.
10. Rothe F, Ignatiadis M, Chaboteaux C, *et al*. Global microRNA expression profiling identifies MiR-210 associated with tumor proliferation, invasion and poor clinical outcome in breast cancer. PLoS One, 2011, 6: e20980.