Hypoxia-induced autophagy reduces radiosensitivity by the HIF-1α/miR-210/Bcl-2 pathway in colon cancer cells

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Abstract. Autophagy is an evolutionarily conserved cellular response to conditions of stress such as hypoxia, which induce radioresistance in cancer cells. We studied the mechanism of action of hypoxia on autophagy and radiosensitivity in colon cancer cells. In the human colon cancer cell lines SW480 and SW620, autophagosomes were analyzed to evaluate autophagy by flow cytometry. The expression of hypoxia inducible factor-1α (HIF-1α), Bcl-2, and miR-210 was detected by western blotting and quantitative real-time polymerase chain reaction (PCR). HIF-1α and miR-210 inhibition was induced by siRNA transfections. Apoptosis detection and colony assays were performed to determine radiosensitivity. HIF-1α and miR-210 showed a significant increase under hypoxic condition. The inhibition of HIF-1α decreased miR-210 expression and autophagy. Silencing of miR-210 upregulated Bcl-2 expression and reduced the survival fraction of colon cancer cells after radiation treatment. Under hypoxia, HIF-1α induces miRNA-210 which in turn enhances autophagy and reduces radiosensitivity by downregulating Bcl-2 expression in colon cancer cells. Our results imply that autophagy contributes to the reduction of radiosensitivity in hypoxic environment, and the process is mediated through the HIF-1α/miR-210/Bcl-2 pathway in human colon cancer cells.

Introduction

Autophagy is an evolutionarily conserved, multistep cellular response to multiple forms of stress such as deprivation of nutrition and oxygen. Depending on the circumstances, autophagy either inhibits or promotes cell growth (1). To enable cell survival under conditions of stress, autophagy contributes to genomic stability by eliminating damaged organelles and protein aggregates that produce reactive oxygen species which cause DNA damage (2). In cancer cells, autophagy is often caused by hypoxia (3), and leads to resistance to radiotherapy and chemotherapy (4,5).

By virtue of rapid cell proliferation, overwhelming growth, increased oxygen consumption, and insufficient blood supply, intratumoral hypoxia is one of the most important characteristics of solid tumors (6,7). Cellular response to hypoxia is partially orchestrated by the activation of hypoxia-inducible factors (HIFs). During hypoxia, the HIF-1α protein is protected from degradation, following which it translocates to the nucleus and binds to constitutively expressed HIF-1β, thereby activating a mass of HIF target genes involved in proliferation, energy metabolism, erythropoiesis, and angiogenesis in both normal tissues and cancer cells (8,9). Autophagy is also involved in a HIF-1α-mediated cell survival mechanism (10-12).

MicroRNAs are a group of small non-coding RNAs, usually 21-24 nucleotides in length. They are involved in posttranscriptional regulation of genes by pairing with target mRNAs to induce cleavage or translational silencing (13). A number of miRNAs can be up- or downregulated by hypoxia; specifically, microRNA-210 is extremely sensitive to oxygen deprivation. In a hypoxic environment, the expression of miR-210 is upregulated by HIF-1α (9,14,15).

Bcl-2, a potential target of miR-210 (9), forms a complex with Beclin-1, a Bcl-2 homology 3 (BH3) domain-only protein that plays important roles in autophagosome formation, autolysosome fusion (16), and promotion of autophagy. The interaction of Bcl-2 and Beclin-1 inhibits autophagy (17). Therefore, we hypothesized that miRNA-210/Bcl-2 may potentially play a role in autophagy under hypoxia.

In the present study, we investigated the mechanism of hypoxia on autophagy and radiosensitivity in colon cancer cells.

Materials and methods

Cell culture. Human colon cancer cell lines SW480 and SW620 (Cell Bank, Shanghai Institutes for Biological Sciences, China)

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were cultured in L-15 culture media (Sigma, CA, USA) supplemented with 10% fetal bovine serum (Biowest, Loire Valley, France) at 37˚C with free gas exchange with atmospheric air. Hypoxia treatment was performed using a tri-gas incubator (37˚C, 5% CO_2, 93% N_2 and 2% O_2; YCP-50S, Changsha Huaxi Electronic Technology Co., Ltd., Hunan, China) for different periods.

Irradiation. X-ray irradiation (RT) was performed at a dose rate of 3.68 Gy/min using the Small Animal Radiation Research Platform (GulmayMedical, ND, USA) with a total dose of 4 Gy determined according to our preliminary research.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR). Cells were grown to a density of $1x10^7$ and digested with trypsin. Total RNA was isolated using the TRIzol reagent (RNAprep Pure Cell/Bacteria kit, Tiangen, Beijing, China), following the manufacturer's instructions. RNA (500 ng) was used as the template for reverse transcription to obtain cDNA using PrimeScript™ RT Reagent kit (Perfect Real-Time, Takara, Japan). QRT-PCR analysis was performed using the following sequence of the reverse transcription primer of miR-210: 5'-GTCGTATCCAGTGCAGGG TCCGAGGTATTCGCACTGGATACGACTCAGCC-3'. The following primers were used for quantitative PCR: miR-210 primers: sense, 5'-CTGTGCGTGTGACAG-3', and antisense, 5'-GTGCAGGGTCCGAGGT-3'; U6 primers: sense, 5'-CTCG CTTCGGCAGCACA-3', and antisense, 5'-AACGCTTCACG AATTTGCGT-3'; HIF-1α primer, sense, 5'-CGTTCCTTCGA TCAGTTGTC-3', and antisense, 5'-TCAGTGGTGGCAGTG GTAGT-3'. The expression of HIF-1α and miR-210 was evaluated using LightCycler 480 SYBR Green I Master (Roche, Basel, Switzerland).

Western blotting. Cells were lysed with RIPA lysis buffer (Roche) and total protein was quantified using Pierce BCA protein assay kit (Thermo Fisher, MA, USA). For analysis, an equal amount of protein extracts was separated by SDS-PAGE on a 10% (w/v) polyacrylamide gel, followed by electrotransfer onto a BioTrace NT Membrane (Pall, NY, USA). The blots were blocked for 1 h with blocking buffer 5% (w/v) fat-free milk, 0.1% (v/v) Tween-20 in PBS. The following antibodies were used: anti-HIF-1α rabbit monoclonal antibody (Epitomics, CA, USA), anti-LC3 mouse monoclonal antibody (Medical & Biological Laboratories Co., Ltd., Japan), anti-Beclin-1 rabbit monoclonal antibody (Cell Signaling Technology, MA, USA), and anti-Bcl-2 rabbit monoclonal antibody (Cell Signaling Technology), anti-ATG12 rabbit monoclonal antibody (Cell Signaling Technology), anti-GAPDH mouse monoclonal antibody (Cell Signaling Technology) and anti-β-actin mouse monoclonal antibody (Cell Signaling Technology). After incubation with horseradish peroxidase-conjugated secondary anti-rabbit (Millipore, MA, USA) or anti-mouse antibodies (GeneTex, TX, USA), the protein bands were detected using the ECL blotting detection reagent (Thermo Fisher, MA, USA), imaged and quantified using Chemioscope Mini system (ChemiQ 4800, Bioshine, Shanghai, China).

Transfection of HIF-1α and miRNA-210 siRNA. The cells were plated for 24 h before transfection and then transfected with miR-210 siRNA or HIF-1α siRNA using X-tremeGene siRNA reagent (Roche). The siRNAs were synthesized by Shanghai GenePharma Co. and contained the following sequences: miR-210 siRNA sense, 5'-UCAGCCGCUGUCAC ACGCACAG-3'; HIF-1α siRNA sense, 5'-CCACCACUGA UGAAUUAAATT-3', and antisense, 5'-UUUAAUUCAUCA GUGGUGGTT-3'.

Autophagy detection. The cells were digested with trypsin and washed twice with phosphate-buffered saline (PBS, pH 7.4) (Hyclone, UT, USA), centrifuged for 5 min at 400 g and resuspended in a binding buffer. The autophagosomes were marked using Cyto-ID Autophagy Detection kit (Enzo, NY, USA). The marked cells were photographed using fluorescence microscopy (Eclipse Ti-S, Nikon, Japan) and the fluorescence intensity was detected by flow cytometry (FC500 MPL, Beckman Coulter, CA, USA).

Apoptosis detection. The cells were digested and washed twice with phosphate-buffered saline (PBS, pH 7.4; Hyclone), centrifuged for 5 min at 400 g and resuspended in a binding buffer. The cells were then stained using Annexin V-FITC Apoptosis Detection kit (BD Biosciences, NJ, USA), followed by the addition of propidium iodide (BD Biosciences). The samples were analyzed by flow cytometry (FC500 MPL, Beckman Coulter).

Colony assay. Irradiated cells were counted, diluted in L-15 culture medium, and reseeded in flasks, followed by incubation for 14 days. After this period, the cells were fixed with polyoxymethylene (Sinopharm Chemical Reagent Co., Ltd., China) and stained with crystal violet. The stained cells were viewed under microscope (Eclipse Ti-S, Nikon) and colonies with >50 cells were calculated. Each experiment included at least 3-6 replicates and all the experiments were repeated 2-3 times.

Statistical analysis. All the data are presented as mean ± standard deviation of more than 3 independent experiments. Statistical analysis was performed using Student's t-test with SPSS, $P<0.05$ was considered to be statistically significant.

Results

Effect of hypoxia on the expression of HIF-1α and miR-210. In the cell lines SW480 and SW620, the relative expression of miR-210 increased significantly after >12 h of hypoxic treatment, compared to the normoxic treatment (Fig. 1A, $P<0.05$). The mRNA expression of HIF-1α remained unchanged under hypoxic treatment compared to normoxic treatment in the two cell lines (Fig. 1B, $P>0.05$). HIF-1α protein was increased after 12 h of hypoxic treatment in both cell lines (Fig. 1C).

Relationship between HIF-1α and miR-210 after hypoxic treatment. Under hypoxic conditions, the protein expression of HIF-1α decreased after transfection with HIF-1α siRNA in both the cell lines (Fig. 2A). The expression of miR-210 also decreased significantly from 24 h after transfection with HIF-1α siRNA under hypoxic treatment (Fig. 2B, $P<0.05$).

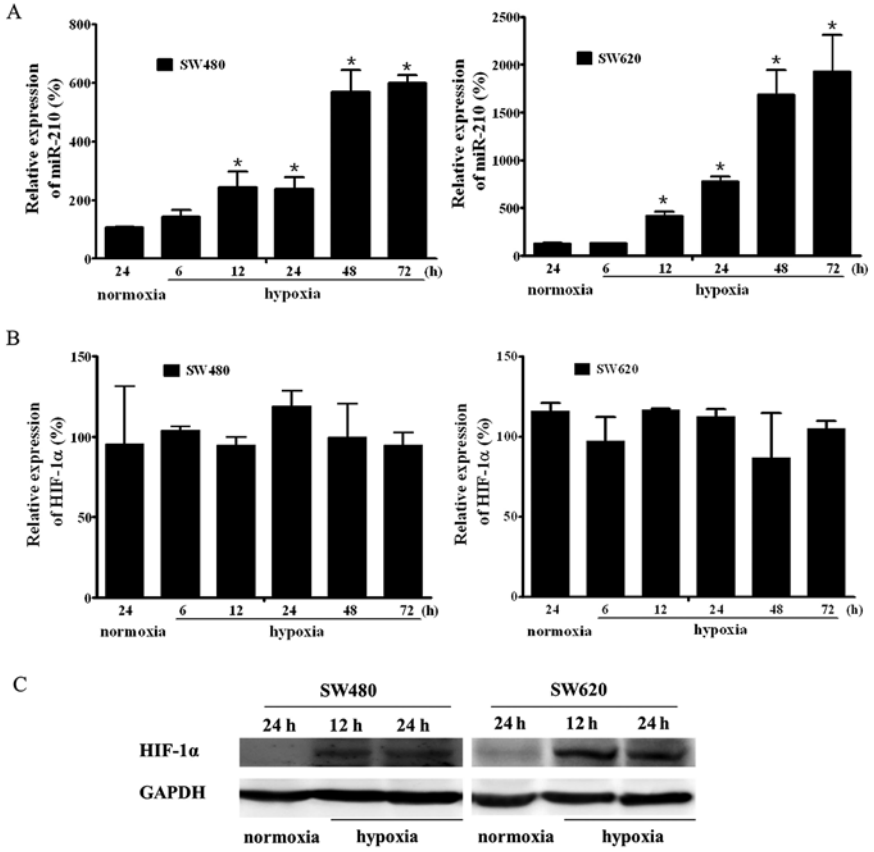

Figure 1. The expression of (A) miR-210 mRNA, (B) HIF-1α mRNA, and (C) HIF-1α protein in colon cancer cells after hypoxic (2% O_2) treatment. Data are expressed as the relative value of the target gene normalized to GAPDH and U6 as the standard and presented as the mean ± standard deviation. *P<0.05 when compared to normoxic (21% O_2, 24 h) treatment control.

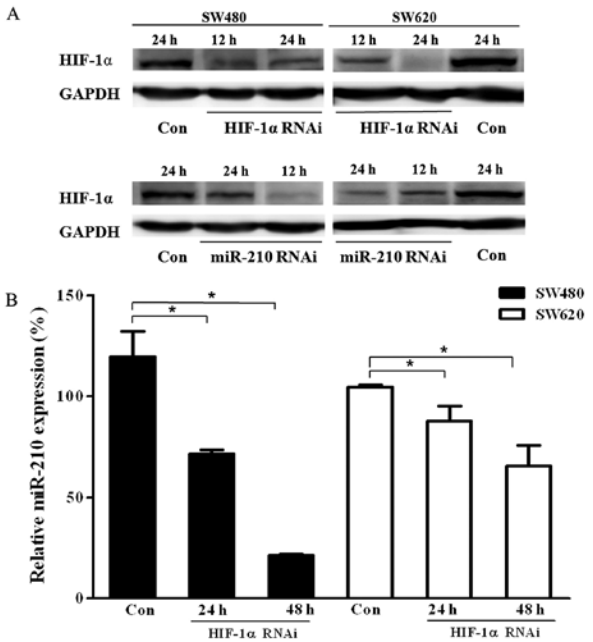

Figure 2. The relationship between HIF-1α and miR-210 expression. (A) The expression of HIF-1α protein after transfection of HIF-1α and miR-210 siRNA in colon cancer cells under hypoxic (2% O_2, 24 h) treatment. (B) The expression of miR-210 after transfection of HIF-1α siRNA in colon cancer cells under hypoxic (2% O_2, 24 h) treatment. Data are expressed as the relative value of the target gene normalized to U6 as the standard and presented as the mean ± standard deviation. Con, hypoxic (2% O_2, 24 h) treatment only control; HIF-1α RNAi, HIF-1α siRNA transfection; miRNA-210 RNAi, miR-210 siRNA transfection. *P<0.05.

In addition, HIF-1α protein was decreased after miR-210 expression was downregulated (Fig. 2A), suggesting that the expression of miR-210 may have a positive feedback effect on the expression of HIF-1α.

Relationship between the expression of miR-210, Bcl-2, and autophagy under hypoxic condition. Compared to normoxic treatment, hypoxic conditions led to a significant increase in autophagy. However, autophagy induced by hypoxia was attenuated significantly after miRNA-210 siRNA transfections (Fig. 3, P<0.05).

Under hypoxic condition, Bcl-2 mRNA expression was significantly increased after miRNA-210 was downregulated by siRNA in both the cell lines (Fig. 4A, P<0.05). Bcl-2 protein expression was decreased significantly in SW620 cells after hypoxic treatment. However, the change of bcl-2 protein expression after hypoxic treatment in SW480 cells was not found. Autophagy-related proteins including Beclin-1, ATG12 and LC3II as well as the ratio of LC3II/LC3I were decreased after miRNA-210 siRNA treatment under hypoxic treatment compared with hypoxic treatment only group (Fig. 4B). Although autophagy induced by the hypoxic treatment was attenuated by the downregulation of miRNA-210, ablation of both miR-210 and Bcl-2 by siRNAs remarkably rescued this attenuation in both SW480 and SW620 cells (Fig. 4C, P<0.05).

Effect of miR-210 and Bcl-2 on radiosensitivity of colon cancer cells under hypoxic condition. Under hypoxic conditions,

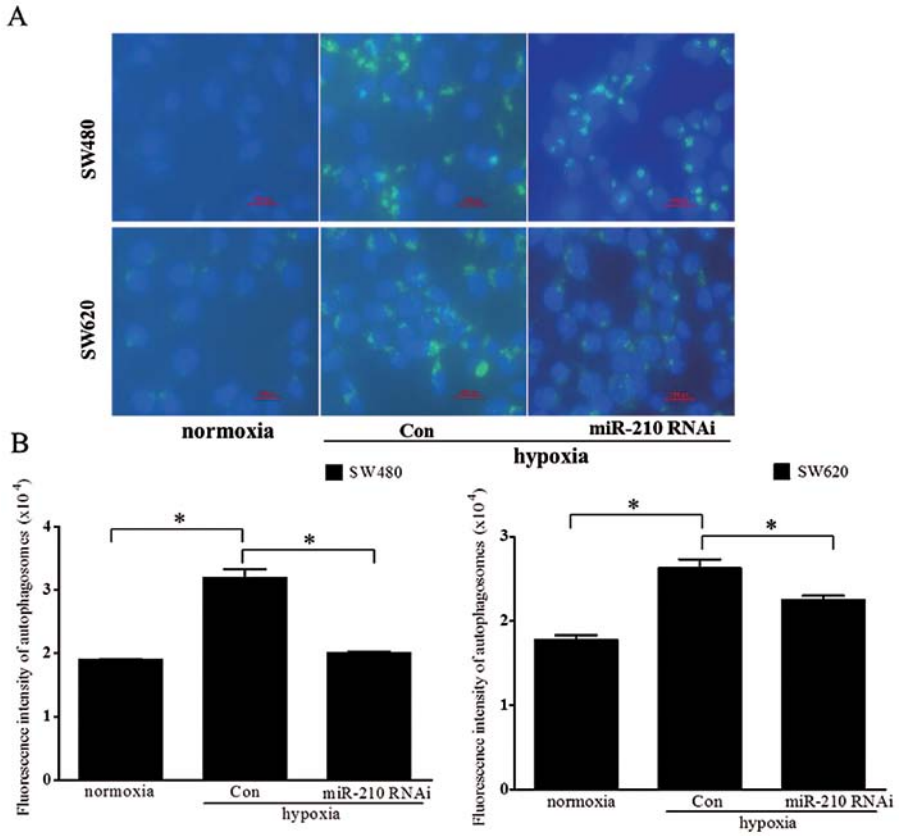

Figure 3. Effect of miR-210 on autophagosome formation after hypoxic (2% O_2, 24 h) treatment in colon cancer cells. (A) Representative image of the autophagosome, which was labeled with green color. (B) The rate of autophagosome formation was measured quantitatively using the fluorescence intensity assay. Independent images per point were analyzed. Data are presented as the mean ± standard deviation. Con, hypoxic (2% O_2, 24 h) treatment only control; miRNA-210 RNAi, miR-210 siRNA transfection. *P<0.05.

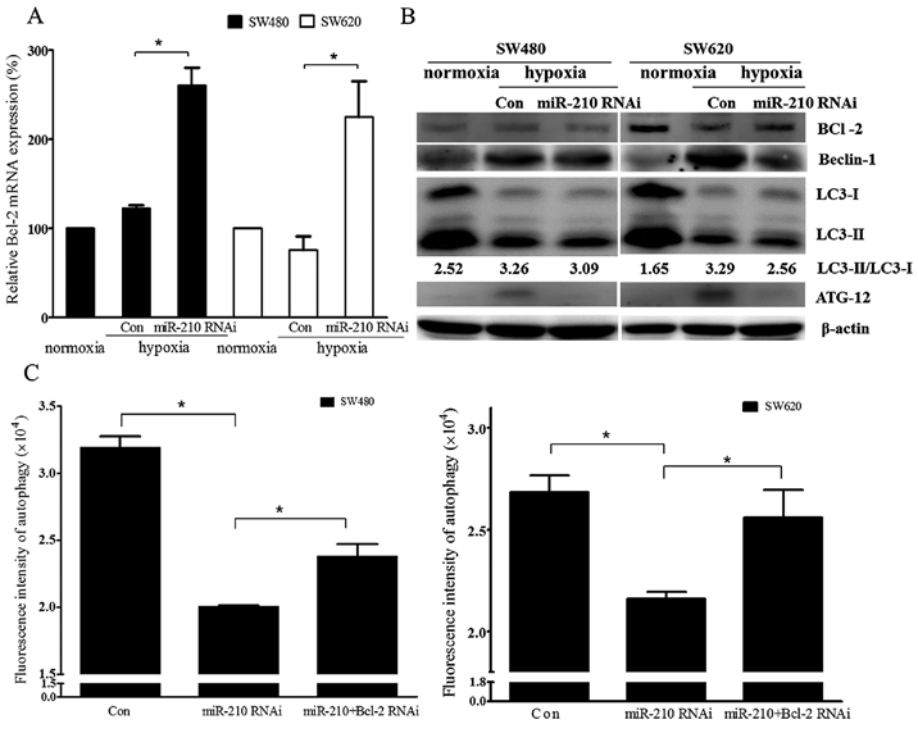

Figure 4. The relationship between miR-210 and Bcl-2 and the effect of miR-210/Bcl-2 on autophagosome formation. (A) Expression of Bcl-2 mRNA in colon cancer cell lines after miR-210 siRNA transfection under hypoxic (2% O_2, 24 h) treatment. Data are expressed as the relative value of the target gene compared with GAPDH as the standard. (B) Levels of autophagy-related proteins after miRN-210 siRNA transfection after hypoxic treatment. (C) The level of autophagosome formation was measured quantitatively using fluorescence intensity assay. Three independent images per point were analyzed. Con, hypoxic (2% O_2, 24 h) treatment only control; miRNA-210 RNAi, miR-210 siRNA treatment; Bcl-2 RNAi, Bcl-2 siRNA treatment. Data are presented as the mean ± standard deviation. *P<0.05.

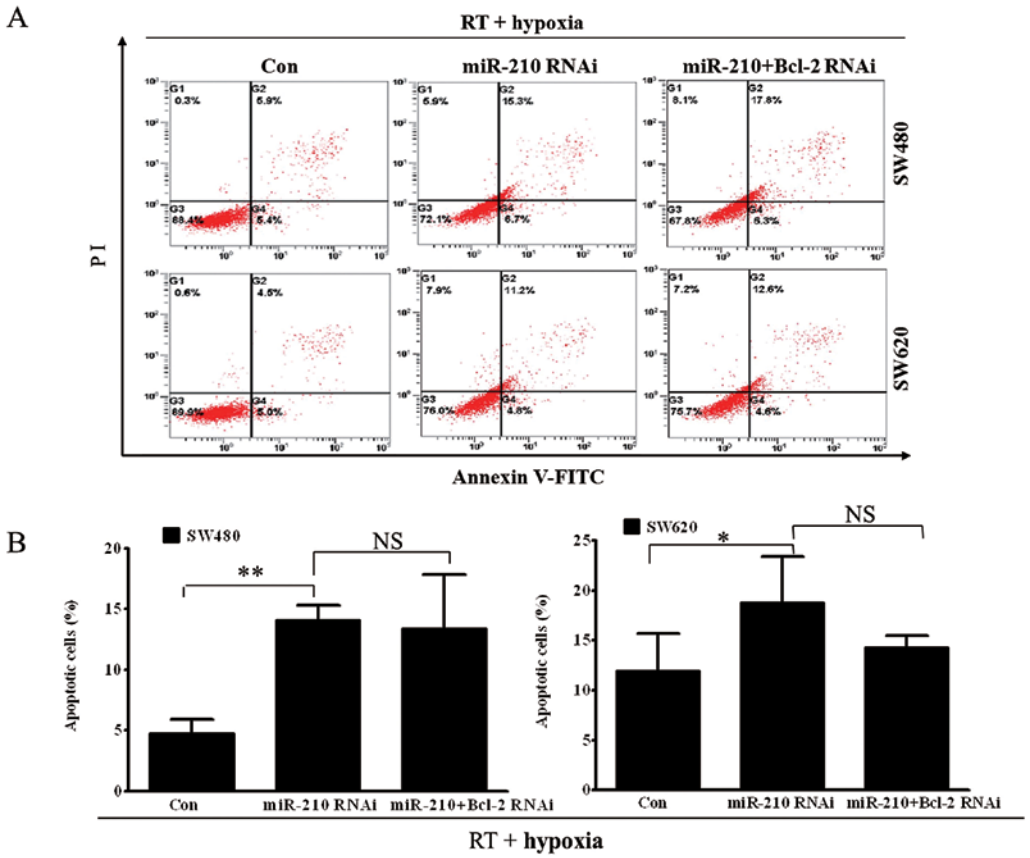

Figure 5. Effect of miR-210 and Bcl-2 on radiation-induced apoptosis in colon cancer cells after hypoxic treatment. (A) Apoptotic cells were stained with Annexin V-FITC/PI and analyzed by flow cytometry. (B) The percentage of apoptotic cells. Data are presented as the mean ± standard deviation. Con, hypoxic (2% O_2, 24 h) treatment only control; RT, radiation treatment with dose of 4 Gy; miR-210 RNAi, miR-210 siRNA-treated group; Bcl-2 RNAi, Bcl-2 siRNA-treated group; miR-210+Bcl-2 RNAi, both miR-210 and Bcl-2 siRNA-treated group. *P<0.05, ** P<0.01. NS, non-significant difference.

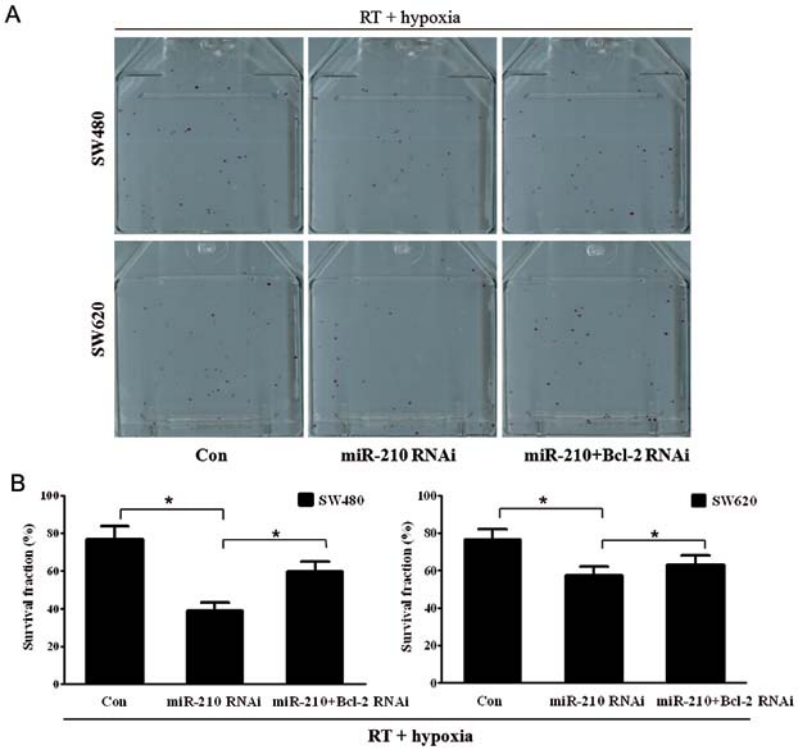

Figure 6. Effect of miR-210 and Bcl-2 on radiation-induced colony formation in colon cancer cells after hypoxia treatment. (A) Representative crystal violet staining of the colonies. (B) The surviving fraction of colon cancer cells after radiation and hypoxic treatment. Data are presented as the mean ± standard deviation. Con, hypoxic (2% O_2, 24 h) treatment only control; RT, radiation treatment with dose of 4 Gy; miR-210 RNAi, miR-210 siRNA-treated group; Bcl-2 RNAi, Bcl-2 siRNA-treated group; miR-210+Bcl-2 RNAi, both miR-210 and Bcl-2 siRNA-treated group. *P<0.05.

apoptosis induced by X-ray irradiation (RT) increased after miR-210 siRNA transfections, compared with RT alone (Fig. 5, $P<0.01$ for SW480 and $P<0.05$ for SW620). However, there was no difference in apoptosis between miR-210 siRNA-transfected group and either the miRNA-210 or the Bcl-2siRNA-transfected group. On the other hand, cell colony formation assay showed that the survival of cells was decreased when miRNA-210 expression was downregulated under hypoxic conditions after RT, while ablation of both miR-210 and Bcl-2 increased the colony formation activity (Fig. 6, $P<0.05$). These findings demonstrate that the inhibition of miR-210 leads to increased radiosensitivity by upregulating Bcl-2 expression under hypoxic conditions, which was not related to apoptosis induced by radiation.

Discussion

Autophagy is an evolutionarily conserved, cellular degradation pathway that eliminates damaged or superfluous proteins and organelles (18,19). Cytosolic material is sequestered in autophagosomes, delivered to lysosomes for degradation, and recycled to sustain cell viability (20). Previous studies demonstrate that autophagy can cause resistance to radio- and chemotherapy in breast, prostate, head and neck, and ovarian cancers (5,21-23).

Hypoxia is a well-known inducer of autophagy (24), one of the most remarkable features of solid tumors. Under these conditions, HIF-1α plays a central role in various cellular responses (25); particularly, it promotes cell proliferation and survival through MYC and PI3K/AKT (26,27); increases angiogenesis through VEGF and PDGF (28); and influences cell metabolism by targeting GLUT1 (29). Hypoxia-induced autophagy is also involved in a HIF-1α-mediated general mechanism of cell survival. It has been identified that BNIP3 and BNIP3L are the downstream targets of HIF-1α-mediated autophagy (10).

In addition, miR-210 acts as downstream of HIF-1α, which is consistently upregulated in multiple cancer cell lines under hypoxic conditions (30-32). It promotes migration and invasion and is proposed as an independent prognostic marker for the overall survival of colon cancer patients (33). Chio *et al* reported that miR-210 could mediate hypoxia-induced neural apoptosis by targeting Bcl-2, and discovered a miR-210-specific binding element in the 3'-UTR of the Bcl-2 mRNA (34).

The anti-apoptotic protein Bcl-2 inhibits apoptosis by binding to Bax or Bak (35). Previous studies demonstrate that the overexpression of Bcl-2 induces resistance to chemo- and radiotherapy in cancer patients (36). Bcl-2 plays a dual role as an anti-apoptotic, anti-autophagic protein, and is closely related to ROS levels (37). BH3 agonists can specifically interrupt the binding of Bcl-2/XL and Bak/Bax by competing for the BH3 domain. Hepatocellular carcinoma cells with increased levels of Bcl-2 exhibit resistance to BH3 agonists by activating the ROS-JNK-autophagy pathway (38). Silencing of Bcl-2 by siRNA induces autophagic cell death, but not apoptosis in MCF-7 breast cancer cells with high level of Bcl-2 expression (39). Similarly, we did not observe any effect of Bcl-2 silencing on apoptosis induced by radiation treatment in colon cancer cells. The crosstalk between apoptosis and autophagy is known to control cell survival and death (40), primarily by JNK1-mediated phosphorylation of Bcl-2. Under conditions of nutrient starvation, low level of Bcl-2 phosphorylation initially occurs and promotes survival by activating autophagy; with prolonged starvation, higher levels of Bcl-2 phosphorylation promote apoptosis (41). This indicates that Bcl-2 acts as a switch for the regulation of apoptosis and autophagy.

In this study, we demonstrated that autophagy is enhanced by hypoxia, and negatively influences radiosensitivity in two colon cancer cell lines. We demonstrated that HIF-1α causes hypoxia-induced overexpression of miR-210, while the downregulation of miR-210 has a negative effect on the expression of HIF-1α. Furthermore, downregulation of Bcl-2 was discovered under hypoxic environment. Beclin-1 is known to regulate the initial steps of autophagy. Beclin-1 possesses a BH-3 domain that binds Bcl-2 and other Bcl-2 homologues such as Bcl-XL and Mcl-1 (42). The association of Bcl-2 and Beclin-1 during normal growth conditions may prevent inappropriate activation of autophagy while disruption of this interaction may induce autophagy (17,41,43).

In conclusion, our study demonstrates that hypoxia induces HIF-1α and its downstream target miR-210, which inhibits the expression of Bcl-2 and enhances autophagy, thereby contributing to radioresistance in colon cancer cells. However, a comprehensive analysis of the interactions between HIF-1α, miR-210, and Bcl-2 remains to be done. Further studies in other cancer cells and animal models are necessary to understand the crosstalk between apoptosis and autophagy. A better understanding of the mechanism of hypoxia-induced autophagy may be of potential value for improving the effectiveness of radiotherapy.

Acknowledgements

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