Research paper

APPBP2 enhances non-small cell lung cancer proliferation and invasiveness through regulating PPM1D and SPOP

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Abstract

Background: The influence of amyloid protein-binding protein 2 (APPBP2) on lung cancer is unknown.
Methods: The function and mechanisms of APPBP2 were investigated in the NSCLC cell lines A549 and H1299. The ectopic expression of APPBP2, PPM1D and SPOP in NSCLS were examined in samples collected from ten pairs of human lung adenocarcinoma cancer tissues and adjacent normal lung tissues. shRNA vector was used for APPBP2 knockdown. Quantitative PCR and western blot assays quantified the mRNA and protein level of APPBP2, PPM1D, and SPOP. Cell proliferation was measured with BrdU, MTT, colony formation assays, and xenograft tumour growth experiments. Cell migration and invasion were analysed with transwell and wound healing assays. Co-Immunoprecipitation assay detected protein–protein interactions.
Findings: APPBP2 was upregulated in NSCLC tissues. Silencing APPBP2 in A549 and H1299 cells resulted in the inhibition of cell proliferation, migration, and invasion, enhancement of apoptosis, and a significant decrease in the expression of PPM1D and SPOP. Overexpression of PPM1D and SPOP attenuated the APPBP2-knockdown inhibition of NSCLC cells. Co-IP assay showed that PPM1D interacted with APPBP2.
Interpretation: The expression level of APPBP2 positively correlates with NSCLC cell proliferation, migration, and invasiveness. APPBP2 contributes to NSCLC progression through regulating the PPM1D and SPOP signalling pathway. This novel molecular mechanism, underlying NSCLC oncogenesis, suggests APPBP2 is a potential target for diagnosis and therapeutic intervention in NSCLC.

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1. Introduction

Lung cancer is the most common cause of malignant tumours worldwide [1]. Of the different types of lung cancer, non-small cell lung cancer (NSCLC) accounts for over 80% of all lung cancer cases. The majority of NSCLC cases are diagnosed at later stages with local invasion or distal metastases, consequently leading to poor effectiveness of surgical or radiotherapeutic interventions [2]. Therefore, there is an urgent need for further understanding of the mechanism underlying NSCLC oncogenesis to support the development of novel therapeutic interventions.

Cancer is the uncontrolled growth of abnormal cells anywhere in the body. Proteins that regulate cell proliferation, apoptosis, and invasion are critically involved in the pathogenesis of cancers. Amyloid protein-binding protein 2 (APPBP2) interacts with microtubules and is functionally associated with beta-amyloid precursor protein transport and/or processing [3,4]. Studies have demonstrated that APPBP2 plays a key role in the oncogenesis of numerous types of cancer. For instance, Hirase et al. demonstrated that APPBP2 is closely associated with malignant phenotypes of ovarian adenocarcinomas [5]. In breast cancer, APPBP2 expression is significantly upregulated, prompting tumour cell

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APPBP2 interacts with microtubules and is functionally associated with beta-amyloid precursor protein (APP) transport and/or processing. Microtubules participate in the formation of the spindle during cell division (mitosis) responsible for cell proliferation. APP is a cell surface protein with signal-transducing properties and controls cells viability, proliferation, migration, and aggressiveness in various cancers. Based on the regulation of microtubules and APP, APPBP2 is found to be involved in the oncogenesis of various types of cancers, such as breast cancer, ovarian clear cell adenocarcinomas, desmoplastic medulloblastomas and neuroblastomas. However, the effects of APPBP2 on non-small cell lung cancer (NSCLC) remains unclear.

In this study, the investigators first demonstrate that APPBP2 expression is significantly enhanced in NSCLC tumours relative to tumour-adjacent normal tissues. Then the investigators provide evidence that APPBP2 controls NSCLC cell proliferation, apoptosis, migration, and invasiveness. Moreover, the investigators discovered that PPM1D and SPOP participate in the molecular mechanism underlying the roles of APPBP2 in NSCLC. Taken together, these findings suggest that APPBP2 contributes to NSCLC progression through regulating the PPM1D and SPOP signalling pathways.

After fixing with 4% formalin and embedding in optimal cutting temperature compound (OCT), tissues were cut into 10 μm sections using a microtome. Subsequently, the slides were treated with 3% H2O2 to block the endogenous peroxidase activity and heated (80 °C) for 10 min in citrate buffer (10 mM; pH 6-0) for antigen retrieval. To reduce nonspecific binding, 10% BSA + 0.4% triton-x100 was applied for 1 h at room temperature (RT). Subsequently, the slides were incubated with primary antibodies (anti-APPBP2, Sigma, RRID: ab151305, 1:500; anti-PPM1D, Sigma, RRID: ab31270, 1:500; anti-SPOP, Abcam, RRID: ab168619, 1:500) at 4 °C overnight, followed by incubating with appropriate secondary antibodies. After that, DAPI (abcam, RRID: b104139) was applied for nucleus staining for 10 min at RT.

2.2. Patients and tissue collection

Between September 2017 and October 2018, ten pairs of lung adenocarcinoma and adjacent normal lung tissues were collected at the First Affiliated Hospital of Bengbu Medical College (Anhui province, China) from patients who underwent surgical resection of lung cancer. The protocol of sample collection was approved by the Ethics Committee of the First Affiliated Hospital of Bengbu Medical College (Bengbu, China). All patients provided informed consent for use of their samples in this study.

2.3. Immunohistochemistry

Targeted therapies show great promise in effectively treating lung cancer patients. Therefore, characterizing and targeting the functionally-relevant molecular aberrations in lung cancer helps to identify new approaches to manage this disease. This research suggests that APPBP2 has a close relationship with NSCLC and contributes to the initiation and progression of NSCLC through regulating the PPM1D and SPOP pathways. Although the implications of APPBP2 in other cancers has been reported, we are the first to clarify the role of APPBP2 in NSCLC and the underlying molecular mechanisms. Therefore, this study provides a novel molecular mechanism underlying the oncogenesis of NSCLC and supports APPBP2 as a potential valuable molecular target suitable for diagnosis and therapeutic intervention in NSCLC.

Research in context

Evidence before this study

APPBP2 interacts with microtubules and is functionally associated with beta-amyloid precursor protein (APP) transport and/or processing. Microtubules participate in the formation of the spindle during cell division (mitosis) responsible for cell proliferation. APP is a cell surface protein with signal-transducing properties and controls cells viability, proliferation, migration, and aggressiveness in various cancers. Based on the regulation of microtubules and APP, APPBP2 is found to be involved in the oncogenesis of various types of cancers, such as breast cancer, ovarian clear cell adenocarcinomas, desmoplastic medulloblastomas and neuroblastomas. However, the effects of APPBP2 on non-small cell lung cancer (NSCLC) remains unclear.

Added value of this study

In this study, the investigators first demonstrate that APPBP2 expression is significantly enhanced in NSCLC tumours relative to tumour-adjacent normal tissues. Then the investigators provide evidence that APPBP2 controls NSCLC cell proliferation, apoptosis, migration, and invasiveness. Moreover, the investigators discovered that PPM1D and SPOP participate in the molecular mechanism underlying the roles of APPBP2 in NSCLC. Taken together, these findings suggest that APPBP2 contributes to NSCLC progression through regulating the PPM1D and SPOP signalling pathways.

Implications of all the available evidence

Targeted therapies show great promise in effectively treating lung cancer patients. Therefore, characterizing and targeting the functionally-relevant molecular aberrations in lung cancer helps to identify new approaches to manage this disease. This research suggests that APPBP2 has a close relationship with NSCLC and contributes to the initiation and progression of NSCLC through regulating the PPM1D and SPOP pathways. Although the implications of APPBP2 in other cancers has been reported, we are the first to clarify the role of APPBP2 in NSCLC and the underlying molecular mechanisms. Therefore, this study provides a novel molecular mechanism underlying the oncogenesis of NSCLC and supports APPBP2 as a potential valuable molecular target suitable for diagnosis and therapeutic intervention in NSCLC.

investigators provide evidence for the interaction of APPBP2 with PPM1D. In short, this study's findings indicate that APPBP2 facilitates the oncogenesis of NSCLC through modulating the PPM1D and SPOP signalling pathway.

2. Materials and methods

2.1. Cell culture and transfection

The immortalized lung adenocarcinoma cell line A549 (RRID: CVCL_0023), non-small cell lung cancer cell line H1299 (RRID: CVCL_0060), and human embryonic cell line HEK293T were purchased from the cell line bank of Bloon (Shanghai, China). Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS, HyClone), 10 mg/ml streptomycin and 10u/ml penicillin. The cell lines were incubated at 37 °C in a 5% CO2 atmosphere.

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2.3.1. Extraction of total RNA, reverse transcription, and real-time polymerase chain reaction analysis

Total RNA was extracted from the cell lines and the paired tissue from ten patients with lung adenocarcinoma using Trizol reagent (Takara, 9108). Quality of the total RNA was determined using RNA gel electrophoresis and NanoDrop. Then, a total of 1 μg of high-quality total RNA were directly processed for synthesizing cDNA using PrimeScript™ RT reagent Kit (Takara, RR047A). The real-time polymerase chain reaction analyses were performed in a 20 μl volume system using SYBR (Takara, RR2010, 10 μl) according to the manufacturer's instruction as follows: Primer mix (0-5 μl, 10 μM/each), template cDNA (1 μl), and sterile water (8-5 μl). The RT-PCR primers for APPBP2, PPM1D, SPOP, and GAPDH were summarized in Supplementary table 1.

2.4. Western blot

Total protein from the human lung adenocarcinoma cancer samples and NSCLC cells were prepared with ice-cold radioimmunoprecipitation assay buffer, quantified using BCA protein assay reagent (Beyotime, P0009, China), and separated by SDS-PAGE. Following electrophoresis, proteins in the gel were transferred to polyvinylidene difluoride membranes (Merck Millipore). After blocking for 1 h with 5% non-fat milk, the membranes were incubated overnight at 4 °C.
with antibodies (anti-APPBP2, Sigma, RRID: ab151305, 1:2000; anti-PPM1D, Sigma, ab31270, 1:2000; anti-SPOP, Abcam, RRID: ab168619, 1:2000), antibody against GAPDH (abcam, RRID: ab181602, 1:5000), or antibody against β-actin (abcam, RRID: ab8226, 1:5000) which was set as the internal standard. This was followed by incubation with a horseradish peroxidase-labelled goat anti-rabbit IgG secondary antibody (abcam, RRID: ab205718, 1:5000) or horseradish peroxidase-labelled goat anti-mouse IgG secondary antibody (abcam, RRID: ab6728, 1:5000) at room temperature for 1 h. Signals were detected with ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences) and visualized using a Chemical Gel Documentation System (Tanon).

2.5. Gene silencing and overexpression

The knockdown of human APPBP2 was performed by shRNA [9]. The investigators set sh-NC and sh-APPBP2 as the control and experimental groups, respectively. The target sequences of sh-NC and sh-APPBP2 are presented in supplementary table 1. sh-NC and sh-APPBP2 were cloned into pLKO.1 (Addgene, 8453) plasmid, respectively. All the plasmids were sequenced. The investigators transfected shRNA-pLKO.1 with helper plasmids (VSVG and delta8-9) into HEK293T cells for 72 h to package lentivirus. The titer of the virus was measured by examining the infectious ability of HEK293T cells. A549 and H1299 cells were infected with the sh-NC and sh-APPBP2 viruses. The investigators screened the stable transfected cell lines with 2 μg/ml puromycin, then RT-PCR and western blotting were applied for biological studies.

For overexpression purposes, human APPBP2, PPM1D, and SPOP CDS were cloned into pCDNA 3·1, which was delivered into the cell lines by lipo-3000. The cells were cultured for 48 h and then examined. For MTT in A549 (or H1299), a modified MTT assay was used in our experiments: The stable cells of sh-APPBP2 infected cells and sh-APPBP2 infected cells were cultured into 12-well plates. When the cells were attached to the wall, the investigators added BrdU to each well with a final concentration of 10 μM and cultured these for another 8 h. They then replaced the BrdU containing medium with normal medium (10% FBS in DMEM) and further cultured for 24 h. The cells were then disassociated and seeded for colony formation in 6-well plates at the appropriate seeding density of 1 × 104 cells/well. While confluent, the cell monolayer was mechanically scarred with a sterile 200 μl pipette tip and incubation was continued for an additional 12 h and 24 h. Images were taken at 0, 12, and 24 h after scarring.

2.9. Wound healing assay

Wound healing assay was performed according to a previously described method with some modifications [11]. A549 (or H1299) stable cells were transplanted into 6-well plates at the appropriate seeding density of 1 × 105 cells/well. After incubation at 37 °C under 5% CO2 for 6 to 10 h, some of the cells had migrated through the porous membrane, whereas the non-migrating cells remained on the upper surface of the filter. The chamber was then immersed in methanol for 10 min to fix the cells, and then scrubbed gently to remove the non-metastatic cells. The migrated cells were stained with 0·5% crystal violet for 10 min and counted under a light microscope. Each experiment was independently performed three times.

2.10. Transwell assay

In vitro invasion capability was measured using 24-well chambers (Millipore, 8 μm, USA) pre-coated with Matrigel (BD, 256234) on the polyethylene terephthalate membrane. In the upper compartment of the transwell chambers, the investigators seeded 5 × 104 stable cells suspended in 500 μl of serum-free medium and filled the lower compartment with complete medium. After incubation at 37 °C under 5% CO2 for 6 to 10 h, some of the cells had migrated through the porous membrane, whereas the non-migrating cells remained on the upper surface of the filter. The channel was then immersed in methanol for 10 min to fix the cells, and then scrubbed gently to remove the non-metastatic cells. The migrated cells were stained with 0·5% crystal violet for 10 min and counted under a light microscope. Each experiment was independently performed three times.

2.11. Establishment of an adenocarcinoma xenograft mouse model

All animal experiments were conducted according to the institutional ethics and safety guidelines (Institutional Animal Welfare and Ethics Committee, First Affiliated Hospital of Bengbu medical college, Anhui, China). In total, forty 8-week-old BALB/c-Foxn1null mice were used in the experiments (Model Animal Research Centre of Nanjing University, Nanjing, China). The mice were randomly divided into four groups. APPBP2 silenced and control xenograft model sets from A549 (or H1299) were established using 5 × 106 cells injected into the right flanks of the subcutaneous tissue of the mice. Tumour length, width and tumour weight were measured every three to four days until the experiment ended. The tumour volume was calculated based on the conventional formula: V = (L × W2)/2, where L and W are the biggest and smallest diameters of the tumour.

2.12. Coimmunoprecipitation (co-IP)

To conduct the experiments, four additional plasmids were constructed. Flag-APPBP2, His, and His-PPM1D were cloned into pCDNA 3·1, respectively. For APPBP2 co-IP in A549 (or H1299) cells,
the cells transformed by lipo-3000, expressing Flag-APPBP2, PPM1D, and SPOP, were cultured for 72 h. Meanwhile, the transformed cells expressing Flag, APPBP2, PPM1D, and SPOP were set as the negative control group. For PPM1D co-IP in the A549 (or H1299) cells, the transformed cells expressing His-APPBP2, PPM1D, and SPOP were cultured for 72 h. The transformed cells expressing His, APPBP2, PPM1D, and SPOP were set as the control group. The cells were lysed for co-IP and were incubated with beads (pierce, 6149) which covalently pre-couple Flag antibody (abcam, ab205606) or His antibody (abcam, ab18184). Some cell lysis, which was incubated with the beads covalently pre-coupled with human IgG, was set as another negative control. The protein binding complex was isolated by centrifuging. The precipitates were diluted with SDS sample buffer, separated on a 10% SDS-PAGE gel and subjected to immunoblotting with the corresponding antibodies.

2.13. Reanalyzing the TCGA database

The investigators explored the mRNA level of APPBP2 in The Cancer Genome Atlas Lung Adenocarcinoma (TCGA-LUAD) cohort. The RNA Seq V2 RSEM data of TCGA-LUAD was obtained using TCGAbiolinks (https://github.com/BioinformaticsFMRP/TCGAbiolinks, R version 3.5). The investigators identified candidate genes which may correlate with the APPBP2 mRNA level using cBioPortal tool [12,13].

2.14. Statistical analysis

Data are presented as mean ± SEM and two-tailed t-test was used to determine statistical significance difference between two groups (except Supplementary Fig. s3b, which adopted one-tailed r-test). One-way ANOVA followed by a Tukey’s Multiple Comparison test was applied for the comparison among groups in the same graph. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. The elevated expression of APPBP2 in human NSCLC

To explore the correlation of APPBP2 with NSCLC, we compared the microarray-based expression data of APPBP2 between the paired normal and tumour tissues in two NSCLC cohorts from Spain (GEO accession: GSE18842) [14] and Taiwan (GEO accession: GSE19804) [15]. In both cohorts, APPBP2 was significantly up-regulated in the tumour tissues compared with the paired normal tissues (paired t-test: p < 0.0001 for the Spain cohort and p = 0.0013 for the Taiwan cohort) (Fig. 1a). In addition, primary cancers and the paired normal tumour-adjacent lung tissues were collected from ten NSCLC patients and subsequently subjected to immunohistochemical and quantitative real-time PCR (qPCR) analysis. As expected, an elevated expression of APPBP2 was observed in NSCLC tumours relative to normal lung tissues (Fig. 1b–e).

Fig. 1. The expression of APPBP2 was elevated in human NSCLC tissues. (a) The APPBP2 expression profile of the NSCLC tissues and the adjacent normal tissues from the GEO database. The left and right panels represent samples from Spain and Taiwan, respectively. (b,c) Immunostaining of APPBP2 of the NSCLC tumour tissues (lower) and the matched normal tissues (upper) from our hospital. Blue signals represent DAPI and brown signals represent APPBP2. (d) Image grey integral optical density (IOD) from immunohistochemistry results. Pink colour and blue colour represent normal tissues and NSCLC tissues, respectively. (e) The APPBP2 mRNA expression levels of ten paired LUAD patients were measured by RT-PCR. Pink colour and blue colour represent normal tissues and LUAD tumour tissues, respectively. Data are presented as mean ± SEM. (Two-tailed t-test for group comparison): ***p < 0.001, **p < 0.01, *p < 0.05 for indicated comparison. Scale bar: 50 μm.
3.2. APPBP2-knockdown suppresses cells proliferation, but induces apoptosis in NSCLC in vitro

As unlimited growth is one of the key hallmarks of cancers [16], we attempted to assess the influence of APPBP2 on cell proliferation and apoptosis in NSCLC. First, lentivirus mediated sh-APPBP2 was applied to silence the APPBP2 expression in both A549 and H1299 cells, with a scrambled shRNA (sh-NC) as the negative control (Fig. 2a). Following the transfection of cells, qPCR and western blotting assays were performed to examine the endogenous APPBP2 expression. The results showed that the expression levels of APPBP2 dropped by about 70% (Fig. S1b–d).

Then BrdU, MTT, colony formation and flow cytometry assays were performed on the stable transfected NSCLC cell lines to detect the proliferation and/or apoptosis. BrdU assays indicated that the silencing of APPBP2 significantly suppressed the proliferation of both A549 (sh-NC: 0.65 ± 0.07, n = 3; sh-APPBP2: 0.28 ± 0.03, n = 3; Fig. 2a,b,e) and H1299 (sh-NC: 0.66 ± 0.06, n = 3; sh-APPBP2: 0.41 ± 0.03, n = 3; Fig. 2c,d,e) cells. This was consistent with the results from MTT (Fig. 2f) and colony formation assays (Fig. 2g–i). Flow cytometry assays revealed that cell apoptosis was dramatically enhanced in two cell lines transfected with sh-APPBP2 compared with sh-NC (Fig. 2j) and Fig. S2a, b), but no significant differences were observed on cell cycles (Fig. 2k and Fig. S2c, d).

3.3. Silencing APPBP2 reduces NSCLC growth in vivo

Xenografts are models of cancer where the tissue or cell lines are implanted into an immunodeficient or humanized mouse, which are used to create an environment that allows for the natural growth of cancer. To assess the action of APPBP2 on NSCLC growth in vivo, we constructed xenograft tumours of A549 and H1299 cells transfected with sh-APPBP2 and sh-NC respectively, followed by the investigation of tumour growth. Interestingly, we found that silencing APPBP2 reduced both the size (Fig. 2m, p) and weight (Fig. 2n, q) of xenografts.

3.4. APPBP2 silencing inhibits cell migration and invasiveness in NSCLC

Metastasis is the leading cause of NSCLC-related mortality, with about 90% of NSCLC patients succumbing to metastasis as opposed to their primary tumours [17]. To elucidate the molecular mechanism(s) underlying NSCLC metastasis, wound healing and transwell assays were performed to assess the roles of APPBP2 in the migration and invasiveness of NSCLC cells. Transwell assays showed that the invasiveness of both A549 and H1299 cells was substantially suppressed by APPBP2 silencing (Fig. 3a–d). Similarly, wound healing assays suggested that the APPBP2 knockdown obviously impaired the migratory activity in both cell lines (Fig. 3e, f).

3.5. APPBP2-knockdown reduced the expression of PPM1D and SPOP in NSCLC

To clarify the molecular mechanism underlying the action of APPBP2 in NSCLC, the investigators analysed a heatmap of potential correlations which synchronized well with APPBP2 expression in human NSCLC samples from the TCGA cohort (TCGA-LUAD). The heatmap revealed several cancerous genes (Fig. 4a), among which the expression of PPM1D and SPOP may be highly correlated with the APPBP2 levels in the TCGA-LUAD cohort (spearman ρ = 0.65 for PPM1D and 0.54 for SPOP; Fig. 4bc). The investigators performed western blotting assays to explore the correlations of APPBP2 with PPM1D and SPOP in ten paired NSCLC tissues and found that three proteins of APPBP2, PPM1D, and SPOP were all enhanced in tumours relative to normal tissues (Fig. S3a,b). This was consistent with the results from the immunohistochemistry and qPCR assays (Fig. 4d–f). Moreover, the investigators found that APPBP2-knockdown significantly reduced the expression of PPM1D and SPOP in both A549 and H1299 cells, as determined by qPCR and western-blot assays (Fig. 4g, h).

3.6. Interaction of APPBP2 with PPM1D and SPOP in NSCLC

Co-immunoprecipitation (co-IP) is a popular technique to identify physiologically relevant protein–protein interactions by using target protein-specific antibodies to indirectly capture proteins that are bound to a specific target protein. To assess the protein interactions among APPBP2, PPM1D, and SPOP, co-IP experiments were performed on NSCLC cells using antibodies anti-Flag (fusion with APPBP2) as well as anti-His (fusion with PPM1D) and revealed that APPBP2 could bind with PPM1D (but not SPOP) in both A549 and H1299 cell contexts (Fig. 4i, j).

3.6.1. Overexpression of PPM1D and SPOP attenuate APPBP2-knockdown inhibition of cell proliferation and invasiveness in NSCLC

To evaluate the mediation of PPM1D and SPOP on the effect of APPBP2 on NSCLC, plasmids containing PPM1D and SPOP expression cassette were transfected into APPBP2-silenced A549 and H1299 cells to enhance the expression of PPM1D and SPOP. This was followed by the examination of cell proliferation, apoptosis, migration, and invasiveness using colony formation, MTT, flow cytometry and transwell assays. APPBP2-knockdown inhibition of biological behaviours in NSCLC cells was strongly attenuated by the overexpression of PPM1D and SPOP. (Figs. 5, 6 and Fig. S3c–e).

4. Discussion

Lung cancer remains the leading cause of cancer related deaths worldwide and targeted therapies are showing great promise in effectively treating certain patients [18,19]. Therefore, new targeted molecular therapeutic strategies for lung cancer are needed to aid patients with this disease. Characterizing and targeting the functionally-relevant molecular aberrations in lung cancer helps to identify new approaches to managing this disease. The investigators report on the oncogenic role of APPBP2 in NSCLC.

APPBP2 interacts with microtubules and is functionally associated with beta-amyloid precursor protein (APP) transport and/or processing [3]. Microtubules participate in the formation of the spindle during cell division (mitosis) responsible for cell proliferation. APP is a membrane protein expressed in many tissues and has been implicated as a regulator of synapse formation [20], neural plasticity [21], and iron export [22]. Interestingly, studies have suggested that APP controls cells viability, proliferation, migration, and aggressiveness in numerous types of cancers [23–26]. Based on these findings, we hypothesized that
APPBP2 could participate in the oncogenesis of cancers through modulating microtubules and/or APP. Actually, the oncogenic roles of APPBP2 in numerous types of cancer has been reported [5–8,27]. However, the roles of APPBP2 in NSCLC are still poorly understood.

Molecular biology studies have demonstrated that cancers are a complexity of multi-stage biological process with the involvement of various factors. This includes the activation of oncogene and the inactivation of tumour suppressor gene, leading to functionally relevant molecular aberrations. In the current study, the investigators compared the expression of APPBP2 mRNA between paired tumour and the adjacent normal tissues in two NSCLC cohorts, discovering a significantly elevated expression of APPBP2 in tumours relative to normal tissues. This result was confirmed in the samples of NSCLC and paired adjacent normal tissues. Similar to our findings in NSCLC, overexpression/amplification of the APPBP2 gene has been reported in other cancers, such as breast cancer [27], ovarian clear cell adenocarcinomas [5], desmoplastic medulloblastomas [7], and neuroblastomas [8]. Taken together, these findings indicate that APPBP2 correlates with the oncogenesis of cancers, including NSCLC.

Uncontrolled proliferation and invasiveness are the key hallmarks of cancer cells and are the leading cause of NSCLC-related mortality. Therefore, the investigators undertook this study to shed light on the association of APPBP2 with the malignant phenotype of NSCLC cells. First, the investigators demonstrated that APPBP2 silencing suppressed cell proliferation and viability while inducing apoptosis in NSCLC cells. Next, the investigators confirmed that knockdown of APPBP2 inhibited
Fig. 4. APPBP2 regulated the expression of PPM1D and SPOP in NSCLC tissues and cells. (a) The heatmap of gene expression profiling organized by APPBP2 expression level by reanalysing the TCGA data. Samples were derived from human LUAD patients. Red circles indicate the genes to be studied. (b,c) PPM1D mRNA level (b) and SPOP mRNA level (c) had a positive linear relationship with the expression of APPBP2. (d) Immunostaining of PPM1D (brown colour) of human LUAD tissues and adjacent normal tissues. DAPI in (d) is indicated as blue colour. (e) Immunostaining of SPOP (brown colour) of human LUAD tissues and adjacent normal tissues. DAPI in (e) is indicated as blue colour. (f) PPM1D mRNA and SPOP mRNA expression level of human LUAD tissues and adjacent normal tissues were measured by RT-PCR. Yellow colour and cyan colour represent normal and tumour samples, respectively. n = 3. (g) RT-PCR results showed PPM1D mRNA and SPOP mRNA expression level in APPBP2 silenced cells of A549 or H1299 (n = 3). (h) PPM1D and SPOP were examined by western blotting on APPBP2 silenced A549 and H1299 stable cells. GAPDH acts as the internal standard. (i) co-IP between the APPBP2-flag and PPM1D or SPOP on A549 or H1299 cell contexts. (j) co-IP between PPM1D-His and APPBP2 or SPOP on A549 or H1299 cell contexts. Data are presented as mean ± SEM. (Two-tailed t-test for group comparison) 0.05 < “p < 0.01, **p < 0.01, ***p < 0.001 for indicated comparison. Scale bars: 50 μm.
Fig. 5. PPM1D and SPOP attenuated the inhibition of cell proliferation induced by APPBP2 knockdown in NSCLC cells. (a) Ectopic expression of PPM1D or SPOP promoted colon formation on APPBP2 silenced A549 cells. Control was set as a blank group without protein overexpression. (b) The statistical results of colony formation indicate the impact of PPM1D and SPOP on NSCLC stable cells. A one-way ANOVA revealed a significant effect of group (F[4,10] = 324.5, p < 0.001 in A549 and F[4,10] = 63.45, p < 0.001 in H1299). (c) The proliferation effects of PPM1D or SPOP expression on A549 stable cells was measured by MTT assay. A one-way ANOVA revealed a significant effect of group (F[6,14] = 106.2, p < 0.001). (d) The proliferation effects of PPM1D or SPOP expression on H1299 stable cells was measured by MTT assay. A one-way ANOVA revealed a significant effect of group (F[6,14] = 393.9, p < 0.001). (e) PPM1D and SPOP had a significant impact on cell apoptosis of A549 stable cells by Annexin V and flow cytometric analysis. A one-way ANOVA revealed a significant effect of group (F[6,14] = 338.4, p < 0.001). (f) PPM1D and SPOP had a significant impact on cell apoptosis of H1299 stable cells by Annexin V and flow cytometric analysis. A one-way ANOVA revealed a significant effect of group (F[6,14] = 310.5, p < 0.001). The addition or non-addition of plasmid or virus is present as “+” and “−”, respectively. Data are presented as mean ± SEM. The difference between means was compared by Tukey’s Multiple Comparison test. *p < 0.05 and ***p < 0.001 for indicated comparison. n.s. refers as not significant.
APPBP2 contains a tetratricopeptide repeat (TPR) structural motif that frequently mediates protein-protein interactions \[28,29\], so the binding of APPBP2 with PPM1D is potentially mediated by the TPR structural motif. Since APPBP2 (PAT1) phosphorylates Mei2 and Ste11 in the controls of sex differentiation \[30\] and functions as a positive regulator of Kinesin motility, resulting in cargo transport \[31\], the modulation of PPM1D by APPBP2 in NSCLC is probably through phosphorylation and/or translocation.

Previous studies have proved the oncogenic roles of PPM1D in numerous types of cancers \[5,6,8,32\], mostly in a p38MAPK/p53/p16 pathway dependent manner \[33,34\]. Notably, H1299 cells have a homozygous partial deletion of the TP53 gene \[35\], suggesting the existence of a P53-independent manner of PPM1D affecting NSCLC cells. Consistently, a P53-independent manner of PPM1D action has been reported in clonal haematopoiesis and myelodysplastic syndrome \[36,37\].

Our findings suggest that SPOP is also a key molecule responsible for the effects of APPBP2 on NSCLC. Although SPOP mutations were identified in numerous types of tumours, especially in prostatic carcinoma \[38\], no SPOP mutations were observed in the analysis of human NSCLC samples from the TCGA cohort in the current study (Fig. 4c). Consistent with our findings, earlier study revealed that somatic mutation of SPOP is rare in lung cancer \[39\]. Interestingly, we found the elevated expression of SPOP in NSCLC (Fig. 4e,f and Fig. S3a,b). APPBP2 promotes a cytoplastmic retention of androgen receptor (AR) \[40\], leading to the dysfunction of AR upregulating the of CDK4 expression in prostatic cancer \[41\]. CDK4 induces the phosphorylation as well as the degradation of SPOP in multiple cancers \[38,42\]. In renal cell carcinoma, SPOP can be activated by HIF and SPOP has very important oncogenic role suppressing PTEN and other genes, as well as activating AKT and ERK \[43\]. Consistently, researchers also found that SPOP, as a strong oncogenic protein, acted as a diagnostic biomarker in renal cell carcinoma \[44–46\]. Based on these reports, it is reasonable to hypothesize that APPBP2 enhances NSCLC proliferation and invasiveness partially through AR/CDK4/SPOP pathway (Fig. 7).

Collectively, APPBP2 is strongly associated with NSCLC and promotes the initiation and progression of tumours, which is, at least partly, mediated by PPM1D as well as SPOP. Our work provides a novel molecular mechanism underlying the oncogenesis of NSCLC and supports APPBP2 as a potential valuable marker suitable for diagnosis and therapeutic intervention in NSCLC.

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**Declarations of interest**

All co-authors agree with the content of this manuscript and report no conflicts of interest related to this study.

**Author contributions**

Conceived and designed the study: X.W., X.G.L., H.G. and F.L.; Performed the experimental procedures: All authors; Analysed the data: F.L. and X.W.; Drafted the Manuscript: F.L. and H.G.; Revised the Manuscript: X.W.; Financial support: X.W.

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**Fig. 6.** PPM1D and SPOP attenuated the inhibition of cell migration and invasiveness induced by APPBP2 knockdown in NSCLC cells. (a) Effects of PPM1D and SPOP on cell migration and invasion of A549 stable cells by transwell, n = 3. (b) Effects of PPM1D and SPOP on cell migration and invasion of H1299 stable cells by transwell, n = 3. (c) The statistical results of PPM1D and SPOP on cell migration and invasion of A549 stable cells by transwell. A one-way ANOVA revealed a significant effect of group \(F(6,14) = 35.7, p < 0.001\). (d) The statistical results of PPM1D and SPOP on cell migration and invasiveness of H1299 stable cells by transwell. A one-way ANOVA revealed a significant effect of group \(F(6,14) = 72.58, p < 0.001\). The addition or non-addition of plasmid or virus is present as “+” and “−”, respectively. Data are presented as mean ± SEM. The difference between means was compared by Tukey’s Multiple Comparison test. 0.001 < **p < 0.01 and ***p < 0.001 for indicated comparison. n.s. refers as not significant. Scale bars: 50 µm.

**Fig. 7.** Graphical signalling pathway of the effect of APPBP2 on NSCLC via PPM1D and SPOP. The blue lines indicate the activation of the targets according to our experiments or published researches. The red lines indicate the possible inhibition of targets according to our reasonable speculation. AR: androgen receptor.
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.05.028.

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