ORIGINAL RESEARCH

Mechanistic Study of CRABP2: Accelerating Lung Cancer Migration and Metastasis through Regulation of the ROS/Src Signaling Pathway

Guang Yang, MD; Huining Liu, PhD; Qifan Yin, MD; Ziqiang Tian, PhD

ABSTRACT

Objective • This study aims to establish a theoretical foundation for the clinical treatment of lung cancer by investigating the regulatory role of CRABP2 in the ROS/Src signaling pathway, specifically in accelerating the migration and metastasis of lung cancer.

Methods • Lung cancer mouse models were established using BALB/c-nu mice, randomly assigned to the control group (NC group) and the experimental group (mimic group). Tumor volume was precisely observed. The impact of CRABP2 on lung cancer migration and metastasis was analyzed through hematoxylin and eosin (H&E) staining and histochemical staining observation. Protein expression analysis was employed to assess CRABP2, ESR1, NOX1, NOX4, p-Src, and p-FAK levels, shedding light on the underlying mechanism. CRABP2's influence on lung cancer migration and metastasis was further investigated using scratch and Transwell experiments.

Results • The findings revealed that the mimic group, with enhanced CRABP2 expression, exhibited a higher proliferation rate and increased migration and metastasis capabilities in lung cancer. Protein expression analysis demonstrated that CRABP2 and ESR1 positively influenced the ROS/Src pathway, promoting lung cancer migration and metastasis. Scratch and Transwell's experiments supported the fact that CRABP2 significantly accelerated lung cancer migration and metastasis.

Conclusions • CRABP2 plays a crucial role in expediting lung cancer migration and metastasis by upregulating ESR1 expression, consequently activating the ROS/Src pathway. This study introduces a novel therapeutic avenue for the clinical treatment of lung cancer, offering a theoretical framework for advancing lung cancer treatment strategies. (*Altern Ther Health Med.* 2025;31(2):70-75).

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INTRODUCTION

Lung cancer, referred to as primary bronchogenic carcinoma, encompasses malignant tumors that primarily arise in the trachea, bronchus, and lung.[1] A mechanism study on lung cancer involves investigating the intricate processes and pathways underlying the development and progression of this disease.[2] In the past decades, lung cancer has been the most commonly diagnosed cancer globally.[1,2] Lung cancer is the most common malignant tumor in the world and the most significant cause of cancer deaths globally, with 1.6 million deaths annually.[3] Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancers and is the leading cause of cancer-related deaths globally.[4]

Over the past two decades, molecular targeted therapy and immunotherapy have markedly enhanced the efficacy of treating NSCLC.[5] Clinically, a limited proportion of NSCLC patients receive early-stage diagnoses (stage I or stage II), allowing for surgical resection of the tumor.[6] However, most advanced NSCLC cases resist existing treatment modalities, ultimately leading to disease progression.[5] The recurrence rate and mortality associated with NSCLC are alarmingly high, reflecting an overall 5-year survival rate of less than 15%.[7] Notably, NSCLC patients in clinical stage I exhibit a more favorable 5-year survival rate exceeding 60%, while those in stage III face a significantly lower rate of less than 30%.[8-10]

In China, lung cancer stands as the primary cause of cancer-related deaths, experiencing a substantial rise in incidence and mortality in recent years.[10] The diversity in lifestyle and socio-economic development contributes to regional and gender disparities. Attribute risk analysis reveals associations between lung cancer and factors such as smoking, air pollution, and occupational exposures.[11]

The body's normal immune system possesses immune monitoring functions. In the presence of tumors, the immune system can identify and selectively eliminate these "non-self" cells through immune mechanisms, thereby resisting tumor development.[12] Despite the increasing visibility of immunotherapy with advancements in medical technology, clinical research in this domain is relatively limited, and theoretical support is insufficient. Cellular retinoic acid binding protein 2 (CRABP2), an intracellular lipid-binding protein that binds to retinoic acid, is thought to regulate retinoic acid signal transduction in cells.[13]

Its primary physiological role is participating in retinoic acid's intracellular transport, absorption, and metabolism (RA). Positioned on the human chromosome 1q21-23, CRABP2 is a relatively small protein with a molecular weight ranging from 15 000 to 16 000,[14] consisting of 138 amino acids. It is widely expressed in various human cancers, including breast cancer, ovarian cancer, prostate cancer, bladder tumors, neuroblastoma, glioma, head and neck squamous cell carcinoma, acute promyelocytic leukemia, and renal cell carcinoma.[15]

Reports indicate that abnormal expression of CRABP2 is linked to tumorigenesis.[16] However, there is a scarcity of studies examining the relationship between CRABP2 and non-small cell lung cancer in China and internationally. Consequently, a thorough investigation into the molecular-level role and mechanism of CRABP2 in tumorigenesis and development is warranted. Such an inquiry holds the potential to offer novel insights for early diagnosis, treatment monitoring, and prognosis evaluation in the context of tumors. Therefore, this study delves into the role of CRABP2 in hastening the migration and metastasis of lung cancer through its regulation of the ROS/Src pathway. The objective is to establish a theoretical foundation for the clinical management of non-small cell lung cancer and offer a practical guide to enhance the survival rates of lung cancer patients.

MATERIALS AND METHODS

Study Design

The study involved acquiring 20 healthy SPF BALB/c-nu mice, with an equal distribution of males and females, weighing 20±4g. These mice were sourced from Henan Skybest Biotechnology Co., Ltd. Additionally, A549 cells, serving as representative human alveolar epithelial cells in lung adenocarcinoma, were employed for the experiments. The DMEM high glucose medium necessary for the study was procured from Gibco, Inc.

Experimental Setup for Inducing Lung Cancer in Nude Mice

The mice were randomly assigned to either the control group (NC group) or the experimental group (mimic group) to establish lung cancer mouse models. In the mimic group, CRABP2 and mimic were subcutaneously injected into the lower part of the axilla. The NC group received injections of CRABP2 and an equal volume of saline. After the injections, the skin wounds of the nude mice were gently clamped with sterile tweezers, and the mice were returned to their cages for continued care. Mice volumes were observed and recorded weekly throughout the experiment. Following the experiment, mice were anesthetized with 3% chloral hydrate and euthanized by cervical dislocation. Tumor pathological sections were then obtained for further analysis.

Histopathological Analysis

Hematoxylin and Eosin (H&E) Staining. The tumor tissue sections underwent standard benzophenone dewaxing, dehydration, and washing processes before being stained with hematoxylin for 5 minutes. After a thorough rinse, water absorption occurred, followed by differentiation with 1% hydrogen chloride ethanol. The sections were washed with running water, causing them to turn blue for approximately 15 minutes, and were then stained with 1% eosin for an additional 5 minutes.

Ethanol and xylene gradient dehydration ensued to achieve transparency, and the sections were sealed with neutral gum. Microscopic observation was carried out to assess the morphological structure of the tumor tissue, and cell counts were performed to investigate the impact of CRABP2 on tumor cell proliferation. Grouping criteria were consistent with those employed in the nude mice tumor formation experiment.

Histological Staining Experiment. The cells were randomly allocated into three groups: the CRABP2 expression-promoting group, the Estrogen Receptor 1 (ESR1) expression-promoting group, and the NC group. Paraffin-embedded tissue sections underwent gradient dehydration and restoration then placed in a light-proof wet box. The subsequent day involved the addition of the secondary antibody, and cells were subsequently washed with PBS. Cell counts were carefully observed and recorded, aiming to investigate the impact of CRABP2 on tumor cell proliferation.

Protein Expression Analysis

***In Vivo* Protein Expression Analysis.** The protein expression levels of CRABP2, ESR1, NADPH Oxidase 1 (NOX1), NADPH Oxidase 4 (NOX4), Phosphorylated Src (p-Src), and Phosphorylated Focal Adhesion Kinase (p-FAK) were detected and observed using Western Blot technology. This analysis aimed to investigate the impact of CRABP2 on regulating the ROS/Src signaling pathway in nude mice.

***In Vitro* Protein Expression Analysis.** Employing Western Blot technology, protein expressions of CRABP2, ESR1, NOX1, NOX4, p-Src, and p-FAK were detected and observed under 1% O_2 hypoxic conditions. This analysis aimed to study the effects of CRABP2 on the ROS/Src signaling pathway. A549 cells were categorized into the mimic group, NC group, IS-mimic group, and IS-NC group. The mimic group received an addition of mimic, while the NC group served as the blank control group with the addition of the same volume of saline. The Mimic group and the NC group received an equal amount of Isuzinaxib, leading to the formation of the IS-NC group and the IS-mimic group.

Cellular Migration and Metastasis Assessment through Scratch and Transwell Tests

Scratch Test. On the *in vitro* cultured monolayer of A549 iron wall cells, a trace was created in the central area using a micro gun head. The central portion of the cells was then removed to observe the repairability of A549 cells and ascertain whether the cells were able to regenerate in the central scratch area. This analysis aimed to evaluate the repair ability of A549 cells and investigate the effects of CRABP2 on the migration and metastasis of lung cancer.

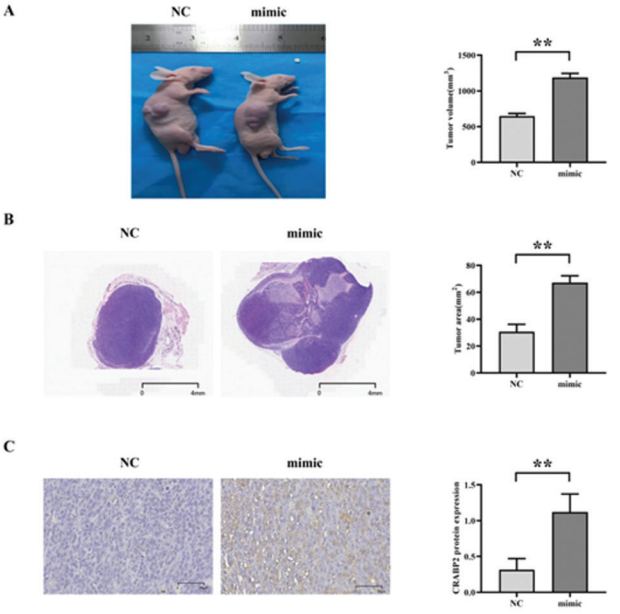
Transwell Test. Utilizing the protein expression analysis method, the migration of A549 cells was quantified, establishing a correlation between protein expression and the number of migrating A549 cells. This analysis aimed to investigate the impact of CRABP2 on the migration and metastasis of lung cancer.

RESULTS

Impact of *CRABP2* Expression on Tumor Volume in Nude Mice

An evident pattern emerged through the detailed observation of tumor formation in nude mice, revealing that the tumor volume within the mimic group surpassed that of the NC group. The experimental outcomes demonstrated that promoting CRABP2 expression significantly accelerates the proliferation of lung cancer, see Figure 1.

Figure 1. Tumor Volume Observation in Nude Mice



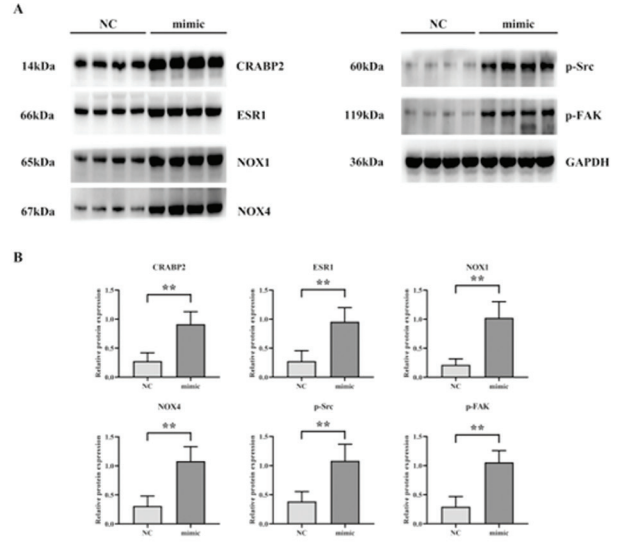
Note: Figure 1 depicts the observation of tumor volume in nude mice, comparing the NC group with the mimic group. Figure A illustrates the tumor volume observation in both groups, while Figure B presents the statistical analysis of the tumor volume observations in the NC group and mimic group. Figure 1C showcases cellular images, providing insights into the cellular composition.

CRABP2 and Tumor Cell Proliferation

H&E Staining. A careful examination of H&E staining data showed a distinct pattern, showcasing a notable increase in the proliferation of tumor cells within the mimic group compared to the NC group. These observations strongly suggest that elevating CRABP2 expression markedly expedites the proliferation of lung cancer cells, as illustrated in Figure 2.

Histological Staining. Through comprehensive histochemical staining observation, it was evident that the number of proliferated tumor cells was higher in both the CRABP2 expression group and the ESR1 expression group compared to the NC group. These results underscored that promoting either CRABP2 or ESR1 expression significantly accelerates the proliferation of lung cancer, refer to Figure 2.

Figure 2. Histopathological Analysis of Lung Cancer Cells



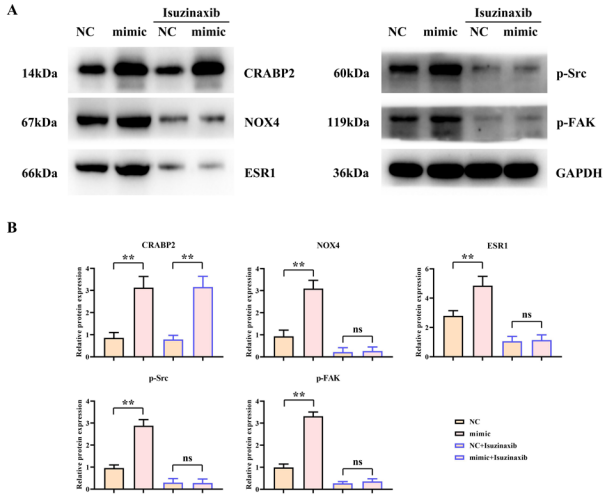
Note: Figure 2A showcases the observation and statistical analysis of H&E staining in lung cancer cells, comparing the NC and mimic groups. In Figure 2B, the study examines the histological staining, providing observations and statistical analyses for the NC group, the CRABP2 expression-promoting group, and the ESR1 expression-promoting group.

CRABP2 and Protein Expression Enhancement

***In Vivo* Protein Expression Analysis.** During *in vivo* Western Blot results, a clear trend emerged, indicating elevated expression levels of CRABP2, ESR1, NOX1, NOX4, p-Src, and p-FAK in the mimic group compared to the NC group. These findings underscore that promoting CRABP2 expression significantly enhances the expression of ESR1, NOX1, NOX4, p-Src, and p-FAK, refer to Figure 3.

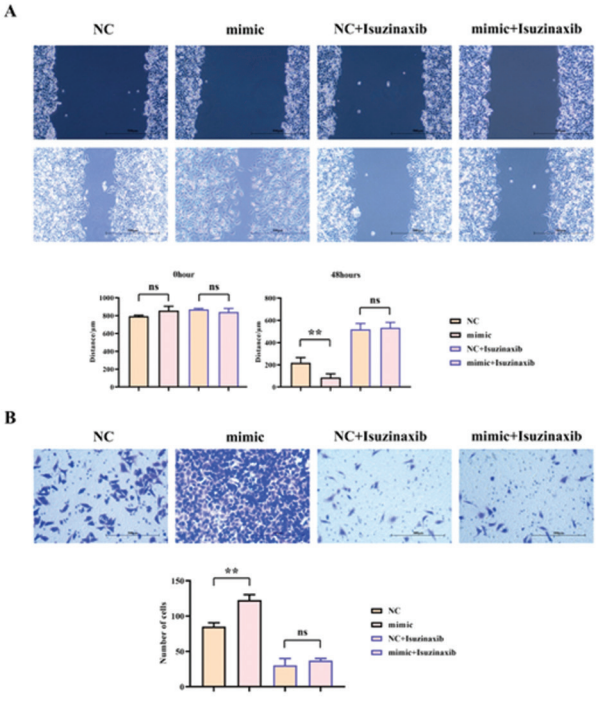
***In Vitro* Protein Expression Analysis.** Analysis of the *in vitro* Western Blot results revealed heightened expression levels of CRABP2, ESR1, NOX1, NOX4, p-Src, and p-FAK in the mimic group compared to the NC group. Notably, in the IS-NC group, expression levels of NOX1, NOX4, p-Src, and p-FAK were lower than in the NC group, with no impact on CRABP2 and ESR1 expression. In the IS-mimic group, expression levels of CRABP2 and ESR1 were higher than in the mimic group, while the expression of NOX1, NOX4, p-Src, and p-FAK remained unaffected. These outcomes affirm that promoting CRABP2 expression effectively enhances the expression of ESR1, NOX1, NOX4, p-Src, and p-FAK, refer to Figure 3.

Figure 3. Protein Expression Analysis in Lung Cancer Cells



Note: Figure 3A presents the protein expression analysis with statistical insights for the NC and mimic groups. Figure 3B provides a comprehensive exploration of protein expression analysis, including statistical analysis for the NC group, mimic group, IS-NC group, and IS-mimic group.

Figure 4. Cell Proliferation and Migration Analysis



Note: Figure 4A showcases the observations and statistical analyses of the Scratch test for the NC group, mimic group, IS-NC group, and IS-mimic group. In Figure 4B, the Transwell test observations and statistical analyses are presented for the NC group, mimic group, IS-NC group, and IS-mimic group.

Impact of CRABP2 Expression on A549 Cell Proliferation and Migration

Scratch Test. The scratch test results revealed that tumour cells' proliferation ability exhibited no significant difference between the IS-mimic group and the mimic group. However, the experimental outcomes unequivocally demonstrated that promoting CRABP2 expression significantly accelerated the overall proliferation of tumor cells, refer to Figure 4.

Transwell Experiment. The Transwell experiment revealed that tumor cells in the CRABP2 expression group exhibited heightened migration ability compared to the NC group. Conversely, the migration ability of tumor cells was diminished in the IS-NC group compared to the NC group. Notably, no significant difference in migration ability was observed between the IS-mimic and mimic groups. These findings underscore that promoting CRABP2 expression distinctly promotes the migration of tumor cells, refer to Figure 4.

DISCUSSION

Lung cancer stands as a formidable malignant tumor, witnessing over 2 million new cases globally each year.[17] Categorized predominantly into NSCLC and small cell lung cancer (SCLC), the former constitutes approximately 80-85% of all reported cancer cases.[18] The therapeutic resource against non-small cell lung cancer encompasses diverse modalities such as surgery, radiotherapy, chemotherapy, targeted therapy, immunotherapy, and other clinically proven methods.[19]

Surgery stands as a primary modality in the comprehensive treatment of lung cancer,[20] encompassing essential procedures such as lobectomy, lobectomy with lymph node dissection, lobectomy with hilar lymph node dissection, and pneumonectomy. The selection of a specific surgical approach hinges upon considerations of the tumor's location, size, lymph node metastasis, and the overall physical condition of the patient, among other factors.[21] While surgical resection proves efficacious in controlling localized lesions, its effectiveness diminishes notably in cases of lung cancer characterized by lymph node metastasis.

Current research predominantly leans towards targeted therapy and immunotherapy for lung cancer. Immunotherapy, a treatment methodology centered on leveraging the immune system to eradicate tumor cells, attains therapeutic efficacy by either stimulating the cytotoxic capabilities of immune cells or impeding the evasion of tumor cells from the immune system.[22-24] This innovative approach can effectively apply to molecular targets like PD-1, PD-L1, and CTLA-4.

CRABP2, also recognized as cellular retinol-binding protein 2, is a retinol-binding protein situated on the cell membrane, functioning as a transmembrane protein. The primary role of CRABP2 lies in transporting retinol from the cytoplasm to the nucleus, facilitating its binding with other proteins. Recent investigations have unveiled that CRABP2 extends its involvement to diverse biological processes, including cell cycle regulation and apoptosis. Notably, a discernible correlation exists between CRABP2 and the proliferation and migration of NSCLC .[25,26] ESR1, denoting estrogen receptor 1, is a pivotal protein exerting a significant regulatory role within the human body. ESR1 and SRC actively participate in endocrine regulation, while MMP2 and MMP9 regulate compound kinase, thus intricately contributing to cell proliferation and migration.[27]

Our in-depth exploration into *in vitro* protein expression reveals compelling observations. In contrast to the NC group,

the overexpression of CRABP2 markedly and positively influences all protein expressions in the experimental setup. This finding affirms its pivotal role in enhancing the expression of ESR1. Conversely, the IS-NC group displays reduced expressions of NOX1, NOX4, p-Src, and p-FAK while maintaining unaffected CRABP2 and ESR1 expressions. This divergence suggests that the inhibition of CRABP2 expression exerts a suppressive effect on the expression of pathway proteins, ultimately leading to the inhibition of the ROS/Src pathway.

A thorough examination of the scratch and Transwell experiments unveiled notable patterns. The mimic group exhibited increased proliferation and migration capabilities compared to the NC group, thereby substantiating CRABP2's role in promoting these attributes in lung cancer cells. Conversely, the IS-NC group demonstrated diminished proliferation and migration abilities, signifying that inhibition of CRABP2 by Isuzinaxib weakened these cellular functions in lung cancer cells compared to the NC group.

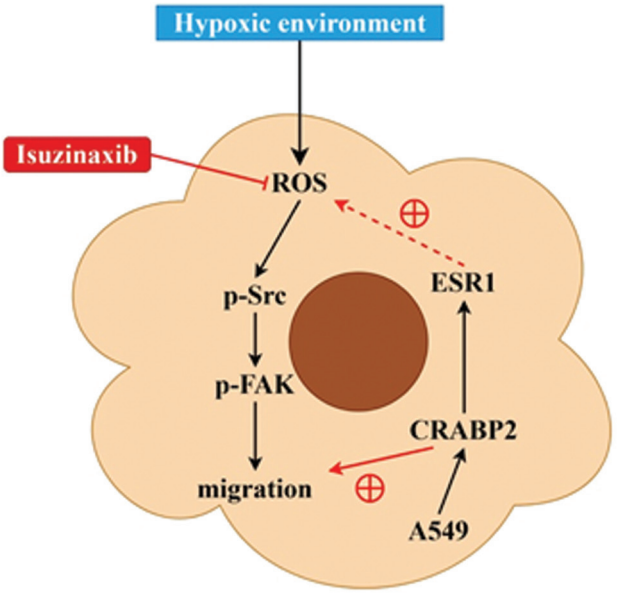
The ROS/Src pathway initiation typically results from cellular oxidative stress reactions, characterized by an excess of reactive oxygen molecules (ROS). ROS, in turn, triggers a cascade of reactions culminating in the activation of the Src protein. This activation plays a critical role in propelling cellular processes such as proliferation, migration, and invasion. Notably, ESR1 contributes to the activation of the ROS/Src pathway, leading to the upregulation of downstream Src.[28] Furthermore, CRABP2 indirectly stimulates the ROS/Src pathway, initiating downstream Src activation FAK phosphorylation and subsequently fostering lung cancer cell proliferation and migration (see Figure 5).

These findings underscore the crucial role of CRABP2 in promoting the proliferation and migration of lung cancer cells through the activation of the ROS/Src pathway. The study reveals the complex relationship between CRABP2, ESR1, and the pathway proteins, providing valuable insights into potential therapeutic targets for mitigating lung cancer progression.

Study Limitations

A few limitations should be acknowledged in interpreting the results of this study. The experimental focus was primarily on *in vitro* and mice models, limiting direct extrapolation to human clinical scenarios. Additionally, the study predominantly explored the role of CRABP2 without extensively considering other potential molecular factors influencing lung cancer progression. The sample size of the *in vivo* experiments was modest, and further studies with larger cohorts are warranted to validate and generalize the observed effects. Moreover, the study did not investigate potential variations in response among different subtypes of non-small cell lung cancer, which could provide a more nuanced understanding of CRABP2's impact. These limitations emphasize the need for cautious interpretation and warrant future investigations to enhance the validity and applicability of the findings.

Figure 5. Mechanistic Insights into CRABP2-Mediated Lung Cancer Progression



Note: Figure 5 illustrates the mechanism by which CRABP2 activates the ROS/Src pathway. This activation occurs by promoting ESR1 expression, leading to increased downstream protein expression. The resultant effect accelerates both the proliferation and migration of lung cancer cells.

CONCLUSION

In conclusion, our study reveals an important role for CRABP2 in regulating lung cancer proliferation and migration. Elevated expression of CRABP2 correlates with increased abilities of lung cancer cells to proliferate and migrate. Consequently, inhibiting the expression of CRABP2 or its downstream effector, ESR1, emerges as a potential strategy to limit the proliferation and migration of lung cancer. These findings present a promising avenue for novel therapeutic interventions in the clinical treatment of lung cancer. This study sheds light on lung cancer progression mechanisms by targeting CRABP2 or its associated pathways. The findings offer a prospective means to enhance the survival outcomes for individuals grappling with this challenging malignancy.

COMPETING INTERESTS
The authors report no conflict of interest.

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None.

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None.

AVAILABILITY OF DATA AND MATERIALS
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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