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Regular paper

CRABP2 involvement in a mechanism of Golgi stress and tumor dry matter in non-small cell lung cancer cells *via* ER dependent Hippo pathway

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Objective: The paper aimed to explore the mechanism of cellular retinoic acid binding protein 2 (CRABP2) involvement in Golgi stress and tumor dryness in non-small cell lung cancer (NSCLC) cells through the estrogen receptor (ER) dependent Hippo pathway. Methods: Human NSCLC cell line A549 was purchased from ATCC andcultured in RPMI-1640 with 10% FBS. Attractene reagent was used for plasmid transfection. ER (sh) RNA was designed using RNAi Designer. Seventy-six hours after infection, stable cells were obtained after treated with puromycin for 3 weeks. ER silencing cells (with inhibited ER expression) were compared to the control cells (normal cultured NSCLC cell line A549, CRABP2 normal expression). CRABP2 and ER expression levels were detected by RT-PCR. MTT assay was used to detect cell proliferation, and the cell localization of ER and Golgi was observed by confocal microscopy. The invasion and metastasis of cells were analyzed by Boden chamber invasion and migration assays. Western blotting assays was used for detecting the protein expression of *E*-cadherin, vimentin, ZO-1 protein and epithelial-mesenchymal transition (EMT) related factors. Results: The lower expression level of mRNA was detected in the ER-silencing group compared to the control group ($P<0.05$). We also found a higher proliferation level of cells, the number of invading and metastatic cells, the expression of vimentin, p-Lats1T1079, Lats1 and p-YAPS127 mRNA in the control group compared to the ER silencing group ($P<0.05$). And the expression level of protein kinase RNA-like endoplasmic reticulum kinase (PERK), phosphorylate eukaryotic initiation factor 2 (p-eIF2 alpha), activating transcription factor 4 (ATF4) and C/EBP-homologous protein (CHOP) in the control group was higher than that in the ER silencing group ($P<0.05$). Adversely, a lower expression level of *E*-cadherin and ZO-1 protein was found in the control group compared to the ER silencing group ($P<0.05$). Conclusion: The expression of CRABP2 in NSCLC cells was regulated by ER, and cell proliferation and invasion were regulated by the Hippo pathway. At the same time, it was found that decreased expression of CRABP2 enhanced endoplasmic reticulum/Golgi stress response.

Keywords: CRABP2; ER; Hippo pathway; non-small cell lung cancer cells; Golgi apparatus; tumor stem

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Abbreviations: ATF4, ctivating transcription factor 4; CHOP, C/EBP-homologous protein; CRABP2, regenerated human intracellular retinoic acid binding protein-2; HCC, hepatocellular carcinoma; EMT, epithelial-mesenchymal transition; ER, estrogen receptor; FGF, fibroblast growth factor; *FGFR1*, fibroblast growth factor receptor 1; NSCLC, non-small cell lung cancer; p-eIF2 alpha, phosphorylate eukaryotic initiation factor 2; PERK, protein kinase RNA-like endoplasmic reticulum kinase; RA, retinoic acid; RAR, retinoic acid receptor

INTRODUCTION

Regenerated human intracellular retinoic acid binding protein-2 (CRABP2) is an intracellular lipid binding protein associated with retinoic acid, which is regarded as a key regulator of retinoic acid signal transduction in cells (Zhang *et al*., 2019b). CRABP2 takes part in cell proliferation, apoptosis, invasion and metastasis through transporting retinoic acid (RA) to retinoic acid receptor (RAR) in the nucleus (Feng *et al*., 2019, Zhu *et al*., 2019). Several studies indicate that CRABP2 can act as a transcription coactivator (Wei *et al*., 2019). In addition, CRABP2 can involve in biological behavior independent of RA or its receptor (Sung *et al*., 2019). Abnormal expression of CRABP2 is associated with human malignancy (Zhang *et al*., 2019a). Decreasing the level of CRABP2 will inhibit the movement of cancer cells and down-regulate the number of receptors on the surface of CRABP2 expression, thereby limiting cell proliferation in vitro (Wu *et al*., 2019). It will induce apoptosis and block cell migration (Xia *et al*., 2019) in esophageal squamous cell carcinoma. Previous studies have reported that estrogen receptor alpha (ERα or ER) regulates CRABP2 transcription in some way (Liu *et al*., 2019). High level of CRABP2 mRNA is associated with the prognosis of patients with non-small cell lung cancer (NSCLC) (Weng *et al*., 2019). It is not clear whether CRABP2 is involved in regulating the invasion and metastasis of NSCLC (Chen *et al*., 2020). The Hippo pathway controls the development of organs by regulating cell apoptosis and cell proliferation. Recently, it has been confirmed that the Hippo pathway is closely related to the proliferation, survival, invasion and metastasis of NSCLC cells (Zhang *et al*., 2020). ER is a nuclear steroid receptor, expressed in approximately 75% of breast cancers. Some reports have shown that estrogen can promote the growth of NSCLC (Yang *et al*., 2019). However, studies have shown that the loss of ER can lead to epithelial-mesenchymal transition (EMT) and the occurrence of tumor metastasis. This study focused on exploring the mechanism of CRABP2 involvement in Golgi stress and tumor stem growth in NSCLC cells through ER dependent Hippo pathway.

MATERIALS AND METHODS

Cells and gene silencing

The human NSCLC cell line A549 with 10% FBS was used for cell culture, purchasing from ATCC. RPMI-1640 (Gibco, USA). Attractene reagent (Qiagen, Germany) was used for plasmid transfection (0.12 μg per well in 6-well plates). ER and control siRNA were obtained from Dharmacon (GE Healthcare, USA). SiRNA (0.2 nmol/well in 6-well plates) was transfected with DharmaFECT (GE Healthcare, USA). ER (sh) RNA was designed using RNAi Designer (US Invitrogen): shER, 5'-GAATGTCAGACAGACGCGAAAATT-3'. pENTR/U6 was used to generate vectors encoding shRNA. Empty plasmid was used as the negative control. All cells were infected with lentivirus for sh-NC expression (sh-NC) or control lentivirus. The stable cells were obtained after 76 hours treated with puromycin for 3 weeks. Stable transfected cells were selected through culture of (Cayman Chemical, Ann Arbor, USA). The cells were selected with G418 (Sigma) at a concentration of 0.5 mg/mL. In the experiment, the cells were divided into the control group (normal cultured NSCLC cell line A549, CRABP2 normal expression) and the ER silencing group (cells with inhibited ER expression).

METHODS

Analysis of migration and invasion of cells

The Boyden chamber was consisted by two compartments separated by a polycarbonate membrane. The membrane contains pores of uniform size of 8.0 μm. To carry out migration analysis, 200 μL serum-free medium containing 2×10^5 cells was placed in the upper compartment, and a complete medium with 20% FBS was placed in the lower compartment. The cell chamber was removed and the extra cells were removed by gently cleaning the upper surface with a cotton swab after 12 hours of regular incubation,. The entire filter membrane was immersed in a fixed solution (4% POM) for 30 minutes. The hematoxylin eosin staining kit (Solarbio) was used for staining. The number of stained cells was determined under a microscope at ×200 magnification in 5 fields of view and the average count was calculated. The microporous filter membrane, coated with Matrigel (BD BioCoat) to form a bioactive three-dimensional matrix, was used for invasion assay. Besides the incubation time of 24 hours, the procedure was the same as the migration assay. After removing the cells above the top chamber, the cells were photographed and counted.

RNA separation and real time RT-PCR. The total RNA extract was obtained by using RNA kit I (Omega Bio-Tek, Inc., Norcross, Georgia, USA) and PrimeScript™RT Master Mix (Takara Biotechnology (Dalian) Co., Ltd., Liaoning, China). The cells were seeded in 12-well plates overnight at a density of 5×10^4/well and treated for 24 hours with DIM (25 and 50 uM). The SYBR-Green I kit (M Dalian Co., Ltd., Dalian, China) was used for Real time polymerase chain reaction (RT-PCR). RNAiso Plus reagent (Takara, Dalian, China) was used for Total RNA isolation. Ct was normalized to endogenous beta actin (ΔCt=Cttarget–Ctβ-actin) and compared with the calibrator using the ΔΔCt method (ΔΔCt=Δ Ctsample- Δ Ctcontrol) for quantifying the relative expression of each gene.

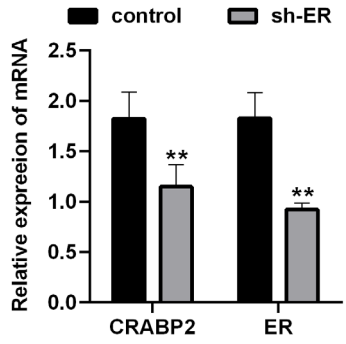

Figure 1. The mRNA expression levels of CRABP2 and ER in a control group and ER silencing group.
sh-ER, ER silencing group. *P<0.05, **P<0.01, ***P<0.001.

Preparation and protein blotting of cell extracts. Proteins were separated using SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) (Bio-Rad, California, USA). The membranes were sealed with 5% skim milk for 2 hours. Lamin A/C and GAPDH served as controls. Cell grading analysis was carried by nuclear and cytoplasmic extraction agents (pioneer Biotech Corp, Xi'an, China). Mst2, p-Lats1T1079, Lats1, p-YAP S127, *E*-cadherin and ZO-1 antibodies were purchased from CST (US Cell Signaling Technology). Lamin A/C, GAPDH, and vimentin were purchased from Proteintech, China. CRABP1, CRABP2 and anti-ER were obtained from Abcam, UK. The protein bands were visualized by image Master II scanner (GE Healthcare, Milwaukee, WI, USA). *E*-cadherin, vimentin, E-calcium sticky eggs, protein kinase RNA-like endoplasmic reticulum kinase (PERK), phosphorylate eukaryotic initiation factor 2 (p-eIF2 alpha), activating transcription factor 4 (ATF4) and C/EBP-homologous protein (CHOP) antibodies were used. ImageJ Pro was used for protein quantification. Relative protein expression was standardized with the level of β-actin in each lane.

Determination of MTT. Cell proliferation was measured by MTT. Transfected 5×10^4 A549 cells were incubated for 96 hours at 37°C in 96 well plates. Cells were incubated at 37°C for 4 hours after 20 μL of 5 mg/mL MTT (Sigma, St. Louis, MO, USA) solution was added,. The medium was removed from each well and the resulting MTT was dissolved in 150 μL DMSO. The results were quantified by spectrophotometry at 490 nm.

Statistical analysis. All experiments were repeated at least three times *in vitro*. Data were analyzed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). These results were displayed as mean ± S.D. The comparison between the two groups was conducted by t test. Two-way ANOVA and Dunnett's multiple comparison test were used in two-way comparison. All statistical tests were two-sided. P<0.05 was considered statistically significant.

Table 1. The mRNA expression level of CRABP2 and ER in control groups and ER silencing group.

Group	CRABP2	ER
Control	1.83±0.26	1.83±0.25
Sh-Er	1.15±0.22	0.92±0.07

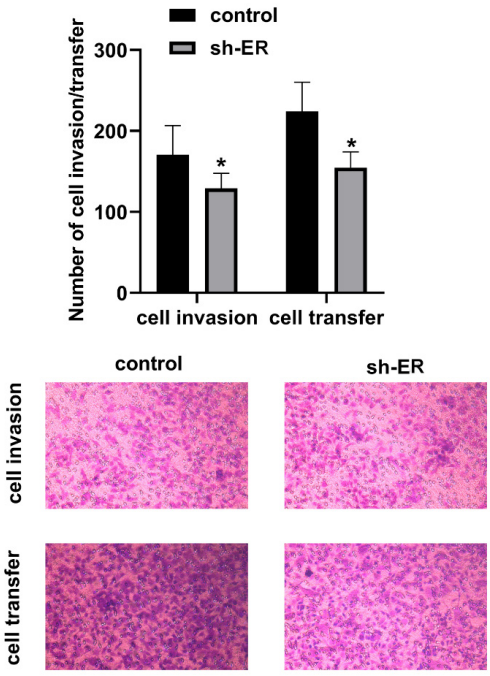

Figure 2. The outcomes of cell invasion and cell transfer in a control group and ER silencing group.
sh-ER, ER silencing group. *P<0.05, **P<0.01, ***P<0.001.

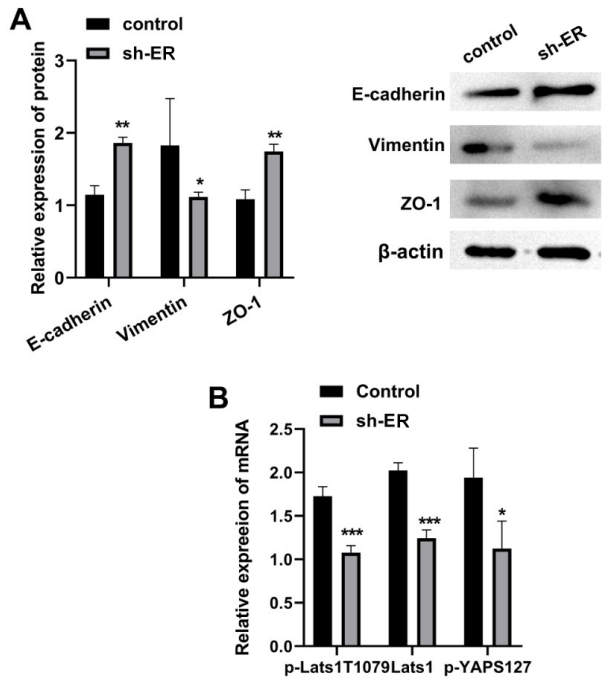

Figure 3. (A) The protein expression of *E*-cadherin, vimentin and ZO-1 in a control group and ER silencing group. (B) The mRNA expression levels of p-Lats1T1079, Lats1 and p-YAPS127 in a control group and ER silencing group.
sh-ER, ER silencing group. *P<0.05, **P<0.01, ***P<0.001.

RESULTS

ER regulates the mRNA expression of CRABP2

The mRNA expression levels of CRABP2 and ER in cells were detected by RT-PCR. Compared with the control group, the mRNA expression levels of CRABP2 and ER decreased in the ER silencing group (P<0.05). These results indicated that CRABP2 expression depended on ER, which promoted the expression of CRABP2 (Fig. 1, Table 1).

CRABP2 promoted cell migration and invasion

Using a lentiviral vector, the function correlation of CRABP2 gene expression restricted cells was tested. Compared with the control group, the number of invasive and metastasized cells was decreased in the ER silencing group (P<0.05) (Fig. 2, Table 2).

Table 2. The outcomes of cell invasion and cell transfer in control groups and ER silencing group.

Group	Cell invasion	Cell transfer
Control	170.67±36	224±36.00
Sh-Er	129±18.52	154.67±19.50

Table 3. The outcomes of cell proliferation assay in control groups and ER silencing group.

Group	24 hours	48 hours
Control	178.08±16.87	234.95±15.72
sh-Er	100.29±11.33	116.70±31.77

Cell proliferation assay

MTT analysis showed that the proliferation of cells cultured for 24 hours and 48 hours in the control group was significantly higher than the ER silence group (P<0.05) (Supplementary Fig. 1 at https://ojs.ptbioch.edu.pl/index.php/abp/, Table 3).

CRABP2 promotes cell EMT production

The outcomes of western blot analysis showed that the expression of *E*-cadherin, vimentin and ZO-1 protein in the ER silencing group was higher than that in the control group (P<0.05), and the expression of vimentin in the ER silencing group was lower than that in the control group (P<0.05). The results indi-

Table 4. The protein expression of E-cadherin, vimentin and ZO-1 in control groups and ER silencing group

Group	*E*-cadherin	Vimentin	ZO-1
Control	1.14±0.13	1.83±0.65	1.08±0.13
sh-Er	1.86±0.09	1.12±0.06	1.74±0.10

Table 5. The mRNA expression levels of p-Lats1T1079, Lats1 and p-YAPS127 in control groups and ER silencing group.

Group	p-Lats1T1079	Lats1	p-YAPS127
Control	1.73±0.12	2.0±0.09	1.94±0.34
sh-Er	1.1±0.08	1.24±0.10	1.12±0.32

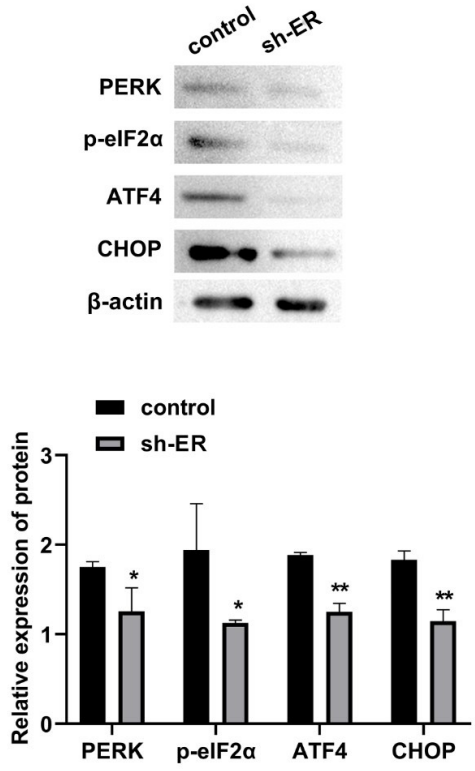

Figure 4. The protein expression of protein kinase like endoplasmic reticulum kinase (PERK), eukaryotic initiation factor 2 (p-eIF2α), activated transcription factor 4 (ATF4), and C/EBP homologous protein (CHOP) in a control group and ER silencing group.
sh-ER, ER silencing group. *P<0.05, **P<0.01, ***P<0.001.

cated that CRABP2 promoted the occurrence of cell EMT (Fig. 3, Table 4).

CRABP2 activates the Hippo pathway

The mRNA expression of factors related to Hippo pathway was analyzed by real time PCR. The expression level of p-Lats1T1079, Lats1 and p-YAPS127 mRNA in the control group was higher than the ER silencing group (P<0.05), indicating that CRABP2 promoted the occurrence of EMT, metastasis and invasion by activating the Hippo pathway (Table 5).

Effect of CRABP2 on endoplasmic reticulum/Golgi stress in cells

Considering the subcellular localization of endoplasmic reticulum and Golgi apparatus, we examined whether CRABP2 affected endoplasmic reticulum/Golgi apparatus stress regulatory protein. The expression levels of protein kinase like endoplasmic reticulum kinase (PERK), eukaryotic initiation factor 2 (p-eIF2α), activated transcription factor 4 (ATF4), and C/EBP homologous protein (CHOP) were detected by Western blotting assays. The expression levels of PERK, p-eIF2, ATF4 and CHOP in cytoplasm of the control group were higher than the ER silencing group (P<0.05) (Fig. 4, Table 6).

Table 6. The protein expression of protein kinase like endoplasmic reticulum kinase (PERK), eukaryotic initiation factor 2 (p-eIF2α), activated transcription factor 4 (ATF4), and C/EBP homologous protein (CHOP) in control groups and ER silencing group.

Group	PERK	p-eIF2α	ATF4	CHOP
Control	1.75±0.06	1.94±0.52	1.88±0.03	1.83±0.10
sh-Er	1.26±0.26	1.13±0.03	1.25±0.10	1.15±0.13

DISCUSSION

Lung cancer is one of the leading causes of death in the worldwide. The high mortality rate of the disease could be caused by delayed diagnosis due to unobvious symptoms. According to a study, about 57% of lung cancer would metastasize and affect the normal function of other organs and tissue (Huang *et al.*, 2019). The lung cancer is commonly divided into NSCLC and small cell lung cancer (SCLC) (Masciale *et al.*, 2019). NSCLC is the most common type of lung cancer, accounting for 80–85% of all patients. Usually, it is a slow and persistent tumor that will metastase and spread to nearby tissues and cells eventually (Guo *et al.*, 2019). SCLC is characterized by rapid initial transmission, with more smokers than non-smokers. NSCLC and SCLC cause about 1.5 million deaths every year. However, due to the heterogeneity and metastasis of NSCLC, most studies have been carried out on NSCLC. According to statistics, only 5% of NSCLC patients can survive after stage IV. Clinical choices such as surgery, chemotherapy, radiotherapy and other cancer therapies have been used to treat such cancers, but there is still a need for better curative effects to cure cancer, especially at the advanced stage. Because of unknown symptoms, the prognosis of NSCLC is very difficult. In early stage NSCLC, most patients do not have symptoms. Therefore, diagnosing the disease at the initial stage is challenging. The formation of tumor biomarkers is very important for early diagnosis, prognosis and individualized treatment of cancer patients (including NSCLC patients) (Piao *et al.*, 2019). In recent years, the new strategy of identifying sensitive and reliable biomarkers for accurate diagnosis and reliable prediction of recurrence has aroused great interest.

CRABP212 has two isoforms (CRABP1 and CRABP2), which have high affinity for all RA trans ligands (Cortesi & Ventura, 2019). These same isoforms are highly conserved among species, displaying 74% sequence identity in humans. The destruction of any CRABP subtype is highly correlated with mild limb defects (Miao *et al.*, 2019). CRABP1 is a passive carrier that binds and releases its ligand according to the concentration gradient. CRABP2 can transmit RA to RAR and the channelization of RA between CRABP2 and RAR will increase overall RAR formation and RAR transcription (Gong *et al.*, 2019). The anticancer mechanism of CRABP2, which does not depend on RA, has been suggested. The anticancer effect of CRABP2 has been suggested in astrocytomas, head and neck tumors. It is generally considered that CRABP2 enhancement of RA signal is an anti-tumorigenic activity. However, there are still some conflicting reports about the exact role of CRABP2 in tumorigenesis, which need to be further studied. Recent proteomic analysis found that high levels of CRABP2 are a poor prognostic marker for ER-negative breast tumors, while another study found that CRABP2 is a subtype-specific biomarker for ovarian cancer. The expression in serous ovarian cancer is upregulated, and CRABP2 expression is positively correlated with tumor grade and stage of cancer. In addition, it has

been found that RNA-Seq in NSCLC cancer tissue is 4.9 times higher than that in normal lung tissue, and 72.1% of NSCLC samples show the expression of CRABP2 by immunohistochemistry (Han *et al*., 2014).

YAP is the major transductor of the Hippo pathway. It induces target genes including Connective tissue growth factor (*CTGF*), which can induce cell proliferation and apoptosis. Previous studies have shown that Yap overexpression will result in the growth and invasion of lung cancer cells and is associated with poor survival as well (Zhao *et al*., 2020). The clear need for more personalized treatments has led to the development of molecularly targeted drugs. Targeted therapy is more effective than non-targeted therapy for patients with lung adenocarcinoma. As a single drug, individual targeted therapy is only clinically useful in patients with known molecular abnormalities and cancer drivers. In order to provide more effective treatment for a larger group of patients, we must identify other targeted carcinogenic pathways and/or selection criteria. A reasonable combination of such therapies that can interact with signal transduction pathways is also a potential option to improve clinical utility. The fibroblast growth factor (FGF) family consists of 18 ligands and 5 receptors, involving in cellular basic activities, including proliferation, wound healing and angiogenesis. It has been shown that aberrant signaling of this pathway promotes tumorigenesis. The amplification of fibroblast growth factor receptor 1 (*FGFR1*) gene in NSCLC occurred in 22% of squamous cell carcinoma and 4% of adenocarcinoma, which is related to poor overall survival and short disease-free survival (Weiss *et al*., 2010). Recently, an activation mutation has been found in the *FGFR2* and *FGFR3* genes of squamous cell lung cancer. The co-expression of FGFR and its corresponding ligands, such as FGF2 and FGF9, has been found in NSCLC, indicating the autocrine mechanism of the proliferation signaling pathway (Ware *et al*., 2013). In this study, the mRNA expression of Hippo pathway related factors in PCR cells was analyzed by RT-PCR. The expression levels of p-Lats1T1079, Lats1 and p-YAPS127mRNA in the control group were higher than the ER silencing group, indicating that CRABP2 promoted EMT, metastasis and invasion through activating the Hippo pathway.

The occurrence and proliferation of lung tumors is associated with ER pathway. In this study, MTT analysis showed that the proliferation of cells cultured for 24 and 48 hours in the control group was higher than the ER silencing group. A population study has shown the role of E2 in the development of lung cancer, which links hormone replacement therapy to increased lung cancer mortality (Gasperino, 2011). Protection measures against lung cancer deaths have also been observed in breast cancer survivors receiving endocrine therapy. It has been previously shown that the major ER homologous estrogen receptor β-1 (ER β) found in NSCLC is responsible for mediating the proliferation of estrogen, while the full-length ERα protein is usually not expressed. In preclinical models, estrogen blocking drugs have been studied for the treatment of lung cancer, and the way to show interaction with ER in lung cancer may be the common target.

In the study, changes of cellular endoplasmic reticulum and Golgi induced by the absence of CRABP2 expression occurred simultaneously with the expression of endoplasmic reticulum/Golgi stress regulatory protein. TEM results showed that ER silencing expanded endoplasmic reticulum and Golgi body structure. Therefore, the effects of endoplasmic reticulum/Golgi stress on endoplasmic reticulum and Golgi Cytopainter were examined. The endoplasmic reticulum and Golgi were dyed red and green, respectively. In the control group, the endoplasmic reticulum tubules were initially localized in the perinuclear region of the control cells. While in the ER silencing cells, the cytoplasm related to endoplasmic reticulum was redistributed. The Golgi apparatus in the control group was strictly localized in the perinuclear region, and in the ER silencing cells the Golgi signal was redistributed in the cytoplasm. The expression levels of PERK, p-eIF2α, ATF4, and CHOP were analyzed by Western blotting assays. The expression levels of PERK, p-eIF2 alpha, ATF4 and CHOP in the control group were higher than those in the ER silencing group. The treatment of NSCLC has made great progress, but for most patients, the conventional treatment method cannot effectively lead to recurrence. Therefore, it is important to understand the occurrence and development of NSCLC, so as to promote treatment and improve the cure rate of patients.

In conclusion, the expression of CRABP2 in NSCLC cells was regulated by ER, and cell proliferation and invasion were regulated by the Hippo pathway. Meanwhile, the decrease of CRABP2 expression enhanced endoplasmic reticulum/Golgi stress response.

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