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Original Articles

Interferon regulatory factor-1 reverses chemoresistance by downregulating the expression of P-glycoprotein in gastric cancer

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ABSTRACT

The emergence of multiple drug resistance (MDR) is the main cause of chemotherapy failure in gastric cancer. In this study, to generate MDR gastric cancer cell lines, we exposed MKN45 and AGS gastric cancer cells to cisplatin, fluorouracil, and adriamycin. Through transcriptome sequencing, we found that interferon regulatory factor-1 (IRF-1) was expressed at significantly lower levels in the MDR cell lines than in the parental cell lines. We then established stable clones of MKN45 and SGC7901 cells with a doxycycline-inducible IRF-1 expression system and confirmed that IRF-1 overexpression efficiently reversed the MDR. Further analyses indicated that IRF-1 suppresses P-glycoprotein (P-gp) expression in vitro and in vivo, leading to an increase in chemotherapy drug retention. The results showed that IRF-1 bound to the promoter regions of P-gp gene and inhibited P-gp transcription. IFN-γ induced IRF-1-mediated downregulation of P-gp in gastric cancer cells. Finally, we demonstrated that the clinical correlation between IRF-1 and P-gp expression and that IRF-1 serves as an independent prognostic factor for patients with gastric cancer. We conclude that IRF-1 reverses the MDR trait of gastric cancer by downregulating P-gp, and this mechanism has potential treatment implications and is clinically actionable.

1. Introduction

Gastric cancer (GC) is currently the fifth most common malignancy after lung cancer, breast cancer, colorectal cancer and prostate cancer, and it is the third leading cause of cancer-associated mortality worldwide [1,2]. Because early-stage GC is not associated with typical clinical symptoms, the majority of GC patients are already at advanced stages of the disease at the time of their initial diagnosis. For GC patients who can undergo surgical resection, perioperative chemotherapy can prevent or delay tumour recurrence and metastasis [3], whereas chemotherapy can improve the quality of life and prolong the survival of GC patients who cannot undergo operations or who have metastases of advanced-stage GC [4]. The efficacy of chemotherapy is directly related to the prognosis of patients with GC. Although many chemotherapy drugs and regimens are available for clinical use, chemotherapy does not induce a satisfactory therapeutic effect in patients with GC, and the five-year survival rate of patients with advanced GC remains less than 30% [5].

The emergence of multiple drug resistance (MDR) is the main cause of chemotherapy failure in GC. MDR refers to the drug resistance extending to several antitumour drugs with diverse structures and various mechanisms after tumours develop resistance to certain chemotherapeutic drugs [6]. Abnormal expression of the ABC transporter family on the cell membrane is one of the major causes of MDR in GC and reduces the therapeutic response by decreasing drug influx and increasing drug efflux [7]. P-glycoprotein (P-gp), a 170-kD protein encoded by the ABCB1 gene, was the first ABC transporter to be discovered [8]. P-gp is expressed at low levels in almost all normal tissues [9], but a previous study revealed that P-gp is highly expressed in GC tissues and that its expression level has a significant negative correlation with sensitivity to chemotherapy [10]. In addition to its active role in transporting intracellular drugs and toxic substances to the extracellular space, P-gp also causes MDR by participating in the regulation of apoptosis and proliferation [11]. At present, the reversal of MDR by targeting P-gp is mainly achieved by inhibiting the expression or activity of P-gp [12].

Interferon regulatory factor-1 (IRF-1) is a nuclear transcription regulator that serves as an activator of genes implicated in the

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regulation of interferon expression, the regulation of both innate and acquired immunity, and lymphocyte differentiation [13]. IRF-1 is also a tumour suppressor and plays an important role in cell proliferation, apoptosis and the DNA damage response by regulating the expression of various genes, such as P21, P53, and caspases [13,14]. Although IRF-1 has been extensively studied in studies aiming to inhibit tumour cell proliferation and apoptosis, few studies have investigated the role of IRF-1 in tumour cell chemoresistance, and their conclusions are contradictory. Pavan et al. found that cisplatin induces IRF-1 expression in ovarian cancer cells and thereby reduces the chemosensitivity of cells to cisplatin [15]. However, a study on melanoma found that interferon (IFN)-α enhances the sensitivity to chemotherapeutic drugs through the IRF-1-mediated signalling pathway [16]. Sakai et al. suggested that the expression of IRF-1 can enhance the sensitivity of pancreatic cancer cells to chemotherapy drugs [17].

In the present study, we identified the role of IRF-1 in reversing MDR in GC. We found that IRF-1 was expressed at low levels in MDR GC cell lines and that IRF-1 overexpression restored the responsiveness of GC cells to chemotherapy drugs. In addition, the IRF-1-mediated transcriptional inhibition of P-gp expression in vitro and in vivo led to an increase in chemotherapy drug retention. Moreover, the clinical significance of the correlation between IRF-1 and P-gp expression was assessed using GC specimens. We also discovered that IFN-γ induces the IRF-1-mediated suppression of P-gp in GC cells. In conclusion, our study contributes to the understanding of the mechanisms through which IRF-1 affects GC chemoresistance and provides further compelling evidence showing that IRF-1 reverses MDR in GC by downregulating P-gp expression, which has potentially clinically actionable treatment implications.

2. Materials and methods

2.1. Clinical sample collection

In total, 54 GC patients who underwent surgery and uniform follow-up at Union Hospital (Wuhan, China) between August 2014 and August 2015 were enrolled in this research study. The diagnosis of GC was confirmed by the original histopathological reports. The GC tissue samples were fixed in formalin and embedded in paraffin. All patients provided written informed consent. This study was approved by the Ethics Committee at the Academic Medical Centre of the Huazhong University of Science and Technology.

2.2. Cell culture and reagents

Human GC cell lines (MKN45, AGS and SGC7901) were purchased from the Bena Culture Collection (Beijing, China). All the cell lines were cultured in RPMI 1640 medium (Gibco, NY, USA) supplemented with 10% foetal bovine serum (FBS) (ScienCell, CA, USA) and 1% penicillin and streptomycin (HyClone, UT, USA) and were grown in a humidified air atmosphere containing 5% CO_2 at 37 ˚C. The drugs cisplatin (CDDP), fluorouracil (5FU), and adriamycin (ADR) were purchased from Selleck (Houston, TX, USA). MDR cell lines from the MKN45 and AGS parental cells were cultured according to the flow diagram shown in Fig. 1A and B. The cytokine IFN-γ was purchased from PeproTect (NJ, USA).

2.3. Transcriptome sequencing

The Illumina HiSeq™ 2000 system (Genergy, Shanghai, China) was used for the de novo transcriptome analysis of the parental GC cells (MKN45 and AGS) and chemotherapy-resistant (MKN45/MDR and AGS/MDR) GC cells. Three samples of the parental and chemotherapy-resistant GC cells were used for sequencing.

2.4. Construction and infection of Lv-IRF-1

The human IRF-1 gene was inserted between the AgeI and EcoRI sites of the GV308 vector to create IRF-1 lentivirus (Lv-IRF-1). A lentivirus with the empty GV308 vector (Lv-Null) was constructed as a control (GeneChem, Shanghai, China). The above recombinant lentiviruses were stably transfected into all GC cell lines according to the manufacturer's instructions. IRF-1 expression was then induced by the addition of doxycycline (Dox) to the medium (4 μg/mL), and the cells were cultured in this medium for 48 h and then subjected to additional assays. All the reagents used for the experiments described in this section were purchased from GeneChem (Shanghai, China).

2.5. Transfection

IRF-1 siRNA and negative siRNA (Neg siRNA) controls were constructed by RiboBio (Guangzhou, China) and transfected at a final concentration of 50 nM. A P-gp-overexpression vector (P-gp OE) and an empty vector were constructed by GeneChem (Shanghai, China) and transfected at a density of 2 μg per 12-well plates. Lipofectamine 3000 reagent (Invitrogen, Carlsbad, MA, USA) was used for cellular transfection of the siRNA and vectors according to the manufacturer's instructions. The cells were then cultured for an additional 48 h and then used in the subsequent assays.

2.6. MTT assay

The sensitivity of the cells to chemotherapy drugs was measured by the MTT assay as previously described [18].

2.7. Apoptosis assay

Cell apoptosis was evaluated using an Annexin V/FITC apoptosis detection kit (AntGene, ant003, Wuhan, China) as previously described [18,19].

2.8. Western blotting

Protein lysates from the cultured cells or tumour tissues were prepared using RIPA buffer (Sigma-Aldrich, Darmstadt, Germany) containing 1% phenylmethanesulfonyl fluoride (PMSF) and phosphatase inhibitors. Western blotting was then performed as previously described [18,19]. Primary antibodies against IRF-1 (Abcam, ab186384, Cambridge, UK, 1:1000 dilution), P-gp (Abcam, ab170904, Cambridge, UK, 1:750 dilution), ABCG2 (Proteintech, 10051-01-AP, Wuhan, China, 1:750 dilution), ABCC1 (Abcam, ab233383, Cambridge, UK, 1:600 dilution), Stat1 (Proteintech, 10144-2-AP, Wuhan, China, 1:750 dilution) and GAPDH (Proteintech, 10494-1-AP, Wuhan, China, 1:5000 dilution) were used. The bands were then detected with secondary antibodies (HRP-conjugated AffiniPure Goat Anti-Rabbit IgG(H + L), Proteintech, SA00001-2, Wuhan, China, 1:2500 dilution) and visualized using an enhanced chemiluminescence detection kit (Meilunbio, Dalian, China). GAPDH was used to normalize the protein level.

2.9. qRT-PCR

Total RNA from the cultured cells or tumour tissues was extracted using the TRIzol reagent kit (Takara, Dalian, China). Reverse transcriptions and quantitative real-time PCR (qRT-PCR) were then performed as previously described [18,19]. GAPDH was used as a loading control. The primers for all the genes were synthesized by RiboBio (Guangdong, China) (Supplementary Table 1).

2.10. Immunofluorescence

The cells were grown on coverslips (WHB, WHB-12-CS, Shanghai,

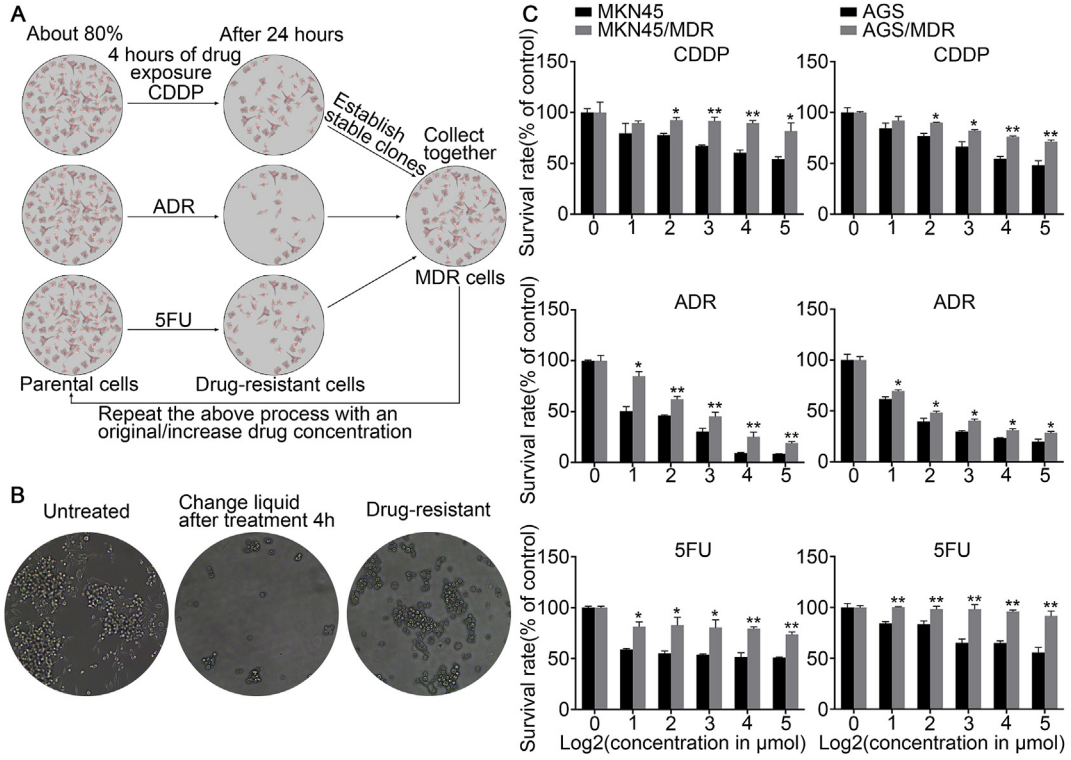

Fig. 1. Establishment of multiple drug-resistant GC cell lines. (A) Taking MKN45 as an example, the cells were seeded into a 12-well plate and grown to approximately 80% confluence, and the first dose of the three chemotherapeutic drugs was then applied at a concentration of 10 μmol/mL. The medium was changed after 4 h of drug exposure, and the cells that were alive 24 h after the removal of the drug were considered resistant cells. Three chemotherapeutic drug-resistant cells were collected and cultured until they resumed proliferating and established stable clones. The drug-resistant cells were further inoculated into a 12-well plate, and the above process was performed until the cells no longer exhibited significant apoptosis during exposure to increasing concentrations of the drugs (the concentration of the drug was increased by 10 μmol/mL). (B) Bright field images of the untreated cells, the cells after 4 h of drug exposure and the residual cell proliferation. (C) Survival rates of the chemotherapy-resistant (MKN45/MDR and AGS/MDR) cell lines compared with those of the parental GC cells (MKN45 and AGS) after treatment with various concentrations of CDDP, 5FU, and ADR. Three independent experiments (N = 3) were performed in triplicate. The data are presented as the means ± standard deviations (SDs). *P < 0.05 and **P < 0.01.

China), fixed with 4% paraformaldehyde (Sigma-Aldrich, Darmstadt, Germany) for 15 min, and then permeabilized with 0.1% Triton X-100 (Biofroxx, Guangzhou, China) for 10 min. After three washes with PBS, the samples were blocked with normal goat serum for 1 h at room temperature. IRF-1 (Abcam, ab186384, Cambridge, UK, 1:100 dilution) and P-gp (Abcam, ab170904, Cambridge, UK, 1:100 dilution) antibodies were subsequently added, and the cells were incubated overnight at 4 °C. After three washes with PBS, the cells were incubated with the secondary antibody (BosterBio, BA1105, Wuhan, China) in the dark at room temperature for 1 h. The cell nuclei were stained with DAPI (blue), and the cells were then observed under a fluorescence microscope (Olympus) at the same exposure.

2.11. Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed using the SimpleChIP Enzymatic Chromatin IP Kit (CST, #9003, MA, USA) following the manufacturer's recommended protocol. Chromatin extracts containing DNA fragments (approximately 150–900 bp) were immunoprecipitated with 2 μg of monoclonal anti-IRF-1 antibody (Santa, sc-74530, CA, USA). The PCR primers for potential IRF-1-binding sites are listed in Supplementary Table 2.

2.12. Luciferase assay

The wild-type and mutant P-gp promoter regions were inserted into pGL3-based vectors. MKN45 and SGC7901 cells plated in 24-well plates were co-transfected with P-gp luciferase reporter constructs and IRF-1 plasmids using the Lipofectamine 3000 reagent. Forty-eight hours after transfection, the cells were harvested and lysed, and the firefly luciferase activity of the lysate was measured up to 48 h after transfection using the dual luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's recommended protocol. The luciferase readings were then normalized to Renilla luciferase activity.

2.13. Xenograft assay

MKN45/Lv-IRF-1 cells (8×10^6 cells/mouse, five mice per group) were suspended in 150 μL of RPMI-1640 medium and subcutaneously injected into the right flanks of 6-week-old BALB/c female nude mice (HFK Bio-Technology, Beijing, China). Throughout the experiment, the mice were administered 2 mg/mL of Dox in drinking water. The tumour volumes were measured with a Vernier calliper every 7 days according to the following formula: volume = 0.5 × length × $(\text{width})^2$. Two weeks after the injection, the mice were intraperitoneally injected with PBS containing CDDP, 5FU or ADR once per week. Twenty-eight days after the initial injection, the mice were humanely sacrificed according to institutional ethics guidelines, and the weights of the tumours were recorded. All the experiments were conducted according to protocols approved by the Animal Research Committee of the Academic Medical Centre at Huazhong University of Science and Technology. All the experimental procedures were conducted in accordance with the guidelines of the Institutional and Animal Care and Use Committees.

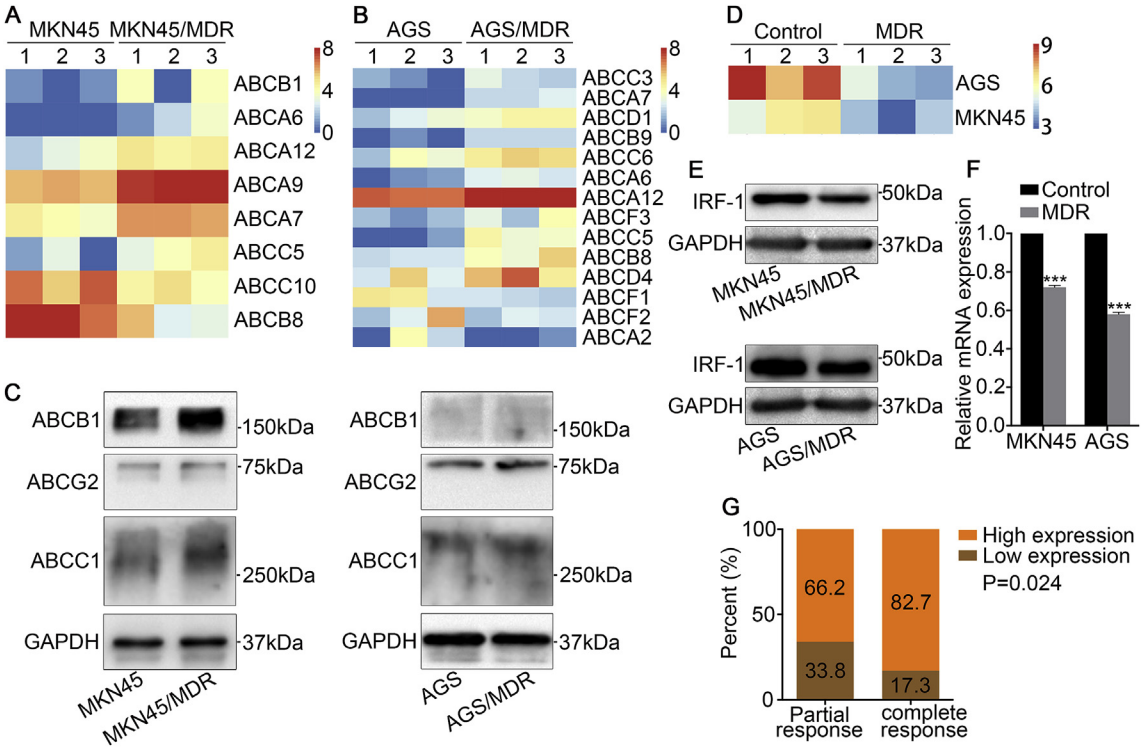

Fig. 2. Analyses and validation of gene expression profiles. (A) Heatmap of transcripts of the ABC gene family in MKN45/MDR cell lines compared with the MKN45 cell line. (B) Heatmap of transcripts of the ABC gene family in AGS/MDR cell lines compared with the AGS cell line. (C) ABCB1 (P-gp), ABCG2, and ABCC1 protein expression patterns in MDR cell lines (MKN45/MDR and AGS/MDR) and parental GC cells (MKN45 and AGS) revealed by western blotting. (D) Heatmap of IRF-1 transcripts in MDR cell lines versus the parental GC cells. (E) IRF-1 protein expression patterns in MDR and parental GC cells revealed by western blotting. (F) IRF-1 mRNA expression patterns in MDR and parental GC cells revealed by qRT-PCR. ***P < 0.001. In C, E and F, three independent experiments (N = 3) were performed in triplicate. The data are presented as the means ± SDs. (G) Low IRF-1 expression was correlated with a partial response to chemotherapy drugs in GC patients based on TCGA datasets.

2.14. Immunohistochemistry

Paraffin-embedded mouse tumour tissues and clinical GC tissues were cut into 5-μm-thick sections, placed on glass slides and stained with haematoxylin and eosin or subjected to immunohistochemistry (IHC). Tissue sections were deparaffinised, subjected to antigen retrieval using 0.01 M citric acid buffer (pH 6.0) at 95 °C for 15 min and incubated overnight at 4 °C with primary antibodies against IRF-1 (Abcam, ab186384, Cambridge, UK, 1:100 dilution) and P-gp (Abcam, ab170904, Cambridge, UK, 1:50 dilution). After three washes with TBS, the sections were incubated with HRP-conjugated AffiniPure Goat Anti-Rabbit IgG (Proteintech, SA00001-2, Wuhan, China, 1:5000 dilution) for 1 h at room temperature. The IHC results for human tissues were scored by two independent observers according to both the percentage of positively stained cells (scored from 0 to 3) and the staining intensity (scored from 0 to 3), and the final immunoreactivity score (IRS, range 0–9) was obtained by multiplying the two scores. The expression levels of IRF-1 and P-gp were classified as low if the score was less than 5 and as high if the score was 5 or higher.

2.15. Bioinformatics analysis

The raw data from GC patients for whom RNA sequencing (RNA-Seq) and clinical information were obtained from The Cancer Genome Atlas (TCGA). The TCGA data and our own sequencing data were analysed using R version 3.3.1 (https://www.r-project.org/). Genes that met the following criteria were considered differentially expressed genes (DEGs): $|\log_2(\text{fold change})| > 1$ and false discovery rate (FDR) < 0.05. The IRF-1 expression value of the lower quartile in the TCGA database was set as the cut-off value for distinguishing between high and low expression levels. In addition, JASPAR (http://jaspar.genereg.net) [20,21] and PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) [22] are open-access databases that are used for the detection of transcriptional regulatory elements in known gene promoters, and we thus utilized these databases to predict potential IRF-1-binding sites in the P-gp gene promoter.

2.16. Statistical analysis

The statistical analyses were performed using SPSS 24.0, and depending on the experiment type, Student's *t*-test or one-way ANOVA was used for the analysis. Chi-square tests were used to analyse the clinical correlations of IRF-1 expression with clinicopathological features. The significant prognostic factors found in the univariate analysis were further subjected to multivariate analysis using the Cox proportional hazards regression model. The Kaplan-Meier method was used to estimate survival, and a log-rank test was used to assess the differences between survival curves. The statistical significance was evaluated based on P values, and P < 0.05 was considered to indicate statistical significance.

3. Results

3.1. Low expression of IRF-1 in GC chemotherapy-resistant cells

To investigate the molecular events associated with the development of chemotherapy MDR in GC, we exposed the human GC cell lines MKN45 and AGS to CDDP, 5FU, and ADR according to the regimen diagrammed in Fig. 1A and B. After six months of drug exposure, an MTT assay was performed to determine the sensitivity of the MDR and parental cell lines to chemotherapeutic drugs. As shown in Fig. 1C, compared with the parental GC cell lines, both MDR cell lines exhibited

longer survival times after exposure to CDDP, 5FU, and ADR. To analyse the mRNA expression profiles of the MDR GC cells, we performed transcriptome sequencing (sequencing results shown in Supplementary Table 3) to identify mRNAs that were differentially expressed between the parental (MKN45 and AGS) and the chemotherapy-resistant (MKN45/MDR and AGS/MDR) GC cells, and our attention was first drawn to the difference in the expression of the ABC gene family. In the MKN45/MDR cell line, six and two ABC genes were upregulated and downregulated, respectively (Fig. 2A), whereas in the AGS/MDR cell line, eleven and three ABC genes were upregulated and downregulated, respectively (Fig. 2B). Specifically, the transcript levels of ABCA6, ABCA7, ABCA12, and ABCC5 were significantly increased in both MDR cell lines compared with the parental cells, whereas the transcript level of ABCB8 was increased in the MKN45/MDR cell line and reduced in the AGS/MDR cell line (Fig. 2A and B). In addition, a western blotting analysis showed that the well-known ABC genes that are responsible for MDR, including ABCB1, ABCG2 and ABCC1, showed different degrees of elevated expression in the MDR cells compared with their parental GC cells (Fig. 2C). Notably, we found that the IRF-1 transcript levels were significantly downregulated in the MDR cell lines compared with their parental GC cells (Fig. 2D), and this abnormal expression of IRF-1 was further validated by western blotting and qRT-PCR (Fig. 2E and F). We then analysed the TCGA GC data repository and found that low IRF-1 expression levels were highly correlated with a partial response to chemotherapy drugs in patients with GC (Fig. 2G).

3.2. IRF-1 regulates the chemotherapy tolerance of GC cells

Cell proliferation assays were performed to examine how the growth inhibition of GC cells affects chemotherapeutic drugs following the change in IRF-1 expression. The induction of IRF-1 expression in MKN45/Lv-IRF-1 cells resulted in a significantly reduced cell survival rate after treatment with chemotherapeutic drugs for 24 h, and the growth of MKN45 cells was inhibited in a dose-dependent manner (Fig. 3A). The opposite results were observed in the MKN45 cell line after the knockdown of IRF-1 (Fig. 3B). In addition, cell apoptosis is an important antitumour pathway involved in chemotherapy [23], and we thus investigated whether IRF-1 overexpression increases chemotherapy-induced apoptosis in GC. As shown in Fig. 3C, the apoptotic cell fraction was significantly increased following IRF-1 overexpression and treatment with chemotherapy drugs compared with that found after chemotherapy drug treatment alone, and the most pronounced increase in apoptosis was observed in the CDDP-treated group. Furthermore, to identify whether a change in the intracellular drug concentration was responsible for the increased sensitivity of GC cells to chemotherapeutic drugs following the increased IRF-1 expression, the function of IRF-1 was assessed by measuring ADR retention through immunofluorescence. The results showed that MKN45/Lv-IRF-1 pretreatment with Dox increased the retention of ADR, and a similar result was obtained with SGC7901 cells (Fig. 3D). These findings suggest that IRF-1 overexpression reverses the MDR in GC and that the role of IRF-1 in mediating chemosensitivity is dependent on membrane transporters.

3.3. IRF-1 regulates P-gp expression in GC cells

To explore the potential mechanisms through which IRF-1 reverses MDR in GC, we map possible transcriptional regulatory targets of IRF-1 using the PROMO database [20,21], cross-aligned the targets with the JASPAR database [22], and selected P-gp as a potential IRF-1 target. We first verified that IRF-1 expression gradually decreased and P-gp expression gradually increased during the different chemotherapeutic exposure periods (Supplementary Figs. 1A and B). To confirm the role of IRF-1 regulation, the GC cell lines MKN45 and SGC7901 were infected with Lv-IRF-1 and the empty vector Lv-Null. After Dox induction, Lv-IRF-1 substantially inhibited the expression of P-gp but not that of Lv-Null (Fig. 4A). An immunofluorescence analysis also confirmed that the mechanism through which increased IRF-1 expression reverses the MDR in GC is associated with decreased expression of cell-membrane P-gp and increased intracellular ADR retention (Fig. 4B). The inhibition of IRF-1 led to an inverse change in P-gp expression (Fig. 4C). To further validate the role of P-gp in the IRF-1-mediated reversal of MDR in GC, we investigate the effect of P-gp in the presence of IRF-1 overexpression through a “rescue” assay. We delivered the P-gp-overexpression vector and empty vector into MKN45/Lv-IRF-1 and SGC7901/Lv-IRF-1 cell lines, and then induced IRF-1 overexpression for 48 h. We first verified that the P-gp expression level was not affected by the empty P-gp vector (Fig. 4D). Subsequently, a Western blot analysis indicated that the IRF-1-mediated inhibition of P-gp expression was reproducibly increased after transfection with the P-gp-overexpression vector (Fig. 4D). MTT arrays demonstrated that the forced expression of P-gp partially restored the growth inhibition of MKN45/Lv-IRF-1 and SGC7901/Lv-IRF-1 cells (Fig. 4E). Collectively, our data indicate that P-gp is required for the IRF-1-mediated reversal of MDR in GC.

3.4. IFN-γ inhibits P-gp expression in GC cells

Our previous studies demonstrated that IFN-γ is one of the strongest inducers of IRF-1 expression in GC cells and that AGS cells are Stat1-deficient, which prevents the induction of IRF-1 by IFN-γ [19]. We subsequently confirmed the expression of Stat1 in the MKN45 and SGC7901 cell lines (Supplementary Fig. 2), and demonstrated that IFN-γ increases IRF-1 expression and inhibits P-gp protein in a dose-dependent manner in both cell lines (Fig. 5A). We conducted MTT assays and found that the cell inhibition rate was substantially increased after IFN-γ pretreatment (Fig. 5B). An immunofluorescence analysis demonstrated that the role of IFN-γ in the reversal of MDR in GC cells is associated with decreased P-gp expression on the cell membrane and increased intracellular drug retention (Fig. 5C). Furthermore, MKN45 and SGC7901 cells were pretreated with a siRNA against IRF-1 and then treated with IFN-γ. We found that the knockdown of IRF-1 expression by IRF-1 siRNA eliminated the inhibition of P-gp expression (Fig. 5D), which confirms that IRF-1 mediates the inhibition of P-gp expression by IFN-γ.

3.5. IRF-1 inhibits P-gp promoter activity

Two potential IRF-1 transcriptional binding sites (site 1 and site 2) in the P-gp gene promoter region were identified through sequence analysis (Fig. 6A). A ChIP assay was utilized to analyse the binding of IRF-1 to the P-gp promoter in MKN45 and SGC7901 cells. As shown in Fig. 6B, using the qRT-PCR reads resulting from the ChIP analysis, the immunoprecipitation of IRF-1 with fragmented chromatin revealed that the use of specific primers for regions surrounding the putative binding site 1 in MKN45 and SGC7901 cells increased the PCR products. Following Dox treatment, a significant increase in PCR products was observed in Lv-IRF-1 cells (Fig. 6C), which indicated an increase in the binding of IRF-1 to binding site 1. We subsequently used a luciferase reporter to explore whether IRF-1 inhibits the transcriptional activity of the P-gp gene promoter. Smaller fragments containing site 1 and site 1 mutations were cloned into the P-gp vector or control vector (Fig. 6D). The luciferase activity of these reporter constructs was examined in MKN45 and SGC7901 cells after IRF-1 induction. Forced IRF-1 expression resulted in decreased luciferase activity compared with that of the controls, and this suppression was reversed by mutation of the target sequences of P-gp (Fig. 6E). These results suggest that the site 1 sequence in the P-gp gene promoter is largely responsible for the inhibition of IRF-1 transcription.

3.6. IRF-1 inhibits P-gp expression in vivo

Because IRF-1 reverses P-gp-mediated MDR in vitro, we then investigated whether elevated IRF-1 expression reverses MDR in vivo. We

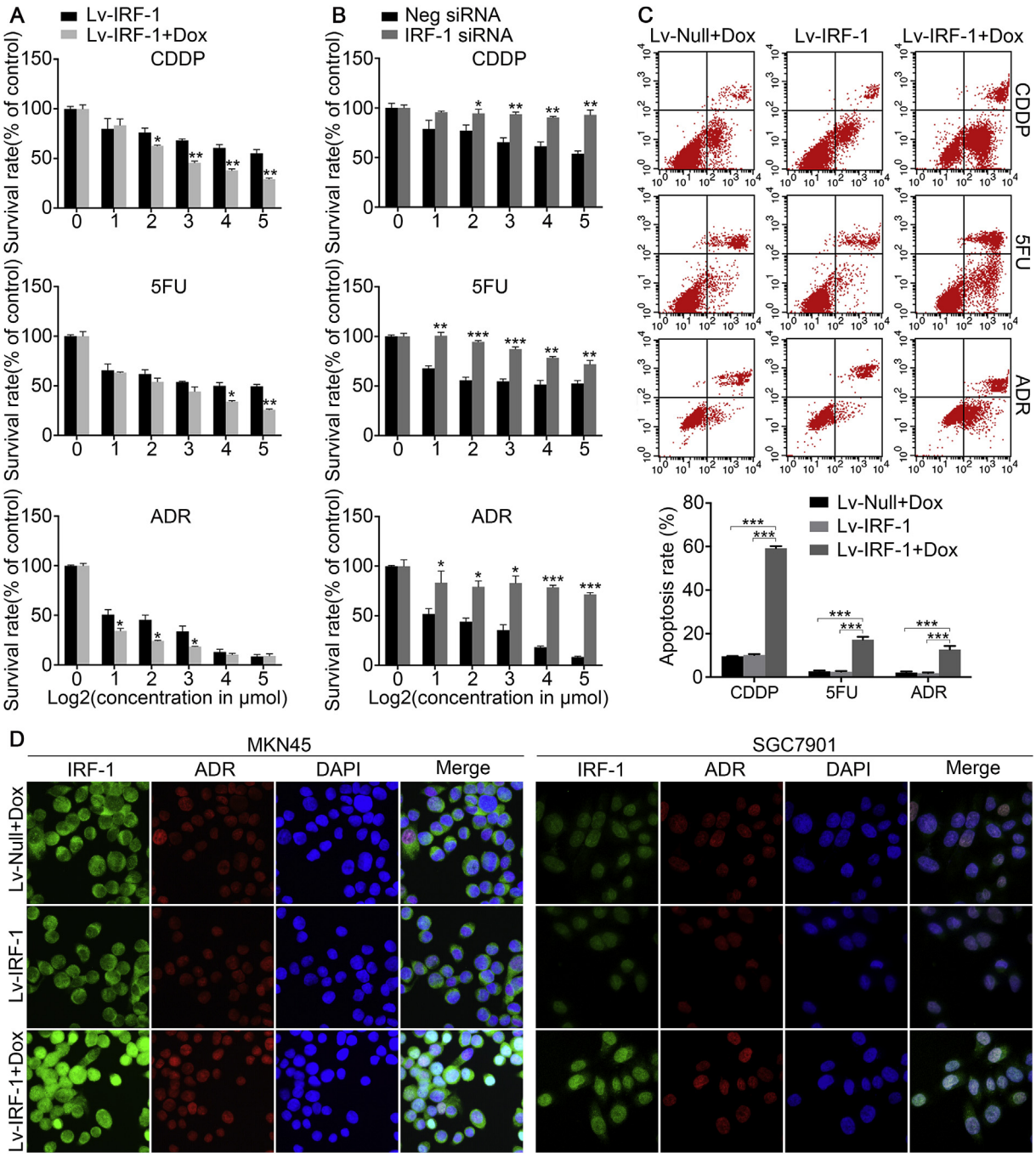

Fig. 3. GC cell lines with high IRF-1 expression are sensitive to chemotherapeutic drugs. (A) Survival rates of MKN45/Lv-IRF-1 cells in the presence or absence of 4 μg/mL Dox 24 h after treatment with various concentrations of CDDP, 5FU, and ADR. (B) Cell survival rates of MKN45 cells transfected with negative control siRNA (Neg-siRNA) or IRF-1 siRNA after treatment with various concentrations of CDDP, 5FU, and ADR. (C) MKN45/Lv-IRF-1 cells were treated with 5 μmol/L CDDP, 5 FU, and ADR for 48 h, and MKN45/Lv-Null and MKN45/Lv-IRF-1 cells were treated with 4 μg/mL Dox and 5 μmol/l CDDP, 5 FU, and ADR for 48 h. The apoptotic cells after the treatments were detected by flow cytometry. In A, B and C, the data are presented as the means ± SDs. *P $<$ 0.05, **P $<$ 0.01 and ***P $<$ 0.001. (D) Lv-IRF-1 cells were treated with 5 μmol/L ADR for 30 min, and Lv-Null and Lv-IRF-1 cells were treated with 4 μg/mL Dox for 48 h and then with 5 μmol/l ADR for 30 min. The IRF-1 expression level and ADR retention after the treatments were detected by immunofluorescence. For all the above experiments, three independent experiments (N = 3) were performed in triplicate.

therefore conducted studies with a xenogeneic model established using MKN45/Lv-IRF-1 cells (Fig. 7A). After the administration of water containing 2 mg/mL Dox, we found that the growth of xenogeneic tumours was not significantly affected by IRF-1 overexpression, whereas the volumes and weights of the tumours in the Dox-fed groups were markedly reduced after treatment with different chemotherapeutic drugs (Fig. 7B and C), which indicated that ectopic IRF-1 expression can reverse MDR. On day 28, the xenogeneic transplanted tumours were isolated, and western blotting and PCR results showed that the expression of P-gp in the Dox-fed groups was significantly lower than that in the control groups due to increased IRF-1 expression (Fig. 7D and E). Further IHC assays yielded consistent results (Fig. 7F). These findings indicate that a high expression level of IRF-1 reverses MDR in GC cells by inhibiting P-gp expression in vivo, which is in agreement with the results obtained in vitro.

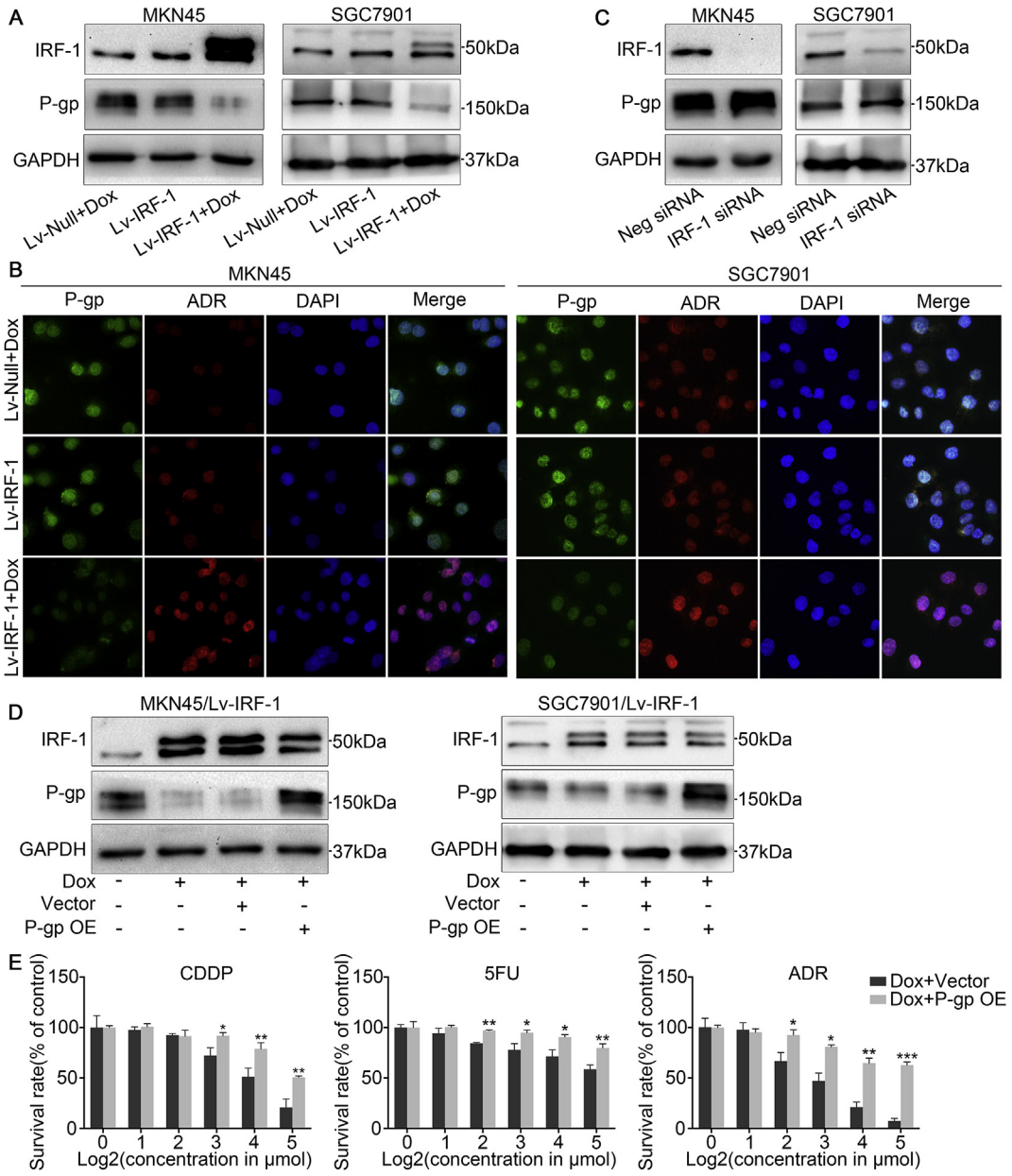

Fig. 4. IRF-1 inhibits P-gp expression. (A) MKN45 and SGC7901 cells were transfected with Lv-IRF-1 or Lv-Null after treatment with the indicated amount of Dox, and the IRF-1 and P-gp protein expression levels were then detected by western blotting. (B) MKN45 and SGC7901 cells were transfected with Lv-IRF-1 or Lv-Null after treatment with ADR for 30 min, and the P-gp expression levels and ADR retention were then detected by immunofluorescence. (C) MKN45 and SGC7901 cells were transfected with Neg-siRNA or IRF-1 siRNA, and the IRF-1 and P-gp protein expression levels were then detected by western blotting. (D) In the presence or absence of the indicated amount of Dox, the IRF-1 and P-gp protein expression levels in MKN45/Lv-IRF-1 and SGC7901/IRF-1 cells transfected with the empty vector (Vector) or the P-gp-overexpression vector (P-gp OE) were detected by western blotting. (E) Survival rates of MKN45/Lv-IRF-1 cells in the presence or absence of the indicated amount of Dox after transfection with the empty vector or the P-gp-overexpression vector. For all the above experiments, three independent experiments (N = 3) were performed in triplicate. In E, the data are presented as the means ± SDs. *P < 0.05, **P < 0.01 and ***P < 0.001.

3.7. Low IRF-1 expression is associated with elevated P-gp and poor prognosis in GC patients

To evaluate the correlation between the IRF-1 and P-gp expression levels in GC tissues, we first conducted an IHC assay with 54 GC specimens (Fig. 8A). As expected, the results revealed that low IRF-1 expression was associated with elevated P-gp expression (Fig. 8B). Furthermore, a univariate analysis indicated that the IRF-1 expression level, histologic grade, tumour stage and lymph node metastasis were unfavourable predictors of the OS of GC patients (Supplementary Table 4). The Kaplan-Meier survival analysis confirmed that the 1- and 3-year OS rates of the low-IRF-1-expression group were significantly worse than those of the high-IRF-1-expression group (Fig. 8C). Further multivariate analysis demonstrated that IRF-1 was an independent prognostic factor of the OS of GC patients (Supplementary Table 5).

To further confirm the clinical value of IRF-1, we investigated the IRF-1 expression level in 238 GC samples based on RNA-Seq data in TCGA. Systematic analysis of the clinicopathological features of these samples revealed that high IRF-1 expression was significantly correlated with chemotherapy response, histological type, distant metastasis and microsatellite instability (Supplementary Table 6). Furthermore, a univariate analysis demonstrated that IRF-1 expression, chemotherapy response, histologic grade, pathologic stage, distant metastasis, lymph node metastasis and microsatellite instability were unfavourable

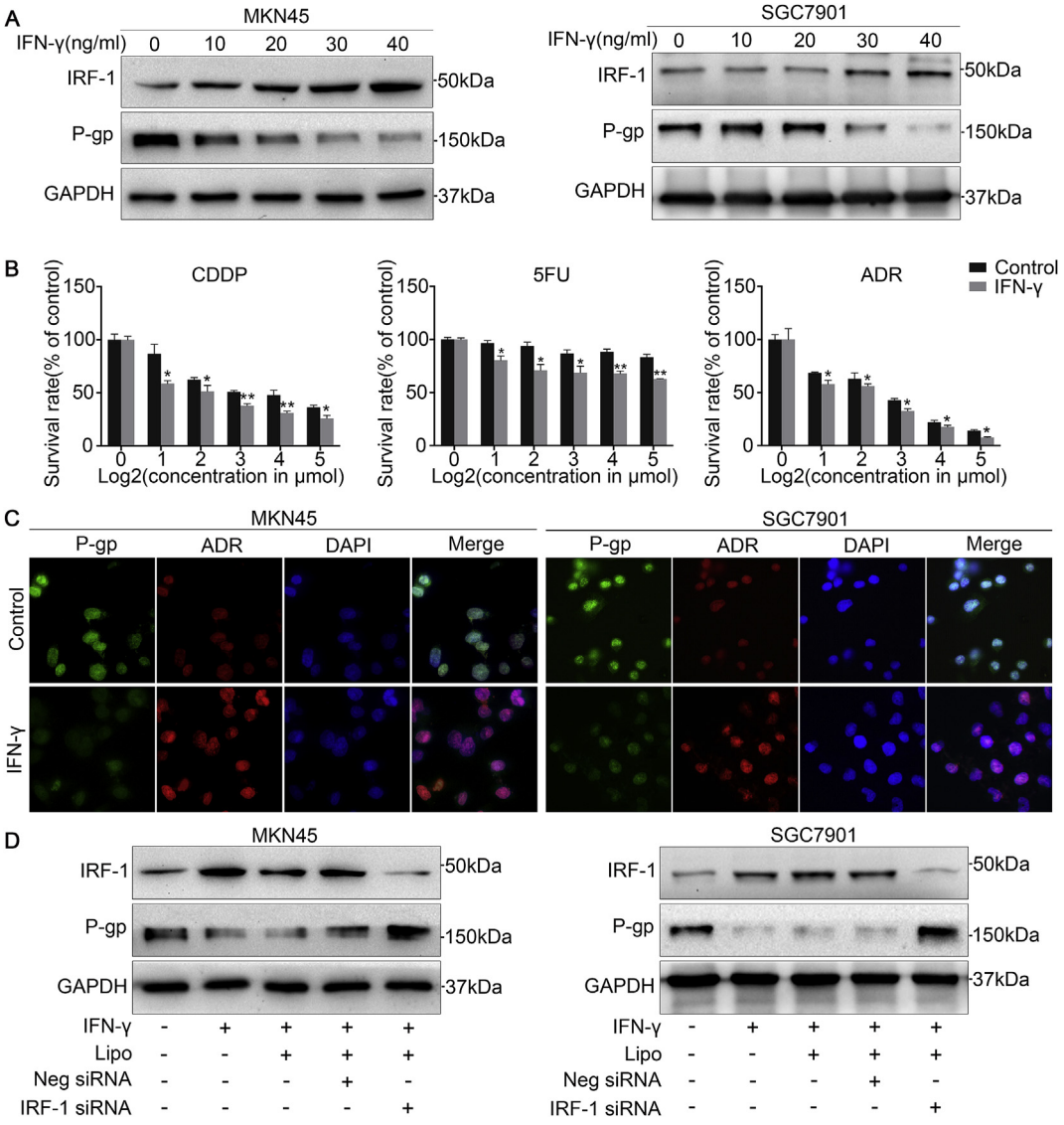

Fig. 5. IFN-γ inhibits P-gp expression through IRF-1. (A) MKN45 and SGC7901 cells were treated with the indicated amount of IFN-γ for 48 h, and the expression levels of IRF-1 and P-gp were analysed by western blotting. (B) Survival rates of MKN45 cells treated with 40 ng/mL IFN-γ for 48 h and then treated with various concentrations of CDDP, 5FU, and ADR. The data are presented as the means ± SDs. *P < 0.05, **P < 0.01 and ***P < 0.001. (C) MKN45 and SGC7901 cells were treated with 40 ng/mL IFN-γ for 48 h and then treated with ADR for 30 min. The P-gp expression levels and ADR retention were then detected by immunofluorescence. (D) MKN45 and SGC7901 cells were treated with no siRNA (transfection agent alone, Lipo), Neg-siRNA or IRF-1 siRNA, cultured with IFN-γ (40 ng/mL, 48 h), and then used for western blotting analysis of IRF-1 and P-gp expression. For all the above experiments, three independent experiments (N = 3) were performed in triplicate.

predictors of the OS of GC patients (Fig. 8D and Supplementary Tables 7) and a further multivariate analysis revealed that IRF-1 was an independent prognostic factor of the OS of GC patients (Supplementary Table 8).

4. Discussion

The emergence of MDR is the main cause of chemotherapy failure in patients with GC [24]; therefore, reversing MDR has become crucial to improving the efficacy of chemotherapy, and it is profoundly important to improve the cure rate and survival rate of patients with GC. MDR in GC is a complex process mediated by multiple factors, and P-gp-mediated MDR, which is also known as the classic drug resistance pathway, is the most thoroughly studied drug resistance mechanism to date [7,25]. Here, we showed that IRF-1 reverses MDR in GC by inhibiting P-gp expression. Our study supports the important role of IRF-1 in chemoresistance in GC and the use of strategies to augment IRF-1 expression or activity as a therapeutic option for GC.

Previous studies have revealed that NF-κB binds to the distal −6092-bp DNA sequence of the P-gp gene promoter and thereby activates gene transcription and upregulates P-gp expression [26]. The protein kinase C (PKC) family member PKCα activates transcription of the −29-bp site of the P-gp gene promoter, and PKCθ activates the transcription of the −982- and −612-bp sites of the P-gp gene promoter [27]. Moreover, the gene promoter of P-gp and its adjacent regions contains CCAAT boxes and GC-rich regions that could be recognized by diverse transcription factors [28]. The mitogen-activated protein kinase (MAPK) family contains multiple members, and the related ERK pathway upregulates P-gp expression by activating the phosphorylation site of the P-gp gene promoter [29]. Additionally, both c-Jun N-terminal kinase (JNK) and P38 signalling can also promote P-gp overexpression and chemoresistance [30,31]. In recent years, the inhibition of P-gp expression for the reversal of tumour chemotherapy resistance has gradually gained attention. For example, miR-506 and

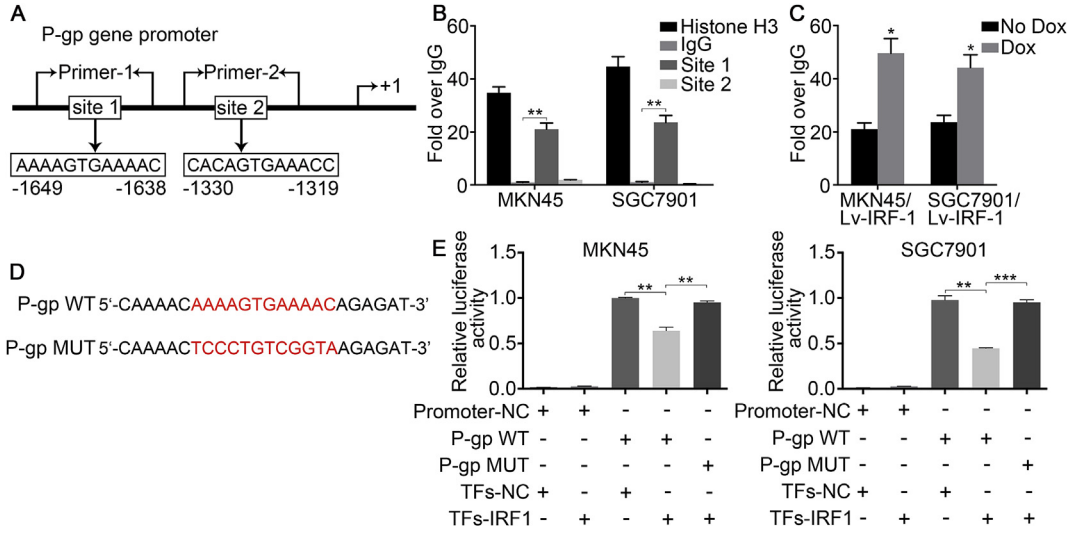

Fig. 6. IRF-1 suppresses P-gp promoter activity. (A) Schematic representation of the IRF-1-binding sites in the P-gp promoter. (B) MKN45 and SGC7901 cells were infected with Lv-IRF-1 and were then utilized in a chromatin immunoprecipitation (ChIP) assay to characterize the recruitment of IRF-1 to the P-gp promoter. IgG was used as a control. (C) MKN45/Lv-IRF-1 and SGC7901/IRF-1 cells were treated with 4 μg/mL Dox for 48 h, and a ChIP assay was then performed to assess the binding of IRF-1 to the P-gp promoter. (D) Schematic diagram of the reporter constructs of the wild-type (WT) and mutant (MUT) P-gp promoter binding site 1 fragment. (E) The indicated P-gp reporter constructs were co-transfected into MKN45 and SGC7901 cells with IRF-1 plasmids (TRs-IRF-1) or normal control plasmids (TRs-NC), and the luciferase activities were then measured and analysed. In B, C and E, three independent experiments (N = 3) were performed in triplicate. The data are presented as the means ± SDs. *P < 0.05 and **P < 0.01.

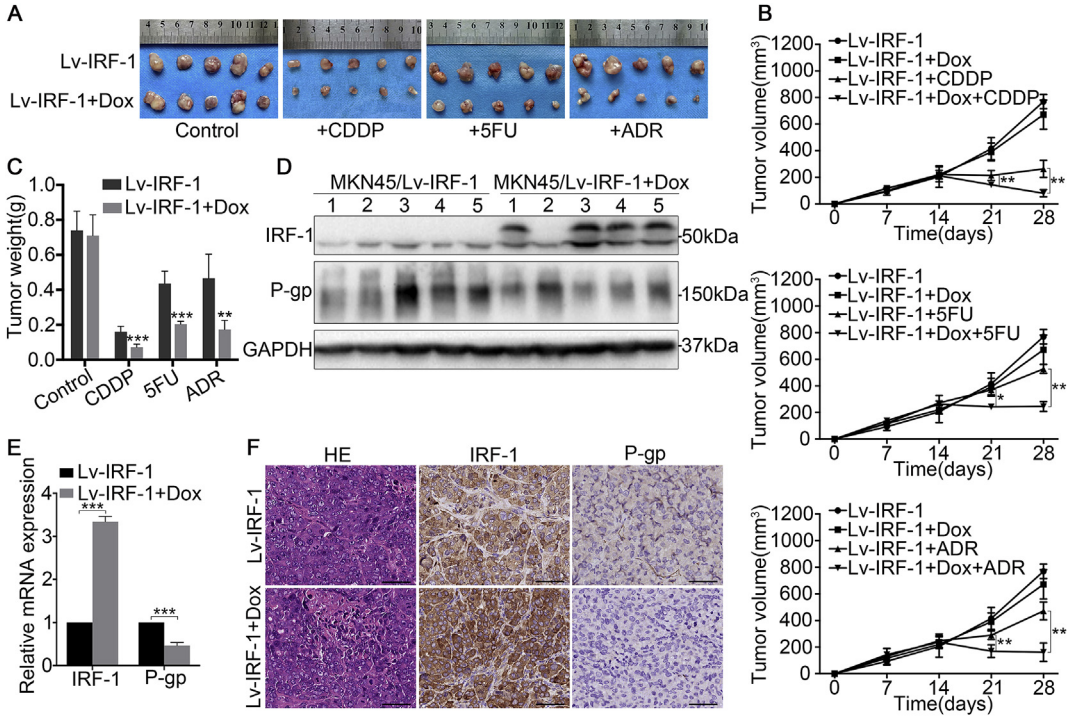

Fig. 7. IRF-1 reverses the MDR of MKN45/Lv-IRF-1 cells in vivo. (A) MKN45/Lv-IRF-1 cells were transplanted into the right flanks of mice, and the differences in tumour growth after chemotherapy are shown. (B) The tumour volumes were measured at the indicated time points. (C) The tumour weights were measured after isolation of the tumours from the mice. The expression levels of IRF-1 and P-gp were detected by (D) western blotting, (E) qRT-PCR, and (F) immunohistochemistry. Scale bars, 50 μm. Each experiment included N ≥ 3 biological replicates. The data are presented as the means ± SDs. *P < 0.05, **P < 0.01 and ***P < 0.001.

miR-508-5p overexpression can enhance chemotherapy sensitivity by inhibiting P-gp expression [32,33]. The drug nelfinavir and the piperine analogue Pip1 exhibit high affinity for the active functional regions of P-gp and inhibit P-gp efflux function [34,35].

Our previous studies showed that enhancing the expression of IRF-1 in GC cells results in increased sensitivity to chemotherapeutic drugs [18]. To further elucidate the potential mechanisms through which IRF-1 affects the chemoresistance of GC, we cultured two MDR cell lines of GC and found that IRF-1 downregulates the expression of P-gp. Furthermore, we analysed the promoter region of P-gp and found two different sequences (site 1, AAAAGTGAAAAC; and site 2, CACAGTGA AACC) of the IRF-1-binding site in the P-gp gene promoter. Our previous studies showed that IRF-1 binds to different sites in the PUMA promoter and thus upregulates its expression [19]. Moreover, Kirchhoff

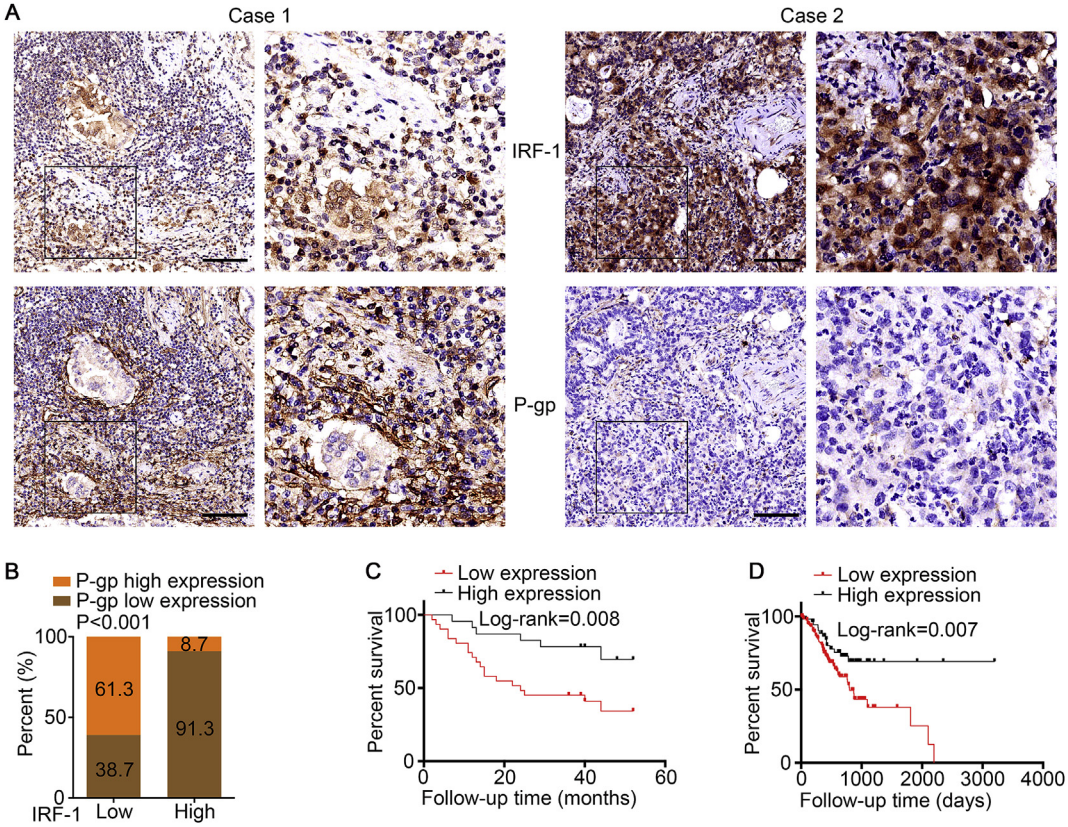

Fig. 8. Low IRF-1 expression levels are significantly associated with elevated levels of P-gp and a poor prognosis. (A) Representative IHC images of IRF-1 and P-gp expression in 54 GC tissues. Case 1 is a representative image from the low-IRF-1-expression group, and Case 2 is a representative image from the high-IRF-1-expression group. Scale bars, 100 μm. The right-hand panels show magnified views of the boxed area in the corresponding left-hand panels. (B) Inverse correlation between the IRF-1 and P-gp expression levels. (C) The prognostic significance of IRF-1 expression was assessed by Kaplan-Meier survival curves and log-rank tests. (D) Kaplan-Meier curves were used to assess the correlation between IRF-1 expression and overall survival based on TCGA datasets.

et al. reported that IRF-1 forms a homodimer in vitro and thereby plays pivotal roles in the gene promoters containing tandem repeat IRF-1-binding sites rather than single sites [36]. Thus, we hypothesized that IRF-1 can play regulatory roles in P-gp expression through these two sites simultaneously, but a ChIP assay confirmed that the site 1 sequences serves as the binding site for IRF-1 and that the product obtained from the site 1 primer was increased after IRF-1 overexpression. Luciferase activity assays further demonstrated that the site 1 sequence is important for IRF-1 inhibition.

The low expression of IRF-1 in cancer has been widely reported, but the underlying mechanisms have not yet been fully elucidated. On the one hand, IRF-1 is regulated by various of cytokines, such as prolactin, leukaemia inhibitory factor (LIF), interleukin-1 (IL-1) and IL-6 [37], and on the other hand, the low expression of IRF-1 is also associated with the deletion, mutation and rearrangements of its gene [38]. Recent studies have shown that miRNAs play an important role in the regulation of IRF-1. For example, miR-23a and miR-502-5p could bind to the 3′-UTR of IRF-1 mRNA, resulting in IRF-1 downregulation and loss of transcriptional activity [39,40]. However, whether other non-coding RNAs, such as lncRNAs and circRNAs, play regulatory roles in IRF-1 expression has not been reported. More importantly, our results showed that low IRF-1 expression was significantly associated with chemotherapy resistance in GC and that the expression level of IRF-1 in MDR cell lines was lower than in their parental GC cells, which indicates that IRF-1 expression was further inhibited by the development of GC chemotherapy resistance. Therefore, further studies are needed to investigate the precise mechanism of IRF-1 downregulation in MDR GC cells.

The tumour immune response plays important roles in the chemotherapy resistance of tumour cells. Chemotherapy triggers the host inflammatory immune response and eliminates immune surveillance by reducing immunosuppressive cell populations, such as $CD4^+CD25^+$ regulatory T cells [41]. Dosset et al. demonstrated that 5FU plus oxaliplatin results in complete tumour elimination when combined with immunotherapy, even though each monotherapy fails in mice [42]. The main purpose of immunotherapy is to enhance the antitumour immunity of the host [41,43]. The addition of immunotherapy during chemotherapy can synergistically improve the clinical outcomes [44]. Studies have shown that the addition of chemotherapeutic drugs to immunotherapy can trigger the host to produce durable and effective tumour-resistant gene-specific T lymphocytes and synergistically optimize the antitumour effects [43]. Using bioinformatics analyses, our previous studies also revealed that tumour immunity-related signalling pathways play important roles in GC chemotherapy resistance [45]. The type II interferon IFN-γ is an important mediator of the immune response to tumourigenesis and viral infection [46,47]. Here, our study provides the first demonstration that IFN-γ can attenuate P-gp expression in an IRF-1-mediated manner and thereby links tumour chemoresistance to the immune response of cancer cells. Therefore, detection of the IFN-γ and IRF-1 expression levels might be helpful for predicting chemotherapy resistance and patient outcomes.

CDDP and 5FU are the first-line clinical drugs for GC chemotherapy [48]. As shown in Fig. 4E, the role of 5FU resistance reversal appeared to be less than that of CDDP and ADR after P-gp expression was increased, which further indicated that membrane transporters are one of the main factors affecting chemoresistance. Moreover, CDDP and ADR are more dependent on P-gp transport than 5FU during the development of chemotherapy resistance, but this finding needs further study. In addition, as shown in Fig. 2C for the AGS cell line, although some GC cells exhibit a low baseline level of P-gp expression, the exogenous

expression of IRF-1 can also affect the MDR of GC cells. This phenomenon indicates that the mechanisms through which IRF-1 affects the MDR in GC might also be associated with other regulatory mechanisms in addition to the regulation of P-gp expression. Prost et al. reported that the deficiency or inhibition of IRF-1 leads to a decrease in DNA repair, which promotes the accumulation of gene mutations, and this finding indicates that IRF-1 plays important roles in DNA repair [49]. Thus, in AGS cells, we explored whether increased expression levels of IRF-1 could affect the DNA damage repair capacity of the cells by regulating the expression of RAD51 and KU80 (our unpublished data).

Our study has important therapeutic implications for GC. The importance of P-gp for tumour chemotherapy tolerance has been increasingly recognized, and an increasing number of studies have shown that tumour MDR can be reversed by regulating P-gp expression [50]. In our xenograft model, we found that the induction of IRF-1 overcomes MDR in GC, which indicates that IRF-1 has clinical potential as a therapeutic target. Moreover, previous studies have shown that IRF-1 could be used as an important biomarker [51,52]. Our clinical data and TCGA data indicate that the IRF-1 expression level is associated with tumour prognosis and multiple clinicopathological parameters of GC patients, which further supports the view that IRF-1 might be an important target for reversing MDR in GC and could serve as an independent prognostic factor for GC patients.

In conclusion, our study showed that IRF-1 expression is downregulated in GC chemoresistant cells, and that increased IRF-1 expression could reverse MDR in GC. Further analyses revealed that IRF-1 reverses GC chemoresistance by transcriptionally inhibiting P-gp gene expression. Our study enriches the knowledge of the mechanisms of chemotherapy resistance in GC and provides a novel strategy for reversing chemotherapy resistance in GC that has potential clinical application value.

Conflicts of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Jingsheng Yuan: Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing - original draft. **Zhijie Yin:** Formal analysis, Validation, Writing - review & editing. **Lulu Tan:** Investigation, Formal analysis. **Wenzhong Zhu:** Investigation, Formal analysis. **Kaixiong Tao:** Methodology, Resources, Supervision, Writing - review & editing. **Guobing Wang:** Methodology, Resources, Supervision, Writing - review & editing. **Wenjia Shi:** Investigation, Supervision, Writing - review & editing. **Jinbo Gao:** Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.05.006.

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