# AMPD2 as a novel regulator of ferroptosis

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## Abstract

# Purpose

Ferroptosis is a newly discovered programmed cell death, but its molecular mechanism remains largely unknown. AMPD2 is a protein, which related to oxygen metabolism in non-small cell lung cancer (NSCLC). In this study, we aimed to clarify whether AMPD2 is a regulatory factor of ferroptosis, which may provide a new theoretical basis for inducing ferroptosis to treat tumors.

# Methods

we constructed AMPD2 overexpression and knockdown cells lines using lentivirus packaging technique. Immunohistochemistry was used to detect the expression of AMPD2 in cancerous tissue and adjacent normal tissue. DCFH-DA probe was used to detect the changes of reactive oxygen species(ROS). MDA assay was used to detect the changes of lipid peroxidation. CCK-8 assay was used to detect cell viability. Correlations of AMPD2 with Ferroptosis-related signaling pathways were analyzed using GEPIA. Western blot assay was used to detect changes in Ferroptosis-related molecular markers and signaling pathway.

# Results

AMPD2 is higher expressed in NSCLC patient's tissues. The Overexpression of AMPD2 significantly reduces intracellular ROS and MDA in NSCLC cells, inhibits ferroptosis induced by RSL3 (ferroptosis activator), enhances the expression of p38, p-p38 and GPX4, whereas knockdown of AMPD2 reverse the results. Moreover, treatment with AMP will increase ferroptosis sensitivity medicated by AMPD2.

# Conclusions

AMPD2 regulates cellular ferroptosis by modulating the level of AMP affecting the p38 pathway and GPX4.

## Introduction

Lung cancer is the most common malignancies and the leading cause of cancer related death in the world(Siegel, et al. 2021). Non-small-cell lung cancer (NSCLC) accounts for over 80% of all lung cancer cases(Sung, et al. 2021). Despite recent advances in the development of new therapies, the overall 5-year survival rate of NSCLC remains less than 20%(Siegel, et al. 2021; Sung, et al. 2021). The efficacy of current therapeutic therapies is crippled by inherent and acquired drug-resistance or severe off-target effects. Therefore, a better understanding of the underlying molecular mechanisms and development of innovative and effective therapeutic approaches are critically desired for NSCLC.

Ferroptosis is a form of regulated cell death that is driven by accumulation of lipid peroxidation and lethal reactive oxygen species(Chen, et al. 2021). Recently, ferroptosis is reported to mediate the occurrence and development of lung cancer(Zhang, et al. 2022; Wu, et al. 2021), which presents a new promising target for NSCLC. However, the regulatory networks underlying ferroptosis are still unclear. Thus, it is imperative to further investigate the regulatory mechanism of ferroptosis and its role in NSCLC.

AMPD2, an important enzyme, regulates the metabolism of adenosine monophosphate (AMP) to inosine monophosphate (IMP) and plays an important biological role in the purine nucleotide cycle(Gao, et al. 2020; Akizu, et al. 2013). AMPD2 was reported to have significant effects on the nephrotic syndrome, hypercholesterolemia(Helmering, et al. 2014), physiological and pathological processes(Kortum, et al. 2018). In addition, over expression of AMPD2 has been proven to be correlated with poor prognosis in caner(Gao, et al. 2020; Akizu, et al. 2013). Despite recent advances in understanding the biological function of AMPD2, the role of AMPD2 in NSCLC remains largely unknown.

In this study, the significance of AMPD2 in NSCLC was investigated with samples of both tumor and normal. Our findings demonstrate that the expression levels of AMPD2 are elevated in NSCLC. AMPD2 might be a regulator of ferroptosis, which provides a new biomarker for poor NSCLC prognosis and a promising therapeutic strategy for the lethal NSCLC.

# Materials And Methods

### Chemicals

The chemicals dissolved in dimethylsulfoxide (DMSO) were: Neflamapimod (synonyms: VX745, Code: HY-10328, MedChemExpress, Monmouth Junction, NJ, USA), adenosine monophosphate (Synonyms: AMP, Code: HY-A0181, MedChemExpress, Monmouth Junction, NJ, USA), RSL3 (Code: S8155, Selleck Chemicals, Houston, TX, USA) and Lipeoxstatin-1 (Code: S7699, Selleck Chemicals, Houston, TX, USA).

### Cell culture

Human lung adenocarcinoma cell lines (A549 and H1299) were purchased from the American Type Culture Collection (Manassas, VA, USA). The cell lines were verified by STR profiling before distribution. The cell lines were grown in RPMI 1640 medium (ThermoFisher Scientific ,Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco, Shanghai, China) in a humidified 5% $CO_2$ atmosphere at 37 °C.

### Plasmids

For knockdown of AMPD2, target shRNA sequences were subcloned into pLKO.1-puro (AgeI/EcoRI) shRNA Lentivector (GENERAY). shAMPD2-1: 5’-GCACGUCUAUGGAUGGCAATTUUGCCAUCCAUAGACGUGCTT-3’,Sh-AMPD2-3:5’-CCAUCGCUUUGACAAGUUUTTAAACUUGUCAAAGCGAUGGTT- 3’. **Cell viability assay**

The cell viability tests were analyzed by the standard cell counting kit-8 (CCK-8) assay method. CCK-8 (K1018) was purchased from APEXBIO (Houston, TX, USA). A549 and H1299 cells were seeded into the 96-well plate with a density of $4\times10^4$ cells/well. After 12 h, the medium was replaced with 100 μL of fresh medium and incubated overnight. Then, 10 μL CCK-8 solution was added to each well and the cells were subsequently incubated at 37℃ for 1 h. Absorbance was measured at 450 nm using the microplate reader (Thermo, USA).

### Western blotting

All cells were washed with PBS and the protein were extracted using RIPA Lysis buffer (Thermo, USA). The protein concentration was quantified with BCA Protein Assay Kit (Beyotime, China). The protein was separated by SDS-PAGE and then transferred to NC membranes. The protein levels were analyzed via western blotting using the corresponding antibodies and were normalized to GAPDH expression. The antibody to AMPD2 (ab137598), Glutathione Peroxidase 4 (ab125066) were obtained from Abcam (Cambridge, MA, USA). The antibody to phospho-p38 MAPK (4511) was purchased from CST (Danvers, MA, USA). The antibody to GAPDH (10494-1-AP) and p38MAPK (14064-1-AP) were obtained from proteintech (Wuhan, China).

### RNA isolation and quantitative real-time PCR (RT-qPCR)

Total cellular RNA was isolated from cells using Trizol reagent (TaKaRa Bio, Dalian, China) and then reversely transcribed into cDNA using PrimeScriptRT Reagent Kit (TaKaRa Bio, Dalian, China) according to the manufacturer's instructions. Real-time PCR was performed using QuantiTect SYBR Green PCRkit (TaKaRa Bio, Dalian, China) in Stratagene MX3000P (Agilent Technologies, Santa Clara, CA, USA). The sequences of the primers were as follows: GAPDH, forward 5'-GCACCGTCAAGGCTGAGAAC-3', reverse, 5'-ATGGTGGTGAAGACGCCA GT-3', AMPD2, forward, 5'-ATGATCCCTTGCAGTTCACTT-3', reverse, 5'-GGCTCTTTACCTTGTGCGAGAA-3'. The expression level was normalized to the internal control and determined by a 2-ΔΔCT method.

### MDA kit

The MDA amount can reflect the degree of lipid peroxidation in the body, which indirectly indicates the extent of cell damage. We detected the MDA content according to manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The absorbance at 532 nm was detected by a microplate reader (Thermo, USA).

### ROS Detection

A DCFH-DA probe Reactive Oxygen Species Assay Kit (Beyotime, China) was used to determine intracellular ROS levels according to the manufacturer's instructions. Briefly, A549 and H1299 cells were attached to 6-well plates. Then, DCFH-DA in serum-free RPMI 1640 medium was loaded with 10 μM at

37℃ for 40 min. After washing three times with serum-free medium, the fluorescence was recorded with a confocal scanning laser microscope (DP72, Olympus, Japan) and quantified with Image J® software.

### Tissue microarray and immunohistochemistry

NSCLC tissue microarrays (TMA), including 30 paired cancerous tissue and adjacent normal tissue were purchased from Shanghai Outdo Biotech (Shanghai, China). TMA were deparaffinized in xylene and rehydrated in series of ethanol solutions and then boiled in a citrate buffer (pH 6.0) in a microwave oven for antigen retrieval and incubated in 3% hydrogen peroxide ($H_2O_2$) to deactivate potential endogenous peroxide activity. The sections were subsequently incubated with 5% normal goat serum and then with a rabbit polyclonal AMPD2 antibody overnight at 4 °C in a wet box. Next day, the sections were washed with PBS thrice and incubated with pv-9000 kit (ZSGB-Bio, Beijing, China) and then subjected to color development using 3,3-diaminobenzidine (ZSGB-Bio, Beijing, China) and counterstained with hematoxylin. The percentage (%) of positively stained cells was classified as 0 (0%–25%), 1 (26%–50%), 2 (51%–75%), or 3 (76%–100%), while the staining intensity was classified as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). We then plus these two scores to reach a staining index.

### Gene Correlation Analysis in GEPIA

Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/index.html), an online database was used to evaluate the correlation between AMPD2 expression and p38 MAPK(MAPK14) expression in LUAD, LUSC and paracancer tissues. GEPIA is a web server for analyzing the RNA sequencing expression data of 9736 tumors and 8587 normal samples from the TCGA and the GTEx projects, using a standard processing pipeline(Tang, et al. 2017).

### Statistical analysis

All statistical calculations were completed using GraphPad Prism (version 9.0). All results were presented as the mean±standard deviation (SD). The differences between the two groups were performed by the Student's t-test.

# Results

### 1. AMPD2 is commonly overexpressed in NSCLC tissues

In this study, the AMPD2 expressions in NSCLC and normal lung tissues were evaluated, the overexpression of AMPD2 was validated in 30 paired (One sample damaged) cancerous tissue and adjacent normal tissue (Fig.1a-b). the results showed that AMPD2 is statistically overexpressed in NSCLC tissues.

### 2. AMPD2 inhibits ferroptotic cell death in NSCLC

To determine the role of AMPD2 in ferroptosis, the stably overexpressed and knocked down AMPD2 in A549 and H1299 cell lines were constructed using lentivirus. The protein expression and mRNA levels of AMPD2 were examined by western blotting and RT-qPCR. As shown in Fig.2a-b, the protein expression levels of AMPD2 were significantly increased in AMPD2 overexpression group, but decreased in AMPD2 knockdown group, compared with the control group. The results of RT-qPCR further indicate the AMPD2 overexpression and knockdown A549 and H1299 cell lines were successfully constructed (Fig. 2c-d), which is consistent with the western blotting assay.

As reactive oxygen species (ROS) and lipid peroxidation are the driving factors and well-established indicators of ferroptosis, DCFH-DA probe and MDA assay kit were employed to explore the effect of AMPD2 on ferroptosis. Reduced cellular ROS production with low fluorescence intensity was observed both in AMPD2 overexpressed A549 and H1299 cell lines (Fig. 3a), whereas in the knockdown group, the results were reversed (Fig. 3b). What's more, MDA assay revealed that compared with that in control group, MDA contents were lower in AMPD2 overexpressed group (Fig.3c), and higher in the knockdown groups (Fig.3d). In addition, Liproxstatin -1(Lip-1), a specific inhibitor of ferroptosis(Fan, et al. 2021, Zhang, et al. 2021) could effectively inhibit the changes of ROS and MDA levels in stable knockdown AMPD2 cells (Fig.3b, d).

RSL3(Sui, et al. 2018, Yang, et al. 2021), a potent ferroptosis-triggering agent, which could promote ferroptosis by inhibition the activity of GPX4. To further identify whether AMPD2 inhibits ferroptosis in NSCLC, the AMPD2 overexpressed and knockdown cell lines were treated with RSL3 (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 μM) for 24 h, and the cell viability was assessed by the Cell Counting Kit-8 (CCK-8). As shown in Fig.4a, overexpression AMPD2 could rescue the ferroptotic cell death induced by RSL3 treatment, while knockdown AMPD2 made cells more sensitive to ferroptosis (Fig.4b). Our results demonstrated that AMPD2 is a key determinant in ferroptotic cell death, which might provide a promising strategy to for the treatment of NSCLC patients.

### 3. Phosphorylated p38 inhibits ferroptotic cell death in NSCLC

The p38 mitogen-activated protein kinase (MAPK) pathway is a vital regulator in cellular response to oxidative stress, which has been reported to be involved in ferroptosis (Hattori, et al. 2017, Lin, et al. 2022). The activation of phosphorylated p38 was reported to resist ferroptosis in Hs578t (breast cancer cells), MDA-MB-231S (breast cancer cells), SF295 (malignant glioma cells), and NCI-H1975 (NSCLC) (Brown, et al. 2021). To explore the effect of phosphorylated p38 on ferroptosis in NSCLC cell lines A549 and H1299, ROS and MDA were detected by DCFH-DA probe and MDA assay kit, respectively. We found that the levels of ROS (Fig.5a) and MDA (Fig.5b) were significantly increased compared to the control group (untreated group), after inhibition of p38 MAPK activity with VX-745 for 24 h. We then added equal amounts of RSL3 (1 μM) and different concentrations of VX-745 (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 μM) to A549 and H1299 wild-type cells for 24 h, and detected cell viability by CCK8. The results showed that the cell activity decreased with the increase of the concentration of the VX-745 (Fig.5c). These results suggest that phosphorylated p38 regulates ferroptosis in A549 and H1299.

### 4. AMPD2 can affect the p38 pathway and regulate Gpx4

To understand the mechanism by which AMPD2 negatively regulates ferroptosis, we first sought to determine whether AMPD2 is correlated with p38 MAPK using TCGA database provided by GEPIA website. As shown in Fig.6a, the expression of AMPD2 was positively correlated with that of p38 MAPK (P=0, R=0.25). Furthermore, the involvement of p38 MAPK in AMPD2 mediated ferroptosis and the impact of AMPD2 on the expression of Gpx4, an important regulator of ferroptosis reported in cancers(Xu, et al. 2022, Bersuker, et al. 2019), was also examined. As shown in Fig.6b, the expression of total p38, phosphorylated p38 and Gpx4 were significantly increased after AMPD2 overexpression, whereas in the knockdown group, the results were reversed (Fig.6c). Taken together, these results suggested that AMPD2 might inhibit ferroptosis by targeting the p38/GPX4.

### 5. AMP restore ferroptosis sensitivity in AMPD2 overexpression cell lines

As AMPD2 catalyzes the metabolism of AMP to IMP(Akizu, et al. 2013, Ehlers, et al. 2021), and mediate ferroptosis through p38/GPX4, the effect of AMP on ferroptosis in AMPD2 overexpressed cell lines was investigated. As shown in Fig.7a-c, After treatment with AMP for 24 h (10 μM), the levels of ROS (Fig.7a) and MDA (Fig.7b) were significantly improved in the AMPD2 overexpression group. Furthermore, the sensitivity of ferroptosis could be restored by addition of AMP or VX-745 in the AMPD2 overexpression group even under inducement by RSL3 (Fig.7c). In addition, lower expression of total p38, phosphorylated p38 and Gpx4 could be observed after AMP treatment in the AMPD2 overexpression group (Fig.7d). All these results indicate that AMPD2 medicate ferroptosis by regulating AMP levels in NSCLC.

## Discussion

Lung cancer is the leading cause of cancer-related mortality in the world(Siegel, et al. 2021; Sung, et al. 2021), and NSCLC accounts for most lung cancer with poor prognosis. Targeted therapy has emerged as an important mean of the disease management for patients with NSCLC(Yuan, et al. 2019). However, a better understanding of patient's molecular features is great demanded to guide the therapeutic decisions for whom may be of targeted therapy benefit.

AMPD2 is a critical enzyme, which catalyzes energy supply and metabolism. Over expression of AMPD2 has been reported to be correlated with poor prognosis in colorectal caner(Gao, et al. 2020) and metabolic dysregulation(Akizu, et al. 2013; Helmering, et al. 2014; Kortum, et al. 2018; Hudoyo, et al. 2017). The present study uncovered that the expression of AMPD2 is statistically higher in NSCLC tissues compared to adjacent tissues according to the IHC analysis from 30 pairs of NSCLC and normal lung tissues,which could provide a new target for intervention in the treatment of NSCLC.

Ferroptosis is a form of regulated cell death which is characterized by accumulation of lipid peroxidation and lethal oxidation(Chen, et al. 2021). Recently, a growing number of research have confirmed that activation of ferroptosis effectively prevents NSCLC progression and enhances the efficacy of targeted

therapy(Yang, et al. 2021; Dong, et al. 2021). Currently, the molecular mechanisms by which ferroptosis affects tumour therapy are still poorly understood.

Our results revealed that overexpression of AMPD2 reduced cellular ROS production and MDA expression, whereas in the knockdown group, the results were reversed. In addition, overexpression of AMPD2 could inhibit RSL3 induced ferroptosis, whereas knockdown of AMPD2 increase RSL3 induced ferroptosis. Thus, our results suggested that AMPD2 might be a negative regulator of ferroptotic cancer cell death, it is important to explore the possible role of AMPD2 in the regulation of the ferroptotic therapy for NSCLC.

Emerging evidence demonstrates that the activation of p38 MAPK pathway is mediated by ferroptosis(Hattori, et al. 2017; Ye, et al. 2020). In this study, we observed that the expression of p-p38, p38 and GPX4 increased in AMPD2 overexpressed NSCLC cells, while decreased in AMPD2 silenced NSCLC cells. Furthermore, we discovered that AMPD2 improves the ferroptotic sensitivity after treatment with the p38 inhibitor of VX-745. These results indicate that AMPD2 might inhibit ferroptosis by targeting p38/GPX4.

AMPD2 is a catalytic enzyme, which catalyzes the metabolism of AMP to IMP. Our results indicate that AMP treatment could enhance the sensitivity of ferroptosis in the AMPD2 overexpression group even under inducement of RSL3. The expressions of total p38, phosphorylated p38 and GPX4 were also reduced. Together, these observations suggest the crucial role of AMPD2 as metabolic enzymes of AMP in regulating ferroptosis may serve as a biomarker for NSCLC.

In conclusion, the outcomes of the present study suggest that AMPD2 acts as a critical regulator of ferroptotic cancer cell death, which may offer direction for a new strategies for possible combinational cancer therapy targeting both AMPD2 and ferroptosis.

## Declarations

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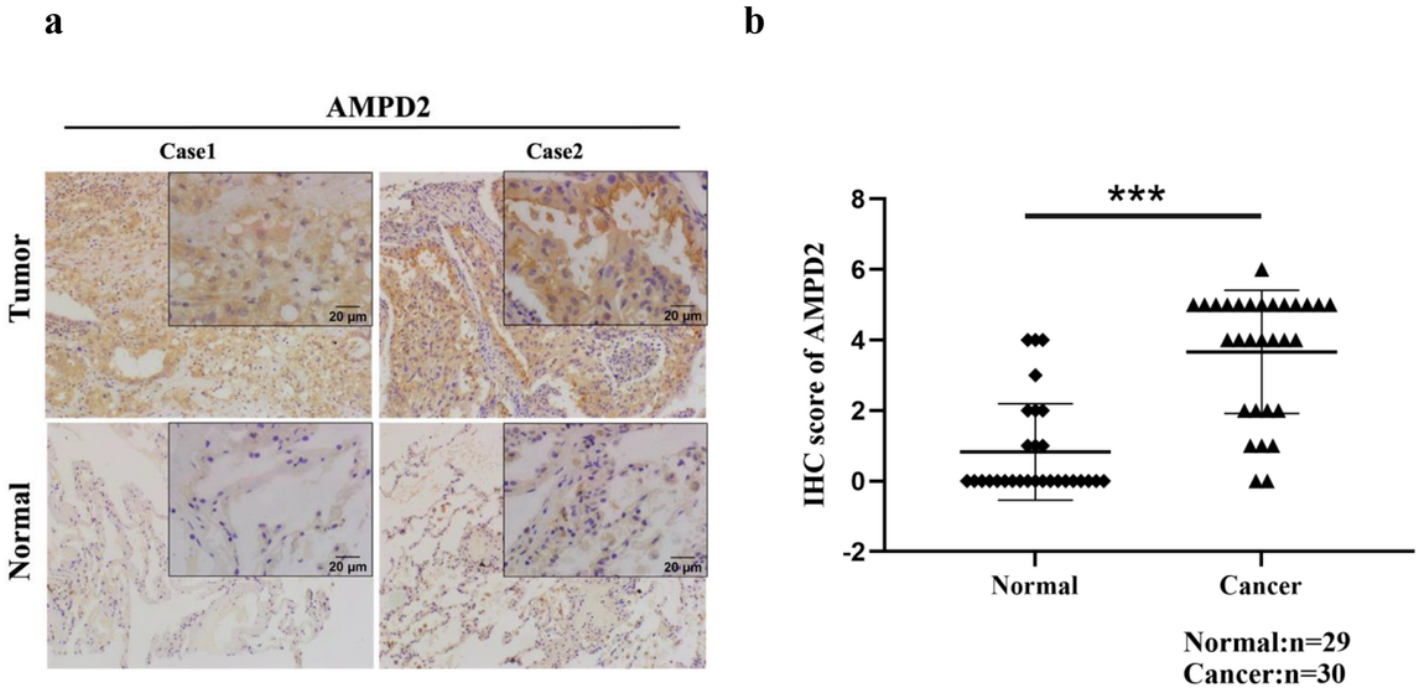
# Figures

Fig.1



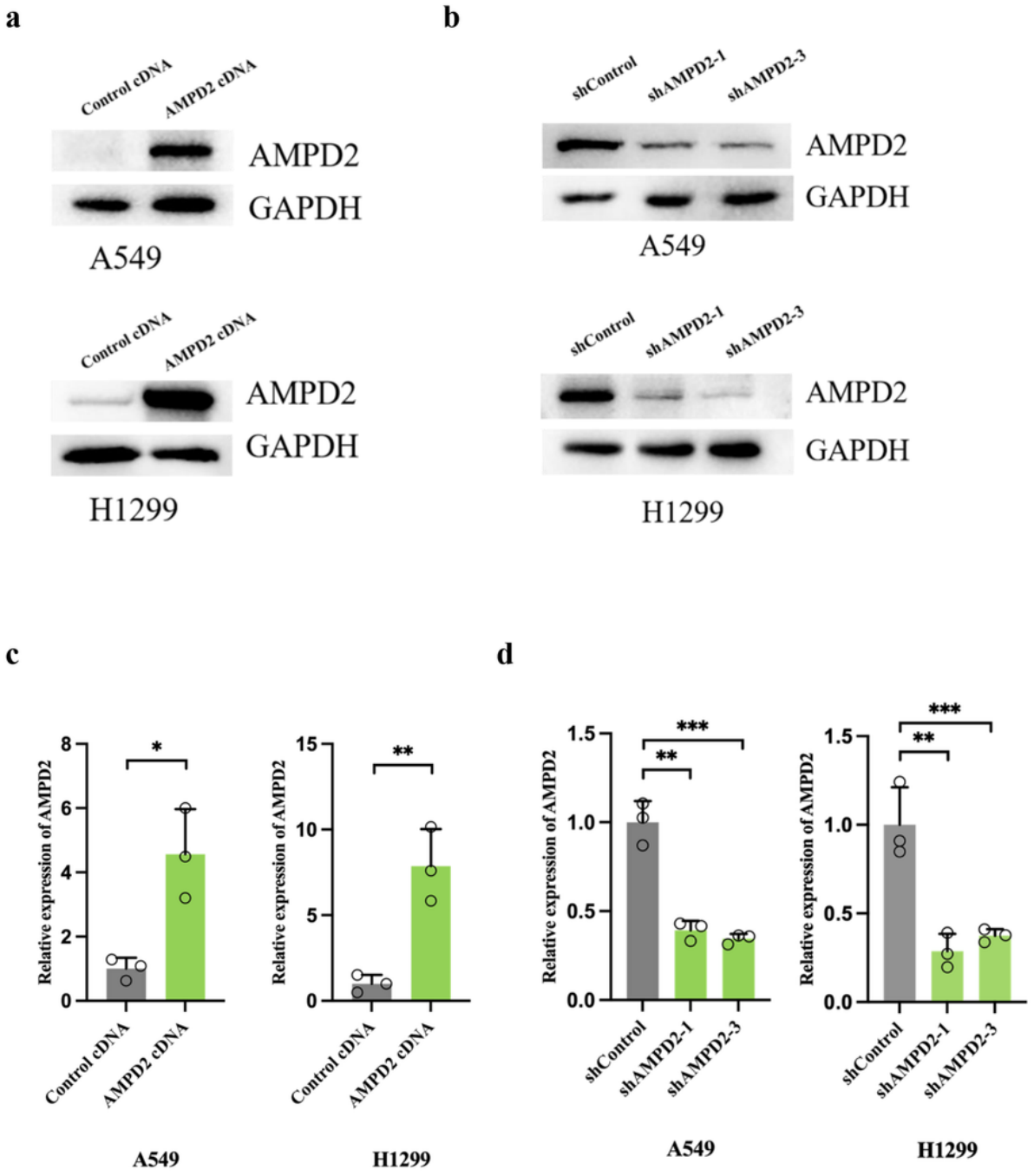

## Figure 1

### AMPD2 is upregulated in NSCLC tissues.

(a) The expression levels of AMPD2 in NSCLC tissues and adjacent normal tissue were measured by immunostained tissue microarray. Scale bar: 20 μm.

(b) Comparison of AMPD2 immunostaining score between 59 cases of NSCLC tissues and adjacent normal tissues. ***P<0.005.

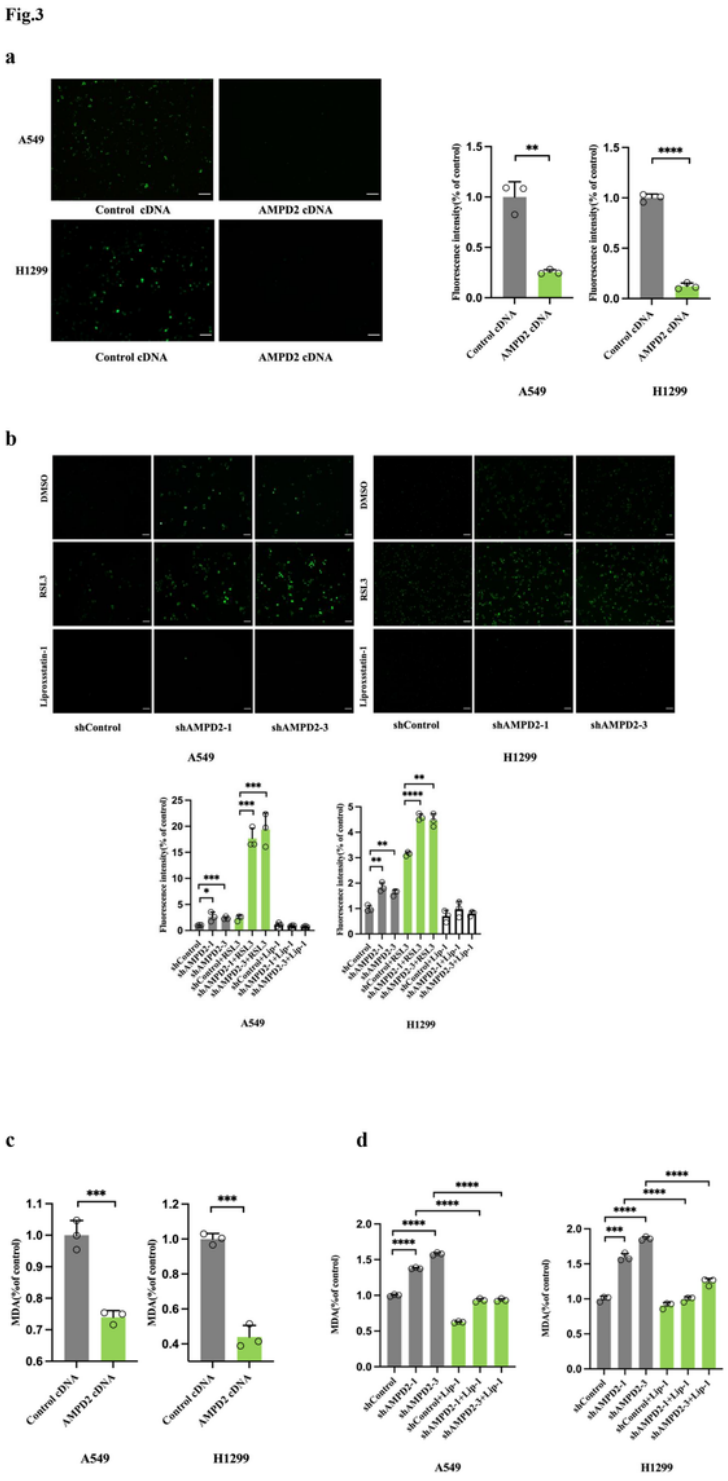
Fig.2




## Figure 2

### Construction of stable AMPD2 overexpression and knockdown cell lines.

The AMPD2 protein (a-b) and mRNA (c-d) expression were detected by western blot analysis and qPCR in the AMPD2 overexpression and knockdown cell lines, respectively. ***P<0.005, **P<0.01, *P<0.05, ±SD, n=3.



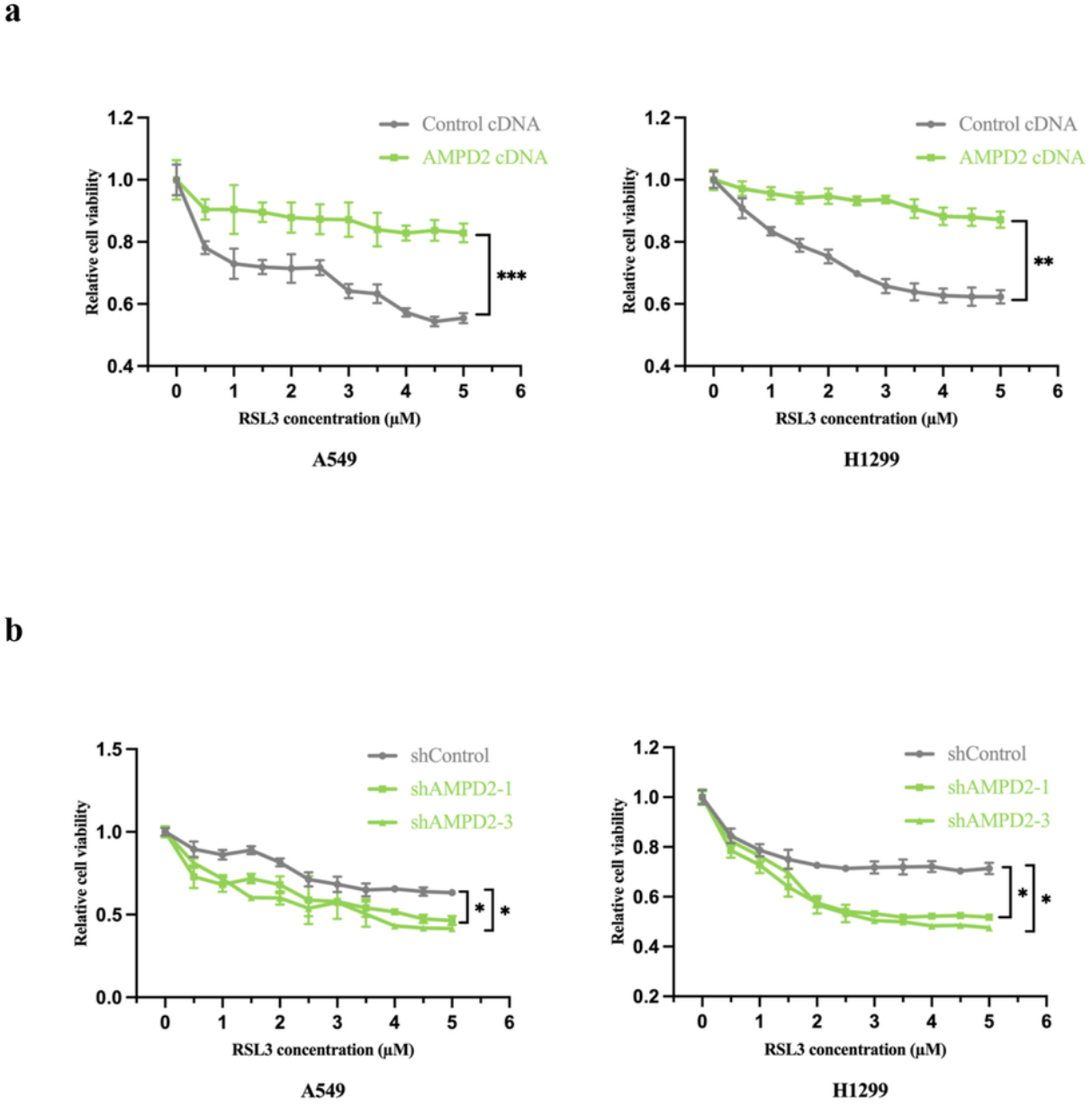

## Figure 3

### AMPD2 inhibits intracellular ROS and MDA.

The levels of ROS production detected by DCFH-DA probe in AMPD2 overexpression cell lines (a) and knockdown cell lines after treatment with RSL3 (1 μM), Liproxstatin-1 (10 μM) or DMSO, for 24 h (b), scale bar: 100 μm, ****P<0.0001, **P<0.01, *P<0.05, ±SD, n=3. The level of MDA detected by MDA kit in

AMPD2 overexpression (c) and knockdown (d) cell lines, compared with control group. ****P<0.0001, ***P<0.005±SD, n=3.

**Fig.4**




## Figure 4

**AMPD2 inhibits RSL3-induced ferroptosis.**

Changes of cell viability in AMPD2 overexpression (a) and knockdown (b) group following treatment with RSL3 (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 μM) for 24 h. ***P<0.005, **P<0.01, *P<0.05,±SD，n=3.

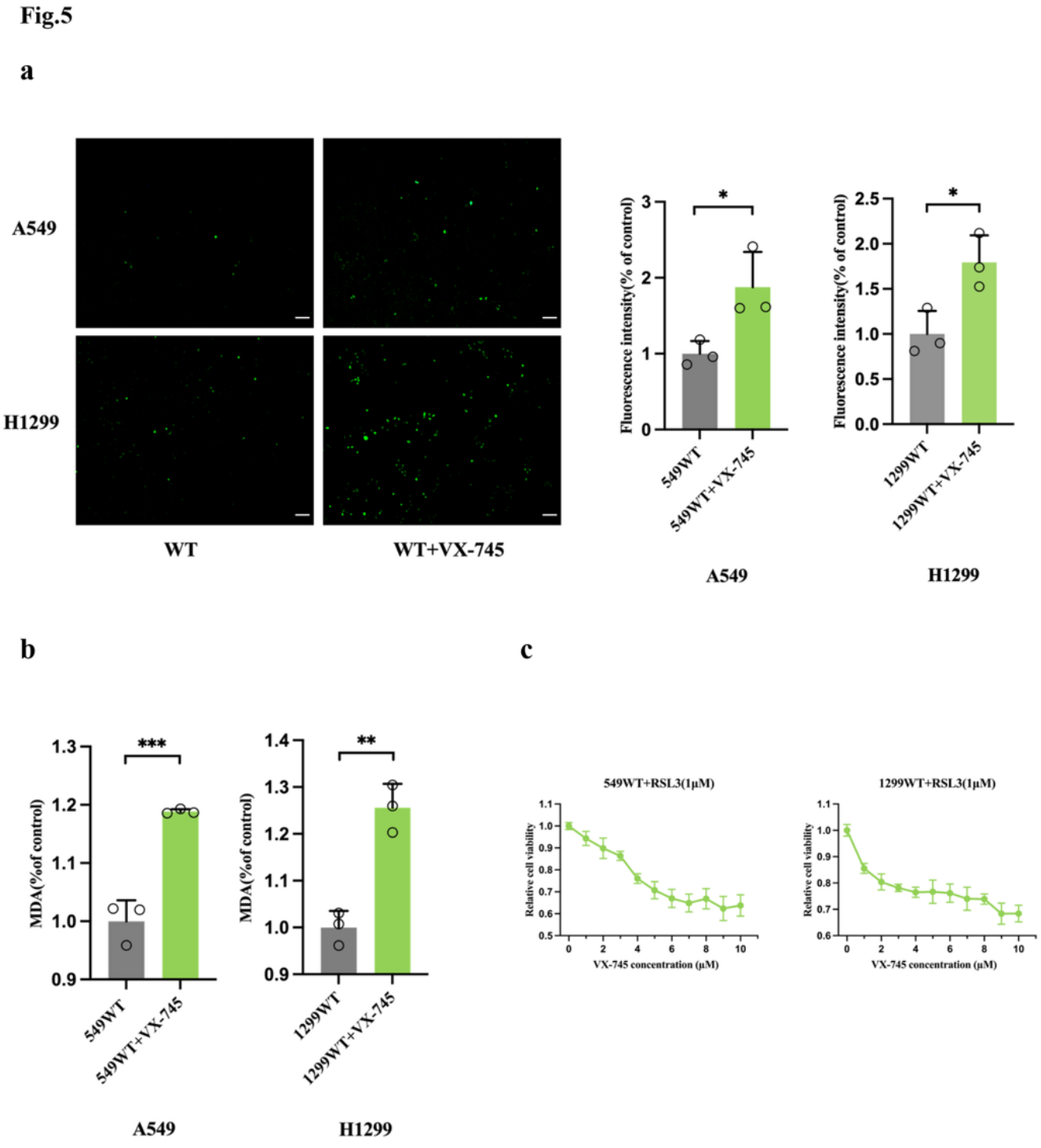
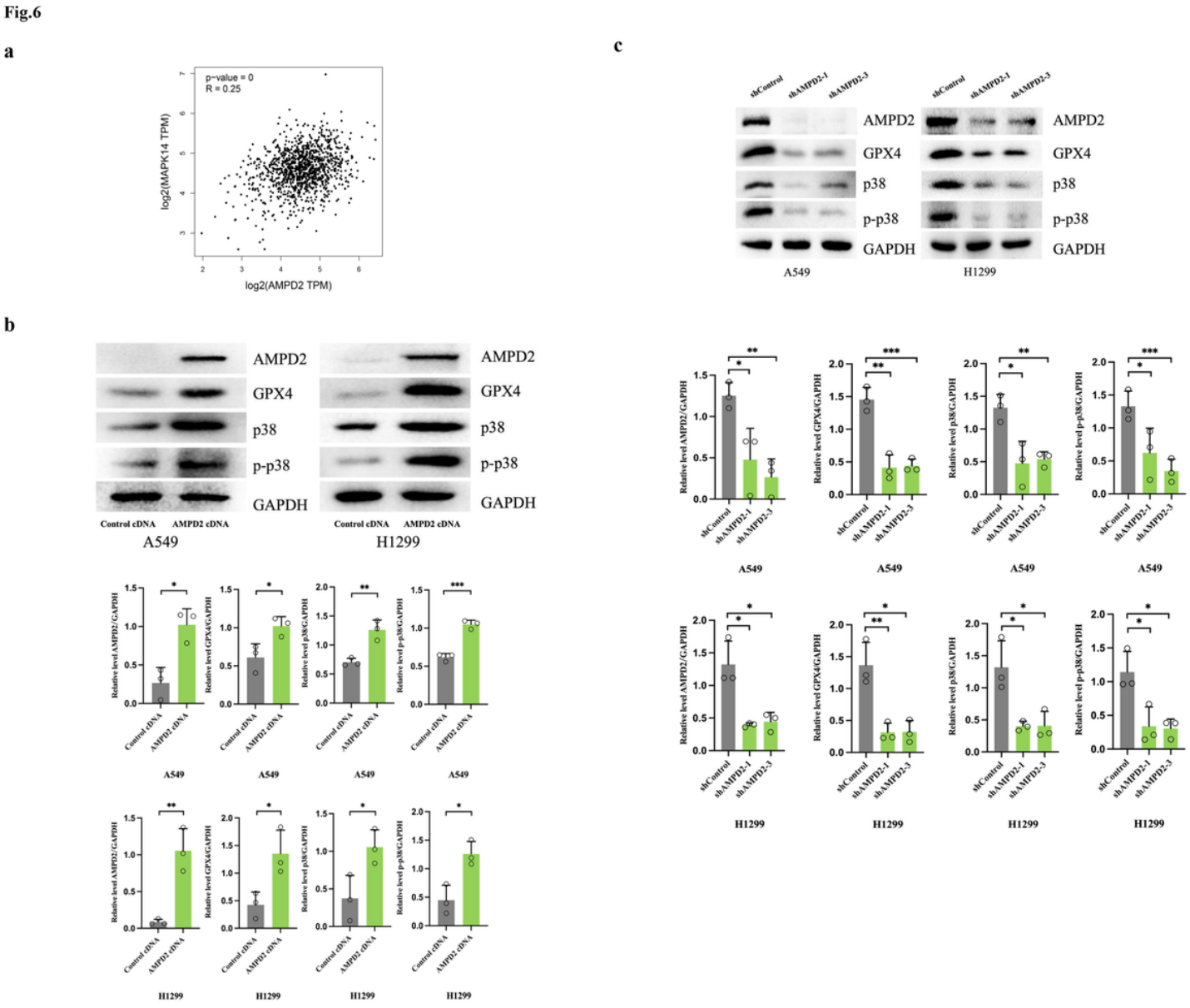



Figure 5

## Phosphorylated p38 inhibits ferroptotic cell death in NSCLC.

The level of ROS detect by DCFH-DA probe (a), the level of MDA detected by MDA kit (b) in wild-type A549 and H1299 upon VX-745 treatment (10 μM) for 24 h. Changes in cell viability detected by CCK8 following treatment with RSL3 (1 μM) and VX-745 (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 μM) for 24 h in wild-type A549 and H1299 (c). Scale bar: 100 μm, ***P<0.005, **P<0.01, *P<0.05, ±SD, n=3.

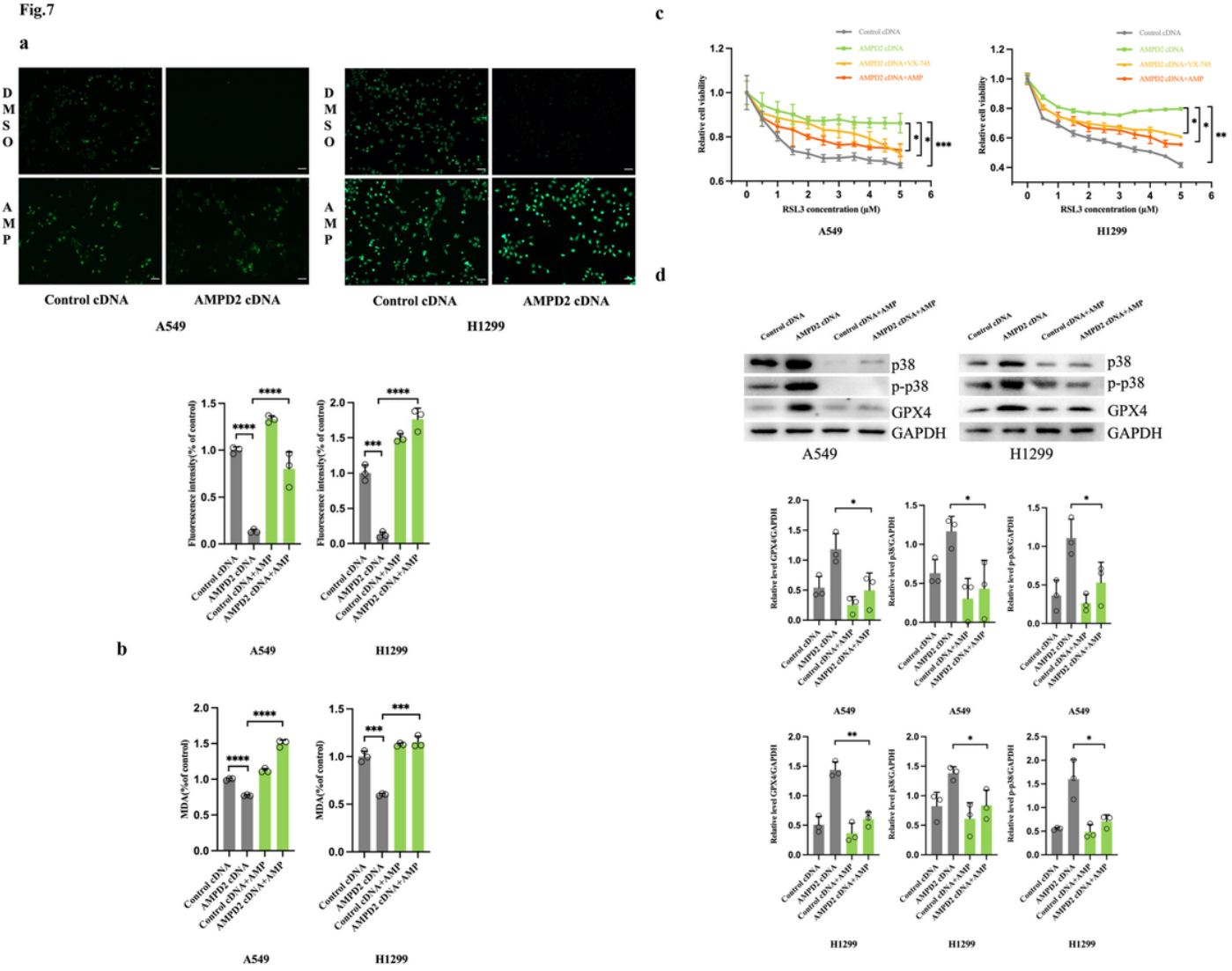



## Figure 6

## AMPD2 can affect the p38 pathway and regulate GpxPX4

(a) The correlation between AMPD2 and p38 in LUAD, LUSC and paracancer tissues. (GEPIA, the "Correlation" function provides paired gene Correlation analysis for a given TCGA data, P=0, R=0.25).

The protein expression of AMPD2, p38, p-p38 and GPX4 in AMPD2 overexpression (b) and knockdown (c) cell lines detected by western blot analysis. ***P<0.005, **P<0.01, *P<0.05, ±SD, n=3.




## Figure 7

### AMP restore ferroptosis sensitivity in AMPD2 overexpression cell lines.

(a) The level of ROS detect by DCFH-DA probe. (b) The level of MDA detected by MDA kit in AMPD2 overexpression cell lines following treatment with AMP (10 μM) or DMSO for 24 h. (c) Changes in cell viability of AMPD2 overexpression group following treatment with VX-745 (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 μM) and AMP (10 μM) for 24 h. (d) The protein expression of p38, p-p38 and GPX4 in AMPD2

overexpression cell lines following treatment with AMP (10 μM) for 24 h, detected by western blot analysis. Scale bar: 100 μm, ****P<0.0001, ***P<0.005,**P<0.01, *P<0.05, ±SD, n=3.