

# The mechanism of lipid raft mediating chemoresistance of cervical cancer

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## ABSTRACT

**الأهداف:** التحقق من ميكانيكية عمل العلاج الكيميائي للأغشية الدهنية (lipid raft mediating chemotherapy) لدى الخلايا المصابة بسرطان عنق الرحم.

**الطريقة:** أُجريت هذه التجربة في كلية تونجي الطبية، جامعة هوازونج للعلوم والتقنية، ووهان، الصين وذلك خلال الفترة من يونيو 2010م إلى فبراير 2011م. لقد قمننا بتقسيم خلايا هيلا إلى 6 مجموعات كالتالي: مجموعة الشاهد (Ctrl)، ومجموعة السيسبلاتين (Cis)، ومجموعة العلاج الكيميائي للأغشية الدهنية (MCD)، ومجموعة الإنزيم (Apo) (NADPD oxidase)، ومجموعة العلاج الكيميائي للأغشية الدهنية + السيسبلاتين (MCD+Cis)، ومجموعة الإنزيم (NADPD oxidase) + السيسبلاتين (Apo+Cis). ولقد قمننا بعد علاج خلايا عنق الرحم بهذه العوامل لمدة 24 ساعة بقياس الخلايا المقاومة وذلك باستخدام جهاز عد الخلايا (CCK-8)، فيما تم قياس مستويات معاملة ألفا المحفز بنقص التأكسج باستخدام اختبار ويسترن. بالإضافة إلى ذلك فقد تم قياس المشتقات التفاعلية للأوكسجين.

**النتائج:** لقد كان قل نمو الخلايا في MCD (نسبة الخلايا المقاومة 62% بالمقارنة مع Ctrl)، كما كان لمجموعة Apo نفس التأثير (65% في Ctrl)، وفي Cis كان 49%، وفي MCD+Cis كان 21%، وفي Apo+Cis كان 23%. بالمقابل فقد نقصت مستويات معاملة ألفا المحفز بنقص التأكسج والمشتقات التفاعلية للأوكسجين في المجموعات MCD، Apo، Cis، و MCD+Cis، و Apo+Cis، وذلك بالمقارنة مع المجموعة Ctrl. حيث قلت مستويات معاملة ألفا المحفز بنقص التأكسج في MCD بنسبة 69.9%، وفي Apo بنسبة 60.2%، وفي Cis بنسبة 55.47%، وفي MCD+Cis بنسبة 21.09%، وفي Apo+Cis بنسبة 25.4%. فيما قلت مستويات المشتقات التفاعلية للأوكسجين في MCD بنسبة 38.6%، وفي Apo بنسبة 35.3%، وفي Cis بنسبة 24%، وفي MCD+Cis بنسبة 12.3%، وفي Apo+Cis بنسبة 12.8%.

**خاتمة:** أظهرت الدراسة بأن الأغشية الدهنية قد تعمل على تنظيم ارتفاع مستويات المشتقات التفاعلية للأوكسجين، ومستويات معاملة ألفا المحفز بنقص التأكسج وذلك عن طريق تفعيل نشاط الإنزيم (NADPD oxidase). وهذا من شأنه أن يعمل على تقوية دور العلاج الكيميائي في مقاومة سرطان عنق الرحم.

**Objective:** To investigate the mechanism of lipid raft mediating chemotherapy resistance in cervical cancer.

**Methods:** This experiment was carried out in the Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China from June 2010 to February

2011. Hela cells were divided into 6 groups: control group (Ctrl), cisplatin group (Cis), lipid raft interference agent group (MCD), NADPH oxidase inhibitor group (Apo), lipid raft interference agent combined with cisplatin group (MCD+Cis), and NADPH oxidase inhibitor combined with cisplatin group (Apo+Cis). After the cervical cancer cells were treated with a correspondent agent for 24 hours, the number of surviving cells were measured utilizing cell counting kits-8 (CCK-8), and the hypoxia inducible factor-1alpha (HIF-1 $\alpha$ ) levels were detected by Western blotting. Reactive oxygen species (ROS) levels were measured indirectly by detection of dichlorodihydrofluorescein fluorescence activity.

**Results:** The cell growth of MCD slowed down (survival cells was 62% compared with the Ctrl group), with the Apo group showing a similar effect (65% in the control group), and 49% for the Cis group, MCD+Cis was 21%, and Apo+Cis was 23%. While the level of HIF-1 $\alpha$  protein and ROS of the MCD group, Apo group, Cis group, MCD+Cis group and Apo+Cis group were decreased significantly compared to the control group. The level of HIF-1 $\alpha$  of MCD group decreased by 69.9%, Apo group by 60.2%, Cis group was 55.5%, MCD+Cis group by 21.1% and Apo+Cis group by 25.4%, while the level of ROS also decreased in the MCD group by 38.6%, Apo group by 35.3%, Cis group by 24%, MCD+Cis group by 12.3% and Apo+Cis group by 12.8%.

**Conclusion:** Lipid raft may up-regulate ROS level and HIF-1 $\alpha$  expression through activating NADPH oxidase, and thus promote chemotherapy resistance in cervical cancer.

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Chemotherapy can obviously improve the cure rate of cervical cancer. However, clinical doctors found that the effect of chemotherapy drugs could fail to kill tumor cells gradually and even invalid after administrated for a period of time, which is exactly a chemoresistance phenomenon. Chemoresistance is the result from tumor cells' adapting to chemotherapeutic environment. A growing number of researchers begin to explore the mechanism of chemoresistance phenomenon. There already has much evidence showing that cervical cancer chemoresistance is closely related to the excessive activation of an important transcriptional factor, hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ).<sup>1</sup> Currently, multi-drug resistance 1 (MDR-1) is accepted as the main human tumor drug-resistant gene,<sup>2</sup> which is one member of the downstream factors of HIF-1 $\alpha$ . Study showed that cervical cancer cells over-expressing MDR-1 were not sensitive to either chemotherapy or radiotherapy<sup>3</sup> for over-expression of HIF-1 $\alpha$  elevating the expression of MDR-1 gene and its product P-gp.<sup>3</sup> Therefore, excessive activation of HIF-1 $\alpha$  is the leading cause of chemoresistance in cervical cancer, and leads to the enhancement of survival ability.<sup>4</sup> Lipid raft is a micro membrane section rich in sphingomyelin and cholesterol. It plays a very important role in promoting and maintaining cells' reactive oxygen species (ROS), which is derived from nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase. Research show that lipid raft damaging can prevent the activation of this oxidase, and the stimulation of lipid raft assembling in the cell membrane can recruit each subunits of NADPH oxidase, activate itself, and increase intracellular ROS.<sup>5,6</sup> The destruction of the structural integrity of the lipid raft can effectively reduce the generation of ROS, which is strongly associated with HIF-1. These evidence indicate that the generation of ROS is closely related to the activation of lipid raft, so a growing number of studies called this signal event "lipid raft redox signaling".<sup>7</sup> In relation to a previous study,<sup>8</sup> we also speculate that lipid raft may activate in vivo ROS through NADPH oxidase, and promote the abnormal expression of HIF-1 $\alpha$  resulting in chemoresistance. In this study, we aim to investigate the mechanism of lipid raft mediating chemotherapy resistance in cervical cancer.

**Methods.** Hela cells was received from Huazhong University of Science and Technology affiliated to Tongji Medical College's Laboratory. Dulbecco modified Eagle's medium (DMEM) was bought from

Gibco-BRL company in Grand Island, New York, USA. Trypsin, fetal bovine serum (FBS) were bought from the United States Hyclone company. Both lipid raft interference agent Methyl- $\beta$ -cyclodextrin (M- $\beta$ -CD) and NADPH oxidase inhibitor Apocynin was the product of Sigma company, St. Louis, Missouri, USA. Rat anti-human polyclonal antibody of HIF-1 $\alpha$  was purchased from the Santa Co, California, USA. Cell counting kit-8 (CCK-8) was from Dojindo Company, Shanghai, China. Cisplatin was bought from Shandong Qilu pharmaceutical factory, Shandong, China. The 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), serum RPMI 1640 were all from the Sigma Company, St. Louis, Missouri, USA. Inverted microscope was from Japan Olympus, Tokyo, Japan. The CO<sub>2</sub> cell incubator was from Germany Hemeus, Hanau, Germany. This study was carried out in the Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China from June 2010 to February 2011. All experiments were implemented in vitro and were approved by the Research Ethics Committees of our hospital. Hela cells were divided into 6 groups: control group (Ctrl), cisplatin group (Cis), lipid raft interference agent group (MCD), NADPH oxidase inhibitor group (Apo), lipid raft interference agent combined with cisplatin group (MCD+Cis), and NADPH oxidase inhibitor combined with cisplatin group (Apo+Cis). After the cervical cancer cells were treated with a correspondent agent for 24 hours, the number of surviving cells were measured utilizing cell counting kits-8 (CCK-8), and the hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) levels were detected by Western blotting, reactive oxygen species (ROS) levels were measured indirectly by detection of dichlorodihydrofluorescein fluorescence activity.

**Cell culture.** HeLa cells were cultured in DMEM medium containing 0.1 mmol/L FBS, and passaged in 37°C, 5% CO<sub>2</sub> saturated humidity incubator, and then digested with the mixture of 1.25 g/L pancreatic enzyme, and 0.2 g/L ethylene diamine tetraacetic acid (EDTA). Cells of the logarithmic growth phase were obtained, then was inoculated in 96 orifice plate with 2x10<sup>3</sup> cells/well. After an overnight culture, the control (Ctrl) group culture medium was changed with fresh culture medium, and the M- $\beta$ -CD group with one mmol/L M- $\beta$ -CD, Apo group with fresh medium possessing 3 micromol/L Apocynin, Cis group with fresh medium possessing 10 micromol/L cisplatin, MCD+Cis group with fresh medium possessing 10 micromol/L cisplatin and 1 mmol/L M- $\beta$ -CD, Apo+Cis group with fresh medium possessing 10 micromol/L cisplatin and 3

micromol/L Apocynin, the volume of culture medium for each hole was 100 microL. All experimental procedures were repeated 3 times. The 6 groups were trained in the incubator for 24 hours.

**Cell counting.** After abandoning the culture medium of cells from the well, 110  $\mu$ L fresh DMEM medium containing CCK-8 reagent 10 microL was added, blended and cultured in the incubator for 4 hours. The optical density value was measured after setting 620 nm as wavelength reference. Values were obtained using this formula: living cells percentage of the control group (%) = (experimental group's OD value/control group cells' OD) x 100%.

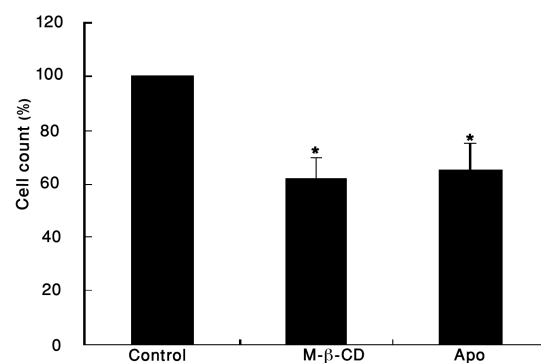
**Detecting the protein expression of HIF-1 $\alpha$  by Western blot.** Under the condition of normoxia, the exponentially growing cells were obtained, and transferred to a culture flask, divided, and cultured them as described previously. After training the cells in changed culture medium for 24 hours, protein was extracted, and the level of HIF-1 $\alpha$  and  $\beta$ -actin were processed according to the kit protocol. The HIF-1 $\alpha$  antibodies were quantified using the Image Gauge V3.3 program (Fujifilm Co., Tokyo, Japan). The ratio of the immunostaining value in the experimental groups to the controls was calculated for each of the 5 liter mate pairs.

**The ROS determination.** Intracellular ROS level was measured by using the cell permeable indicator 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). The procedure was carried out according to the kit protocol. For the determination of ROS,  $2 \times 10^4$  cells were seeded in black 96-well plates, and grown in complete culture medium. Every group cells were rinsed with phosphate buffered saline (PBS) and incubated with 10 micromol/L DCFH-DA in culture medium without FBS for 30 minutes at 37°C. The green fluorescence (oxidized DCFH-DA), indicating the presence of oxidants was measured using a spectrophotometer (Tecan Infinite M200, Crailsheim, Germany) at an excitation wavelength of 495 nm, with an emission at 528 nm. During the entire procedure with DCFH-DA, the plate was kept out of light to avoid fading of the fluoroprobe. Each experiment was performed in triplicate independently.

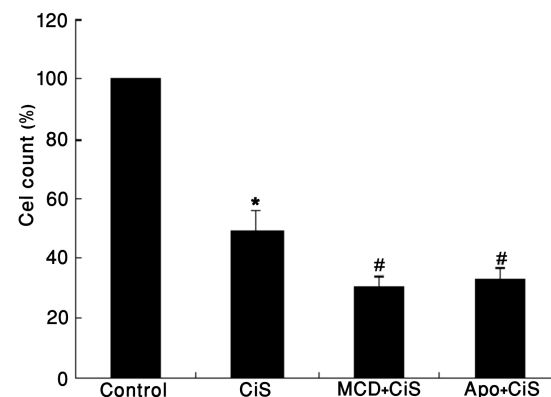
**Statistical analysis.** Statistical analysis was performed using Statistical Package for Social Sciences version 15 (SPSS Inc, Chicago, IL, USA. All data presented are given as the mean  $\pm$  standard deviation. Differences among treatments compared to those of solvent controls were evaluated by student's t test with unequal variance. A difference was considered significant at  $p < 0.05$ .

**Results. Lipid raft interference agent and NADPH oxidase inhibitor alone inhibited the growth of cervical cancer cells.** After treatment with one mmol/L lipid raft interference agent (M- $\beta$ -CD), or with 3 micromol/L Apocynin for 24 hours, the Hela cells growth rate slowed down significantly, and the cell counting of MCD group was 62%, and and Apo group was 65% to the control group. (Figure 1). These suggest that ROS derived from lipid raft related NADPH oxidase correlate to the growth of cervical cancer cells closely.

**Lipid raft interference agent and NADPH oxidase inhibitor strengthen the effect of cisplatin in inhibiting the growth of cervical cancer cells.** After treating cervical cancer cells with 10 micromol/L cisplatin for 24 hours, we counted the living cells by CCK-8 method, we found the counting decreased to 49% compared to the control group, which treated culture medium without Cis for 24 hours. However, if we simultaneously applied lipid raft interference agent M- $\beta$ -CD or NADPH oxidase inhibitors Apocynin as synergistic treatment,



**Figure 1** - The effect of methyl- $\beta$ -cyclodextrin (M- $\beta$ -CD), Apocynin (Apo) on the growth of cervical cancer cells (\* $p=0.034$  versus control (n=5)).



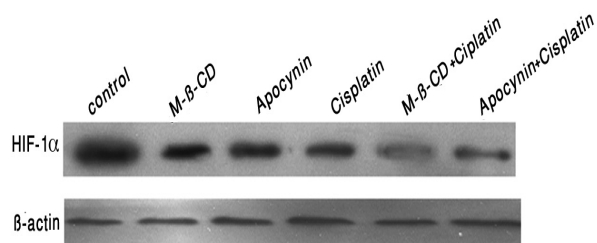
**Figure 2** - A graph showing the repressing ability of Cisplatin on cervical cell versus control group ( $p=0.026$ ), and that Methyl- $\beta$ -cyclodextrin (M- $\beta$ -CD [ $p=0.041$ ]) and Apocynin (Apo [ $p=0.049$ ]) could enhance the repressing effect of Cisplatin.

then the proportion of the cervical cancer cells counting decreased to 31% in the M- $\beta$ -CD group, and 33% in the NADPH group to the control group (Figure 2).

**Lipid raft interference agent and NADPH oxidase inhibitor strengthen the effect of cisplatin in down-regulating intracellular HIF-1 $\alpha$ .** The results revealed that the treatment with lipid raft interference M- $\beta$ -CD and Apocynin alone significantly down-regulated the expression of HIF-1 $\alpha$ , which suggests that lipid raft

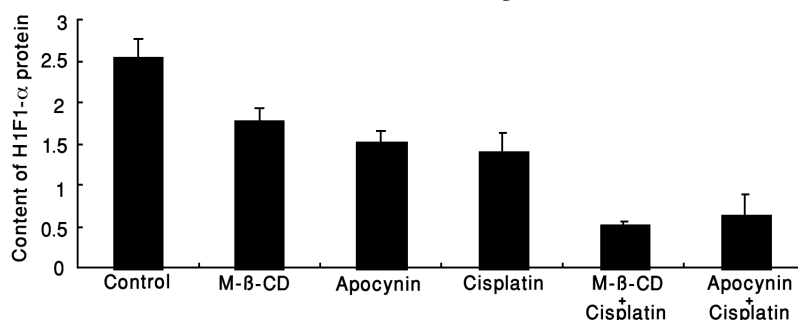
interference and NADPH oxidase inhibitor might repress the growth of cervical cancer through inhibiting the expression of HIF-1 $\alpha$  protein. In this experiment, we also found that these 2 agent combined with cisplatin achieved a more powerful effect on repressing HIF-1 $\alpha$  expression compared to solo application, and the repressing ratio of the M- $\beta$ -CD+Cis group decreased from 55.5 to 21.1% compared to the control group, and Apocynin+Cis group decreased to 25.4% compared to the control group. (Figure 3 & Figure 4).

**Lipid raft interference agent and NADPH oxidase inhibitor strengthen the effect of cisplatin on down-regulating ROS level.** The level of ROS for cisplatin alone was 24% compared to the control group, while when cisplatin was combined with M- $\beta$ -CD or Apocynin, the percentage of ROS decreased to 12.3% and 12.8% to the control group respectively (Figure 5).

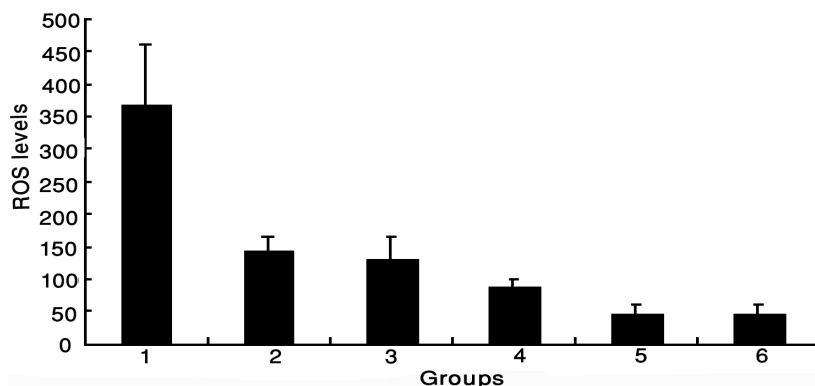


**Figure 3** - Impact of methyl- $\beta$ -cyclodextrin (M- $\beta$ -CD) and Apocynin alone or combined with cisplatin on expression of hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) in cervical cancer cells.

**Discussion.** Cervical carcinoma is a common malignant tumor in women's reproductive system. Not



**Figure 4** - Methyl- $\beta$ -cyclodextrin (M- $\beta$ -CD) and Apocynin (Apo) all showed the ability to down-regulate the expression of hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) versus control group ( $p=0.012$  and  $p=0.019$ ) or versus Cisplatin ( $p=0.027$  and  $p=0.022$ ), especially if M- $\beta$ -CD and Apo is combined with Cisplatin, they all showed synergistic inhibiting effect on the HIF-1 $\alpha$  ( $p=0.025$  and  $p=0.031$ ).



**Figure 5** - The methyl- $\beta$ -cyclodextrin (M- $\beta$ -CD) and Apocynin (Apo) all showed the ability to down-regulate the level of reactive oxygen species (ROS) versus control group ( $p=0.015$  and  $p=0.022$ ), or versus cisplatin ( $p=0.037$  and  $p=0.038$ ) especially, if M- $\beta$ -CD and Apo is combined with cisplatin, they all showed synergistic inhibiting effect on the level of ROS ( $p=0.045$  and  $p=0.042$ ).

only the cervical cancer cells are growing out of control, but also the incidence of MDR after chemotherapy is closely related to the over-activation of an important transcription factor, that is, HIF-1 $\alpha$ .<sup>4</sup> The HIF-1 $\alpha$  is important in determining the prognosis of malignant tumors as its excessive activation can promote tumor cells' growth, invasion, metastasis, and induce tumor cell resistant to chemotherapy and radiotherapy. Clinical studies found that a 2-year survival rate is significantly lower in patients with high expression of HIF-1 $\alpha$  than those with low expression of HIF-1 $\alpha$ .<sup>9,10</sup> Our previous study also confirmed that HIF-1 $\alpha$  expression in cervical cancer tissue was significantly increased, and when the expression of HIF-1 $\alpha$  was significantly reduced by transfecting specific small interfering RNA (shRNA), the growth of cervical cancer slowed down.<sup>11</sup> These indicate that over-activation of HIF-1 $\alpha$  can mediate the development of cervical cancer. Over-activation of HIF-1 $\alpha$  is related to the inhibition of the activity of prolyl hydroxylase enzymes (PHDs), because inactivity of PHDs cannot hydroxylate HIF-1 $\alpha$ , and HIF-1 $\alpha$  can not be degraded through the proteasome body pathway, but translocate to the nucleus, and combine with HIF-1 $\alpha$ , which is almost stably expressed in all cells as a dimer, HIF-1. Then HIF-1 would bind to the hypoxia response element in the position of promoter or enhancer of different genes, and promoting the expression of downstream target genes.<sup>12</sup> These downstream genes participate in tumor angiogenesis, glycolysis, immune escape, chemoresistance, conferring cervical cancer cells more malignant characteristics. Recently, MDR-1 is widely accepted as the most important human MDR gene,<sup>13</sup> whose upstream genes includes HIF-1 $\alpha$ . Studies have shown that increased expression of HIF-1 $\alpha$  could induce the overexpression of MDR-1 and its product P-gp.<sup>14</sup> Thus, we presume here that HIF-1 $\alpha$  is a major cause in promoting the occurrence of chemoresistance during cervical cancer chemotherapy drug implication, and also an important factor leading to the enhancement of the cervical cancer's survival ability.

We have known that high concentration of ROS would injure cells, however, in recent years, many studies have shown that concentration of endogenous ROS produced by cells is low and subtoxic, especially H<sub>2</sub>O<sub>2</sub> in low-concentration, which would not damage the cells but can be used as an important intracellular second messenger, playing an important role in promoting cell growth.<sup>15-19</sup> The ROS could inhibit PHDs and prevent HIF-1 $\alpha$  degradation through the proteasome body pathway, and thereby enhance the stability of HIF-1 $\alpha$ .<sup>20-22</sup> In normoxia, the accumulation of ROS,

especially that generates from intracellular NADPH oxidase (Nox), it becomes an important messenger in maintaining the activity of HIF-1 $\alpha$  at a higher level.<sup>18,19</sup> However, why do NADPH oxidase derived ROS would display such a steady increase in cervical cancer cells? In recent years, research on the field of lipid rafts provides an opportunity to solve this problem. Lipid raft is a micro membrane section rich in sphingomyelin and cholesterol, playing a very important role in promoting and maintaining cells' ROS originating from NADPH oxidase. The latest concept suggests that lipid raft not only exists in the cell membrane, but also can be found in all kinds of cell organelles transmembrane, providing an ideal interactional platform for cellular transmembrane signal transmission and the occurrence of intracellular signal events.<sup>23</sup> As for NADPH oxidase, especially the main Nox<sub>2</sub>, which includes subunits of gp91phox, P22phox, P47phox, P67phox, P40phox, Rac1, and so forth,<sup>4</sup> only when the subunits gather on the lipid raft and assemble as a complex does the oxidase have the activity of catalytic. When the lipid raft is damaged, the gathering of the subunits would be surely prevented, and thus the activation of NADPH oxidase will also be suppressed.<sup>4</sup> Research have shown that the damage of lipid raft can prevent the activation of NADPH oxidase,<sup>5</sup> and the assembling of lipid raft in the cell membrane can recruit each subunits of this oxidase, so as to activate itself and to increase intracellular ROS.<sup>24</sup> However, the destruction of the structural integrity of the lipid raft can effectively reduce the generation of ROS. These evidence demonstrate that the generation of ROS are closely related to lipid raft. There are also reports demonstrating that lipid raft has something to do with cervical cancer.

Prabhakar<sup>15</sup> reported that cervical cancer related human papilloma virus HPV16 E5 could stimulate the epithelial cells of the cervix to increase the content of lipid raft, which included lipid raft marker protein Caveolin-1 and one composition of lipid raft GM1 Ganglioside, further indicating that the expression of lipid raft can be increased through the stimulation of carcinogen in the cervix.

In this study, we found that processing cervical cancer cells with lipid raft interference agent M- $\beta$ -CD could make Hela cells grow at an apparently slower rate, and treatment with NADPH oxidase inhibitor Apocynin showed a similar effect. This suggests that lipid raft and NADPH oxidase related ROS play very important role in the growth of cervical cancer cells, even further indicating that lipid raft obtain this ability through NADPH oxidase pathway, and that lipid raft

interference agent and lipid raft related NADPH oxidase inhibitor may be an effective way for the treatment of cervical cancer. We also found that when processing HeLa cells with lipid raft interference agent M- $\beta$ -CD, the ROS level declined obviously, and at the same time, HIF-1 $\alpha$  expression showed a similar result. We speculate that the tumor cells growth repressing effect from lipid raft interference agent and NADPH oxidase inhibitor result probably from the expression of intratumor oxygen-related factor HIF-1 $\alpha$ . We do confirm that these 2 agents could down-regulate HIF-1 $\alpha$  level, and thus inhibit the growth of tumor cells. These further shows that lipid raft and NADPH oxidase are very important in activating HIF-1 $\alpha$  in cervical cancer cells, that is, lipid raft might promote the generation of endogenous ROS through NADPH oxidase, and thereby inhibiting HIF-1 $\alpha$ 's degradation, and ultimately resulting in the continuous high expression of HIF-1. We also found that, compared with the control group, HIF-1 $\alpha$  expression and ROS level in cisplatin group were significantly decreased. Lipid raft interference and NADPH oxidase agents can enhance this kind of effect of cisplatin. At the same time, compared with the implication of cisplatin alone, these 2 drugs both achieve a more pronounced inhibition effect on cervical cancer cell growth when synergistically applied with cisplatin, and the difference is significant. This suggests that chemoresistance is related to HIF-1 $\alpha$ , and can be mediated by intracellular ROS. As lipid raft interference agent and NADPH oxidase inhibitor can reduce intracellular ROS level and reduce HIF-1 $\alpha$  expression in cervical cancer, they are capable of inhibiting the growth of cervical cancer. Accordingly, we speculate here that lipid raft interference agent and NADPH oxidase inhibitor can enhance the cancer cells' sensitivity to chemotherapeutic drug and decrease its resistance to chemotherapy by reducing intracellular ROS levels and inhibiting the expression of HIF-1 $\alpha$ , which inspires a possible new direction of drug development in combination therapy for cervical cancer, especially for the chemotherapy drug resistant patients.

In conclusion, we confirm that lipid rafts associated and NADPH oxidase derived ROS is related to the activity of intracellular HIF-1 $\alpha$ , and we also proved that by interfering the lipid raft-NADPH oxidase-ROS signaling pathway, we can prevent the growth of cervical cancer cells, increase their sensitivity to chemotherapeutic drugs, reduce the occurrence of MDR, and through this, provide a new promising direction for the joint treatment of cervical cancer. There are some limitations in this study as we did not conduct our hypothesis in a multi-angle way. Further studies is needed to clarify the

exact mechanism regarding lipid raft in regulating the chemoresistance of cervical cancer.

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