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Effect of multidrug resistance 1/P-glycoprotein on the hypoxia-induced multidrug resistance of human laryngeal cancer cells

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Abstract. In a previous study, it was demonstrated that hypoxia upregulated the multidrug resistance (MDR) of laryngeal cancer cells to chemotherapeutic drugs, with multidrug resistance 1 (MDR1)/P-glycoprotein (P-gp) expression also being upregulated. The present study aimed to investigate the role and mechanism of MDR1/P-gp on hypoxia-induced MDR in human laryngeal carcinoma cells. The sensitivity of laryngeal cancer cells to multiple drugs and cisplatin-induced apoptosis was determined by CCK-8 assay and Annexin-V/propidium iodide staining analysis, respectively. The accumulation of rhodamine 123 (Rh123) in the cells served as an estimate of drug accumulation and was evaluated by flow cytometry (FCM). MDR1/P-gp expression was inhibited using interference RNA, and the expression of the MDR1 gene was analyzed using reverse transcription-quantitative polymerase chain reaction and western blotting. As a result, the sensitivity to multiple chemotherapeutic agents and the apoptosis rate of the hypoxic laryngeal carcinoma cells increased following a decrease in MDR1/P-gp expression ($P<0.05$). Additionally, FCM analysis of fluorescence intensity indicated that the down-regulated expression of MDR1/P-gp markedly increased intracellular Rh123 accumulation ($P<0.05$). Such results suggest that MDR1/P-gp serves an important role in regulating hypoxia-induced MDR in human laryngeal carcinoma cells through a decrease in intracellular drug accumulation.

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Introduction

Laryngeal carcinoma is the second most prevalent malignancy located in the head and neck region (1). Chemotherapy is a common therapeutic approach for patients with advanced or metastatic laryngeal carcinoma; however, multidrug resistance (MDR) has been a major contributor to the failure of chemotherapy in patients presenting with this disease (2,3). The pathogenetic mechanisms underlying the regulation of MDR in laryngeal cancer cells currently remain unclear.

Hypoxia has been considered as an essential feature in human solid tumors, such as laryngeal cancer, and it affects a number of biological cell behaviors, including angiogenesis, apoptosis and resistance to therapy, occurring through the regulation of gene expression and modification of certain proteins (4). It has been reported that hypoxia induces drug resistance in various solid tumors (5-7). Similarly, it was confirmed in a previous study that hypoxia has an effect on the regulation of MDR in human laryngeal carcinoma cells (8). Therefore, hypoxia serves as a major obstacle in the development of cancer chemotherapy. Currently, further research is required to fully understand the regulatory mechanisms of MDR in hypoxic laryngeal cancer cells.

Multidrug resistance 1 (MDR1)/P-glycoprotein (P-gp), a plasma membrane glycoprotein encoded by the MDR1 gene, is a member of the adenosine triphosphate-binding cassette (ABC)-type transporter family. In a previous study, the upregulation of MDR1/P-gp expression was associated with a poorer prognosis in patients with human laryngeal cancer (9). It is understood that MDR1/P-gp serves a crucial role in regulating intracellular drug concentrations, consequently determining the drug sensitivity of human cells. Recently, MDR1/P-gp has been elucidated to be involved in the regulation of MDR in human laryngeal cancer cells (10,11), with the inhibition of MDR1/P-gp expression having been confirmed as an effective method to reverse MDR in such cells (11). Furthermore, previous data have demonstrated that MDR1/P-gp expression is significantly upregulated in response to hypoxia in laryngeal cancer cells (12). To the best of our knowledge, there are currently no existing studies that focus on the function and mechanism of MDR1/P-gp in the hypoxia-induced MDR observed in laryngeal cancer cells.

The present study aimed to investigate the role of MDR1/P-gp in the hypoxia-induced MDR of human laryngeal carcinoma cells and elucidate the mechanisms underlying this.

Materials and methods

Cell culture. Human laryngeal cancer Hep-2 and AMC-HN8 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and the Asan Medical Center, Ulsan University College of Medicine (Seoul, Korea), respectively. Each cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 100 IU/ml penicillin and 100 IU/ml streptomycin at 37˚C with 5% CO_2.

Exposure to hypoxia. For hypoxic exposure, the Hep-2 and AMC-HN8 cells were incubated in a NU-4750 US Autoflow CO_2 Water-Jacketed Incubator (NuAire, Plymouth, MN, USA) at 37˚C, with 1% O_2 and 5% CO_2 balanced by nitrogen.

Transfection of small interfering RNA (siRNA). The double strand siRNA oligonucleotide obtained to target the human MDR1 gene (sense, 5'-GGAAAAGAAACCAACUGUCTT-3'; and anti-sense, 5'-GACAGUUGGUUUCUUUUCCTT-3') was synthesized by Shanghai GenePharma Co. Ltd. (Shanghai, China), as previously described (13). As a negative control, the cells were transfected with a non-specific control siRNA (sense, 5'-UUCUCCGAACGUGUCACGUTT-3'; and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'; Shanghai GenePharma Co. Ltd.), which had no homology to any human transcripts on record. The cells were cultured in antibiotic-free medium for 24 h prior to transfection with 100nM siRNA using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were harvested and examined following a 24-h transfection period.

Reverse transcription-quantitative polymerase chain reaction (PCR) analysis. Total RNA was extracted from the laryngeal carcinoma cells using TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Subsequently, the isolated RNA was reverse-transcribed into complementary DNA (cDNA) using the PrimeScript® RT Reagent kit (Takara Bio, Inc., Otsu, Japan), as previously described (14). The primers used were as follows: MDR1 sense, 5'-CTTCAGGGTTTCACATTTGGC-3' and antisense, 5'-GGTAGTCAATGCTCCAGTGG-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense, 5'-CATCTTCCAGGAGCGAGA-3' and antisense, 5'-TGTTGTCATACTTCTCAT-3'. PCR amplification was performed in a 20-μl final reaction mixture, including a diluted cDNA solution, 10 μM of each primer and 10 μl SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Inc.) using the ABI 7900HT Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermal cycling conditions were as follows: One cycle at 95˚C for 10 min and 40 cycles at 95˚C for 15 sec and 60˚C for 60 sec. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method, as previously described (15).

Western blot analysis. The laryngeal cancer Hep-2 and AMC-HN8 cells were harvested and lysed with cold radioimmunoprecipitation assay buffer (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. Equal amounts of protein extract (25 μg total protein) were loaded into sodium dodecyl sulfate polyacrylamide gel electrophoresis (5% stacking gel and 8% separating gel), followed by separation at 80 V for ~2 h and transferal onto Immobilon®-P PVDF Membranes (EMD Millipore, Billerica, MA, USA), blocked with 4% skimmed milk for 1.5 h at room temperature. Protein extracts were incubated overnight at 4˚C with the primary antibodies [mouse monoclonal anti-human MDR1/P-gp (catalog no., ab3366; 1:200; Abcam, Cambridge, UK) and mouse monoclonal anti-human GAPDH (1:1,000; Sigma-Aldrich)], followed by incubation for 1 h with the secondary antibodies [horseradish peroxidase-conjugated anti-mouse secondary antibodies (1:5,000; Sigma-Aldrich)]. Finally, protein bands were detected by electrogenerated chemiluminescence according to the manufacturer's protocols (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

Cell cytotoxicity assay. The sensitivity of the Hep-2 and AMC-HN8 cells to fluorouracil (5-FU), cisplatin, doxorubicin, paclitaxel and gemcitabine was determined using a cell counting kit 8 (CCK-8) assay. The cells were plated in 96-well culture panels (Hep-2, $5x10^3$ cells/well; and AMC-HN8, $8x10^3$ cells/well), and following 12 h, the cells were treated with relevant doses of chemotherapeutic agents (5-fluorouracil: 0.1, 1, 10, 100 and 1000 μg/ml; gemcitabine: 0.032, 0.32, 3.2, 32 and 320 μg/ml; cisplatin: 0.03, 0.3, 3.0, 30 and 300μg/ml); adriamycin: 0.004, 0.04, 0.4, 4 and 40 μg/ml); and paclitaxel: 0.09, 0.9, 9, 90 and 900 μg/ml) and incubated for 48 h under normoxic or hypoxic conditions. The sensitivity of the cells to each agent was detected using CCK-8 assay, and the IC_{50} was calculated as the concentration of each drug required to produce a 50% reduction in the number of cells.

Annexin-V/propidium iodide (PI) apoptosis assay. The apoptotic indices of the Hep-2 and AMC-HN8 cells were examined by flow cytometry (FCM). Cells in the log phase were grown in 6-well plates (Hep-2, $3x10^5$ cells/well; and AMC-HN8, $4x10^5$ cells/well) and cultured overnight at 37˚C. The culture medium was then renewed, and the cells were incubated in normoxic or hypoxic conditions for 12 h. Cisplatin was added to each well with a final concentration of $2.5x10^{-9}$ M, and the cells were cultured in normoxic or hypoxic conditions for 48 h. Subsequently, 5 μl (50 μg/ml) Annexin-V-fluorescein isothiocyanate (FITC) was added to each well, and the cells were incubated for a further 10 min. The cells were washed twice with DMEM and then resuspended in 190 μl Tris-hydrochloride buffer. Next, 5 μl (20 μg/ml) PI was added to the resuspended cells and cultured at 4˚C for a further 10 min. The mean fluorescence intensity of Annexin-V-FITC/PI was measured by FCM. Finally, the apoptotic indices of the Hep-2 and AMC-HN8 cells were calculated by the mean fluorescence intensity.

Rhodamine 123 (Rh123) accumulation assay. Rh123 accumulation in the Hep-2 and AMC-HN8 cells was analyzed by FCM, which was performed as previously described (16).

Table I. Effect of MDR1/P-gp expression inhibition on the chemosensitivity of hypoxic Hep-2 cells.

	IC_{50}, μg/ml		
Drug	Untreated control	Negative control	MDR1-siRNA
Paclitaxel	$3.90x10^{-2}$±$0.21x10^{-2}$	$3.85x10^{-2}$±$0.26x10^{-2}$	$7.61x10^{-3}$±$0.39x10^{-3}$[a]
5-FU	240.81±0.32	238.90±0.21	78.24±0.48[a]
Doxorubicin	3.94±0.15	3.96±0.24	2.24±0.27[a]
Gemcitabine	39.26±0.16	39.18±0.28	28.26±0.35[a]
Cisplatin	9.10±0.10	8.82±0.12	4.48±0.36[a]

Mean ± standard deviation of three individual experiments. [a]P<0.05 vs. untreated control and negative control. IC_{50}, half maximal inhibitory concentration; MDR1, multidrug resistance 1; siRNA, small interfering RNA; 5-FU, fluorouracil.

Table II. Effect of MDR1/P-glycoprotein expression inhibition on the chemosensitivity of hypoxic AMC-HN8 cells.

	IC_{50}, μg/ml		
Drug	Untreated control	Negative control	MDR1-siRNA
Paclitaxel	$35.54x10^{-3}$±$0.36x10^{-3}$	$36.04x10^{-3}$±$0.17x10^{-3}$	$7.23x10^{-3}$±$0.58x10^{-3}$[a]
5-FU	230.34±0.64	232.48±0.92	71.53±0.82[a]
Doxorubicin	3.72±0.22	3.87±0.35	2.12±0.19[a]
Gemcitabine	36.40±0.32	35.58±0.29	21.48±0.13[a]
Cisplatin	8.83±0.58	8.64±0.26	3.35±0.27[a]

Mean ± standard deviation of three individual experiments. [a]P<0.05 vs. untreated control and negative control. IC_{50}, half maximal inhibitory concentration; MDR1, multidrug resistance 1; siRNA, small interfering RNA; 5-FU, fluorouracil.

The cell suspension was detected by a FACSCalibur Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using 488 nm excitation. All the data were analyzed using CellQuest™ software (BD Biosciences).

Statistical analysis. Each experiment was repeated at least three times, and the data are presented as the mean ± standard deviation. Comparisons of quantitative variables were performed using Student's t-test or a one-way analysis of variance using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Inhibition of MDR1 gene expression by RNA interference. In a previous study, it was confirmed that MDR1 gene expression in human laryngeal cancer cells was upregulated by hypoxia (12). In the present study, the Hep-2 and AMC-HN8 cells were transfected with either non-specific control siRNA (scrambled siRNA) or a double-strand siRNA oligonucleotide that targeted the MDR1 gene (MDR1-siRNA) for 24 h, followed by a 24-h incubation in hypoxic conditions. As presented in Fig. 1, compared with the negative or untreated controls, the protein and mRNA levels of the MDR1 gene in the hypoxic laryngeal cancer cells were significantly reduced following transfection with MDR1-siRNA (P<0.05).

Inhibition of MDR1/P-gp expression suppresses MDR in hypoxic laryngeal carcinoma cells. It has been previously demonstrated that hypoxia upregulates the sensitivity of human laryngeal cancer cells to multiple chemotherapeutic agents (8). In the present study, to allow for the investigation of the role of MDR1/P-gp in hypoxia-induced MDR, the Hep-2 and AMC-HN8 cells were transfected with MDR1-siRNA or scrambled siRNA for 24 h prior to incubation in hypoxic conditions. Subsequently, the drug sensitivity of the MDR1-siRNA group was compared to that of the negative or untreated control groups using a CCK-8 assay. As presented in Tables I and II, the sensitivity of the hypoxic laryngeal carcinoma cells to cisplatin, gemcitabine, paclitaxel, 5-FU and doxorubicin was significantly upregulated following the suppression of MDR1/P-gp expression (P<0.05).

MDR1/P-gp protects hypoxic laryngeal cancer cells from cisplatin-induced apoptosis. A previous study demonstrated that hypoxia enhances the rate of chemotherapy-induced apoptosis in laryngeal cancer cells (8). In the present study, FCM with Annexin-V/PI staining was utilized to assess the role of MDR1/P-gp in the hypoxic protection of Hep-2 and AMC-HN8 cells from cisplatin-induced apoptosis. It was observed that the rate of cisplatin-induced apoptosis was significantly increased under hypoxic conditions in the MDR1-siRNA transfected cells compared with the untreated or negative controls (P<0.05; Fig. 2).

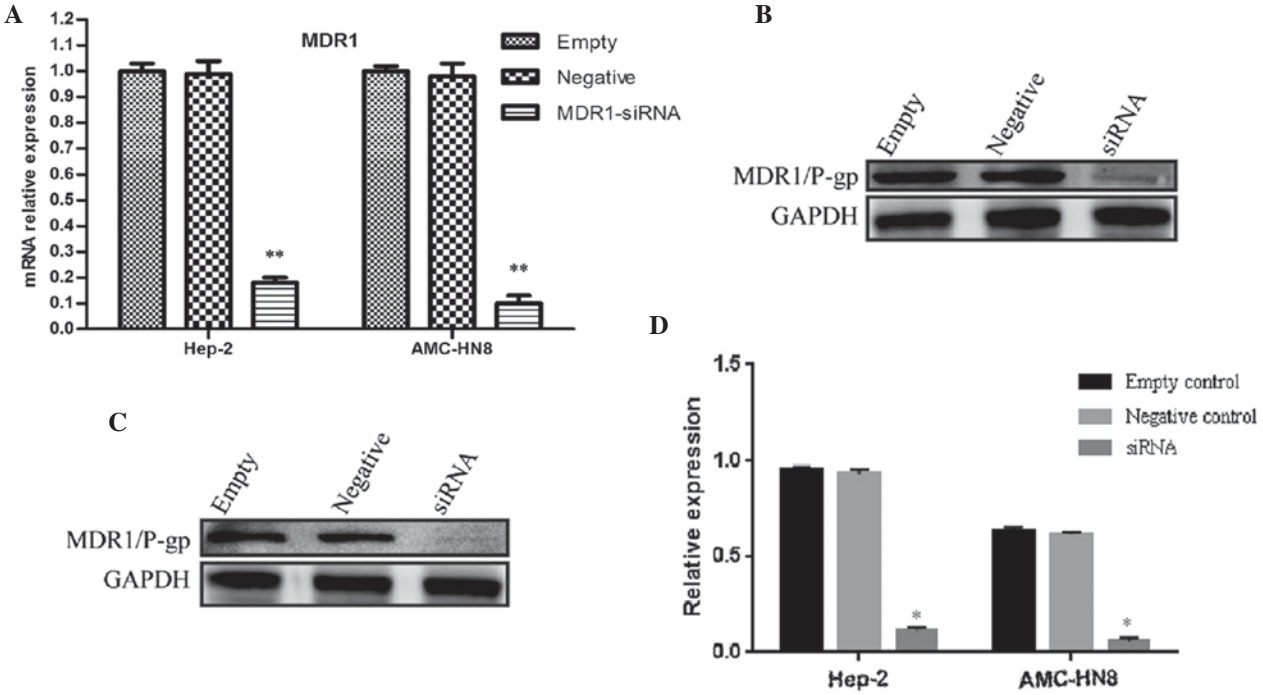

Figure 1. Downregulation of MDR1 gene expression in hypoxic laryngeal cancer cells transfected with MDR1-siRNA. Hep-2 and AMC-HN8 cells were transfected with vectors containing either a MDR1 scrambled or MDR1 targeting sequence (MDR1-siRNA), and were then cultured in hypoxic conditions for 24 h. (A) MDR1 mRNA expression in the cells was analyzed by reverse transcription-quantitative polymerase chain reaction; **P<0.01 vs. empty and negative groups under hypoxia. MDR1/P-gp protein expression in the (B) Hep-2 and (C) AMC-HN8 cells was assessed by western blotting. (D) Quantification of western blotting. *P<0.05 vs. empty and negative groups. MDR1, multidrug resistance 1; siRNA, small interfering RNA; P-gp, P-glycoprotein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

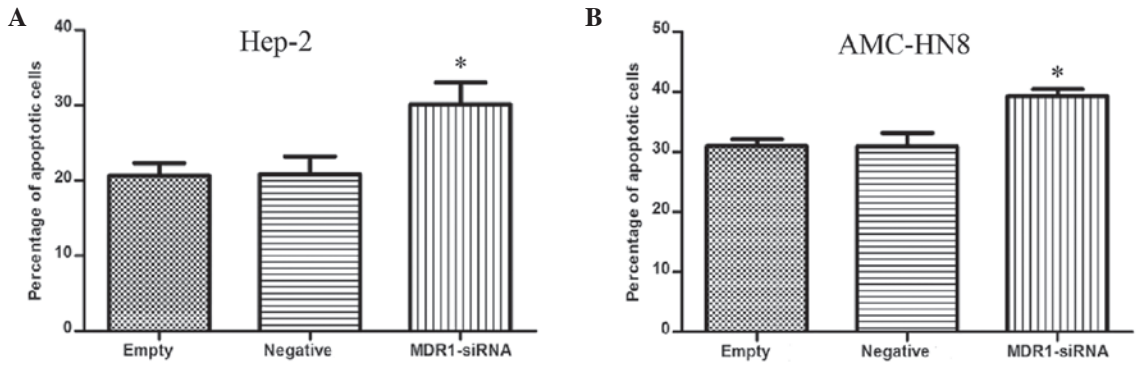

Figure 2. Effect of MDR1/P-glycoprotein expression on the rate of cisplatin-induced apoptosis in hypoxic cells. The apoptotic percentage of the hypoxic (A) Hep-2 and (B) AMC-HN8 cells in the control groups and MDR1-siRNA group pre-treated with cisplatin ($2.5x10^{-9}$ M). Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05 vs. empty and negative groups. MDR1, multidrug resistance 1; siRNA, small interfering RNA.

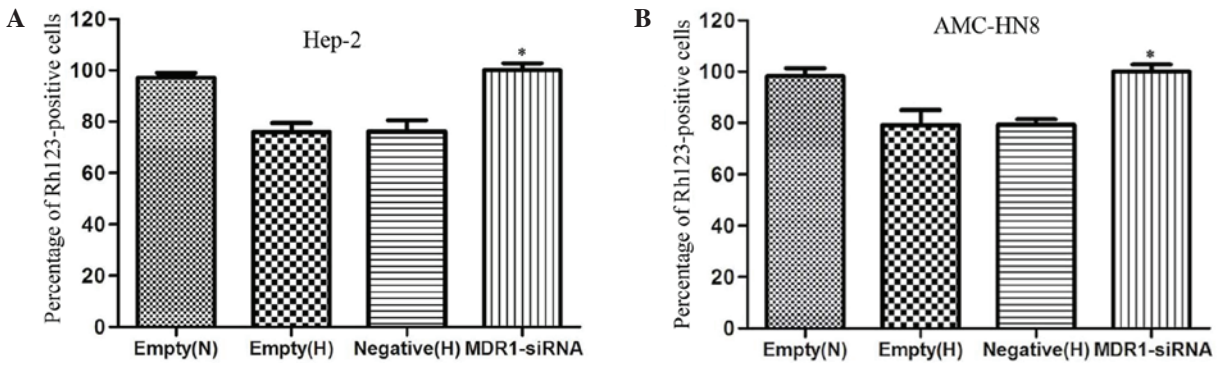

Figure 3. Effect of MDR1/P-glycoprotein on the accumulation of intracellular rhodamine 123 in hypoxic laryngeal cancer cells. (A) Hep-2 controls and Hep-2 MDR1-siRNA-transfected cells in hypoxic conditions. *P<0.05 vs. empty and negative groups. (B) AMC-HN8 controls and AMC-HN8 MDR1-siRNA-transfected cells in hypoxic conditions. *P<0.05 vs. empty and negative groups. Data are presented as the mean ± standard deviation from three independent experiments. N, normoxia; H, hypoxia; MDR1, multidrug resistance 1; siRNA, small interfering RNA.

MDR1/P-gp downregulates drug accumulation in hypoxic laryngeal cancer cells. A previous study demonstrated that hypoxia significantly reduces the intracellular drug accumulation in laryngeal carcinoma cells (8). In the current study, the effect of MDR1/P-gp expression on intracellular drug accumulation in hypoxic Hep-2 and AMC-HN8 cells was evaluated using FCM to detect the fluorescence intensity of intracellular Rh123 accumulation. As presented in Fig. 3, the laryngeal carcinoma cells exposed to hypoxic conditions had significantly decreased Rh123 accumulation ($P<0.05$). Furthermore, the inhibition of MDR1/P-gp expression in the hypoxic Hep-2 and AMC-HN8 cells transfected with MDR1-siRNA significantly increased the intracellular accumulation of Rh123 ($P<0.05$; Fig. 3).

Discussion

Despite the wide application of chemotherapeutic agents for the treatment of human laryngeal cancer, MDR remains a major therapeutic obstacle. The hypoxic microenvironment has been recognized as a common phenomenon in the tissues of human solid tumors. Currently, there is increasing evidence suggesting that hypoxic cells are somewhat resistant to conventional chemotherapy in various human malignancies (7,17,18). A previous study confirmed that the sensitivity of laryngeal cancer cells to multiple chemotherapeutic drugs may be reduced by hypoxia (8). However, the regulatory mechanism underlying hypoxia-induced MDR in human laryngeal carcinoma cells remains to be identified.

MDR1/P-gp is a member of the ABC-type transporter family and has been considered as a biofunctional regulator of MDR in a series of human tumor cells (19-21). In a previous study, it was demonstrated that the expression of MDR1/P-gp in human laryngeal cancer tissues was associated with malignant progression and metastasis (9). Furthermore, several studies have reported that MDR1/P-gp may serve a crucial role in the regulation of MDR in laryngeal cancer cells (10,11). Zhigang *et al* (11) observed that the inhibition of MDR1/P-gp expression served as an effective method to reverse MDR in laryngeal cancer cells. Similarly, certain studies have identified that hypoxia may contribute to MDR through the upregulation of MDR1/P-gp expression in human tumor cells (22,23). By contrast, Song *et al* (24) indicated that MDR1/P-gp may not be involved in hypoxia-induced MDR in non-small cell lung cancer cells. Another previous study confirmed that hypoxia significantly enhances the expression of MDR1/P-gp in laryngeal carcinoma cells (12), and the current study demonstrated that the inhibition of MDR1/P-gp expression significantly increased the sensitivity of laryngeal cancer cells to multiple drugs in hypoxic environments. Therefore, further research has verified that the inhibition of MDR1/P-gp expression upregulates the apoptosis rate of hypoxic laryngeal cancer cells induced by chemotherapy. This suggests that MDR1/P-gp may be partly involved in the hypoxia-induced MDR observed in laryngeal cancer cells, and the suppression of MDR1/P-gp expression may be considered as an effective approach to achieve MDR reversal. The differences noted between the aforementioned studies may be due to intrinsic differences in the regulatory mechanisms of MDR in numerous types of tumor cells.

Theoretically, MDR1/P-gp, as an energy-dependent membrane efflux pump, has an effect on the drug sensitivity of human cells by regulating intracellular drug concentrations (25,26). In a previous study, it was demonstrated that hypoxia markedly reduced drug accumulation in laryngeal cancer cells (8). In practice, the results of the present study indicated that inhibition of MDR1/P-gp expression results in the downregulation of the transport function in hypoxic laryngeal cancer cells, leading to increased intracellular drug concentration and suppression of hypoxia-induced MDR. Similar to the results of Wartenberg *et al* (22) and Xia *et al* (23), the data from the current study suggested that MDR1/P-gp may partly contribute to hypoxia-induced MDR in human laryngeal cancer cells through the regulation of drug transport. However, further research is necessary to determine the regulatory mechanisms underlying MDR1/P-gp expression in hypoxic laryngeal cancer cells.

In conclusion, the present study indicates that MDR1/P-gp expression serves an important role in the mediation of MDR in hypoxic laryngeal carcinoma cells. The results have provided evidence that a decrease in intracellular drug concentration may explain how MDR1/P-gp contributes to the MDR of hypoxic laryngeal cancer cells. Therefore, targeting the MDR1 gene may be a potentially beneficial therapeutic strategy for the reversal of hypoxia-induced MDR in human laryngeal cancer cells.

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