



Deubiquitylating inhibitor b-AP15 induces c-Myc-Noxa-mediated apoptosis in esophageal squamous cell carcinoma

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Published online: 24 July 2019
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Abstract

Esophageal squamous cell carcinoma (ESCC) is one of the most malignant tumors in east Asia. However, the molecular mechanism underlying its progression remains unclear. The ubiquitin–proteasome system (UPS) is a central mechanism for protein degradation and turnover. Accumulating evidence showed that more and more deubiquitinases could serve as attractive anti-cancer target. The expression of USP14 and UCH37 in esophagus squamous cell carcinoma tissues were examined by immunohistochemistry and western blot assays. Effect of b-AP15, a USP14 and UCH37 inhibitor, on ESCC cell growth was evaluated by cell viability assay. After cell lines being treated with b-AP15, cell cycle, apoptosis and the expression of related proteins were further explored to investigate the anti-ESCC mechanism of b-AP15. Results showed that deubiquitinating enzymes (DUBs) USP14 and UCH37 expressed at higher levels in ESCC tissues than in adjacent tissues. b-AP15 could inhibit cell proliferation and induce G2/M cell cycle arrest and apoptosis in ESCC cells. Mechanistically, b-AP15 treatment triggered Noxa-dependent apoptosis, which was regulated by c-Myc. Silencing Noxa and c-Myc could reduce b-AP15-induced apoptosis in ESCC cells. Our results revealed a novel mechanism of anti-tumor activity of b-AP15 in ESCC, and b-AP15 could be used as a potential therapeutic agent in ESCC.

Keywords Esophagus squamous cell carcinoma · b-AP15 · c-Myc · Noxa · Apoptosis

Abbreviations

ESCC	Esophageal squamous cell cancer
UPS	Ubiquitin–proteasome system
DUBs	Deubiquitinating enzymes
USP14	Ubiquitin-specific peptidase 14
UCH37	Ubiquitin C-terminal hydrolase 37
IHC	Immunohistochemistry

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10495-019-01561-9>) contains supplementary material, which is available to authorized users.

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DAB	3,3'-Diaminobenzidine tetrahydrochloride
ROS	Reactive oxygen species
ER	Endoplasmic reticulum

Background

Esophageal cancer is one of the malignant tumors in the digestive system [1, 2], and ranks the sixth most common cancer worldwide [2]. Esophageal squamous cell carcinoma (ESCC) is the major histological form of esophageal cancer in Asian countries, and it ranks the fourth most common cancers in China [3, 4]. The 5-year survival of esophageal cancer is still less than 20% mostly due to the late-stage-diagnosis [5, 6] and limited efficacy of traditionally comprehensive treatment, including surgical resection and standard first-line chemo-radiotherapeutic approaches [5, 7, 8]. These facts underscore the urgent need for early diagnosis and more effective therapeutic strategies, including target therapy and immuno-therapy.

Ubiquitin-specific peptidase 14 (USP14) and Ubiquitin C-terminal hydrolase 37 (UCH37, also known as UCHL5) belong to three proteasome-associated DUBs [9, 10], which bind to the 19S regulatory particle of 26S proteasome [9, 11] and remove ubiquitin from proteasomal substrates, thus regulating the substrates function and participating in various pathophysiology process, including oncogenesis [12]. Recent reports showed that ubiquitin-specific peptidase 14 (USP14) worked as an oncogene and overexpressed in several cancers [13–16], and it was positively correlated with poor prognosis [15, 17] and elevated cancer recurrence [18]. Genetic and pharmacological inactivation of USP14 could inhibit proliferation [19–21], induce cell cycle arrest [19, 21] and trigger apoptosis [15, 19] in cancer cells. Like USP14, UCH37 was also involved in tumor progression. UCH37/UCHL5 was firstly reported overexpressed in cervical carcinoma [22]. Subsequent studies showed that UCH37/UCHL5 expressed higher in ESCC [23], epithelial ovarian cancer [24] and hepatocellular carcinoma [25]. UCH37/UCHL5 could promote tumor progression by regulating several different signaling pathways [26, 27]. These studies suggest that USP14 and UCH37/UCHL5 can be used as potential therapeutic target for cancer.

Several inhibitors targeting DUBs have been discovered as potential therapy strategy in recent years [11, 28–31]. b-AP15 was first reported by D'Arcy et al. and described as an inhibitor of USP14 and UCH37/UCHL5 [32]. Researches have shown that b-AP15 was widely used as anticancer reagent [33–38]. b-AP15 treatment inhibited tumor progression of colon cancer, Lewis lung carcinoma, breast cancer, prostate cancer and multiple myeloma and corresponding xenografts exhibited prolongs survival [33, 34]. However, the effect and mechanism of b-AP15 in ESCC remains unknown. Here, we used b-AP15 as treatment reagent in four

human ESCC cell lines, including EC1, EC109, Kyse510 and Kyse450, to explore its anti-cancer function in ESCC and uncover underlying mechanisms.

Materials and methods

Cell lines and drug sources

Human ESCC cell lines EC1, EC109, Kyse510 and Kyse450 were cultured in DMEM medium (Biological Industries (BI), Biological Industries Israel Beit Haemek LTD., Israel) supplemented with 10% fetal bovine serum (Biological Industries (BI), Israel) at 37 °C in a 5% CO₂ incubator. b-AP15 were purchased from MedChemExpress (MCE, Shanghai, China), dissolved in DMSO, and stored at –80 °C. Diluted working solution was prepared freshly before each experiment.

Immunohistochemical staining

Human ESCC tissue array was purchased from Shanghai Outdo Biotech Co. Ltd (OD-CT-DgEso01-004). The expression of USP14 was detected using immunohistochemistry as previous described [39–41]. The expression of USP14 was detected by IHC staining with specific USP14 antibody (1:70, Cat. No. ab137433, Abcam Trading Company Ltd). Briefly, the tissue array was dehydrated, and peroxidase blocked. Antigen retrieval was undergone using 0.01 mol/L citric acid buffer (pH 6.0) and a pressure cooker. Primary antibodies were added and incubated at 4 °C overnight, followed by staining with a Histostain-Plus kits (SP-9000) and 3,3'-Diaminobenzidine tetrahydrochloride (DAB) (ZLI-9032) (ZSGB-BIO, Beijing, China). The slide was counterstained with hematoxylin. The stained slide was observed, and images were acquired by microscopy. Based on staining intensity, samples were classified into 4 groups, from the lowest intensity (–) to the highest (+++) [42]. Images were scanned by Motic digital slide scanning system (Motic (Xiamen) Electric Group Co., Ltd).

Collection of esophageal cancer tissues and detection of the expression of USP14 and UCH37 in tissues

Fresh primary esophageal cancer tissues and adjacent esophageal tissues were collected from 16 ESCC patients undergoing resection at the Linzhou Cancer Hospital (Linzhou, Henan, China, 2014). Histologic pathological diagnoses were determined in accordance with the American Joint Committee on Cancer manual criteria for esophageal cancer.

Tissues were grinded, and proteins were extracted using RIPA buffer (Cat. No. P0013B, Beyotime Institute

of Biotechnology, Jiangsu, China). Protein concentration was determined using BCA kit (Cat. No. P0012, Beyotime, China). 50 µg proteins were loaded per lane and detected using USP14 antibody (1:1000, sc-398009, Santa Cruz Biotechnology, Santa Cruz, CA) and UCH37 antibody (1:5000, ab124931, Abcam Trading Company Ltd, Shanghai, China), respectively. GAPDH (1:1000, AB-P-R 001, Hangzhou Goodhere Biotechnology Co., LTD, Zhejiang, China) was used as loading control.

Cell proliferation and cell colony assays

For cell proliferation assay, EC1, EC109, Kyse510 and Kyse450 cells in log growth phase were seeded in 96-well plates and treated with indicated concentrations of b-AP15 (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 µmol/L) for 72 h, followed by Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instructions. CCK-8 reagents (KeyGEN BioTECH, Nanjing, China) were added to each well and the plates were further incubated for 1.5 h at 37 °C. Relative viability was quantified by measuring the absorbance at 450 nm using a microplate reader. Cells treated with DMSO (0.1%) were used as control.

For colony assays, cells were seeded in six-well plates (500 cells per well) in triplicate, treated with DMSO (0.1%) or b-AP15 (0.02, 0.05, 0.1 µmol/L) and cultured for 10 days. The colonies were fixed with 4% paraformaldehyde (Beyotime, China) and stained with crystal violet (Beyotime, China). Colonies comprising at least 50 cells were counted.

Cell cycle analysis

EC1 and Kyse450 cells were treated with DMSO (0.1%) or b-AP15 (0.2, 0.4, 0.6 µmol/L) for 24 h and fixed in 70% ethanol at –20 °C overnight, then stained with a propidium iodide solution (50 µg/mL; Sigma, St. Louis, MO) containing RNase A (30 µg/mL; Sigma) at 37 °C for 30 min, analyzed for cell-cycle profile using BD Accuri™ C6 (BD Bioscience, USA). Data were analyzed with ModFit LT software (Verity Software House, Topsham, ME).

Detection of apoptosis and caspase3 activity

EC1 and Kyse450 cells were treated with DMSO (0.1%) or b-AP15 (0.4, 0.6, 0.8 µmol/L) for 72 h. Then apoptosis was detected by Annexin V-FITC/PI Apoptosis Detection Kit (KeyGEN BioTECH, Nanjing, China). AnnexinV⁺ cells were collected as apoptotic cells. Caspase-3 activity was measured by CaspGLOW™ Fluorescein Active Caspase-3 Staining Kit (BioVision, Inc. Milpitas, California) as described in the instruction. Apoptosis and caspase3 activity were analyzed using BD Accuri™ C6 (BD Bioscience, USA).

Mitochondrial membrane potentials assay

JC-1 (Yeasen Inc, Shanghai, China) was employed to measure mitochondrial depolarization in ESCC cells. Briefly, EC1 and Kyse450 cells were seeded into 60-mm dishes and exposed to DMSO (0.1%) or b-AP15 (0.4, 0.6, 0.8 µmol/L) for 48 h. Total cells were collected and incubated with JC-1 for 20 min at 37 °C followed by the procedure. The quantification of fluorescence was detected by flow cytometry (BD Accuri™ C6, BD Bioscience, USA). Cells with intact mitochondria displayed high red fluorescence and appeared in the upper right quadrant of the scatterplots. In contrast, cells that had lost mitochondrial membrane potential (MMP) displayed high green and low red fluorescence and appeared in the lower right quadrant.

Western blot

EC1 and Kyse450 cell lysates treated with DMSO or b-AP15 as indicated concentration were prepared for western blot analysis. 50 µg proteins were loaded per lane and detected using antibodies against p21 (Cat. No. 2947), p27 (Cat. No. 3686), Wee1 (Cat. No. 4936), pWee1 (Cat. No. 4910), pH3 (Cat. No. 3377), cleaved PARP (Cat. No. 5625), cleaved Caspase 3 (Cat. No. 9664), cleaved Caspase 9 (Cat. No. 7237), Bax (Cat. No. 5023), Bak (Cat. No. 12105), Bid (Cat. No. 2002), Bim (Cat. No. 2933), c-IAP1 (Cat. No. 7065), Bcl-x1 (Cat. No. 2764), Mcl-1 (Cat. No. 5453), XIAP (Cat. No. 2045), Survivin (Cat. No. 2808), ATF4 (Cat. No. 11815), CHOP (Cat. No. 5554) (all diluted into 1:1000, Cell Signaling Technology, Inc., Boston, MA, USA), Noxa (1:1000, Cat. No. OP180, Millipore, Billerica, MA), c-Myc (1:1000, Cat. No. sc-40, Santa Cruz Biotechnology, Santa Cruz, CA), FOXO3a (1:2000, Cat. No. ab53287, Abcam Trading Company Ltd). GAPDH (1:1000, AB-P-R 001, Hangzhou Goodhere Biotechnology Co., LTD, Zhejiang, China) was used as loading control. Secondary antibodies, peroxidase-conjugated goat anti-mouse IgG (Cat. No. ZB.2305) and peroxidase-conjugated goat anti-rabbit IgG (Cat. No. ZB.2301) were purchased from ZGSB.Bio, Inc., (Beijing, China). The membrane was incubated with corresponding secondary antibodies (1:3000) for 2 h at room temperature and detected using an ECL Kit (Cat. No. P0018, Beyotime, China).

Real-time polymerase chain reaction

EC1 and Kyse450 cells were treated with DMSO (0.1%) or b-AP15 (0.6 µmol/L) for 0 h, 12 h, 24 h, and 48 h. Total RNA was isolated using the Trizol reagent and Ultrapure RNA kit (CW Biotech, China) according to the manufacturer's instructions. RNA (1.0 µg) was purified and reversely transcribed by PrimeScript™ RT reagent Kit with gDNA Eraser (Cat. No. RR047A, Takara, Dalian, China) following

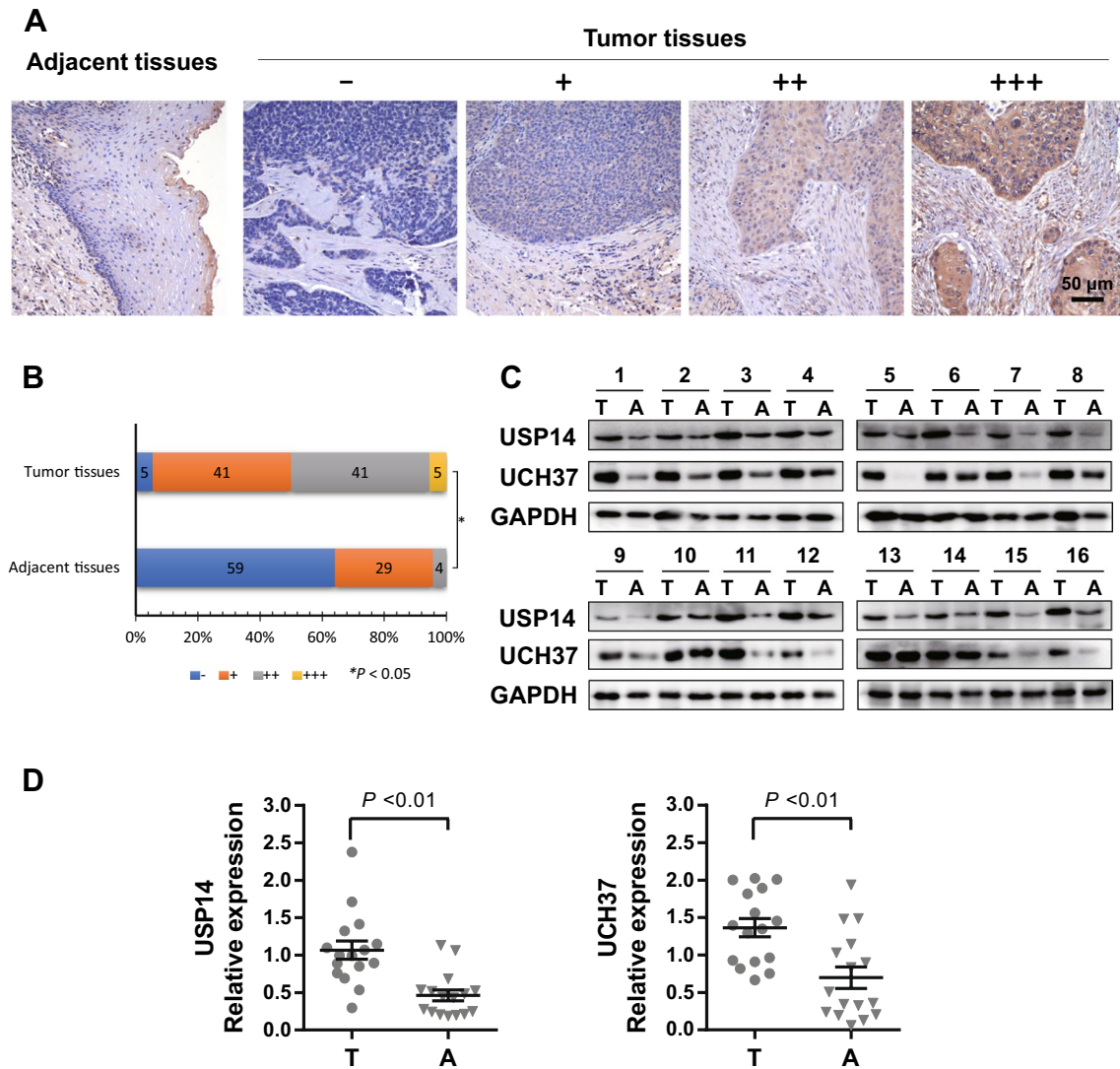


Fig. 1 Overexpression of USP14 and UCH37 in ESCC tissues. **a** Analyze the expression of USP14 by IHC staining of human ESCC tissues array. Samples were divided into four groups according to staining intensity from the weakest (–, group 1) to the strongest (+++, group 4). **b** Classification and statistical analysis of IHC staining intensity by Mann–Whitney Test ($P < 0.01$). **c** Expression of

USP14 and UCH37 in 16 pairs of ESCC tissues and corresponding adjacent esophageal tissues by western blot analysis. **d** Quantification of proteins expression of western blot. The results of a total of 16 pairs of tissues (tumor vs. adjacent normal tissues) were analyzed ($P < 0.01$). Horizontal lines indicated means \pm standard deviations (Error bar = S.D.). A adjacent tissues; T tumor tissues

the manufacturer’s instructions. The cDNA was quantified by real-time quantitative PCR using QuantiNova™ SYBR Green PCR Kit (Cat. No. 208054, QIAGEN, Germany) and a Real-time PCR system (Applied Biosystems, Foster City, Calif.). For each sample, the mRNA abundance was normalized to the amount of GAPDH. Primers are as follows:
 Noxa: forward, 5'-GGAGATGCCTGGGAAGA-3',
 Noxa: reverse, 5'-TTCTGCCGGAAGTTCAGT-3';
 GAPDH: forward, 5'-AAAGGGTCATCATCTCTG-3',
 GAPDH: reverse, 5'-GCTGTTGTCATACTTCTC-3'.

Gene silencing using small interfering RNA (siRNA)

EC1 and Kyse450 cells were transfected using Lipofectamine 2000 (Invitrogen, life technologies) with siRNA mixture targeting Noxa or c-Myc for 48 h and further treated with b-AP15 (0.8 μ mol/L) for another 48 h. Then cells were collected for apoptosis and western blot assays. The sequences of the siRNA are as follows:
 siNoxa#1: GUAAUUAUUGACACAUUC
 siNoxa#2: GGUGCACGUUCAUCAAUUUG
 sic-Myc#1: AACGUUAGCUUCACCAACA
 sic-Myc#2: CGAGCUAAAACGGAGCUU

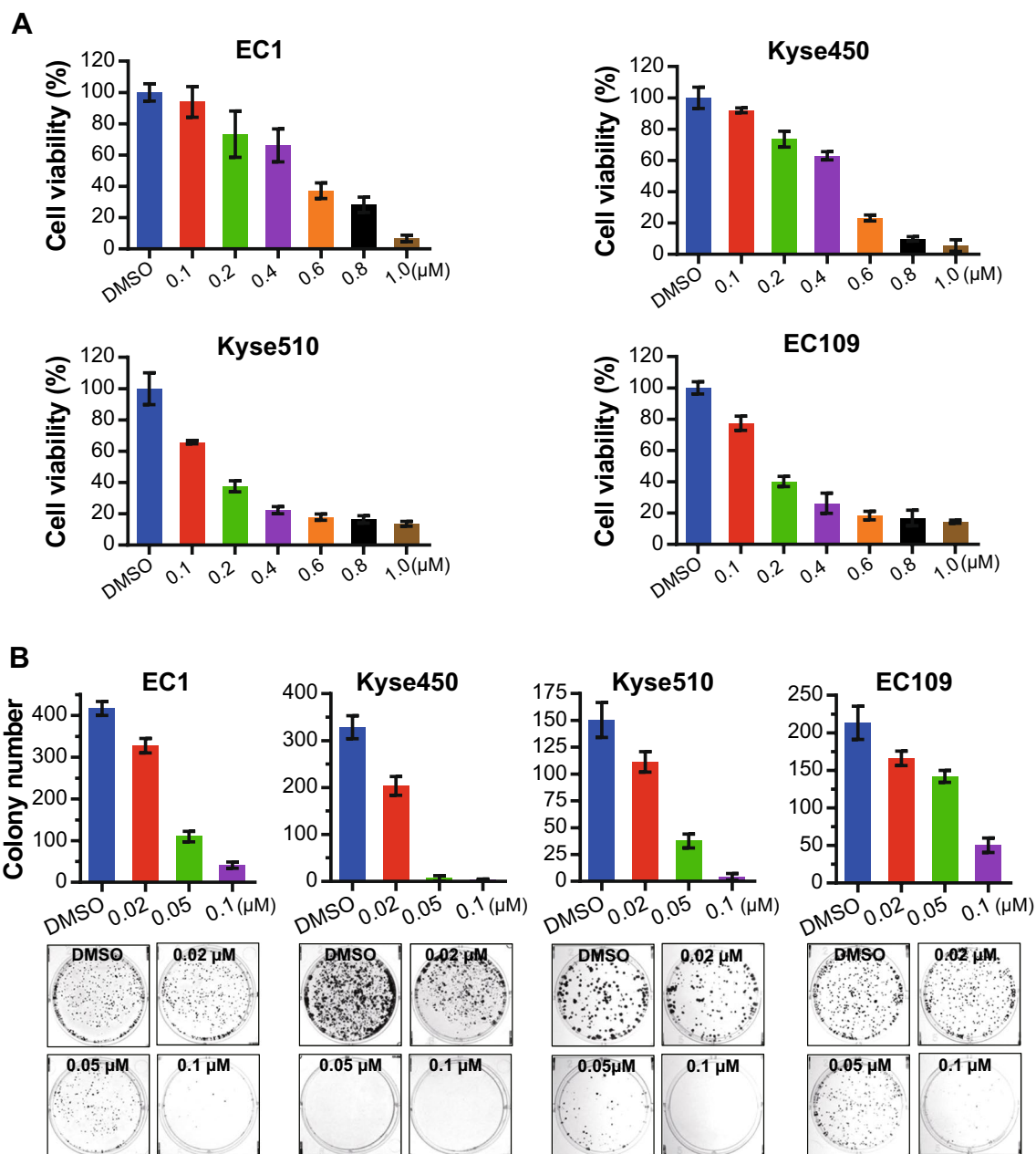


Fig. 2 b-AP15 inhibits growth and colony formation of esophageal cancer cells. **a** Effect of b-AP15 on the viability of ESCC cells Kyse450, Kyse510, EC1 and EC109. Cells were treated with DMSO (0.1%) or b-AP15 (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 $\mu\text{mol/L}$) for 72 h and

viability was detected with the CCK-8 kit. **b** Efficacy of b-AP15 on colony formation of ESCC cells. ESCC cells were treated with b-AP15 at different concentrations for 10 days, and then fixed, stained and counted as described in Materials and Methods

siControl: UUCUCCGAACGUGUCACGU

Statistical analysis

The expression of USP14 in IHC assay was analyzed by Mann–Whitney Test using SPSS software. All other statistical tests were performed in GraphPad Prism 5 software. *P* values less than 0.05 were considered statistically different. *means $P < 0.05$, **means $P < 0.01$.

Results

USP14 and UCH37 overexpressed in ESCC tissues

To investigate whether USP14 or UCH37 could be used as anti-cancer target in ESCC, we first examined their expression in tissue microarray with ESCC and adjacent tissues. Results from Immunohistochemistry (IHC) assay showed that the expression of USP14 expressed higher in ESCC

tissues than that in adjacent normal tissues (Fig. 1a, b). The expression of UCH37 in ESCC and adjacent normal tissues have already been examined by Chen et al. and the results showed that UCH37 also expressed higher in ESCC tissues than that in adjacent normal tissues [23]. Results from western blot assay from 16 paired ESCC and adjacent normal tissues further confirmed these results ($P < 0.01$) (Fig. 1c, d).

b-AP15 inhibited the proliferation of ESCC cells

Cell proliferation assay and colony formation assay were used to explore the anti-cancer effect of b-AP15 in ESCC cells. Results showed that ESCC cell growth was inhibited (Fig. 2a) and colony formation ability was compromised after b-AP15 treatment (Fig. 2b). Since similar growth inhibition effect of b-AP15 were shown on Kyse450, Kyse510, EC1 and EC109, we selected Kyse450 and EC1 cell lines in the following study.

b-AP15 induced G2/M cell cycle arrest

To investigate how b-AP15-induced ESCC cell growth inhibition, we first examined the effect of b-AP15 on cell cycle. Flow cytometry analysis showed that after being treated with b-AP15 for 24 h, EC1 and Kyse450 cells cycles were arrested in G2/M phase (Fig. 3a, b). Expression of cell cycle-related proteins was further examined by western blot assay. The results revealed that b-AP15 treatment increased the expression of G2/M phase related proteins p21, p27 and pWee1 (Fig. 3c). Meanwhile, the M phase marker protein p-H3 [43] also increased in a concentration-dependent manner (Fig. 3c), indicating that b-AP15-treated-cells were arrested at the mitosis phase.

b-AP15 triggered mitochondrial apoptosis

ESCC cells that being treated with b-AP15 shrank and turned round (data not shown), implying that b-AP15 treatment could trigger apoptosis. This hypothesis was confirmed by Annexin V-FITC/PI assay and caspase3 activity assay, which showed that b-AP15 treatment led to increased Annexin V⁺ cells (Fig. 4a) and caspase3 activity (Fig. 4b) in a dose-dependent manner. In accordance with the FACS results, b-AP15 treatment also increased the cleavage of caspase9, caspase3 and poly ADP-ribose polymerase (PARP) (Fig. 4c). Meanwhile, b-AP15 induced the loss of mitochondrial membrane potential ($\Delta\Psi_m$), a classical marker of the activation of intrinsic apoptosis (Fig. 5a). These results suggested that b-AP15 could trigger mitochondrial apoptosis in ESCC cells.

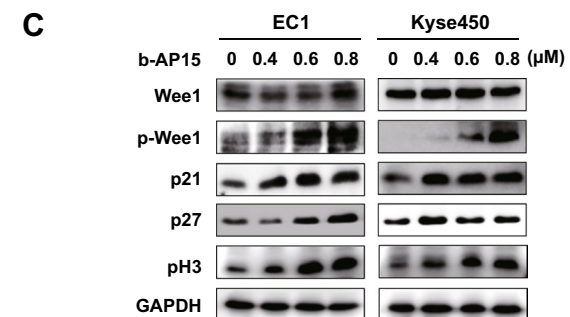
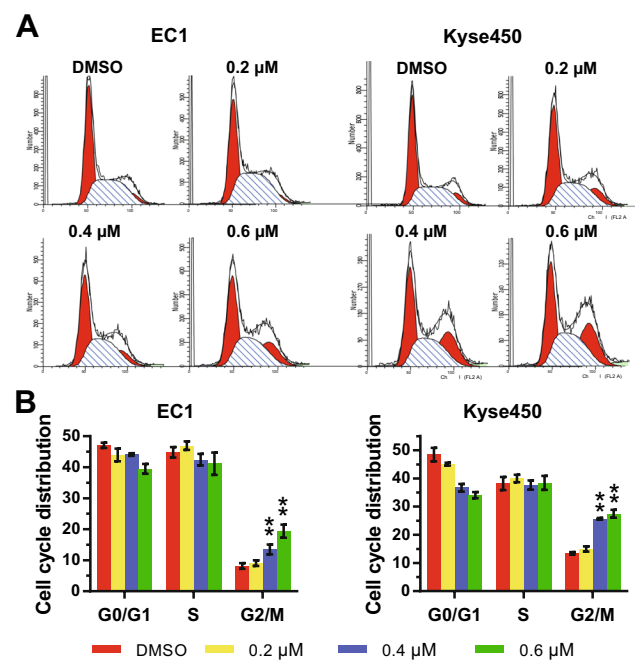
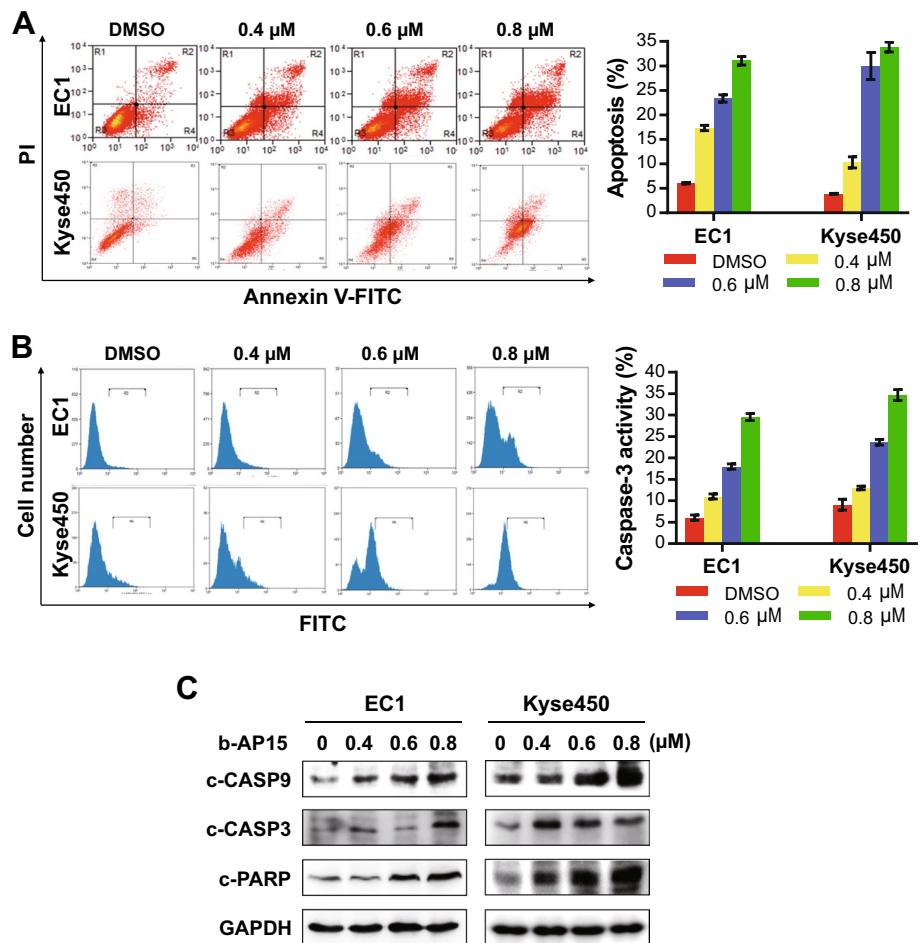


Fig. 3 b-AP15 triggers G2/M cell cycle arrest in esophageal cancer cells. **a–b** b-AP15 induced G2/M cell cycle arrest. EC1 and Kyse450 cells were treated with b-AP15 at different concentration for 24 h, followed by PI staining and FACS analysis for cell cycle profile (upper panel). Distribution was analyzed by Modifit and Graphpad software (lower panel). **c** Effect of b-AP15 on the expression of cell-cycle-related proteins. EC1 and Kyse450 cells were treated with b-AP15 at indicated concentrations and subjected to western blot subsequently. GAPDH was used as loading control

Noxa was transactivated by c-Myc and plays an important role in b-AP15 induced apoptosis

b-AP15 induced the loss of mitochondrial membrane potential (Fig. 5a), implying that b-AP15 induced intrinsic apoptosis. So we examined the effect of b-AP15 treatment on the expression of anti-apoptotic and pro-apoptotic proteins. Results showed that Noxa was upregulated in both EC1 and Kyse450 cells, while other proteins slightly changed (Fig. 5b). Furthermore, we found that the expression of Noxa was upregulated in both mRNA and protein levels in a time-course dependent manner (Fig. 6a). Silencing Noxa rescued b-AP15-induced apoptosis

Fig. 4 b-AP15 induces apoptosis. EC1 and Kyse450 cells were treated with b-AP15 for 72 h. **a** Apoptosis was determined by FACS analysis using Annexin V-FITC/PI double-staining kit and Annexin V⁺ cell populations were defined as apoptosis. **b** CASP3 activity was also analyzed with FACS. All data were representative of at least three independent experiments. **c** Treatment with b-AP15 increased the expression of cleaved CASP9, CASP3, and PARP. EC1 and Kyse450 were treated with b-AP15 for 72 h and cell lysates were assessed by western blot with primary antibodies against cleaved CASP9, CASP3, or PARP. GAPDH was used as loading control



(Fig. 6d). The expression of Noxa-related transcription factor was also investigated. Results showed that polyubiquitin was accumulated after treated with b-AP15 in a concentration-dependent manner (Fig. 6c). Meanwhile, except for up-regulated c-Myc, all the other transcription factors slightly changed (Fig. 6b). Knock-down of c-Myc reduced b-AP15-induced apoptosis (Fig. 6d), down-regulated the expression of Noxa (Fig. 6d) and the cleavage of PARP (Fig. 6d) in both EC1 and Kyse450 cells. These observations indicated that c-Myc-mediated up-regulation of Noxa play a major role in b-AP15 induced apoptosis in ESCC cell lines.

Discussion

DUBs have been reported to contribute to tumorigenesis by regulating several different pathways [12]. Targeting DUBs play important and therapeutically exploitable roles in the treatment of several different types of cancers [9, 42, 44, 45]. USP14 and UCH37/UCHL5 -are reported to be over-expressed in several cancers [13–18, 22] and DUB inhibitor b-AP15 can selectively induce cell growth inhibition in several different types of tumor cell lines, including

colon carcinoma, prostate cancer, leukemia, mantle cell lymphoma (MCL) and multiple myeloma model (MM) [33–38]. However, whether b-AP15 has similar effects in ESCC cell lines and the underlying mechanisms remains uninvestigated. Here, we examined the expression of USP14 and UCH37 in ESCC and found that they were both overexpressed in ESCC tissues, which were consistent with most recent reports [17]. Moreover, inactivation of both USP14 and UCH37 with b-AP15 induced effective cell growth inhibition in four different ESCC cell lines. These results indicate that USP14 and UCH37 can serve as anti-ESCC target and b-AP15 have potential to be used as anti-ESCC therapeutics.

b-AP15 was first identified by D'Arcy et al. as an inhibitor for USP14 and UCH37/UCHL5, which could block the deubiquitylating activity of both deubiquitinates without affecting proteolytic activities of the 20S core particle [32]. Reports showed that b-AP15 could effectively inhibit tumor cell growth and metastasis by interfering different pathway. Several studies have shown that b-AP15 induced cell cycle arrest in a tumor-specific manner, while others have shown that b-AP15 triggered apoptosis or regulated proliferation signaling pathway of cancer cells. However,

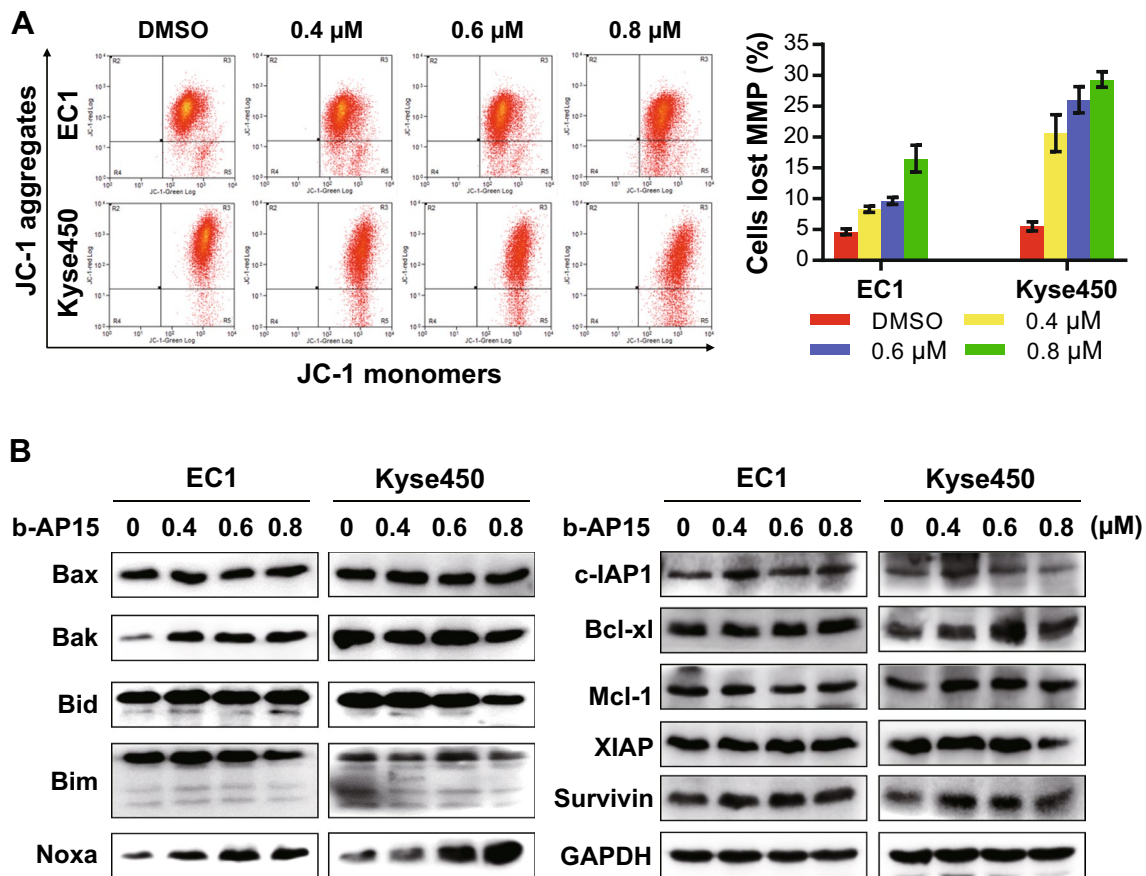


Fig. 5 Effect of b-AP15 on the expression of pro-apoptotic and anti-apoptotic proteins. **a** b-AP15 induced mitochondrial membrane depolarization. EC1 and Kyse450 cells were treated with b-AP15 as indicated. Cells with intact mitochondria displayed high red fluorescence and appeared in the upper right quadrant of the scatterplots. In contrast, cells that had lost mitochondrial membrane potential (MMP) displayed high green and low red fluorescence and appeared

in the lower right quadrant (left panel). Percentage of cells that had lost mitochondrial membrane potential is shown in the right panel. **b** Effect of b-AP15 on the expression of pro-apoptotic and anti-apoptotic proteins. Cells were treated with b-AP15 with the indicated concentrations for 72 h. Cell extracts were prepared for western blot analysis. GAPDH was used as a loading control

the mechanisms by which b-AP15 inhibit ESCC cell growth are largely unknown.

Recent reports showed that DUBs worked as an integral component of the core cell cycle machinery and cell cycle checkpoints. They regulated cell-cycle-related proteins, such as cyclins, cyclin-dependent kinases and checkpoint proteins, which play an important role by their mis-regulation in cancer. b-AP15 treatment increased the number of hypodiploid cells and induced G2/M arrest in multiple myeloma cell lines [33] and colon cancer cell line HCT116. Mechanistically, b-AP15 treatment could decrease the expression of G2/M phase cell cycle regulatory proteins *cdc25c*, *cdc2*, and cyclin B1 [33] and upregulate p21 and p27 [33]. Being consistent with these results, our results found that b-AP15 treatment could lead to G2/M cell cycle arrest by increasing the expression of p21, p27 and pWee1, meanwhile, the M phase marker protein p-H3 [43] also increased in a concentration-dependent manner (Fig. 3c), indicating that

b-AP15-treated esophageal cells were arrested at the mitosis phase. However, Cai and his co-workers found that b-AP15 dramatically induced G0/G1 cell cycle arrest in both AR-responsive and AR-irresponsive prostate cancer cells by significantly decreasing the expression of cyclin D1, CDK6, CDK4, and phospho-Rb, while increasing the expression p27 [34]. These results imply that b-AP15 treatment might induce cell cycle arrest in a cell-specific manner.

It was previously reported that apoptosis was an important anti-tumor mechanism employed by b-AP15 [33–35, 46]. b-AP15 could enhance DR5 activation-induced apoptosis through stabilizing DR5 [35]. Besides, b-AP15 exhibited cytotoxicity to both androgen receptor-dependent and -independent prostate cancer cells by increasing reactive oxygen species (ROS) generation, endoplasmic reticulum (ER) stress and triggered apoptosis [34]. In view of recent studies showing that b-AP15 induced apoptosis in triple negative breast cancer, natural killer cells and multiple myeloma through

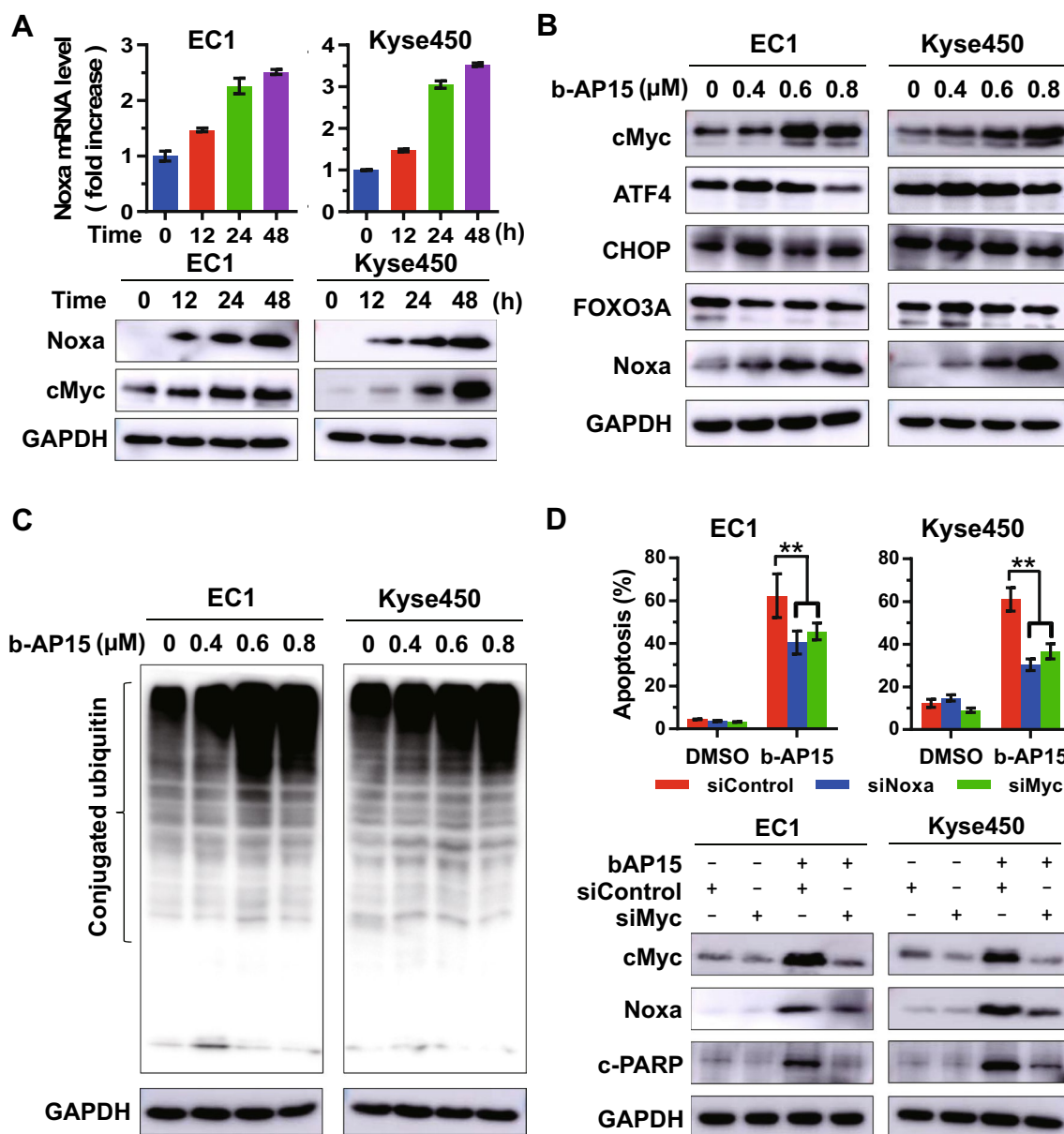


Fig. 6 Noxa is transactivated by c-Myc and participates in b-AP15-induced apoptosis in ESCC cells. **a** b-AP15 increased both the mRNA and protein level of Noxa. Cells were treated with b-AP15 (0.6 $\mu\text{mol/L}$) and collected at the indicated time points. Total RNAs were isolated. qPCR analysis was used to analyze the mRNA level of Noxa (normalized to GAPDH) (top panel). Cell extracts were prepared for western blot analysis of Noxa (bottom panel). **b** Screen of Noxa-related transcription factors. EC1 and Kyse450 cells were treated with b-AP15 for 72 h and cell lysates were assessed by western blot with specific antibodies. GAPDH was used as a control. **c** b-AP15 treatment accumulated polyubiquitin in a concentration-

dependent manner. EC1 and Kyse450 cells were treated with b-AP15 for 72 h and cell lysates were assessed by western blot with specific antibodies. GAPDH was used as a control. **d** The expression of c-Myc was responsible for b-AP15-induced apoptosis in ESCC cells. After transfected with the control siRNA, Noxa siRNA mixture or c-Myc siRNA mixture for 48 h, EC1 and Kyse450 cells were further treated with b-AP15 (0.8 $\mu\text{mol/L}$) for 48 h. Apoptosis was examined by Annexin V-FITC/PI double-staining analysis (top panel). Knock-down efficiency, expression of Noxa and cleaved PARP were assessed by western blot analysis. GAPDH was used as loading control (bottom panel)

mechanisms distinct from extrinsic apoptosis and unfolded protein response [38, 47, 48]. Several reports showed that besides death-reporter pathway, b-AP15 or its analogue could induce mitochondrial apoptotic pathway in different manners [32, 49, 50]. D'Arcy et al. and Brnjic et al. reported

that b-AP15 induced BAX and BAK independent apoptosis [32, 50]. b-AP15 and its analogue F6 induces apoptosis in BCL2 overexpressing cells [32, 49]. F6 induced mitochondrial apoptotic pathway accompanied by the upregulation of Noxa [49], which is consistent with our results. Our findings

showed that b-AP15 treatment upregulated the expression of Noxa in a time- and concentration-dependent manner. Silencing Noxa rescued the b-AP15 induced apoptosis. Due to previous reports that c-Myc was a prime transcription factors to transactivate Noxa [51, 52], we hypothesized that the accumulation of Noxa would be related to a function of c-Myc after b-AP15 treatment. Our studies confirmed that b-AP15 treatment induced accumulation of c-Myc in EC1 and Kyse450 cells. Knockdown of c-Myc reduced the accumulation of Noxa protein. These results indicated that b-AP15-induced apoptosis was in a tumor-specific manner.

Conclusion

In summary, our studies have identified a novel mechanism of the deubiquitinase inhibitor b-AP15-induced apoptosis in ESCC. This compound requires c-Myc to increase Noxa expression and trigger the mitochondrial apoptotic pathway, which might give an insight into the anti-tumor effect and mechanism of b-AP15 in ESCC.

Acknowledgements And the authors would also like to thank those who contributed their tissue samples to support scientific research in medical fields.

Authors' contributions Conceptualization, Tao Hu and Pei Li; Methodology, Beibei Sha, Xiaoyu Chen, Miaomiao Li and Longhao Wang; Validation, Beibei Sha, Xiaoyu Chen, Miaomiao Li, Longhao Wang and Han Wu; Results interpretation, Beibei Sha, Xiaoyu Chen, Miaomiao Li and Xingge Liu; Manuscript preparation: Ping Chen and Jianxiang Shi; Supervision, Tao Hu and Pei Li; Project administration, Tao Hu and Pei Li; Funding acquisition, Ping Chen, Tao Hu and Pei Li.

Funding This research was funded by the National Natural Science Foundation Grant of China (Grant Nos. 81001102, 81101894, 81672421, U1604189), Natural Science Foundation of Henan Province (Grant No. 162300410302), Outstanding Young Talent Research Fund of Zhengzhou University (Grant Nos. 51999223, 32210449) and Program for Science & Technology Innovation Talents in Universities of Henan Province (Grant No. 18HASTIT046).

Data availability Data sharing not applicable to this article as no datasets were generated or analyzed during the current study. Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Ethical approval All experiments were approved by the Institutional Review Board of Zhengzhou University.

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