ANTIOXIDANT ABILITY CONTRIBUTES TO THE CHEMOTHERAPEUTIC RESISTANCE IN LUNG CANCER CELL UNDER HYPOXIA

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Abstract - Tumor cells situated rapidly changed microenvironment, including oxygen loss. Oxygen loss, which is also called hypoxia, is reported to raise the chemotherapeutic resistance in breast cancers through upregulating the antioxidant ability resulted from the activation of the major antioxidant regulator, Nrf2, and the following phase II enzymes, GCLC, GCLM, and NQO1. However, whether Nrf2 and phase II enzymes are the hinge for hypoxia-induced drug resistance in lung cancer still remain unknown and need further investigations. This study aims to explore the cellular responses under hypoxia and search for the therapeutic targets for hypoxia-induced drug resistance in lung cancer. With the *in vitro* cell culture model, the activation of Nrf2 and phase II enzymes under hypoxia was confirmed. The inhibition of phase II enzymes can efficiently increase the sensitivity to the commonly used chemotherapeutic drug, cisplatin (CDDP). These results provided more combined therapeutic options for chemotherapy in lung cancers.

Key words - Phase II antioxidant enzyme, Chemotherapeutic resistance, lung cancer, hypoxia, cisplatin

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I. INTRODUCTION

Tumor cells were characterized as rapid growth cells. During the process of solid tumor growth, the central cells encounter the situation of rapidly loss of oxygen, which is called hypoxia condition[1]. Researchers had found that cancer cells become more metastatic and drug resistant under hypoxia[2], thus start an era exploring how cancer cells respond to hypoxia. We have reported previously that the antioxidant ability rises rapidly under hypoxia through activating of transcription factor, Nuclear factor (erythroid-derived 2)-like 2 (NFE2L2/Nrf2) through conformational change of KEAP1, the endogenous inhibitor of Nrf2[3]. Nrf2 is proved to serve as not only a marker for tumor malignancy[1], but also the main regulator of antioxidant capacity[4]. After release from Keap1, Nrf2 is stabilized and translocated to the nucleus, binds to the ARE (antioxidant response element), and initiates the expression of the following phase II enzymes, including catalytic subunit of glutamate-cysteine ligase (GCLC), glutamate-cysteine ligase regulatory subunit (GCLM), and NAD(P)H dehydrogenase 1 (NQO1). These phase II enzymes contribute to the resistance to the commonly used chemotherapeutic drug, cisplatin (CDDP) in breast cancer cells[3]. However, whether antioxidant ability triggers the drug resistance in lung cancer is still in the mist. Non-small

lung cancer is a serious problem worldwide. There are many strategies targeting lung cancer, but the drug resistance under hypoxia remains a knotty problem in chemotherapy. This study aims to explore whether Nrf2 and the phase II enzymes are key regulators of the drug resistance under hypoxia in lung cancers.

II. MATERIALS & METHODS

Cell culture

A549 lung adenocarcinoma cells were cultured in DMEM containing with 10% FBS in 37℃ and 5% CO_2. The environment in the normal incubator containing around 20% O_2 presented normoxia condition, while 2% O_2 represented hypoxia condition.

MTT assay

$1X10^4$ Cells were seeded in 48 wells for overnight, and then treated with CDDP with or without inhibitors for 24 h. MTT (5μg/ml) was added to medium and incubated for another 3 h. Medium was then removed and 100 μl DMSO was used to dissolve the crystal, and OD (550nm) was measured with ELISA reader. The cell survival was presented by fold of control.

Western blot

Cells were trypsinized and collected after indicated treatments and proteins were extracts from cells with lysis buffer containing protease inhibitor. 20 μg proteins were loaded in 10% acrylamide gel with electroporation and transferred to membranes. Membranes were blotted with indicated primary antibodies and HRP-conjugated secondary antibodies. ECL and X-film were used to get the images of protein expression levels.

Immunocytochemistry (IHC)

Cells were seed on the glass slides and incubated under normoxia and hypoxia conditions for 8 h. Cells were fixed with 4% paraformaldehyde, incubated overnight with Nrf2 antibody, and then 1 h with FITC-conjugated secondary antibody and DAPI (1:20000). Fluorescent images were captured with a fluorescence microscopy (Leica).

III. RESULTS

Hypoxia leads to chemotherapeutic resistance in lung cancer cells.

Cancer cells faces to hypoxia during rapid tumor growth, and hypoxia is proved to decrease the sensitivity to chemotherapeutic drugs in various cancer cells[1]. However, the regulatory mechanism is not clear yet in lung cancer cells. Among all lung cancer cells, A549 is the malignant one and most widely used in studies[5], thus was chosen in this study to explore the underlying mechanisms about how cells respond to hypoxia and increase the drug resistance. First, the cell survival (MTT assay) was evaluated with 24 h administration of the commonly used chemotherapeutic drug, cisplatin (CDDP) in various concentrations under normoxia (20% O_2) or hypoxia (2% O_2) conditions. The cell survival is decreased in a dose dependent manner in both normoxia and hypoxia, however, the cell survivals are higher under hypoxia compared to normoxia in 25 and 50 μg/ml CDDP (Figure 1).

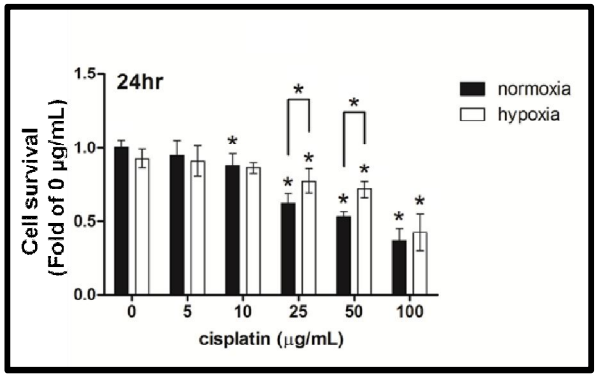


Figure 1. The cell survival with CDDP treatment under normoxia and hypoxia.

The cell was measured by MTT assay and the survival

was higher under hypoxia compared to normoxia with 24 h treatment of 25 and 50 μM CDDP. *, $p<0.05$ compared to 0 μg/ml CDDP or indicated comparison.

Phase II antioxidant enzymes increase under hypoxia condition.

Researchers reported that hypoxia increases the expression levels of anti-oxidant enzymes, especially the phase II enzymes, to resist the cytotoxic effect of CDDP in breast cancer cells [3,6]. Next, whether the phase II enzymes were increased under hypoxia in lung cancer cells was examined. Cells were incubated under hypoxia condition for 0-8 h, and the expression levels of phase II enzymes were detected by western blot. Both GCLC and GCLM were increase in a time dependent manner, and NQO1 only increased at 8 h (Figure 2).

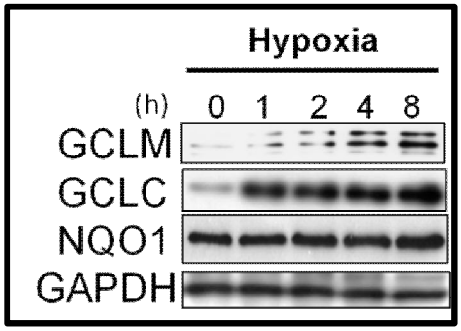


Figure 2. The expression of phase II enzymes under hypoxia.

The expression levels of GCLC, GCLM, and NQO1 were evaluated with western blot under hypoxia with GAPDH as the internal control. GCLC and GCLM increased in a time dependent manner while NQO1 only increased at 8 h.

Hypoxia leads to the activation of Nrf2.

The main regulator of the expression of phase II enzymes is Nrf2, thus the activity of Nrf2 was next studied. The protein expression of Nrf2 was tested by western blot, and the Nrf2 levels were increased in a time dependent manner under hypoxia (Figure 3, upper panel). Nrf2 is bound to Keap1, and send to proteosome by ubiquitinization. Once cells face the oxidative stress, the Keap1 is phosphorylated, and then goes to conformational changes, thus the Nrf2 can be released. The Nrf2 then is phosphrylated, translocated into the nucleus, and then triggered the expression of phase II enzymes by binding to the ARE domain (ref). The Nrf2 was stained by green fluorescence and the nucleus was stained with DAPI (blue). In the normoxia condition, the Nrf2 was expressed mainly in the cytosol, and the nuclear Nrf2 increases significantly with 8 h hypoxia incubation (Figure 3, lower panel).

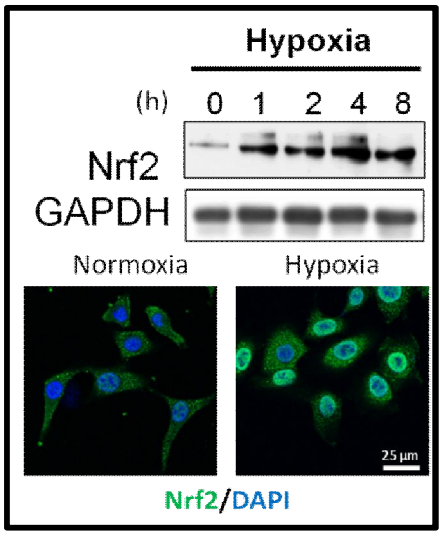


Figure 3. Nrf2 was activated by hypoxia.

The expression level and location of Nrf2 were evaluated by western (upper panel) and ICC (lower panel), respectively. The Nrf2 level increased in a time dependent manner under hypoxia condition. The Nrf2 translocated from cytosol under normoxia to the nucleus under hypoxia. Nucleus were stained with DAPI.

Inhibition of Phase II enzymes enhances the drug sensitivity under hypoxia.

Phase II enzymes and the key regulator, Nrf2, increased under hypoxia, thus in the last part, whether decrease of antioxidant ability by inhibition the phase II enzymes can increase the drug sensitivity was going to be explored. GCLC inhibitor,

L-buthionine-S,R-sulfoximine (BSO) (10 μM), and NQO1 inhibitor, Dicoumorol (DIC, 10 μM), were used to test the drug sensitivity in A549 under hypoxia. After 24 h administration of CDDP with inhibitors under hypoxia, both BSO and DIC significantly decreased the cell survival with CDDP treatment (Figure 4).

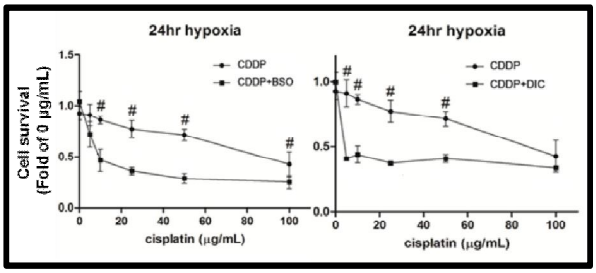


Figure 4. Phase II enzyme inhibitors increased the drug sensitivity of CDDP under hypoxia.

CDDP was used to treat A549 combined with BSO and Dicoumarol (DIC) under hypoxia. Both BSO and DIC significantly increased the drug sensitivity under hypoxia.

DISCUSSION

In this study, the activation of Nrf2 and the following phase II antioxidant enzymes under hypoxia in lung cancer cells was confirmed, and the phase II enzymes can become the therapeutic targets. Both BSO, the inhibitor of GCLC, and dicoumarol, the inhibitor of NQO1, dramatically decreased the cell survival combined with CDDP under hypoxia. These results served as the fundamental knowledge in the cell response under hypoxia and increased the alternative choices of combined chemotherapy in lung cancer treatment.

Although the activation and nuclear translocation of Nrf2 was confirmed, the detailed interaction and the regulation of Nrf2 and Keap1 under hypoxia were not clear yet and needed further examinations. The release of Nrf2 from Keap1 can be regulated by ERK, JNK, p38, and PKC, and these factors can participate in the activation of Nrf2 under hypoxia[7]. Keap1 received DNA methylation modification can also be considered as one regulator of Nrf2 activation[8]. In addition, there is also a Keap1-independent regulation of Nrf2 activity by protein acetylation[9]. Finding the detailed regulatory mechanisms can enrich the knowledge about the cellular response toward hypoxia in lung cancer and increase the potential therapeutic targets. The combined therapy of antioxidant enzyme inhibitors and CDDP can be a better choice in the future clinical usage.

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