Glutathione peroxidase 2 overexpression promotes malignant progression and cisplatin resistance of KRAS-mutated lung cancer cells

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Abstract. Kirsten rat sarcoma viral oncogene homolog (KRAS) aberrations frequently occur in patients with lung cancer. Oncogenic KRAS is characterized by excessive reactive oxygen species (ROS) accumulation, thus, ROS detoxification may contribute to KRAS-driven lung tumorigenesis. In the present study, the influence of glutathione peroxidase 2 (GPX2) on malignant progression and cisplatin resistance of KRAS-driven lung cancer was explored. The RNA sequencing data from TCGA lung cancer samples and GEO database were downloaded and analyzed. The effects of GPX2 on KRAS-driven lung tumorigenesis were evaluated by western blotting, cell viability assay, soft agar assay, Transwell assay, tumor xenograft model, flow cytometry, BrdU incorporation assay, transcriptome RNA sequencing, luciferase reporter assay and RNA immunoprecipitation. In the present study, GPX2 was upregulated in patients with non-small cell lung carcinoma (NSCLC), and positively correlated with poor overall survival. Ectopic GPX2 expression facilitated malignant progression of KRAS G12C-transformed BEAS-2B cells. Moreover, GPX2 overexpression promoted growth, migration, invasion, tumor xenograft growth and cisplatin resistance of KRAS-mutated NSCLC cells, while GPX2 knockdown exhibited the opposite effects. GPX2 overexpression reduced ROS accumulation and increased matrix metalloproteinase-1 (MMP1) expression in KRAS-mutated NSCLC cells. In addition, GPX2 was directly targeted by miR-325-3p, while MMP1 knockdown or miR-325-3p overexpression partially abrogated the effects of GPX2 in NSCLC cells. In conclusion, the results indicated that GPX2 facilitated malignant progression and cisplatin resistance of KRAS-driven lung cancer, and inhibition of GPX2 may be a feasible strategy for lung cancer treatment, particularly in patients with active KRAS mutations.

Introduction

Lung cancer is the second most common cancer and the leading cause of cancer-related deaths worldwide (1). It is estimated that there were 2.2 million newly diagnosed lung cancer cases (11.4% of all new cancer cases) and 1.8 million lung cancer-related deaths (18.0% of all cancer deaths) all over the world in 2020 (1). Tobacco use, occupational exposures to carcinogens, history of respiratory diseases such as chronic obstructive pulmonary disease (COPD) are common risk factors (2). Lung cancer can be divided into two categories: Non-small cell lung carcinoma (NSCLC) and small-cell lung carcinoma (SCLC). NSCLC accounts for nearly 85% of all lung cancers, among which 40% are adenocarcinoma, 25-30% squamous cell carcinoma, and 10-15% large cell carcinomas (3,4). Frequently occurred genetic alternations of NSCLC include aberrations in TP53, EGFR, Kirsten rat sarcoma viral oncogene homolog (KRAS), FGFR1, PTEN, ROS1, ERBB2, BRAF and ALK (5,6). KRAS is one of the most frequently mutated oncogenic drivers for NSCLC, particularly for lung adenocarcinoma (7). At present, only a few treatments are available for KRAS-mutated patients with NSCLC, and there is an urgent need to explore the molecular vulnerability of KRAS-driven lung cancer.

KRAS mutations occur in ~20-40% of lung adenocarcinomas. Unlike other druggable aberrations in EGFR and ERBB2, and rearrangements of ALK and RET in lung cancer, KRAS aberrations have been historically described as ‘undruggable’ targets (8). Recent findings suggest that KRAS<sup>G12C</sup> mutation can be selectively inhibited by a covalent G12C-specific inhibitor ARS-1620, but this is limited to the subset of KRAS<sup>G12C</sup>-driven lung cancers (9). Oncogenic KRAS is characterized by induction of ROS, a key step for cellular transformation and tumorigenesis (10). However, excessive ROS production causes oxidative stress, which is deleterious to
cells. Thus, it is no surprise that ROS detoxification is important for KRAS-driven lung tumorigenesis. For example, inactive mutations of Keap1 are prone to occur in KRAS-mutated lung adenocarcinomas. Loss of Keap1 facilitates KRAS-driven lung tumorigenesis via activating NRF2 (11). Therefore, it is hypothesized that genes involved in ROS detoxification may facilitate KRAS-driven lung tumorigenesis via reduction of ROS accumulation.

Glutathione peroxidase 2 (GPX2) is a member of the glutathione peroxidase family. As a key enzyme of the glutathione peroxidase family, GPX2 plays an important role in alleviating cellular damage caused by oxidative stress. There is increasing evidence demonstrating that GPX2 also has a role in tumorigenesis. GPX2 is upregulated in a variety of cancers, including breast (12), liver (13), and bladder cancer (14). Furthermore, silencing of GPX2 leads to growth inhibition and accumulation of ROS in castration-resistant prostate cancer (15). GPX2 knockdown suppresses migration, invasion and metastasis of liver cancer cells in vitro and in vivo (13). GPX2 is also upregulated in patients with lung cancer (16). However, it is not certain whether GPX2 is involved in KRAS-driven lung tumorigenesis. In the present study, the potential functions of GPX2 were evaluated in KRAS<sup>G12C</sup>-transformed BEAS-2B cells and KRAS-mutated NSCLC cells. It was determined that GPX2 was upregulated in patients with NSCLC and promoted malignant progression of KRAS<sup>G12C</sup>-transformed BEAS-2B cells. Moreover, GPX2 overexpression facilitated proliferation, migration, invasion, tumor growth and cisplatin resistance of KRAS-mutated NSCLC cells, while knockdown of GPX2 exhibited the opposite effects. GPX2 was directly targeted by microRNA (miRNA or miR)-325-3p, and overexpression of miR-325-3p abolished the effects of GPX2 in NSCLC cells. The present study elucidated a novel role of GPX2 in KRAS-driven lung tumorigenesis.

Materials and methods

**Patient samples.** The present study was approved (approval no. CY20160325) by the Ethics Committee of The First Affiliated Hospital of Chongqing Medical University (Chongqing, China). Written informed consents were obtained from all enrolled patients. A total of 120 human NSCLC samples and paired adjacent non-tumor tissues were collected from March 2016 to September 2017 at The First Affiliated Hospital of Chongqing Medical University. There was no significant difference between the sex and ages of patients with NSCLC. No patients received preoperative chemo- or radiotherapy before surgery. Tumor grades and stages were determined according to the guidance of World Health Organization (WHO) and TNM classification of the International Union Against Cancer (UICC). The clinical information of patients with NSCLC was retrieved from the hospital database. Patients were followed up to 48 months post-surgery.

**Cell culture and reagents.** NSCLC cell lines NCIH1385 (ATCC no. CRL-5867), NCIH1573 (ATCC no. CRL-5877), A549 (ATCC no. CCL-185), NCIH358 (ATCC no. CRL-5807), SW1573 (ATCC no. CRL-2170), NCIH2291 (ATCC no. CRL-5939), NCIH1792 (ATCC no. CRL-5895) and NCIH23 (ATCC no. CRL-5800), and an immortalized but non-tumorigenic human bronchial epithelial cell line BEAS-2B (ATCC no. CRL-9609) were purchased from American Type Culture Collection (ATCC). All cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Hyclone; Cytiva) at 37˚C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cisplatin (cat. no. S1166; Selleck Chemicals) was dissolved in phosphate-buffered saline (PBS), thus PBS was used as a vehicle control.

**Plasmid constructs and lentivirus packaging.** KRAS<sup>G12C</sup> mutant was cloned from SW1573 cells and inserted into the pCDH lentivirus vector (cat. no. CD510B-1; System Biosciences, LLC). The primers for the KRAS<sup>G12C</sup> mutant were: KRAS forward, 5'-GCCCTAGCTAGCCACCATGACTGAA TATAAACCTGTTGATGATG-3' and reverse, 5'-ATAAGAATGCGGCCCGACCATGATTATGCCTTAA-3'. GPX2 expression lentiviral vector was constructed by inserting the coding sequence of GPX2 into the pCDH lentiviral vector. The empty pCDH lentiviral vector was used as the empty vector (EV) control. The primers for GPX2 cloning were: GPX2 forward, 5'-GCCCTAGCTAGCCACCATGATTATGCCTTAA-3' and reverse, 5'-ATAAGAATGCGGCCCGACCATGATTATGCCTTAA-3'. To knock down GPX2 or matrix metalloproteinase-1 (MMP1), short hairpin RNAs (shRNAs) targeting GPX2 (sh#1 and sh#2) or MMP1 (sh#MMP1-1 and sh#MMP2) were cloned into the pLKO.1 plasmid (Sigma-Aldrich; Merck KGaA). The pLKO.1 plasmid inserted with a non-targeting sequence was used as a negative control (sh#NC). MiR-325-3p expression vector was constructed by inserting the mature sequence of miR-325-3p (5'-AACUAUCUUCCAGGAGUAAUU-3') into pCMV-MIR vector (cat. no. PMVimir; OriGene Technologies, Inc.). The empty pCMV-MIR vector was used as the miR-ctrl. Virus particles were produced from 293T cells (ATCC no. CRL-3216) by co-transfecting target plasmids (5 µg) with lentiviral-packaging plasmids psPAX2 (3 µg) and pMD2.G (2 µg) of the 3rd generation system using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Virus particles were collected at 24, 48 and 72 h post-transfection. For virus infection, cells were incubated with virus particles overnight at 37˚C supplemented with 8 µg/ml polybrene (Sigma-Aldrich; Merck KGaA). The multiplicity of infection (MOI) was 10/1. The stable cell lines were used for subsequent experiments 72 h later at least. The shRNA sequences were: sh#1, 5'-TCTTTAAGTTGCATTATAGATG-3' and reverse, 5'-TCTTTAAGTTGCATTATAGATG-3'; sh#2, 5'-TCTTTAAGTTGCATTATAGATG-3' and reverse, 5'-TCTTTAAGTTGCATTATAGATG-3'; sh#MMP1-1, 5'-TCTTTAAGTTGCATTATAGATG-3' and reverse, 5'-TCTTTAAGTTGCATTATAGATG-3'; sh#MMP1-2, 5'-TCTTTAAGTTGCATTATAGATG-3' and reverse, 5'-TCTTTAAGTTGCATTATAGATG-3'; sh#MMP2, 5'-TCTTTAAGTTGCATTATAGATG-3' and reverse, 5'-TCTTTAAGTTGCATTATAGATG-3'.

**Transcriptome RNA-sequencing and data analysis.** Total RNA was extracted using TRIzol reagent (Takara Bio, Inc.). For transcriptome RNA sequencing, the mRNA sequencing libraries were generated by NEB Next Ultra RNA Library Prep Kit (Illumina, Inc.). A total of 20 µl of the library was sequenced on a HiSeq 2000 platform using the HiSeq Sequencing Kit (200 cycles; cat. no. FC-401-1001; Illumina Inc.) A total of 50-bp single-end sequenced reads were sequenced on a HiSeq 2000 platform using the HiSeq Transcriptome RNA-sequencing and data analysis. Total RNA was extracted using TRIzol reagent (Takara Bio, Inc.). For transcriptome RNA sequencing, the mRNA sequencing libraries were generated by NEB Next Ultra RNA Library Prep Kit (Illumina, Inc.). A total of 20 µl of the library was sequenced on a HiSeq 2000 platform using the HiSeq Sequencing Kit (200 cycles; cat. no. FC-401-1001; Illumina Inc.) A total of 50-bp single-end sequenced reads were sequenced on a HiSeq 2000 platform using the HiSeq Transcriptome RNA-sequencing and data analysis. Total RNA was extracted using TRIzol reagent (Takara Bio, Inc.). For transcriptome RNA sequencing, the mRNA sequencing libraries were generated by NEB Next Ultra RNA Library Prep Kit (Illumina, Inc.). A total of 20 µl of the library was sequenced on a HiSeq 2000 platform using the HiSeq Sequencing Kit (200 cycles; cat. no. FC-401-1001; Illumina Inc.) A total of 50-bp single-end sequenced reads were sequenced on a HiSeq 2000 platform using the HiSeq Transcriptome RNA-sequencing and data analysis. Total RNA was extracted using TRIzol reagent (Takara Bio, Inc.). For transcriptome RNA sequencing, the mRNA sequencing libraries were generated by NEB Next Ultra RNA Library Prep Kit (Illumina, Inc.). A total of 20 µl of the library was sequenced on a HiSeq 2000 platform using the HiSeq Sequencing Kit (200 cycles; cat. no. FC-401-1001; Illumina Inc.) A total of 50-bp single-end sequenced reads were sequenced on a HiSeq 2000 platform using the HiSeq Transcriptome RNA-sequencing and data analysis. Total RNA was extracted using TRIzol reagent (Takara Bio, Inc.). For transcriptome RNA sequencing, the mRNA sequencing libraries were generated by NEB Next Ultra RNA Library Prep Kit (Illumina, Inc.). A total of 20 µl of the library was sequenced on a HiSeq 2000 platform using the HiSeq Sequencing Kit (200 cycles; cat. no. FC-401-1001; Illumina Inc.) A total of 50-bp single-end sequenced reads were sequenced on a HiSeq 2000 platform using the HiSeq Transcriptome RNA-sequencing and data analysis. Total RNA was extracted using TRIzol reagent (Takara Bio, Inc.). For transcriptome RNA sequencing, the mRNA sequencing libraries were generated by NEB Next Ultra RNA Library Prep Kit (Illumina, Inc.). A total of 20 µl of the library was sequenced on a HiSeq 2000 platform using the HiSeq Sequencing Kit (200 cycles; cat. no. FC-401-1001; Illumina Inc.) A total of 50-bp single-end sequenced reads were
filtered by RNA-BisSeq method (17), mapped to hg19 genome using HISAT2 (https://ccb.jhu.edu/software/hisat/index.shtml), and evaluated by Hiseq sequencer. Differentially expressed genes were analyzed by Limma package (version, 3.40.2) of R software (https://bioconductor.org/packages/release/bioc/html/limma.html). All samples were assessed in triplicate.

TCGA, GTEx and GEO data analysis. The RNA sequencing raw data for patients with lung cancer were downloaded from The Cancer Genome Atlas (TCGA), Genotype-Tissue Expression Project (GTEx) and Gene Expression Omnibus (GEO) [GSE32863 (18), GSE40791 (19), GSE75037 (20) and GSE101929 (21)]. PRADA tool (22) and HtSeq V0.6.1 (23) were used to analyze the sequencing data. The differentially expressed genes were evaluated by Limma package (version, 3.40.2) of R software. The expression was calculated using the 2ΔΔCq method (24) and normalized to U6 or GAPDH. The qPCR cycling conditions were as follows: Denaturation at 95°C for 30 sec; and 40 cycles at 95°C for 5 sec and 60°C for 30 sec. The primer sequences were: GPX2 sense, 5'-TCTCTACTCTCATCGATGC-3' and antisense, 5'-TGGATACCAACAGGAG-3'; MMP1 sense, 5'-AGATGTGGAGTGCCTGAT-3' and antisense, 5'-CAGGACGCTTTGTGATATG-3'; GAPDH sense, 5'-TGACCACCACTGCTGAC-3' and antisense, 5'-GGATGAGCAG-3'; U6 sense, 5'-CGCCTCGAGAGCAGCA-3' and antisense, 5'-CGGCCTCGAGACCTTTGCA-3'. Each sample was assessed in triplicate.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA. Complementary DNA (cDNA) was synthesized by PrimeScript RT reagent Kit (Takara Bio, Inc.). The expression level of miR-325-3p and p-Akt (Ser473) rabbit mAb (product no. 4060; all 1:1,000; all from Cell Signaling Technology, Inc.), anti-GAPDH antibody (product code ab28245, 1:2,000), anti-GPX2 antibody (product code ab40130; 1:500), anti-MMP1 antibody (1:500; product code ab52631; Abcam) and anti-Ago (product code ab279392; 1:500; all from Abcam). The secondary antibodies were anti-rabbit IgG HRP-linked antibody (product no. 7074; 1:3,000) and anti-mouse IgG HRP-linked antibody (product no. 96714; 1:5,000) (HRP conjugate) both from Cell Signaling Technology, Inc.

Cell viability assay. Cell viability was assessed using Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology) according to manufacturers' instructions. In brief, BEAS-2B, SW2573, NCIH1792, A549 and NCIH1385 cells (2,500/well) were seeded in 96-well plates and cultured for 1, 3, 5 and 7 days. To evaluate the IC50 of cisplatin, SW2573, NCIH1792, A549 and NCIH1385 cells (2,500/well) were seeded in 96-well plates and treated with 0, 0.63, 1.25, 2.5, 5, 10, 20, 40, 80 and 160 μM cisplatin for 6 days. Next, CCK-8 reagent (10 μl) was added to each well and incubated for 1 h at 37°C. The absorbance at 450 nm was determined by a microplate reader. Each sample was assessed in triplicate.

Soft agar assay. A soft agar assay was performed as previously reported (25). Briefly, cells (10,000/well) were seeded in 0.35% top agar in 6-well plates. The bottom agar was 0.6%. Cells were cultured for 3 weeks, then stained with 0.5 mg/ml MTT (Sigma-Aldrich; Merck KGaA). The absorbance was measured using a microplate reader. The absorbance at 450 nm was determined by a microplate reader. Each sample was assessed in triplicate.

Transwell migration and invasion assays. For the Transwell migration assay, BEAS-2B (7.5x104), SW2573 (5x104), NCIH1792 (7.5x104), A549 (5.2x104) or NCIH1385 cells (7.5x104) were seeded in 500 μl serum free medium and added into a Boyden chamber (8-μm pore size; MilliporeSigma). The chamber was then placed into a 24-well plate filled with 500 μl culture medium containing 10% FBS. Cells were allowed to migrate for 24-48 h at 37°C, then fixed using 4% paraformaldehyde for 10 min at room temperature and stained with 0.5% crystal violet for 20 min at room temperature. Images were captured using a light microscope (Leica Microsystems GmbH). For the Transwell invasion assay, the Boyden chamber was precoated with Matrigel for 30 min at room temperature (BD Biosciences).

Western blotting. Cell lysates were prepared using RIPA buffer (Beyotime Institute of Biotechnology) supplemented with protease inhibitors (Sigma-Aldrich; Merck KGaA). Protein concentration was determined by Quick Start™ Bradford Protein Assay kit. A total of 20 μg proteins were resolved on 8-12% SDS-PAGE gels and transferred to PVDF membranes. Non-specific bindings were blocked by 5% skim milk for 1 h at room temperature. The membranes were then incubated with specific primary antibodies at 4°C overnight and corresponding secondary antibodies at room temperature for 1 h. The bands were detected using a Bio-Rad ChemiDoc XRS system (Bio-Rad Laboratories, Inc.) using the ECL kit (cat. no. RPN2232; Amersham; Cytiva). The specific antibodies were: Anti-KRAS antibody (product code ab275876; 1:500; Abcam), p44/42 MAPK (Erk1/2) rabbit mAb (product no. 4695), phosphorylated (p)-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) rabbit mAb (product no. 4370), Akt antibody (product no. 9272), and p-Akt (Ser473) rabbit mAb (product no. 4060; all 1:1,000; all from Cell Signaling Technology, Inc.), anti-GAPDH antibody (product code ab28245, 1:2,000), anti-GPX2 antibody (product code ab40130; 1:500), anti-MMP1 antibody (1:500; product code ab52631; Abcam) and anti-Ago (product code ab279392; 1:500; all from Abcam). The secondary antibodies were anti-rabbit IgG HRP-linked antibody (product no. 7074; 1:3,000) and anti-mouse IgG HRP-linked antibody (product no. 96714; 1:5,000) (HRP conjugate) both from Cell Signaling Technology, Inc.

Tumor xenograft model. Animal studies were conducted according to the protocol approval (approval no. CY20160325) by the Institutional Animal Care and Use Committee of The First Affiliated Hospital of Chongqing Medical University. For animal studies, BEAS-2B cells were transduced with EV, GPX2 and KRASG12C lentivirus as indicated, SW1573 cells were transduced with EV and GPX2 lentivirus, while A549 cells were transduced with sh#1 and sh#NC lentivirus. Next, BEAS-2B cells (2x106) were subcutaneously injected into 16 six-week-old female BALB/c nude mice. SW1573 cells (2x106) were subcutaneously injected into 10 six-week-old female BALB/c nude mice. The average weight of the mice was 20 g. Following cell injection, the mice were randomly divided
into groups. The mice were housed in individually ventilated cages under specific pathogen-free conditions under a 12-h light/dark cycle, 20-26°C and 50-80% humidity. Mice were allowed access to sterilized water and feed ad libitum. Tumor xenografts were allowed to grow for 4 weeks. The tumor volume was measured every three days using a caliper and calculated by the formula: (length x width²)/2. At the end of the experiment, the mice were anaesthetized using 3% isoflurane and sacrificed by cervical dislocation. The tumor xenografts were then dissected out and weighed.

The maximum tumor volume in the study was <2,000 mm³, and the maximum tumor diameter in the study was <2 cm.

Assessment of ROS levels and NADPH/NADP⁺ expression. The ROS levels were assessed using Cellular Reactive Oxygen Species Detection Assay Kit (Abcam) according to the manufacturer’s instructions. Briefly, BEAS-2B, SW2573 and NCIH1792 cells were incubated with 20 µM 2',7'-dichlorodihydrofluorescein diacetate for 30 min at 37°C. The oxidized fluorescent compound dichlorofluorescein (DCF) was measured using a FACScan flow cytometer (BD Biosciences) with an excitation wavelength at 488 nm and an emission wavelength at 535 nm. Data was analyzed using Flowjo 6.7 software (BD Biosciences). NADPH/NADP⁺ expression was evaluated by NADPH/NADP-Glo Assay Kit (cat. no. G9081; Promega Corporation) according to manufacturer’s instructions. Each sample was assessed in triplicate.

Bromodeoxyuridine (BrdU) incorporation assay. BEAS-2B, SW2573, NCIH1792, A549 or NCIH1385 cells were incubated with 10 µmol/l BrdU for 4 h at 37°C. The cells were then stained with BrdU Mouse mAb (product no. 5292; 1:200; Cell Signaling Technology, Inc.) at 4°C overnight and goat anti-mouse IgG Alexa Flour 488 conjugated (product code ab238004; 1:300; both from Abcam) at room temperature for 1 h. DAPI (Sigma-Aldrich; Merck) was used to stain the nucleus. Images were obtained using Olympus FV1000 confocal microscopy.

Cell apoptosis analysis. SW1573 and NCIH1792 cells introduced with GPX2 or EV lentivirus, or A549 and NCIH1385 cells introduced with sh#1, sh#2 or sh#NC lentivirus were treated with 2.5 or 10 µM cisplatin for 3 days, and stained with Annexin V-FITC for flow cytometry as follows. In brief, SW2573, NCIH1792, A549 or NCIH1385 cells (1x10⁶) were dispersed as single cell suspension using 0.5% trypsin ( Gibco; Thermo Fisher Scientific, Inc.), and then stained with Annexin-V-FITC (BD Biosciences) and propidium iodide (BD Biosciences) at room temperature for 15 min avoiding light. The apoptotic cells were measured by a FACScan flow cytometer (BD Biosciences) with an excitation wavelength at 488 nm and an emission wavelength at 530 nm. Data was analyzed using Flowjo 6.7 software (BD Biosciences). Each sample was assessed in triplicate.

Luciferase reporter assay. TargetScanHuman 7.2 (https://www.targetscan.org/vert_72/) was used to predict conservative miRNA binding sites for GPX2. The 3'UTR of GPX2 containing the predicted binding sites for miR-325-3p was cloned into the pMIR-REPORT plasmid (GPX2 wt). The binding sites were mutated by Quickchange site-directed mutagenesis kit (Agilent Technologies, Inc.) to generate GPX2 mut vector. Then GPX2 wt or GPX2 mut, miR-325-3p expression vector or miR-ctrl, and a Renilla luciferase plasmid were co-transfected into A549 and NCIH1385 cells at a ratio of 2:1 using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). A Dual Luciferase Reporter Assay System (Promega Corporation) was used to measure luciferase activity at 48 h post-transfection via comparison with Renilla luciferase activity. Each sample was assessed in triplicate.

RNA immunoprecipitation (RIP). Magna RIP RNA-Binding Protein Immunoprecipitation Kit (cat. no. 17-700; Sigma-Aldrich; Merck KGaA) was used for the RIP assay. Briefly, A549 or NCIH1385 cells (1x10⁶) were lysed in RIP lysis buffer (Beyotime Institute of Biotechnology) on ice for 30 min, and then supernatant was incubated with 30 µl of Protein-A/G agarose beads (Roche Diagnostics) supplemented with 2 µg anti-Agol (product code ab279392; 1:300) or anti-IgG (product code ab28004; 1:300; both from Abcam) at 4°C overnight. The beads were washed 5 times with RIP washing buffer (20 mM Tris-Cl pH 7.4, 150 mM NaCl, and 0.5% NP-40), and centrifuged at 2,000 x g for 1 min at 4°C. The bounded proteins were boiled with 1X SDS loading buffer and analyzed by western blotting, and immunoprecipitated RNAs were analyzed by RT-qPCR.

Statistical analysis. GraphPad Prism 8.0 (GraphPad Software, Inc.) was used for statistical analysis. Data were expressed as the mean ± standard deviation (x ± SD). Differences between two groups were evaluated using unpaired Student’s t-test. Differences between three or more groups were analyzed by one-way ANOVA followed by LSD post hoc test. The half maximal inhibitory rate (IC₅₀) of cisplatin was measured using GraphPad Prism 8.0. Overall survival was evaluated by Kaplan-Meier method (with LSD post hoc test). Pearson correlation analysis was used to evaluate the correlation between miR-325-3p and GPX2 expression in patients with NSCLC. P-values <0.05 were considered to indicate statistically significant differences.

Results

GPX2 is upregulated in patients with NSCLC. To search for potential genes enrolled in the tumorigenesis of NSCLC, the data derived from TCGA NSCLC database and GTEx were analyzed. A total of 289 upregulated genes and 575 downregulated genes were revealed in patients with NSCLC, which were depicted in volcano map (Fig. 1A). Among them, GPX2 was ranked in the top 10 upregulated genes (Fig. 1A and Table SI). GPX2 was significantly upregulated in patients with NSCLC (Fig. 1B). This was further confirmed using the GEO database (GSE32863, GSE40791, GSE75037 and GSE101929) (Fig. 1C). In addition, GPX2 exhibited no association with tumor stages, suggesting that upregulation of GPX2 may be an early event for lung tumorigenesis (Fig. 1D). As GPX2 is a key enzyme of the glutathione redox system (12,13), it was hypothesized that GPX2 may be involved in KRAS-driven lung tumorigenesis via reduction of ROS accumulation. KRAS mutations are predominately accumulated in lung adenocarcinoma (8,9),
thus the expression of GPX2 was evaluated in patients with lung adenocarcinoma. The data from TCGA lung adenocarcinoma database indicated that GPX2 was upregulated in patients with lung adenocarcinoma, particularly those with KRAS mutations (Fig. 1E). In addition, high GPX2 expression was associated with poor overall survival of patients with lung adenocarcinoma (Fig. 1F). The aforementioned results were analyzed from the TCGA, GTEx and GEO data bases. To confirm this, GPX2 expression was also assessed in a cohort of 120 lung adenocarcinoma patients in the present study. The result revealed that GPX2 was evidently upregulated in patients with lung adenocarcinoma (Fig. 1G). The patients with lung adenocarcinoma were divided into a high- or low-GPX2 expression group by using the median expression as the cut-off value. The data confirmed that high GPX2 expression was associated with poor prognosis of patients with lung adenocarcinoma (Fig. 1H). The expression of GPX2 was further evaluated in KRAS-mutated lung cancer cell lines. GPX2 was highly expressed in NCIH1385, NCIH1573 and A549 cells, and expressed at a low level in NCIH1792 and NCIH23 cells (Fig. 1I). Taken together, the aforementioned results indicated that GPX2 was upregulated in patients with NSCLC, particularly those with KRAS mutations.

**Forced GPX2 expression promotes KRAS\textsuperscript{G12C}-driven lung tumorigenesis.** The potential functions of GPX2 in KRAS-driven lung tumorigenesis were evaluated by gain-of-function assays. BEAS-2B is an immortalized but non-tumorigenic epithelial cell line derived from human bronchial epithelium. KRAS\textsuperscript{G12C} is the most commonly occurring KRAS mutation in lung cancer, accounting for as...
much as 40% of all KRAS aberrations (26,27). To evaluate the influence of GPX2 on KRAS-driven lung tumorigenesis, BEAS-2B cells were introduced with KRASG12C expression lentivirus or EV control, and then lysates were collected for western blotting. (B) BEAS-2B cells (2,500/well) transduced with indicated vectors were seeded in 96-well plates, and then cell viability assays were conducted at days 0, 2, 4 and 6. (C and D) BEAS-2B cells (10,000/well) transduced with indicated vectors were seeded in 6-well plates for soft agar assays. (E) Representative images and (D) average number of colonies per well were shown. Scale bar, 500 µm. (E and F) BEAS-2B cells transduced with indicated vectors were used for Transwell migration and invasion assays. (E) Representative images and (F) relative migration or invasion cells are shown. Scale bar, 50 µm. (G-I) BEAS-2B cells transduced with the indicated vectors were presented. *P<0.05. GPX2, glutathione peroxidase 2; KRAS, Kirsten rat sarcoma viral oncogene homolog; EV, empty vector; ROS, reactive oxygen species; DCF, dichlorofluorescein.

Figure 2. Forced GPX2 expression promotes KRASG12C-driven lung tumorigenesis. (A) BEAS-2B cells were transduced with KRASG12C mutant, GPX2 expression lentivirus or EV control, and then lysates were collected for western blotting. (B) BEAS-2B cells (2,500/well) transduced with indicated vectors were seeded in 96-well plates, and then cell viability assays were conducted at days 0, 2, 4 and 6. (C and D) BEAS-2B cells (10,000/well) transduced with indicated vectors were seeded in 6-well plates for soft agar assays. (C) Representative images and (D) average number of colonies per well were shown. Scale bar, 500 µm. (E and F) BEAS-2B cells transduced with indicated vectors were used for Transwell migration and invasion assays. (E) Representative images and (F) relative migration or invasion cells are shown. Scale bar, 50 µm. (G-I) BEAS-2B cells transduced with the indicated vectors were presented. *P<0.05. GPX2, glutathione peroxidase 2; KRAS, Kirsten rat sarcoma viral oncogene homolog; EV, empty vector; ROS, reactive oxygen species; DCF, dichlorofluorescein.
was analyzed by flow cytometry. KRAS<sup>G12C</sup>-transformed BEAS-2B cells exhibited an increased level of oxidized DCF compared with non-transformed BEAS-2B cells, but this was relieved by GPX2 overexpression (Fig. 2J and K). Moreover, GPX2 significantly increased the ratio of NADPH/NADP<sup>+</sup> in KRAS<sup>G12C</sup>-transformed BEAS-2B cells (Fig. 2L). Collectively, the results indicated that forced GPX2 expression promoted GPX2<sup>G12C</sup>-driven lung tumorigenesis, and this may be due to the alleviation of KRAS-induced oxidative stress.

**GPX2 overexpression facilitates malignant progression and cisplatin resistance of KRAS-mutated NSCLC cells.**

The influence of GPX2 on the malignant properties of KRAS-mutated NSCLC cells was evaluated. SW1573 and NCIH1792 exhibited low endogenous levels of GPX2 and oncogenic KRAS mutations. These two cell lines were selected for gain-of-function assays in the present study. Forced GPX2 expression successfully upregulated the protein levels of GPX2 in SW1573 and NCIH1792 cells (Fig. 3A). In the cell viability assay, GPX2 overexpression increased the cell growth of SW1573 and NCIH1792 cells (Fig. 3B). In the BrdU incorporation assay, GPX2 overexpression increased the number of BrdU-positive cells compared with the EV control (Fig. 3C and D). In the Transwell assay, GPX2 overexpression increased the number of migrated and invasive SW1573 and NCIH1792 cells (Fig. 3E and F). In addition, forced GPX2 expression promoted tumor xenograft growth of SW1573 cells in nude mice (Fig. 3G-I). Cisplatin is a chemotherapeutic drug known to induce cell death by producing excessive ROS, and ROS elimination has been demonstrated to confer cisplatin resistance (28). In the present study, GPX2 overexpression increased the IC<sub>50</sub> value of cisplatin in SW1573 and NCIH1792 cells (Fig. 3J). Flow cytometric analysis indicated that GPX2 overexpression reduced the number of apoptotic cells induced by cisplatin treatment (Fig. 3K and L). The ROS levels were further evaluated. GPX2 overexpression significantly reduced the levels of oxidized DCF in SW1573 and NCIH1792 cells (Fig. 3M and N). In addition, SW1573 and NCIH1792 cells overexpressed with GPX2 had higher NADPH/NADP<sup>+</sup> ratios (Fig. 3O). Collectively, the data indicated that GPX2 overexpression facilitated malignant progression and cisplatin resistance of KRAS-mutated NSCLC cells.

**Knockdown of GPX2 suppresses malignant progression and increases platinum sensitivity of KRAS-mutated NSCLC cells.**

The potential influence of GPX2 on KRAS-mutated NSCLC cell lines was further evaluated by loss-of-function assays. A549 and NCIH1385 cells exhibited high endogenous GPX2 levels and oncogenic KRAS mutations, thus GPX2 was depleted in these cells using GPX2 specific shRNAs (sh#1 and sh#2). The knockdown efficiency was validated by western blotting (Fig. 4A). Next, the influence of GPX2 knockdown was evaluated. In the cell viability assay, knockdown of GPX2 suppressed the growth of A549 and NCIH1385 cells (Fig. 4B). This was further demonstrated by BrdU incorporation assay, as depletion of GPX2 reduced the number BrdU-positive cells in A549 and NCIH1385 cell lines (Fig. 4C and D). In the Transwell migration and invasion assays, GPX2 knockdown markedly reduced the number of migrated and invasive A549 and NCIH1385 cells (Fig. 4E and F). To evaluate the influence of GPX2 knockdown in vivo, A549 cells were transduced with GPX2 specific shRNA and subcutaneously injected into nude mice. The data indicated that GPX2 knockdown impaired tumor growth of A549 cells, with reduced tumor volumes and weights (Fig. 4G-I). The effects of GPX2 knockdown on cisplatin sensitivity of A549 and NCIH1385 cells were evaluated. It was determined that depletion of GPX2 reduced the IC<sub>50</sub> values of cisplatin (Fig. 4J). Moreover, GPX2 knockdown significantly increased the number of apoptotic cells in A549 and NCIH1385 following cisplatin treatment (Fig. 4K and L). Taken together, the results indicated that knockdown of GPX2 suppressed the malignant progression and increased the platinum sensitivity of KRAS-mutated NSCLC cells.

**Knockdown of MMP1 abolishes the effects of GPX2 in KRAS-mutated NSCLC cells.**

There is increasing evidence indicating that antioxidants can promote metastasis of lung tumors (29,30). In the present study, GPX2 overexpression promoted the migration and invasion of KRAS<sup>G12C</sup>-transformed BEAS-2B cells and KRAS-mutated NSCLC cells, corresponding with previous studies (29,30). To explore the potential downstream targets which were influenced, GPX2 was overexpressed in SW1573 cells and subjected to transcriptome RNA sequencing. A total of 110 dysregulated genes were identified (Fig. 5A and Table SII). Among them, MMP1 was significantly upregulated by GPX2 overexpression (Fig. 5A). This was further validated by RT-qPCR and western blotting. GPX2 overexpression increased the mRNA and protein expression of MMP1 in SW1573 and NCIH1792 cells (Fig. 5B and C). MMP1 has been implicated in the migration and invasion of lung cancer cells (31,32). Thus, it was hypothesized that GPX2 may enhance the migration and invasion of NSCLC cells partially through the upregulation of MMP1. To confirm this, MMP1 was knocked down by MMP1 specific shRNAs (sh#MMP1-1 and sh#MMP1-2) in NSCLC cells (Fig. 5D). In the Transwell assay, GPX2 significantly increased the number of migrated and invasive SW1573 and NCIH1792 cells, but this was completely abrogated by MMP1 knockdown (Fig. 5E and F). Collectively, the results indicated that knockdown of MMP1 abolished the effects of GPX2 in KRAS-mutated NSCLC cells.

**GPX2 is directly targeted by miR-325-3p.**

MiRNAs are small non-coding RNAs that can promote mRNA degradation by base pairing with target mRNAs (33). Accumulated studies indicate that miRNAs are dysregulated in lung cancer and play important roles in lung tumorigenesis (34). In the present study, it was hypothesized that miRNAs may regulate GPX2 expression in NSCLC cells to some extent. TargetScanHuman 7.2 was used to predict conservative miRNA binding sites for GPX2. In the present study, GPX2 was identified to have conservative binding sites for miR-325-3p (Fig. 6A). The interaction between miR-325-3p and GPX2 in NSCLC cells was evaluated by luciferase reporter assay. MiR-325-3p overexpression significantly reduced the luciferase activity of GPX2 in A549 and NCIH1385 cells compared with miR-ctrl (Fig. 6B). In addition, forced miR-325-3p expression markedly reduced GPX2 expression in A549 and NCIH1385 cells.
RIP assays were conducted to evaluate mRNA enrichment by the Ago/RNA‑induced silencing (RISC) complex after miR‑325‑3p overexpression. The Ago/RISC complex was successfully pulled down by Pan‑Ago antibody (Fig. 6D). The expression level of miR‑325‑3p was upregulated while GPX2 was downregulated in the Ago/RISC complex following miR‑325‑3p overexpression (Fig. 6E). In addition, miR‑325‑3p expression was negatively correlated with GPX2 in patients with NSCLC (Fig. 6F). Collectively, the results indicated that GPX2 was directly targeted by miR‑325‑3p in NSCLC cells.

Figure 3. GPX2 overexpression facilitates malignant progression and cisplatin resistance of KRAS‑mutated NSCLC cells. (A) SW1573 and NCIH1792 cells were introduced with GPX2 or EV lentivirus, and then lysates were collected for western blotting. (B) SW1573 and NCIH1792 cells (2.5×10⁵/well) introduced with GPX2 or EV lentivirus were seeded in 96‑well plates, and then cell viability was evaluated at days 0, 2, 4, and 6. (C‑F) SW1573 and NCIH1792 cells introduced with GPX2 or EV lentivirus for (C and D) BrdU incorporation assays and (E and F) Transwell migration and invasion assays. Scale bar, 50 µm for C and E. (G‑I) SW1573 cells (2×10⁶) introduced with GPX2 or EV lentivirus were subcutaneously injected into nude mice, and then tumor xenografts were allowed to grow for 4 weeks. (G) Tumor growth curves, (H) representative images and (I) tumor weight are presented. (J) SW1573 and NCIH1792 cells (2.5×10⁵/well) introduced with GPX2 or EV lentivirus were seeded in 96‑well plates and treated with 0, 0.63, 1.25, 2.5, 5, 10, 20, 40, 80 and 160 µM cisplatin for 6 days, and then the relative cell viability was evaluated by CCK‑8 assay. (K, L) SW1573 and NCIH1792 cells introduced with GPX2 or EV lentivirus were treated with 2.5 or 10 µM cisplatin for 3 days, and then (K) the cells were stained with Annexin V‑FITC for flow cytometry. (L) The percentages of apoptotic cells are presented. (M and N) ROS levels were evaluated by flow cytometry. (M) Oxidative DCF‑positive cells and (N) relative FACS values are presented. (O) NADPH/NADP⁺ ratio of SW1573 and NCIH1792 cells introduced with GPX2 or EV lentivirus are shown. *P<0.05. GPX2, glutathione peroxidase 2; KRAS, Kirsten rat sarcoma viral oncogene homolog; NSCLC, non‑small cell lung cancer; EV, empty vector; BrdU, bromodeoxyuridine; CCK‑8, Cell Counting Kit‑8; ROS, reactive oxygen species; DCF, dichlorofluorescein; PI, propidium iodide.

(Fig. 6C). (G‑F) RIP assays were conducted to evaluate mRNA enrichment by the Ago/RNA‑induced silencing (RISC) complex after miR‑325‑3p overexpression. The Ago/RISC complex was successfully pulled down by Pan‑Ago antibody (Fig. 6D). The expression level of miR‑325‑3p was upregulated while GPX2 was downregulated in the Ago/RISC complex following miR‑325‑3p overexpression (Fig. 6E). In addition, miR‑325‑3p expression was negatively correlated with GPX2 in patients with NSCLC (Fig. 6F). Collectively, the results indicated that GPX2 was directly targeted by miR‑325‑3p in NSCLC cells.

**MiR‑325‑3p overexpression abrogates the effects of GPX2 in KRAS‑mutated NSCLC cells.** As GPX2 is a downstream
target of miR-325-3p, it was hypothesized that miR-325-3p may suppress KRAS-driven lung tumorigenesis via inhibiting GPX2. In the present study, ectopic expression of miR-325-3p reduced GPX2 expression in A549 and NCIH1385 cells,

Figure 4. Knockdown of GPX2 suppresses malignant progression and increases platinum sensitivity of KRAS-mutated NSCLC cells. (A) A549 and NCIH1385 cells were introduced with sh#1, sh#2 or sh#NC lentivirus, and then lysates were collected for western blotting. (B) A549 and NCIH1385 cells (2,500/well) introduced with sh#1, sh#2 or sh#NC lentivirus were seeded in 96-well plates, and then cell viability was determined at days 0, 2, 4, and 6. (C-F) A549 and NCIH1385 cells introduced with sh#1, sh#2 or sh#NC lentivirus were used for (C and D) BrdU incorporation assay and (E, F) Transwell migration and invasion assays. Scale bar, 50 µm for C and E. (G-I) A549 cells (2x10^6) introduced with sh#1 or sh#NC lentivirus were subcutaneously injected into nude mice, and then tumor xenografts were allowed to grow for 4 weeks. (G) Tumor growth curves, (H) representative images and (I) tumor weights are shown. (J) A549 and NCIH1385 cells (2,500/well) introduced with sh#1, sh#2 or sh#NC lentivirus were seeded in 96-well plates and treated with 0, 0.63, 1.25, 2.5, 5, 10, 20, 40, 80 and 160 µM cisplatin for 6 days, and then relative cell viability was evaluated by CCK-8 assay. (K, L) A549 and NCIH1385 cells introduced with sh#1 or sh#2 or sh#NC lentivirus were treated with 2.5 or 10 µM cisplatin for 3 days, and then (K) cells were stained with Annexin V-FITC for flow cytometry. (L) The percentages of apoptotic cells are presented. *P<0.05. GPX2, glutathione peroxidase 2; KRAS, Kirsten rat sarcoma viral oncogene homolog; NSCLC, non-small cell lung cancer; sh, short hairpin; NC, negative control; BrdU, bromodeoxyuridine; CCK-8, Cell Counting Kit-8; PI, propidium iodide.
but this was reversed by introducing with GPX2 expression lentivirus (Fig. 7A). MiR-325-3p overexpression inhibited cell growth of A549 and NCIH1385 cells, but this was partially abrogated by GPX2 restoration (Fig. 7B). In the BrdU incorporation assay, the number of BrdU-positive cells were reduced by miR-325-3p overexpression but partially replenished by the introduction of GPX2 expression lentivirus (Fig. 7C and D). In the Transwell assay, the number of migrated and invasive cells in A549 and NCIH1385 were downregulated by miR-325-3p overexpression but reversed by GPX2 overexpression (Fig. 7E and F). In addition, miR-325-3p overexpression reduced the IC₅₀ of cisplatin in A549 and NICH1385 cells, while GPX2 overexpression abolished this effect. Collectively, the results indicated that miR-325-3p overexpression abrogated the effects of GPX2 in KRAS-mutated NSCLC cells.

Discussion

As a key member of the glutathione peroxidase family, accumulated studies have indicated that GPX2 is involved in lung tumorigenesis and chemoresistance. For instance, high GPX2 expression was revealed to be correlated with worse overall survival of patients with NSCLC (35). In addition, GPX2 was vital for the tumor suppressive function of YAP1 in lung squamous cell carcinoma via regulation of ROS accumulation (36). Long non-coding RNA NLUCA1 promotes malignant progression of lung adenocarcinoma partially through upregulation of GPX2 (37). In the present study, it was demonstrated that GPX2 was upregulated in patients with NSCLC, especially those with KRAS mutations. Ectopic expression of GPX2 promoted KRASG12C-driven lung tumorigenesis in a non-tumorigenic epithelial cell line BEAS-2B. Forced GPX2 expression facilitated proliferation, migration, invasion, tumor xenograft growth and cisplatin resistance of KRAS-mutated NSCLC cells, while knockdown of GPX2 exhibited the opposite effects. The results demonstrated an oncogenic role of GPX2 in KRAS-driven lung cancer.

In the present study, GPX2 overexpression markedly reduced ROS accumulation in KRASG12C-transformed BEAS-2B cells and KRAS-mutated NSCLC cells, while GPX2
knockdown exhibited the opposite effects. It was hypothesized that GPX2 facilitated KRAS-driven lung tumorigenesis via alleviating KRAS-induced oxidative stress. Numerous studies have demonstrated that cells with active KRAS mutations are vulnerable to increasing ROS levels, despite the fact that KRAS controls the expression of a panel of ROS detoxification mediators. For example, SLC7A11 is a cysteine/glutamate antiporter responsible for cysteine uptake. Silencing of SLC7A11 was demonstrated to selectively kill KRAS-mutated lung adenocarcinoma cells via increasing oxidative stress and ER stress-induced cell apoptosis (38). Moreover, vitamin C was revealed to selectively kill KRAS-mutated colorectal cancer cells via depletion of glutathione and increasing ROS accumulation (39). Thus, it is no surprise that antioxidants can promote KRAS-driven tumorigenesis. SLC25A22, a member of the mitochondrial glutamate transporter family SLC25, facilitates KRAS-driven colorectal cancer progression via increasing intracellular synthesis of aspartate and reducing oxidative stress (40). In addition, long term treatment with antioxidants such as N-acetylcysteine and vitamin E increases KRAS-driven lung tumor metastasis by stabilizing the transcriptional factor BACH1 (29). Corresponding with this line, it was demonstrated that GPX2 promoted migration and invasion of KRAS-mutated NSCLC cells. In addition, GPX2 overexpression upregulated the expression of MMP1, and silencing of MMP1 partially abrogated the effects of GPX2 in KRAS-mutated NSCLC cells. MMP1 has long been identified to regulate the metastasis of cancer cells (41). In the present study, it was determined that GPX2 promoted the migration and invasion of NSCLC cells partially through the upregulation of MMP1.

There are previous studies demonstrating that GPX2 is involved in drug resistance of cancers. In HCC, inhibition of CD13 promoted cytotoxicity of chemotherapeutic drugs partially through downregulation of GPX2 and subsequently increasing ROS production (42). In gastric cancer, CD44 was positively correlated with GPX2 expression and facilitated chemoresistance via reducing intracellular ROS accumulation (43). It is also reported that upregulation of GPX2 confers cisplatin resistance of lung adenocarcinoma cells (16). In the present study, GPX2 overexpression promoted cisplatin resistance of KRAS-mutated NSCLC cells, while silencing of GPX2 exhibited the opposite effects. The results demonstrated a role of GPX2 in the chemoresistance of lung tumors, corresponding with the aforementioned previous studies.

In the present study, GPX2 was directly targeted by miR-325-3p, and forced miR-325-3p expression abolished the effects of GPX2 on KRAS-mutated NSCLC cells. Most of the protein coding genes are targeted by miRNAs, including GPX2, glutathione peroxidase 2; miR-325-3p, microRNA-325-3p; wt, wild-type; mut, mutant; ctrl, control; RIP, RNA immunoprecipitation; RT-qPCR, reverse transcription-quantitative PCR; RISC, RNA-induced silencing; n.s., not significant.
GPX2 was upregulated in patients with NSCLC and promoted KRAS-driven lung tumorigenesis. GPX2 overexpression reduced ROS accumulation and increased MMP1 expression in KRAS-mutated NSCLC cells. In addition, GPX2 was directly targeted by miR-325-3p, and miR-325-3p overexpression abrogated the effects of GPX2 in KRAS-mutated NSCLC cells. The results demonstrated an oncogenic role of GPX2 in KRAS-driven lung tumorigenesis, and inhibition of GPX2 may be a feasible strategy.
for lung cancer treatment, particularly in patients with KRAS mutations.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
All authors guarantee the integrity of the entire study. The experiments were conducted by MW, GF and XC. Clinical studies were conducted by GF. Data was analyzed by MW and MG. The manuscript was prepared and reviewed by MG. MG and MW conceived and designed the study and confirm the authenticity of all the raw data. All authors have read and approved the manuscript.

Ethics approval and consent to participate
The present study was approved (approval no. CY20160325) by the Ethics Committee of The First Affiliated Hospital of Chongqing Medical University (Chongqing, China). Written informed consents were obtained from all enrolled patients. The protocol of the present study concerning human subjects adhered to the ethical standards of the institutional committee and to the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. The protocol for the animal studies adhered to the ethical standards of and was approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital of Chongqing Medical University.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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