SPANDIDOS PUBLICATIONS

Inhibition of proliferation and apoptosis induced by a Na^+/H^+ exchanger-1 (NHE-1) antisense gene on drug-resistant human small cell lung cancer cells

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Abstract. The goal of this study was to evaluate the effect of the Na^+/H^+ exchanger-1 (NHE-1) antisense gene on drug-resistant human small cell lung cancer (SCLC) cell proliferation and apoptosis. A recombinant NHE-1 antisense gene was transfected into drug-resistant human SCLC H446/CDDP cells. Intracellular pH (pHi) was measured with fluorescence spectrophotometry. Cell proliferation was assayed cytometrically, and expression of the apoptosis gene caspase-3 was assayed using immunohistochemistry. Apoptosis and the cell cycles were imaged using a flow cytometer. pHi decreased significantly in transfected cells compared with control cells transfected with an empty vector (6.86±0.01 and 7.25±0.02, respectively, $P<0.01$). Cell proliferation began to decrease 48 h after antisense gene transfection, and the expression of the caspase-3 was stronger in transfected cells compared to the control group. The drug resistant exponent was significantly decreased ($P<0.01$), and there were more cells in G1 in the transfected group compared to the control group (70 and 57%, respectively, $P<0.05$). The rate of apoptosis in transfected cells was significantly higher than in the control group (12.18±1.86 and 2.37±0.33%, respectively, $P<0.01$). The NHE-1 antisense gene was able to induce drug-resistant human SCLC H446/CDDP cells to become acidified and apoptotic, which could provide a novel therapy for multi-drug resistance SCLC.

Introduction

Small cell lung cancer (SCLC) is a non-resectable and highly metastatic neoplastic disease, accounting for about 20% of all lung cancers (1). It is particularly aggressive and has a poor prognosis, with the 5-year survival rate at diagnosis rarely exceeding 10% (2). SCLC is a chemosensitive and radiosensitive disease; chemotherapy for SCLC typically involves a combination of etoposide, doxorubicin, vincristine, paclitaxel, and platinum-based regimens (3-5). First-line combination chemotherapy (with or without regional radiotherapy) induces a positive response in 70% of patients, with 50% of patients showing complete remission (6,7). However, 90% of these complete remission patients relapse with multi-drug resistant tumours, making chemotherapy ineffective (8,9). Multidrug resistance is a serious clinical problem, which often severely limits the effectiveness of cancer chemotherapy (10). Thus, treating SCLC presents a significant clinical challenge.

However, an effective way to treat SCLC multi-drug resistance has been reported (11). NHE-1 can directly interact with other regulatory cellular signaling pathways and is a multifaceted regulator of cell migration, proliferation, and cell death (12-14). The Na^+/H^+ exchanger NHE-1 isoform is expressed ubiquitously and plays the crucial role of regulating intracellular pH by catalyzing the exchange of one extracellular sodium ion against one intracellular proton. Maintaining a steady-state intracellular pH during physiological conditions requires the continuous cellular export of acidic equivalents (15,16). Na^+/H^+ exchangers (NHE) are membrane transporters that facilitate H^+ efflux in cells (17).

Indeed, activation of NHE-1 and the resulting cellular alkalinization has been described as a key mechanism for the development and maintenance of tumour cells (18). Tumour cells deficient in NHE activity either fail to grow or show severely retarded growth when implanted in immunodeficient mice (19). Previous studies have provided strong evidence that NHE-1 gene expression was consistent with cell survival (13,20). Moreover, decreased NHE-1 expression led to tumour cell growth arrest, acidification of the intracellular milieu, and sensitization to death triggers (21). Intracellular H^+ increases, resulting in a pHi less than 7.0, can trigger cell apoptosis (22). Tumour cells rely on increased NHE-1 gene expression in order to strengthen the Na^+/H^+ exchange, pump out abnormally high levels of intracellular H^+, thus preventing the pHi decline and intracellular acidification (19). As a result, NHE-1 gene expression in tumour

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cells may play an important role in protecting them against apoptosis (23). Thus, NHE-1 is a potential target for intervention in multi-drug resistance SCLC.

Cisplatin is one of the most important chemotherapeutic drugs in clinical practice (24). Although its mode of action of has been under intensive study since its discovery more than 30 years ago, the exact mechanism has yet to be defined (25,26). Cisplatin-induced multi-drug-resistant lung cancer cell lines are used to gain better understanding of SCLC (27). The resistance cell line NCI-H446/CDDP generated in our center has stable cell biological characteristics and is a reliable multi-drug resistance model of SCLC (28,29).

This study aims to uncover the effect of the NHE-1 antisense gene on the drug-resistant SCLC cell line H446/CDDP and to prove that intra-cellular acidification can inhibit resistant lung cancer cell proliferation and cause apoptosis, with the final goal of finding a novel therapy for multi-drug resistance SCLC.

Materials and methods

Construction of the NHE-1 antisense expression vector pCMV-Script and NHE-1 mRNA quantitative analysis. The primer sequences were 5'-TCAAGCTTCAGTCACTTTC CTCAGC-3' and 5-'CGGATCCAACCACCACGAGTA AGG-3'. The 5' primer had an engineered *Hind*III restriction site, and the 3' primer had an engineered *Bam*HI restriction site. We amplified cDNA from the A549 human lung cancer cell line using conventional PCR. Conditions were 95˚C, 3 min, the addition of the Taq enzyme; 94˚C, 40 sec, 60˚C, 40 sec, 72˚C 45 sec, 35 cycles; 72˚C 10 min.

Purified PCR products were cut with *Hind*III + *Bam*HI enzymes and then inserted into the pCMV-Script vector (Promega, USA). Successful integration of the plasmid was evaluated by *Hind*III + *Bam*HI double digestion. The construct was sequenced by the Boya Company (Shanghai, China). The plasmid was transformed into competent XL-Blue cells and the plasmid DNA was purified (Promega) and checked using *Hind*III + *Bam*HI digests.

Cell monolayers were grown to the exponential phase of growth, RNA was extracted, and RT-PCR was performed by the afore-mentioned method. Upper primers: 5'-TGACG GTCAGGTCATCACTATCGGCAATGA-3', lower primers: 5'-TTGATCTTCATGGTGVTAGGAGCGAGGGCA-3'. We determined the strip integral optical density (indicated by grayscale) in order to measure expression of NHE-1.

Cell transfection. Resistant H446/CDDP SCLC cells were cultured in RPMI-1640 medium (containing 10% FBS) at 37˚C in 5% CO_2. The antisense expression vector was transfected into H446/CDDP SCLC cells using a lipid transfection method and called group T. The control group was transfected with an empty vehicle and called group C. Forty-eight hours after transfection, fresh RPMI-1640 medium with 10% FBS and G-418 (final concentration 500 $\mu g \cdot ml^{-1}$) was added (30).

Cell pHi. Tumour cells were digested with 0.25% EDTA and 0.01% trypsin, rinsed with Hank's liquid rinse, and made into a single-cell suspension at a density of $5.0x10^5$ cells/ml (31). Cells (2 ml) were added to 24 test tubes. BCECF-AM (Sigma) was added to a final concentration of 2 μg/ml, and tubes were incubated at 37˚C for 30 min in the dark. After the incubation period and a third Hank's liquid rinse, the cells were centrifuged, and the supernatant was discarded. The 24 tubes were divided into eight groups, and 3 ml of KCl buffer at pH 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, or 7.6 was added. Cells were incubated in a 37˚C waterbath for 10 min then analysed immediately. Fluorescence intensity was measured at optical wavelengths 440, 495 and 525 nm. Fluorescence intensity was obtained at different pH values in order to calculate standard curves and regression equations.

Group T and group C H446/CDDP cancer cells were made into single-cell suspensions, rinsed twice with Hank's liquid rinse, and then re-suspended in NaCl buffer at a density of $2x10^6$ cells/ml. Three samples were taken from each, BCECF-AM was added, cells were incubated for 30 min at 37˚C in the dark, then analyzed immediately. Fluorescence intensity ratios at 495 nm/440 nm were measured, and the average sample pHi was calculated by substituting values into a regression equation.

Cell culture and cell proliferation count. Cells were cultured in DMEM medium supplemented with 10% newborn calf serum (both from Gibco/Invitrogen, NY, USA) in a 37˚C incubator with a humidified atmosphere of 5% CO_2-95% O_2 (32). Cells were grown at a density of $1x10^4$ cells/ml. After 24, 48, 72, 96 or 120 h in culture, the number of drug-resistant H446/CDDP cells and recombinant-transfected cells was counted.

MTT assay for H446/CDDP cell drug sensitivity. The number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (33). Briefly, the cells were cultured in 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) at a density of $1x10^4$ cells/well in the presence of cisplatin, etoposide, vincristine, 5-fluorouracil, or topotecan at the indicated concentrations. Each drug was applied at 5 different concentrations, each 5-fold larger than the next. After a 48-h incubation, MTT (Sigma, St. Louis, MO, USA) dissolved in PBS was added to each well at a final concentration of 5 mg/ml, and the cells were then incubated at 37˚C for 4 h. The water-insoluble dark blue formazan crystals that formed during MTT cleavage in actively metabolizing cells were dissolved in DMSO. The optical density was measured at a wavelength of 490 nm with a Bio-Rad 680 microplate reader (Bio-Rad, CA, USA). All experiments were performed in triplicate. IC_{50}, the 50% inhibitory concentration of each drug, was calculated with a logarithmic plot. Inhibitory rate = $(1\text{-}OD_{drug}/OD_{control}) \times 100\%$.

Caspase-3 immunohistochemical analysis. Cells were trypsinised with 0.25% trypsin, plated on coverslips, incubated for 24 h, and then washed three times with Tris-buffered saline (TBS, Sigma). Cells were then fixed with 4% paraformaldehyde for 30 min, dried at 37˚C, washed three times with TBS, and equilibrated in TBS containing 0.3% Triton X-100 (Sigma). Endogenous peroxide activity was blocked by treatment with 0.3% H_2O_2 (Sigma) in methanol for 30 min. Cells

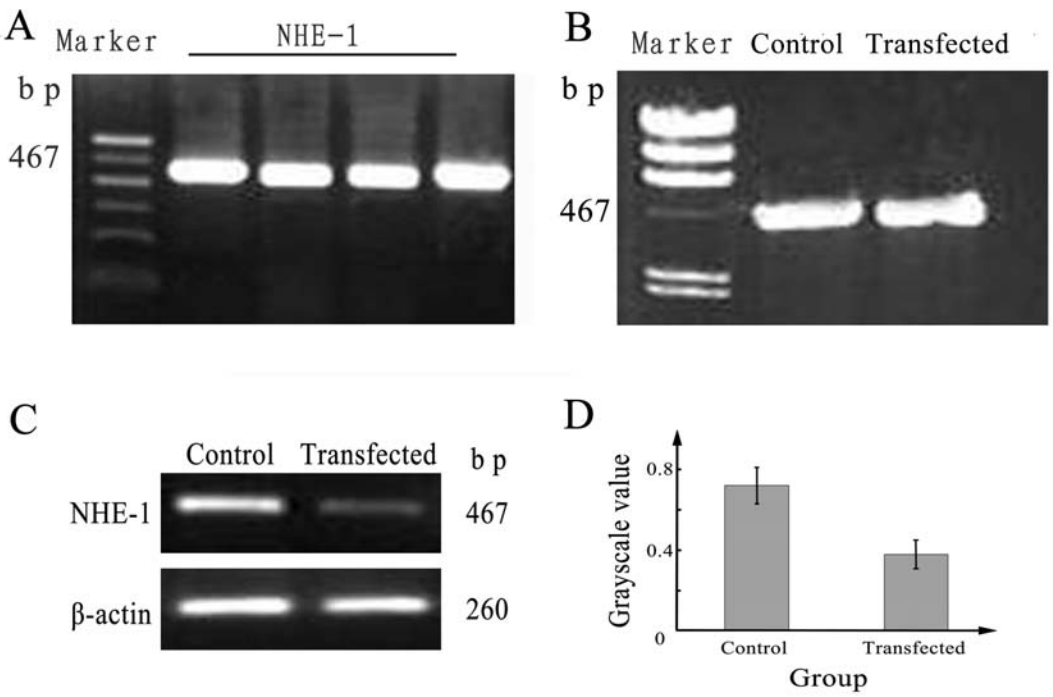

Figure 1. Identification and expression analysis of NHE-1. (A) cDNA from human lung cancer A549 cells was used as a template, and conventional PCR methods were used to amplify a 467 bp (including restriction site) DNA fragment. (B) Amplified ligated plasmid included the expected 467 bp length product, showing that the antisense gene vector was successfully transfected into the cell genomes. (C and D) Expression of NHE-1 in cells after transfection with the antisense gene. The transfected group shows significantly less expression than the control group (set ß-actin value as 1, P<0.01).

were then treated with 0.1% trypsin in 0.05 M Tris and 0.02 M $CaCl_2$ (pH 8.0), and non-specific binding was eliminated by blocking with 1.5% normal goat serum (Santa Cruz Biotechnology, USA) and 0.5% bovine serum albumin (BSA) in TBS. The coverslips were then incubated overnight at 4˚C with a primary antibody (rabbit polyclonal IgG to caspase-3, diluted 1:200, Santa Cruz Biotechnology) in TBS with 0.5% BSA, followed by incubation with a secondary antibody (HRP-conjugated goat anti-rabbit IgG, Santa Cruz Biotechnology) and then with an avidin/biotin complex (Santa Cruz Biotechnology) in TBS with 0.1% BSA for 1 h. Cells were developed with 0.5% 3,3'-diaminobenzidine (Sigma) in 0.1% H_2O_2, 0.05 M Tris, and 0.85% NaCl (pH 7.4) for 5 min. Finally, the coverslips were counterstained with Gill's haematoxylin, dehydrated, and mounted with Permont (Sigma). A negative control, in which the primary antibody was omitted, was used to verify the specificity of the immunohistochemical results.

Cell cycle and apoptosis analysis. Cell cycle distribution apoptosis analysis were analysed by flow cytometry as described (34). Briefly, following incubation for 24, 48, 72, 96, or 120 h, cells were trypsinised with 0.25% trypsin (Sigma), counted, centrifuged at 300 g x for 5 min, and fixed in ethanol at 4˚C overnight. The cells were then washed and centrifuged. The resulting cell pellets were resuspended in an RNase solution (0.02 mg/ml; Sigma) containing propidium iodide (0.02 mg/ml; Sigma) and incubated at 4˚C for 30 min. The DNA content of approximately 1-2x10^5 stained cells was analysed using a FACScan flow cytometer equipped with the FACStation data management system running CellQuest software (Becton-Dickinson, San Jose, CA, USA). The results are expressed as a plot of fluorescence intensity versus cell number.

Statistical analysis. All statistical analyses were carried out using SPSS 14.0 statistical software. Data are expressed as the mean ± the standard error of mean (SEM) of separate experiments. Differences in measured parameters among the groups were analysed by one-factor ANOVA and the least significant difference test. Results with P≤0.05 were considered statistically significant.

Results

Level of NHE-1 mRNA expression after transfection. The NHE-1 antisense gene sequence was correct as shown by sequencing (Boya Company, China). NHE-1 expression in resistant SCLC H446/CDDP cells after the transfection of the antisense gene was significantly lower than in cells transfected with an empty vector and the gray values was 0.38±0.07 and 0.72±0.09, respectively (P<0.01) (Fig. 1).

pHi. The pHi in resistant SCLC H446/CDDP cells after antisense gene transfection was markedly lower than in the control group, in which cells were transfected with an empty vector (6.86±0.01 and 7.25±0.02, respectively, P<0.01).

Cell proliferation. Differences between cell numbers in NHE-1 antisense gene-transfected cells and control cells gradually increased after transfection and became statistically significant at 48 h (P<0.01) (Table I).

Table I. Cell count of H446/CDDP at different time-points.

Group	0	24 h	48 h	72 h	96 h	120 h
C	1.00±0.09	1.46±0.13	2.35±0.26	5.58±0.39	8.46±0.67	12.7±0.78
T	1.32±0.12	1.62±0.15	2.85±0.21[a]	4.78±0.36[a]	6.92±0.55[a]	9.03±0.67[a]

Cell counts of H446/CDDP were determined at 24, 48, 72, 96, 120 h respectively after NHE-1 antisense gene transfected. The values are shown as the mean ± SEM. [a]P<0.01 compared to the control group [least significant difference (LSD) test by ANOVA].

Table II. Multi-drug resistance index of H446/CDDP cell after transfected NHE-1 antisense gene.

Drug	Group C	Group N
Cisplatin	0.7469±0.041	0.2971±0.032[a]
Etoposide	0.5513±0.033	0.2379±0.033[a]
Vincristine	0.4812±0.026	0.2921±0.027[a]
5-Fluorouracil	2.4718±0.271	1.4228±0.156[a]
Topotecan	0.6216±0.076	0.3326±0.057[a]

Drug resistance indexes of H446/CDDP cell to cisplatin, etoposide, vincristine, 5-fluorouracil, topotecan after transfected NHE-1 antisense gene were determined respectively. The values are shown as the mean ± SEM. [a]P<0.01 compared to the control group [least significant difference (LSD) test by ANOVA].

Cell drug sensitivity. MTT methods were used to measure the optical density of cells 48 h after transfection. The resistance index to each drug decreased significantly in cells transfected with the NHE-1 antisense gene compared to cells in the control group (P<0.01) (Table II).

Cell cycle and apoptosis. Flow cytometry was used to determine the cell cycle stage. More cells were in the G1 phase in cells transfected with the NHE-1 antisense gene (G1 phase, 70%; S phase, 17%) than in the control group (G1 phase, 57%; S phase, 30%) (P<0.01) (Fig. 3).

Expression of the pro-apoptotic protein caspase-3 was determined by immunohistochemical analysis. Caspase-3 was present in the cytoplasm and part of the nucleus of NHE-1 antisense transfected cells. In contrast, there was no caspase-3 expression in cells from the control group (Fig. 2).

The apoptosis rate was measured by double-staining flow cytometry analysis. The rate of apoptosis in cells transfected with the NHE-1 antisense gene was 12.18±1.86%, which was significantly higher than the 2.37±0.33% observed in the control cells (P<0.01) (Fig. 3).

Discussion

At present, chemotherapy is still the main treatment for SCLC (35). During and after chemotherapy, cancer cells often become resistant to multiple drugs, which affects the effectiveness of chemotherapy and leads to increased mortality (11,36). The H446/DDP cell line was derived from H446 cells that were made resistant to cisplatin, which is in a category of drugs commonly used in clinical chemotherapy (37). This group includes drugs such as cisplatin, etoposide, vincristine, 5-fluorouracil, and topotecan. The cell cycle of H446/DDP cells is not significantly different from H446 cells, which can reduce errors in the detection of drug

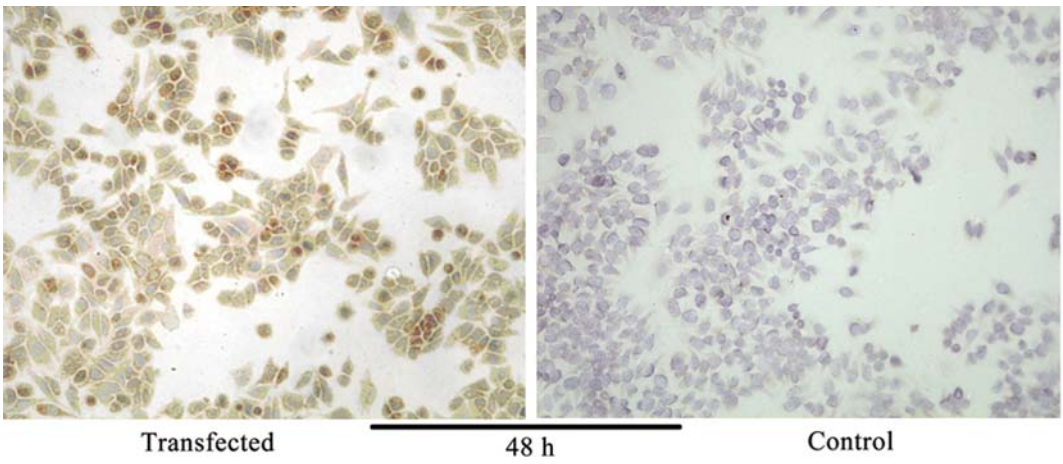

Figure 2. Expression of caspase-3 in cells. Pro-apoptotic gene caspase-3 expression was determined by immunohistochemistry. More yellow-brown positive areas were seen in the cytoplasm of NHE-1 antisense transfected cells, and many nuclei also expressed caspase-3. In contrast, there were more cells in the control, but there was no detectable caspase-3 staining.

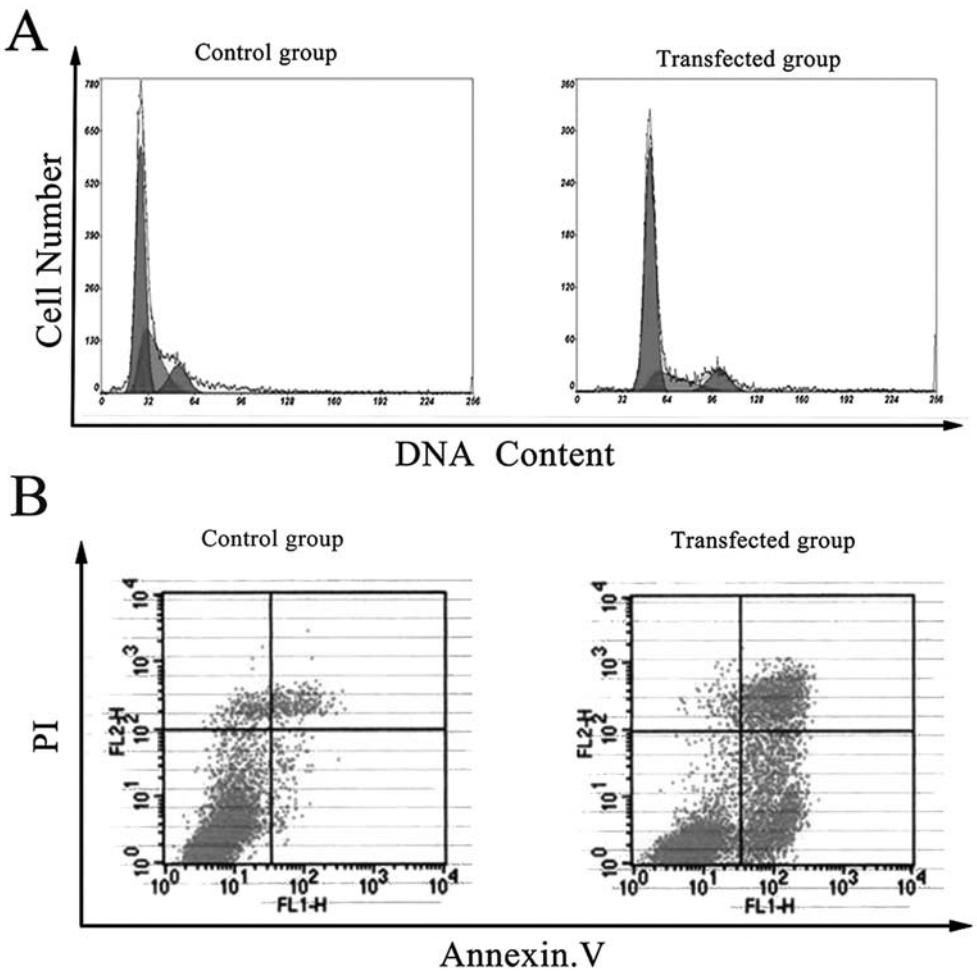

Figure 3. Cell cycle and apoptosis 48 h after transfection. (A) Flow cytometry was used to determine the percentage of cells in the different cell cycle phases. More cells were in the G1 phase in cells transfected with the NHE-1 antisense gene than cells in control group ($P<0.01$). (B) Cell apoptosis rate was measured by double-staining flow cytometry analysis. The rate of apoptosis in cells transfected with the NHE-1 antisense gene was significantly higher than in control group cells ($P<0.01$).

resistance, so we used the H446/DDP cell line to study experimental MDR *in vitro*.

In this study, we transfected the NHE-1 antisense gene into H446/CDDP cells and found that the NHE-1 level in transfected cells was significantly lower than in cells transfected with an empty vector (control group), suggesting that the transfected NHE-1 antisense gene could effectively reduce or inhibit NHE-1 expression. NHE-1 is an 815 amino acid, 105-110 kDa glycoprotein that is expressed in a wide range of eukaryotic cells (38). In physiological conditions, NHE-1 participates in the regulation of sodium and hydrogen ions, pHi, cell-conditioning capacity, and other important physiological functions by mediating Na^+/H^+ exchange in order to maintain the stability of cells and normal membrane potential (18). NHE-1 is the most important pHi regulator; its expression and activity impacts the stability of cellular pHi, which is the most basic condition required for cell growth (39). When altered, pHi can impact cell growth and function directly, so expression and activity of NHE-1 plays an important role in cell growth (40). An inhibitor of NHE-1 could inhibit cell growth by affecting these processes.

Previous studies have shown that the NHE-1 gene is highly expressed in lung cancer cells. NHE-1 plays an important role in maintaining lung cancer cells at a neutral or alkaline pHi (39). NHE-1 can prevent tumour cell death induced by acidity, which has significant effects on tumour cell division, growth and proliferation, invasion, and metastasis (41).

This study showed that the pHi in H446/CDDP cells after NHE-1 antisense gene transfection decreased significantly compared with control cells and that the environment around NHE-1 antisense transfected cells was more acidic ($P<0.01$), which is consistent with previous studies. At the same time, we found that there were fewer cells in the NHE-1 antisense transfected group than in the control group, that the cell doubling time was markedly longer, that cell proliferation was also slower, and that these differences gradually increased as time passed. The number of cells in G1 was significantly increased in the NHE-1 antisense group as compared to the control group 48 h after transfection, while cell number began to have a statistically significant difference ($P<0.01$), which was most obvious at 120 h. The rate of apoptotic cells after NHE-1 antisense transfection was also larger than in the control group ($P<0.01$). Immunohistochemical staining showed that the apoptosis gene caspase-3 was expressed strongly in NHE-1 antisense-transfected H446/CDDP cells compared to the control group. This observation suggests

that pHi in cancer cells plays an important role in cell proliferation and apoptosis; when pHi decreases to a certain level, it inhibits the cell cycle and induces apoptosis. However, this mechanism should be studied in greater detail.

MTT results showed that the resistance index to cisplatin, 5-fluorouracil, vincristine, etoposide, and topotecan was significantly lower in NHE-1 antisense-transfected H446/CDDP cells than in control cells after 48 h of culture ($P<0.01$), which implies that the NHE-1 antisense gene effectively induced drug-resistant SCLC H446/CDDP cells to become apoptotic and increased the sensitivity of cells to chemotherapeutic drugs. In other words, inhibition of NHE-1 could effectively induce drug-resistant SCLC to become apoptotic, which might also be related to decreased pHi in cancer cells.

In conclusion, the above experimental results show that the expression of NHE-1 in drug-resistant SCLC H446/CDDP cells was significantly reduced after cells were transfected with the NHE-1 antisense gene. This transfected NHE-1 antisense gene was able to inhibit the cell cycle, induce cell apop-tosis, and reduce chemotherapy drug resistance. Reduced pHi inside cells may be the mechanism behind this pheno-menon, which provides a theoretical basis and a novel way to prevent and treat drug-resistant SCLC.

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