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HOXA5 Derives the Malignant Progression and Cisplatin Resistance of Esophageal Squamous Cell Carcinoma by Regulating USP18-Mediated IFI27 Deubiquitination

Qiang Wang[1] | Shiheng Ren[1] | Zheng Pan[2] | Yuxin Chen[1] | Xiangyan Liu[1,3]

[1]Department of Thoracic Surgery, Shandong Provincial Hospital, Cheeloo College of Medicine, Shandong University, Jinan, Shandong, China | [2]Department of Thoracic Surgery, The People's Hospital of Laoling City, Dezhou, China | [3]Department of Thoracic Surgery, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, Shandong, China

Correspondence: Xiangyan Liu (liuxiangyan1@163.com)

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ABSTRACT

Background: Chemo-resistance is a major obstacle to the treatment of esophageal squamous cell carcinoma (ESCC). Interferon alpha-inducible protein 27 (IFI27) has been reported to be associated with ESCC progression. This study is designed to explore the role and mechanism of IFI27 in the cisplatin (DDP) resistance of ESCC.

Methods: IFI27 and Ubiquitin-specific peptidase 18 (USP18) levels were detected by real-time quantitative polymerase chain reaction (RT-qPCR). IFI27, multidrug resistance-associated protein 1 (MRP1), USP18, and Homeobox A5 (HOXA5) protein levels were determined using western blot. DDP resistance, cell viability, proliferation, apoptosis, invasion, and migration were assessed using MTT, EdU, flow cytometry, transwell, and wound healing. Interaction between USP18 and IFI27 was verified using Co-immunoprecipitation (CoIP) assay. Binding between HOXA5 and USP18 promoter was predicted by JASPAR and validated using Chromatin immunoprecipitation (ChIP) and dual-luciferase reporter assays.

Results: IFI27 was upregulated in DDP-resistant ESCC tissues and cells. IFI27 knockdown repressed DDP resistance, cell proliferation, invasion, migration, and induced cell apoptosis in vitro. Mechanistically, USP18 induced the deubiquitination of IFI27 and prevented its degradation. Furthermore, HOXA5 was a transcription factor of USP18 and activated the transcriptional activity of USP18 via binding to its promoter region.

Conclusion: USP18 transcriptionally mediated by HOXA5 could promote cell malignant behaviors and DDP resistance through deubiquitinating IFI27, providing a promising therapeutic target for ESCC treatment.

1 | Introduction

As the most common aggressive gastrointestinal malignancy, esophageal cancer (EC) has been identified as a major life-threatening health problem worldwide [1, 2]. Accounting for 90% of ECs, esophageal squamous cell carcinoma (ESCC) occurs in the middle or upper third of the esophagus, and the main risk factors for its development include heavy alcohol consumption and smoking [3]. At present, ESCC is highly prevalent in Asia and sub-Saharan Africa, especially in China, which has a higher incidence than any other country and accounts for about 50% of all diagnosed cases of EC around the world [4, 5]. Because of the asymptomatic characteristics in the early stage and unreliable screening approaches, most ESCC patients are often diagnosed at an advanced stage with a poor prognosis [6]. Compared with surgery alone, the combined therapies incorporating

chemotherapy have improved the overall survival of ESCC patients [7, 8]. With broad-spectrum antitumor activity, cisplatin (DDP) is of utmost importance for ESCC patients [9]. In recent years, DDP-based chemotherapy regimens have been widely adopted as neoadjuvant, radical, and palliative therapies for EC [10, 11]. However, drug resistance is one of the decisive factors for treatment failure [12]. More than 60% of subjects with locally advanced ESCC fail to achieve a pathologically complete response to neoadjuvant chemo-radiotherapy, eventually resulting in tumor recurrence and poor prognosis [13, 14]. Accordingly, further understanding of the molecular mechanism underlying DDP resistance is an important step for improving the prognosis of ESCC patients.

Encoded within chromosome 14q32.12, Interferon alpha-inducible protein 27 (IFI27, also known as IFI27L1, ISG12A) is a hydrophobic mitochondrial protein consisting of 122 amino acids [15]. Located in mitochondria, human IFI27 is implicated to promote cell apoptosis induced by DNA damage [16]. It has been reported that IFI27 is indispensable for mitochondrial function and browning in adipocytes [17]. Earlier studies have shown that IFI27 participates in various biological processes, such as apoptosis, proliferation, and innate immunity [18, 19]. Furthermore, multiple researches have proposed that IFI27 is an oncogene that is aberrantly upregulated in different squamous cell carcinomas (SCC) and is associated with poor survival [20, 21]. For example, IFI27 was highly expressed in tongue SCC, and its silencing could hinder cell growth and migration [22]. Beyond that, overexpressing IFI27 could the migration, invasion, and EMT in head and neck SCC cells [23]. Notably, a recent report has displayed that elevated IFI27 could boost ESCC cell angiogenesis and malignant progression by regulating exosomal miR-21-5p [24]. However, the role and underlying mechanism of IFI27 on DDP resistance in ESCC is far from being addressed.

Convincing evidence has described that ubiquitination is prevalent in the post-translation modifications of proteins, which could influence the structure, function, and stability of target proteins by complex and diverse ubiquitin ligation processes [25]. As a dynamic and reversible process, ubiquitination, is tightly regulated by ubiquitin-ligating (E3) enzymes and can be reversed by deubiquitinating enzymes (DUBs) [26]. Ubiquitination imbalance caused by dysregulation of E3 or DUBs has been reported to impair several cellular physiological processes, which are closely linked to the development of human cancers [27]. Therefore, they have emerged as promising therapeutic targets for tumor management [28]. As an important member of DUBs, Ubiquitin-specific peptidase 18 (USP18, also called USP43) has been confirmed to exert an oncoprotein in many human tumors [29]. For instance, USP18 could catalyze the deubiquitination of Notch1 by interacting with c-Myc, thus enhancing the Notch1-c-Myc axis and boosting pancreatic cancer progression [30]. Moreover, overexpressing USP18 could facilitate the growth and metastasis of colorectal cancer through deubiquitinating and stabilizing the Snail1 protein, and effectively promote cell survival in response to treatment with three different chemotherapy drugs (fluorouracial, doxorubicin, and DDP) [31]. Of note, USP18 functioned as the DUB of ZEB1 protein and enhanced the stability of ZEB1 protein, ultimately expediting ESCC cell invasion and metastasis [32]. Nevertheless, whether USP18 is involved in DDP resistance and its target protein substrates in ESCC cells has been rarely studied.

Herein, our data discovered that IFI27 was upregulated in DDP-resistant ESCC, and USP18 directly bound, deubiquitinize, and stabilized IFI27 protein. In addition, we further revealed the upstream signaling of USP18 and found that Homeobox A5 (HOXA5) could maintain its expression via promoting the binding of HOXA5 to its promoter. Therefore, this study aimed to elucidate whether USP15 transcriptionally mediated by HOXA5 affects ESCC progression and DDP resistance via regulating IFI27 deubiquitination.

2 | Materials and Methods

2.1 | Clinical Samples and Cell Culture

After all participants signed written informed consent, ESCC tumor tissues were provided by the Shandong Provincial Hospital. All samples were immediately frozen in liquid nitrogen and then stored at −80°C until use. According to their responsiveness to DDP-based chemotherapy, 66 subjects were divided into two groups: 37 DDP-resistant patients whose tumors recurred during DDP chemotherapy after R0 resection; 29 DDP-sensitive patients whose tumors had no recurrence during DDP chemotherapy. In addition, the research was approved by the Ethics Committee of Shandong Provincial Hospital.

Human normal esophageal epithelial cell line (HEEC, BNCC359279, BeNa Culture Collection, Beijing, China) and two ESCC cell lines TE-1 (CL-0231, Procell) and KYSE450 (STM-CL-5553, STEM RECELL, Shanghai, China) were cultured in RPMI-1640 medium (Invitrogen, Paisley Scotland, UK). The corresponding DDP-resistant ESCC cells (TE1/DDP and KYSE450/DDP) were established from the parental cell lines by using a repetitive pulsatile treatment with escalating doses of cisplatin, as previously described [33, 34]. To maintain the DDP-resistant phenotype of TE1/DDP and KYSE450/DDP cells, 10 μM DDP was additionally added into the culture medium.

2.2 | Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Based on RNAsimple Total RNA Kit (DP419, Tiangen, Beijing, China), total RNAs were extracted. The quality and purity of RNA were determined by a NanoDrop ND-1000 spectrophotometer. After reverse transcription into cDNA with LunaScript RT SuperMix Kit (E3010, NEB, Ipswich, MA, USA), qPCR reaction was conducted by applying Power SYBR Green Master Mix (4 367 659, Invitrogen). The relative expression of target genes was calculated with the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH. Primer sequences are listed in Table 1.

2.3 | Western Blot Assay

In short, tissues and cells were lysed by ice-cold RIPA buffer (Keygen, Nanjing, China). After being quantified with BCA method, the cell lysates were loaded on 10% SDS-PAGE gel for

TABLE 1 | Primer sequences used for PCR.

Name		Primers for PCR (5′-3′)
IFI27	Forward	CCTTCTTTGGGTCTGGCTGA
	Reverse	CCACACTGGTCACTGCTGAT
USP18	Forward	CATGGCGCTTGAGAGATTCC
	Reverse	CAACCAGGCCATGAGGGTAG
GAPDH	Forward	GGAGCGAGATCCCTCCAAAAT
	Reverse	GGCTGTTGTCATACTTCTCATGG

separation and transferred to PVDF membranes (Millipore, Molsheim, France). To block nonspecific binding, TBS containing 5% skimmed milk was applied to incubate membranes for 1 h. After that, the membranes were incubated with primary antibodies: IFI27 (ab171919, 1:1000, Abcam, Cambridge, MA, USA), MRP1 (#72202, 1:1000, CST, Danvers, Massachusetts, USA), USP18 (#4813, 1:1000, CST), HOXA5 (ab140636, 1:1000, Abcam), and GAPDH (ab9485, 1:2500, Abcam) at 4°C overnight. After incubation with secondary antibody for 2 h, protein bands were visualized with ECL reagent (Millipore) and quantified using Image J.

For protein stability, OE-control or OE-USP18-transfected cells were collected after exposure to 50 μg/mL cycloheximide (CHX, a protein synthesis inhibitor, Sigma-Andrich, Louis, MO, USA) for 0, 5, 10, 20, and 25 h. After extraction, the stability of IFI27 protein level was analyzed with western blot.

2.4 | Cell Transfection

For knockdown system, small interfering RNAs against IFI27 (si-IFI27), USP18 (si-USP18), HOXA5 (si-HOXA5), POU2F1 (si-POU2F1), and negative control (si-NC) were chemically synthesized and provided by GenePharma (Shanghai, China). For overexpression system, the cDNA sequences of USP18 (NM_017414.4) and IFI27 (NM_001130080.3) were respectively amplified by PCR and inserted into pcDNA vector (GenePharma), with empty vector pcDNA used as the negative control (OE-control). All cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen), followed by incubation for 48 h.

2.5 | MTT Assay

In this assay, drug resistance and cell viability were assessed. For drug resistance, transfected DDP-resistant ESCC cells (5×10^3 cells/well) in 96-well plates were incubated for 24 h prior to treatment with different concentrations of DDP for 48 h. After that, each well was mixed with 20 μL of sterile MTT solution (5 mg/mL, Sigma-Aldrich) for 4 h at 37°C. After dissolving formed formazan crystals with 150 μL DMSO, a microplate reader was used for the absorbance at 490 nm. The concentration of DDP causing the half inhibition of growth (IC_{50}) was defined from the relative survival curve. In addition, at the indicated time points (0, 24, 48, and 72 h) after transfection, MTT assay was conducted for cell viability.

2.6 | 5-Ethynyl-2′-Deoxyuridine (EdU) Assay

In short, 4×10^4 DDP-resistant ESCC cells were maintained in 24-well plates for 24 h. Overall, 48 h after transfection, cells were hatched with the fresh medium with EdU solution (20 μM, RiboBio, Guangzhou, China) for 2 h, followed by fixture in 4% formaldehyde solution. After PBS washing, Apollo reaction cocktail was added to each well for 30 min, which then was stained with DAPI (Sigma-Aldrich). Images were photographed with a fluorescence microscope.

2.7 | Flow Cytometry for Cell Apoptosis

Briefly, DDP-resistant ESCC cells were harvested and resuspended in binding buffer. After double-stained with 5 μL Annexin V-FITC and 10 μL PI (Invitrogen) under a dark condition for 15 min. Cell apoptotic rates were evaluated using flow cytometry within 1 h and FlowJo V10.

2.8 | Matrigel Invasion Assay

To determine the invasive ability of DDP-resistant ESCC cells, the upper chamber (BD Bioscience, Heidelberg, Germany) was spread with coated 50 μL Matrigel (Solarbio, Beijing, China) for 30 min prior to cell seeding. Subsequently, 1×10^5 cells in 200 μL serum-free medium were introduced into the upper chamber, whereas lower counterpart was added with 600 μL complete medium. Incubated for 24 h, the invading cells into the lower side were fixed and dyed with 0.1% crystal violet (Beyotime). Under a microscope, five selected fields of view were quantified and photographed for invading cells.

2.9 | Wound Healing Assay

To measure the migratory ability, DDP-resistant ESCC cells at 80% confluency were harvested in six-well plates. The cell monolayer was scraped linearly to introduce an artificial wound using a sterile pipette (record 0 h). After removing the cellular debris, medium was replaced by serum-free fresh medium and cells were cultured for 24 h. Finally, the gap size was imaged at 0 and 24 h using a microscope and analyzed using ImageJ.

2.10 | CoIP

To check the interaction between USP18 and IFI27, this experiment was carried out in DDP-resistant ESCC cells. In short, the cells were suspended in IP Lysis buffer (Invitrogen) for 15 min at 4°C. The whole cell lysates were collected and centrifuged. Then, 10% cleared lysates were retained as input control, whereas the remaining were incubated with indicated antibodies (anti-USP18, anti-IFI27, or anti-IgG antibody) at 4°C for 16 h, followed by mixture with protein-A/G magnetic beads (Millipore) at. After PBS washing, western blot assay was used for the protein complex from agarose beads.

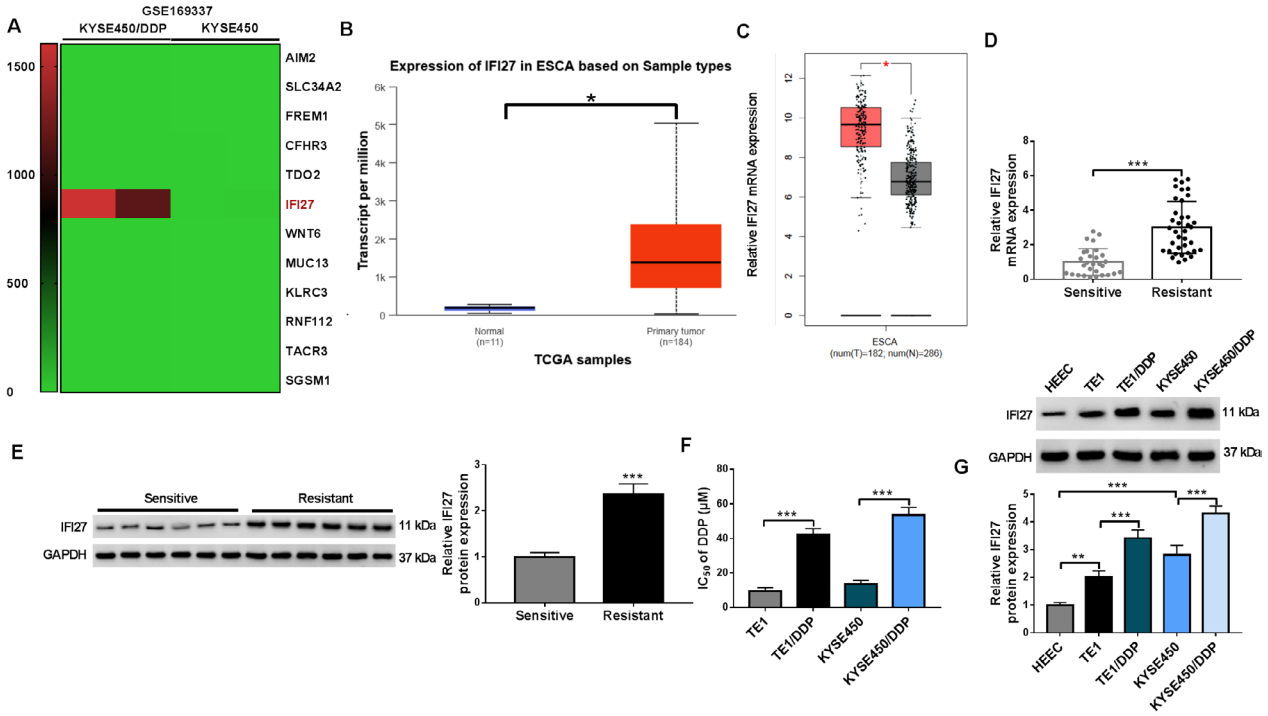

FIGURE 1 | Identification of IFI27 expression in DDP-resistant ESCC tissues and cells. (A) Identification of differentially expressed genes in cisplatin-resistant ESCC cells (KYSE450/DDP) and parental ESCC cells (KYSE450), based on RNA-seq data from GEO dataset (GSE169337). (B and C) TCGA and ENCORI datasets presented the mRNA level of IFI27 in ESCA samples and normal samples. (D) RT-qPCR assay was applied to measure the expression level of IFI27 in 37 treatment-resistant patients and 29 treatment-responsive patients. (E) IFI27 protein level was examined in the drug-resistant group ($n=6$) and the drug-responsive group ($n=6$) using western blot. (F) IC_{50} value of DDP was measured using MTT in TE1, TE1/DDP, KYSE450, and KYSE450/DDP cells. (G) IFI27 protein level was determined using western blot in HEEC, TE1, TE1/DDP, KYSE450, and KYSE450/DDP cells. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

2.11 | Ubiquitylation Assay

In brief, DDP-resistant ESCC cells with si-NC or si-USP18 were washed with PBS. After that, the cells were lysed for immunoprecipitation assay with anti-IFI27 antibody. At last, the IFI27 ubiquitination was determined based on western blot assay and anti-Ub.

2.12 | Chromatin Immunoprecipitation (ChIP)

The putative HOXA5 binding sites (#1: −690~−683, #2: −1179~−1172) in the USP18 promoter were predicted using JASPAR software. Then, ChIP assay was performed to verify the predication in TE1/DDP cells using SimpleChIP Enzymatic Chromatin IP Kit (CST). Briefly, 1×10^6 cells were harvested for ChIP, followed by cross-linking with 1% formaldehyde at 37°C for 30 min. After terminating this cross-linked using glycine, the cells were subjected to washing, centrifugation, and DNA digestion. Then, the cross-linked chromatin preparation was incubated with anti-HOXA5 or normal rabbit IgG overnight at 4°C, followed by RT-qPCR analysis.

2.13 | Dual-Luciferase Reporter Assay

To identify USP18 promoter activity, we cloned the wild-type (WT) sequence possessing the binding site 1 of USP18 or mutant-type (MUT) sequence into pmirGLO plasmids. Then, above-modified plasmids were transfected into DDP-resistant ESCC cells along with si-NC or si-HOXA5. After 48 h, Dual Luciferase Assay Kit (Promega, Madison, WI, USA) was used for the detection of luciferase activities in cell lysates.

2.14 | Statistical Analysis

In this study, all data were statistically analyzed based on GraphPad Prism7. Data comparison was performed using Student's *t*-test or one-way ANOVA with Tukey's tests. When *p* values less than 0.05, differences possess statistical significance. All quantitative data were expressed as mean ± standard deviation (SD).

3 | Results

3.1 | IFI27 Expression Was Upregulated in DDP-Resistant ESCC Tissues and Cells

To reveal differentially expressed genes in cisplatin-resistant and parental ESCC cells, a microarray dataset (GSE160299) was downloaded from the Gene Expression Omnibus (GEO) database and selected to characterize IFI27 expression in KYSE450/DDP and parental ESCC cells (KYSE450). As presented in Figure 1A, IFI27 expression was clearly increased in KYSE450/

DDP cells compared with KYSE450 cells. Furthermore, data from the Cancer Genome Atlas (TCGA) and Encyclopedia of RNA Interactomes (ENCORI) also indicated that IFI27 was highly expressed in primary tumors relative to normal samples in esophageal carcinoma (ESCA) samples (Figure 1B,C). Subsequently, RT-qPCR and western blot results exhibited that IFI27 mRNA level and protein level were significantly upregulated in the DDP-resistant versus DDP-sensitive ESCC tissues (Figure 1D,E). Moreover, we found that IC_{50} value of DDP in TE1/DDP and KYSE450/DDP cells was apparently higher than that in their parental cells (TE1 and KYSE450) (Figure 1F), verifying the production of DDP resistance in TE1/DDP and KYSE450/DDP cells. In addition, western blot results displayed that IFI27 protein level was overexpressed in ESCC cell lines (TE1 and KYSE450) especially in DDP-resistant ESCC cell lines (TE1/DDP and KYSE450/DDP) when compared to HEEC cells (Figure 1G). Together, these results suggested that dysregulated IFI27 may be associated with DDP resistance in ESCC.

3.2 | IFI27 Knockdown Repressed Cell Proliferation and DDP Resistance in DDP-Resistant ESCC Cells

To further explore the functional role of IFI27 on ESCC cell development and DDP resistance, in vitro loss-of-function analyses were performed. As displayed in Figure 2A, IFI27 protein level was obviously reduced in si-IFI27-transfected TE1/DDP and KYSE450/DDP cells compared with the si-NC group, suggesting that the knockdown efficiency of IFI27 is successful. After that, IC_{50} determination presented that IFI27 depletion significantly decreased DDP resistance in TE1/DDP and KYSE450/DDP cells (Figure 2B). It has been reported that MRP1 is an important cause of multidrug resistance in human tumor cells. Hence, we next investigate the effect of IFI27 silencing on MRP1 protein level. As a result, western blot analysis exhibited that MRP1 protein level was obviously reduced by IFI27 downregulation in TE1/DDP and KYSE450/DDP cells (Figure 2C). Subsequently, MTT and EdU assays that cell viability (Figure 2D,E) and EdU-positive cells (Figure 2F) were remarkably blocked after si-IFI27 transfection in TE1/DDP and KYSE450/DDP cells. In parallel, flow cytometry analysis discovered that the lack of IFI27 could evidently induce the apoptosis of TE1/DDP and KYSE450/DDP cells (Figure 2G). In addition, the results Transwell and wound healing assays displayed that IFI27 deficiency led to an apparent decrease in the invasion and migration potential of TE1/DDP and KYSE450/DDP cells (Figure 2H,I). Collectively, these outcomes indicated that IFI27 knockdown diminished cell malignant behaviors and DDP resistance in DDP-resistant ESCC cells.

3.3 | USP18 Stabilized the Expression of IFI27 in DDP-Resistant ESCC Cells

Based on STRING online software prediction, IFI27 was found to interact with USP18, a widely expressed deubiquitinating enzyme (Figure 3A). Then, through analyzing data from TCGA-ESCA and ENCORI-ESCA, we observed that USP18 expression was increased in tumor samples (Figure 3B,C). Besides, the knockdown efficiency of USP18 in TE1/DDP and KYSE450/

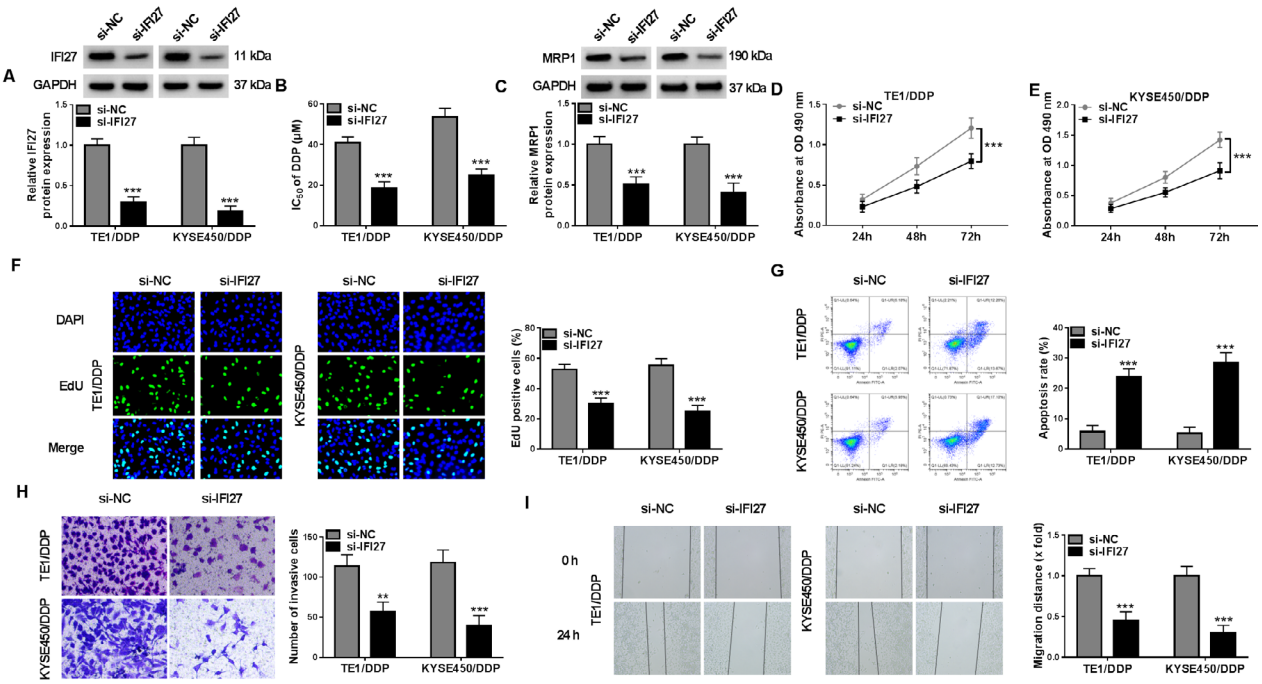

FIGURE 2 | IFI27 silencing-sensitized TE1/DDP and KYSE450/DDP cells to DDP. TE1/DDP and KYSE450/DDP cells were transfected with si-NC or si-IFI27. (A) Western blot analysis of IFI27 protein level in transfected TE1/DDP and KYSE450/DDP cells. (B) DDP resistance of si-NC or si-IFI27-transfected TE1/DDP and KYSE450/DDP cells was assessed using IC_{50} value of DDP by MTT assay. (C) MRP1 protein level was determined by Western blot in transfected TE1/DDP and KYSE450/DDP cells. (D and E) Cell viability was examined using MTT assay in transfected TE1/DDP and KYSE450/DDP cells. (F) EdU-positive cells were assessed using EdU assay in transfected TE1/DDP and KYSE450/DDP cells. (G) Flow cytometry analysis of cell apoptosis rate in transfected TE1/DDP and KYSE450/DDP cells. (H and I) Cell invasion and migration were measured in transfected TE1/DDP and KYSE450/DDP cells using Transwell and wound healing assays. $^{**}p < 0.01$; $^{***}p < 0.001$.

DDP cells was measured and displayed in Figure 3D. After that, western blot assay further showed that IFI27 protein level significantly dwindled through USP18 silencing in TE1/DDP and KYSE450/DDP cells, whereas these effects were partially overturned after proteasome inhibitor MG132 treatment (Figure 3E). Meanwhile, the overexpression efficiency of USP18 was detected and shown in Figure 3F. Next, we asked whether USP18 prevented IFI27 from degradation by using a protein stability assay. As expected, following CHX (a chemical that inhibits protein synthesis) exposure, USP18 upregulation could prolong IFI27 protein half-life in TE1/DDP and KYSE450/DDP cells (Figure 3G). Simultaneously, CoIP assay validated the binding ability of endogenous USP18 to IFI27 in TE1/DDP and KYSE450/DDP cells (Figure 3H). Beyond that, we further confirmed that the ubiquitination level of IFI27 was rapidly increased after the downregulation of USP18 in TE1/DDP and KYSE450/DDP cells (Figure 3I), consistent with the previous results. Overall, these results suggested that USP18 could increase IFI27 expression via deubiquitination by DDP-resistant ESCC cells.

3.4 | USP18 Downregulation Could Improve DDP Sensitivity in DDP-Resistant ESCC Cells by Regulating IFI27 Ubiquitination

Given the regulatory role of USP18 in IFI27 expression in DDP-resistant ESCC cells, we further identified whether the impact of USP18 on cell malignant development and DDP resistance was correlated with IFI27. Then, the overexpression efficiency of IFI27 was measured and presented in Figure 4A. Drug resistance results displayed that USP18 silencing could apparently enhance DDP sensitively in DDP-resistant ESCC cells, which was partly abolished after OE-IFI27 co-transfection

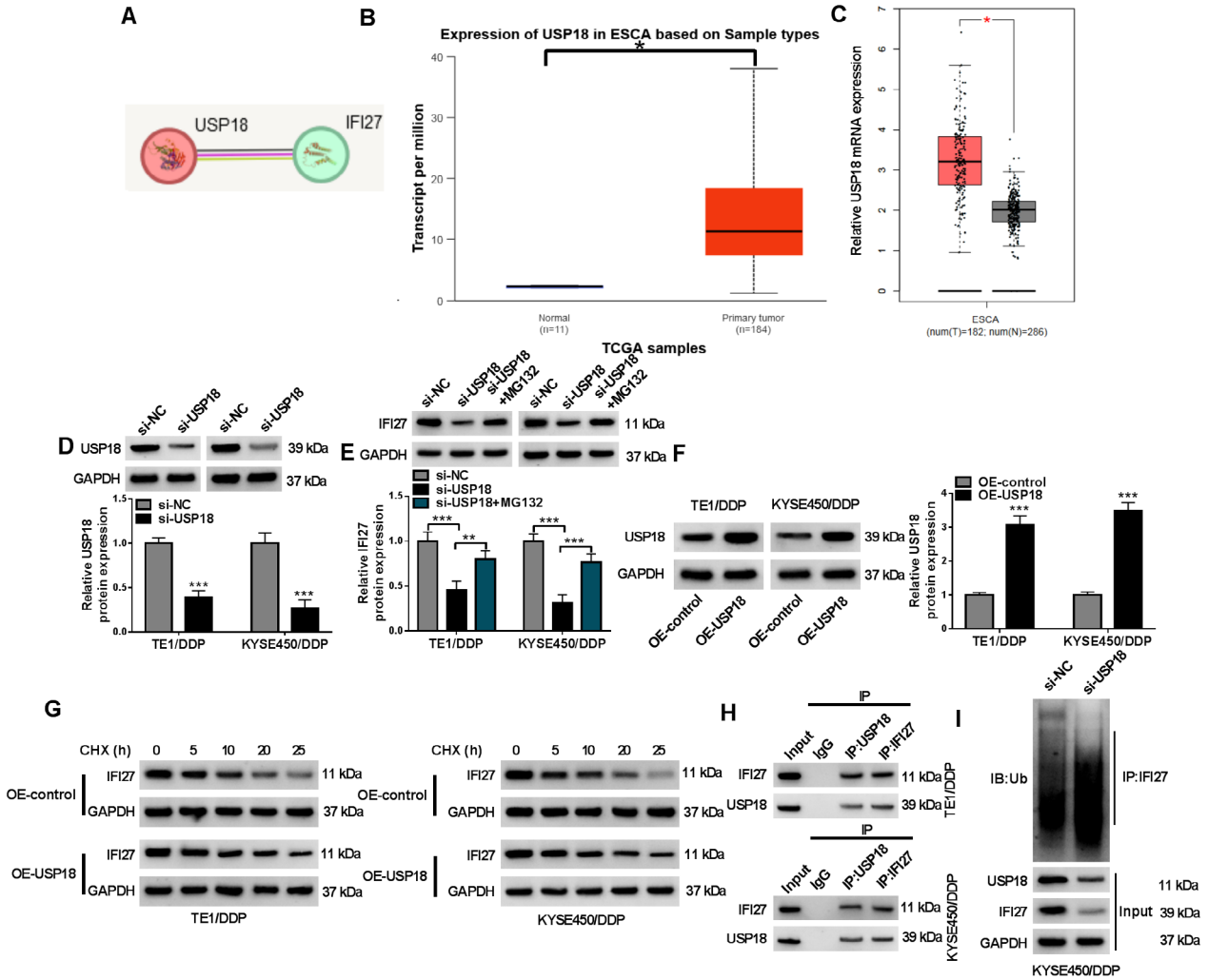

FIGURE 3 | USP18 increased IFI27 expression through deubiquitination. (A) STRING online software was used to analyze the interaction between USP18 and IFI27. (B and C) USP18 expression was determined in ESCA samples and Normal samples using TCGA and ENCORI datasets. (D) USP18 protein level was measured in TE1/DDP and KYSE450/DDP cells transfected with sh-NC or sh-USP18 using western blot assay. (E) Western blot analysis of IFI27 protein level in TE1/DDP and KYSE450/DDP cells transfected with si-NC, si-USP18, or si-USP18+ proteasome inhibitor MG132. (F) USP18 protein level was assessed TE1/DDP and KYSE450/DDP cells transfected with OE-control or OE-USP18 using western blot assay. (G) Influences of USP18 overexpression on IFI27 protein stability after CHX treatment was assessed using western blot assay in TE1/DDP and KYSE450/DDP cells. (H) The CoIP assay was performed to explore the interaction between USP18 and IFI27 in TE1/DDP and KYSE450/DDP cells. (I) IFI27 ubiquitination was detected by CoIP assay in TE1/DDP cells transfected with si-NC or si-USP18. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

(Figure 4B), accompanied by increased MRP1 expression (Figure 4C). Meanwhile, USP18 deficiency-mediated cell viability (Figure 4D,E) and cell proliferation (Figure 4F) inhibition in TE1/DDP and KYSE450/DDP cells was significantly ameliorated by IFI27 upregulation. Beyond that, the promotion of USP18 absence on cell apoptosis in TE1/DDP and KYSE450/DDP cells was partly relieved through IFI27 overexpression (Figure 4G). Besides, transwell and wound healing assays exhibited that the forced expression of IFI27 could distinctly ameliorate the negative effects of USP18 depletion on cell invasion (Figure 4H,I) and migration (Figure 4J) in TE1/DDP and KYSE450/DDP cells. Collectively, these findings indicated that elevated IFI27 reversed the repression of USP18 knockdown on cell malignant development and DDP resistance in DDP-resistant ESCC cells.

3.5 | HOXA5 Directly Bound the Promoter of USP18

Furthermore, we tried to check the upstream molecular mechanism of USP18. As shown in Figure 5A,B, two potential target mRNAs (HOXA5 and POU2F1) of USP18 gene promoter were predicted by Genecards, JASPAR, and PROMO. For further selection, the knockdown efficiency of HOXA5 or POU2F1 in TE1/DDP and KYSE450/DDP cells was determined and shown in Figure 5C,D. After that, these two targets were subjected to RT-qPCR analysis responding to HOXA5 or POU2F1 downregulation. As a result, USP18 was significantly reduced in both TE1/DDP and KYSE450/DDP cells after si-HOXA5 transfection, rather than cells transfected si-POU2F1 (Figure 5E). Thus, we chose HOXA5 for further research. In addition, RT-qPCR results verified that HOXA5 mRNA level was significantly enhanced in the DDP-resistant compared with DDP-sensitive ESCC tissues (Figure S1). According to JASPAR database analysis, HOXA5 had two binding sites to the promoter region of USP18 (Figure 5F). To identify whether HOXA5 affects the promoter activity of USP18, a dual-luciferase reporter assay was conducted in TE1/DDP and KYSE450/DDP cells. As displayed in Figure 5G,H, HOXA5 deficiency could effectively decrease the luciferase activity of WT-USP18, but not that of the mutant groups (Figure 5G,H). Meanwhile, ChIP results further presented that HOXA5 bound to the first site of the promoter of USP18 in TE1/DDP cells (Figure 5I). Additionally, western blot assay exhibited that HOXA5 silencing could prominently dwindle USP18 protein level in TE1/DDP and KYSE450/DDP cells (Figure 5J). All these results indicated that HOXA5 is directly bound to the promoter of USP18 and activated USP18 transcription.

3.6 | Overexpressing USP18 Could Abolish the Effects of HOXA5 Knockdown on Cell Development and DDP Resistance in DDP-Resistant ESCC cells

Next, to further check the effects of HOXA5 and USP18 on cell malignant behaviors and DDP sensitivity in TE1/

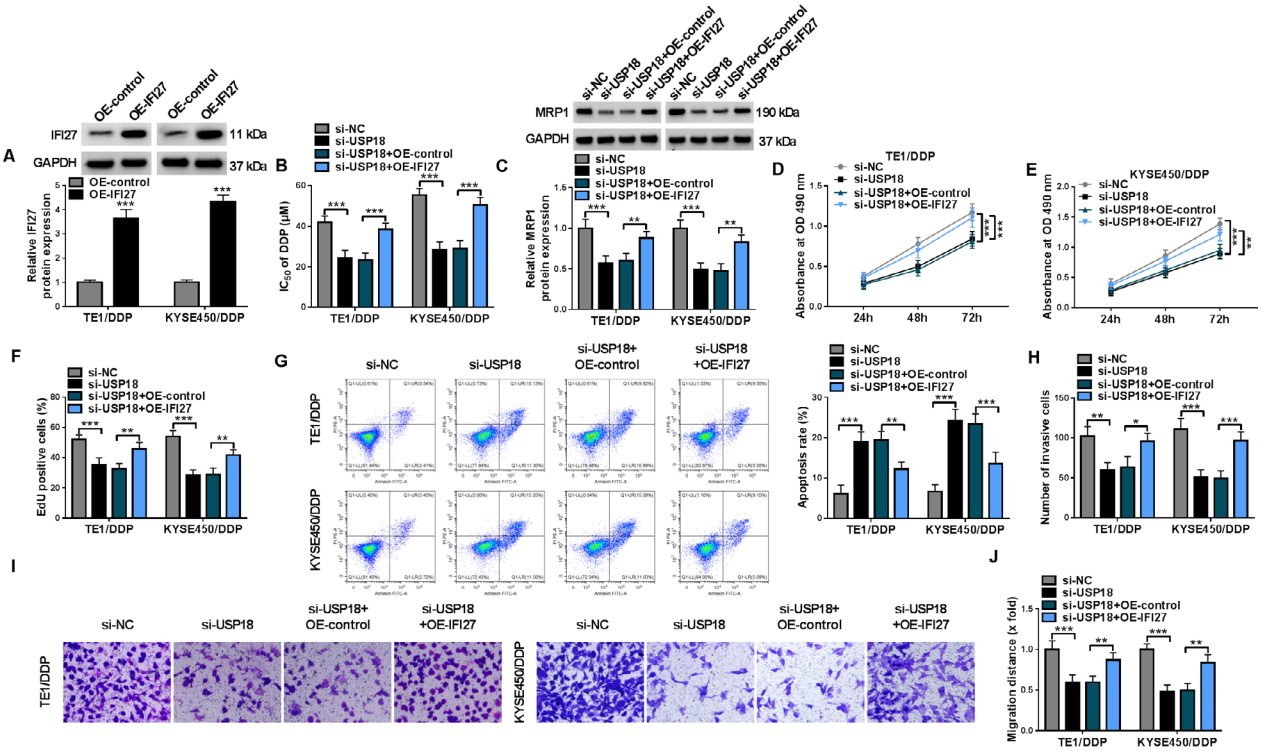

FIGURE 4 | USP18/IFI27 regulated cell malignant behaviors and DDP sensitivity in DDP-resistant ESCC cells. (A) IFI27 protein level was determined in TE1/DDP and KYSE450/DDP cells transfected with OE-control or OE-IFI27 using western blot. (B–J) TE1/DDP and KYSE450/DDP cells were transfected with si-NC, si-USP18, si-USP18 + OE-control, or si-USP18 + OE-IFI27. (B) IC_{50} value of DDP was measured by MTT assay in transfected TE1/DDP and KYSE450/DDP cells. (C) MRP1 protein level was determined by Western blot. (D and E) MTT analysis of cell viability was performed. (F) EdU-positive cells were examined using EdU assay. (G) Cell apoptosis rate was determined using flow cytometry. (H–J) Cell invasion and migration were detected using Transwell and wound healing assays. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

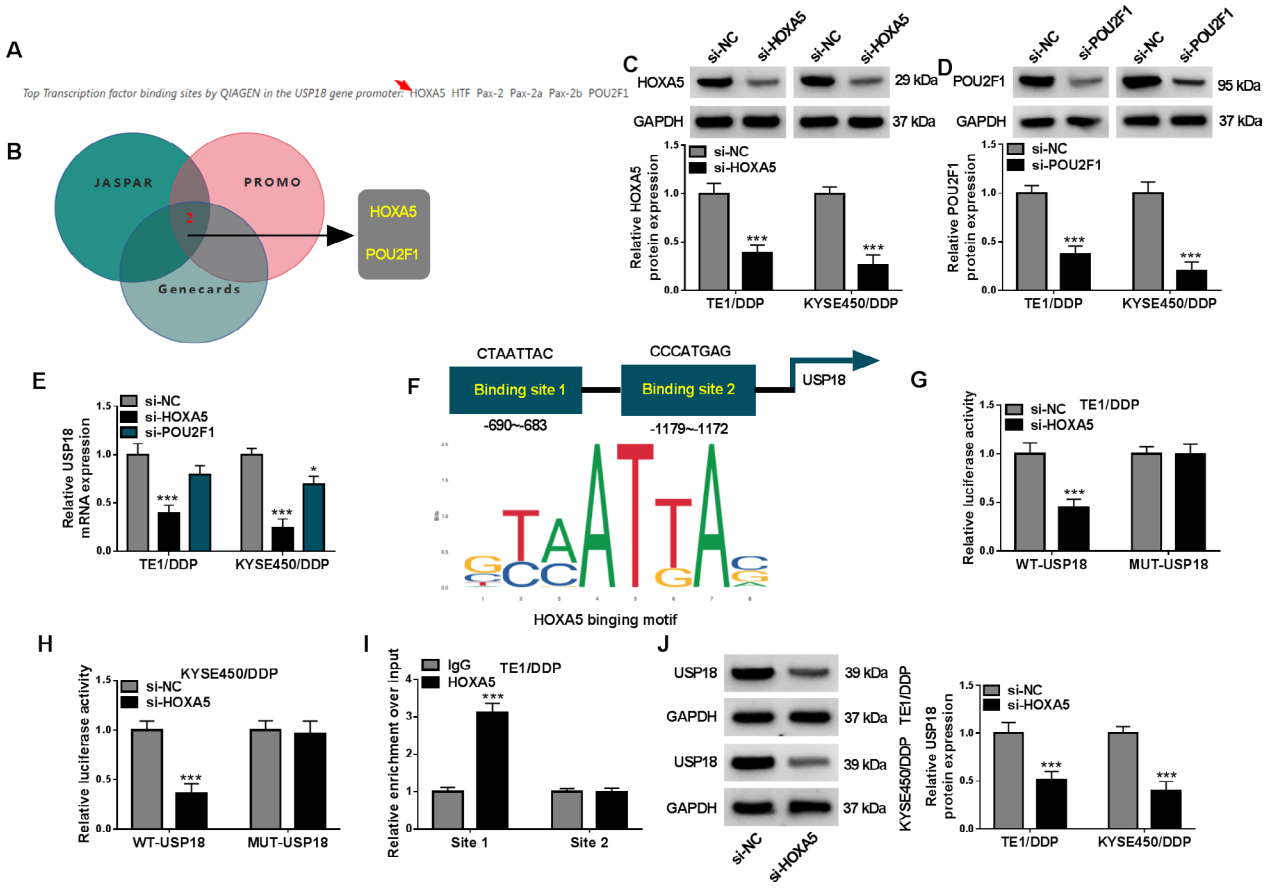

FIGURE 5 | HOXA5 activated the transcription of USP18. (A and B) Two potential target mRNAs of USP18 gene promoter were predicted by Genecards, JASPAR, and PROMO. (C) HOXA5 protein level was determined in TE1/DDP and KYSE450/DDP cells transfected with si-NC or si-HOXA5 using western blot. (D) POU2F1 protein level was assessed in TE1/DDP and KYSE450/DDP cells transfected with si-NC or si-POU2F1 using western blot. (E) Western blot analysis of USP18 protein level in si-NC, si-HOXA5, or si-POU2F1. (F) The JASPAR website predicted the binding of transcription factor HOXA5 to the USP18 promoter. (G and H) A dual-luciferase reporter assay was used to verify the binding between HOXA5 and USP18 promoter in TE1/DDP and KYSE450/DDP cells. (I) ChIP assay was performed to assess the binding between HOXA5 and the two binding sites of the USP18 promoter. (J) USP18 protein level was determined using western blot in TE1/DDP and KYSE450/DDP cells transfected with si-NC or si-HOXA5. $^{*}p < 0.05$; $^{***}p < 0.001$.

DDP and KYSE450/DDP cells, rescue assays were performed. Functionally, IC_{50} determination presented that HOXA5 downregulation-mediated DDP resistance repression was dramatically relieved after OE-USP18 co-transfection (Figure 6A), as evidenced by enhanced MRP1 (Figure 6B). Beyond that, decreased cell viability (Figure 6C,D) and cell proliferation (Figure 6E) were viewed due to the deficiency of HOXA5 in TE1/DDP and KYSE450/DDP cells, which were partially overturned by USP18 upregulation. Meanwhile, flow cytometry analysis exhibited that the addition of OE-USP18 strikingly mitigated the positive effects of HOXA5 silencing on cell apoptosis in TE1/DDP and KYSE450/DDP cells (Figure 6F). In addition, the lack of HOXA5 could dramatically attenuate cell invasion and migration in TE1/DDP and KYSE450/DDP cells, while these impacts were partly abrogated by USP18 overexpression (Figure 6G,H). Overall, these results suggested that HOXA5 knockdown blocked cell malignancy and DDP resistance in DDP-resistant ESCC cells by regulating USP18.

3.7 | Validation of HOXA5/USP18/IFI27 Regulatory Axis in HOXA5/USP18

Based on the above findings, we inferred that HOXA5 could exert its functional role partially through the USP18/IFI27 regulatory pathway. To verify the assumption, we further explored whether HOXA5 could affect the expression of IFI27 by USP18. As shown in Figure 7A,B, the silencing of HOXA5 could apparently block the IFI27 protein level in TE1/DDP and KYSE450/DDP cells, whereas these effects partly relieved these effects. Taken together, these results indicated that HOXA5 could repress IFI27 expression by modulating USP18 in DDP-resistant ESCC cells.

4 | Discussion

Although DDP-based neoadjuvant chemotherapy has been predominantly effective for most solid tumors for decades, acquired or primary drug resistance is becoming a major impediment to

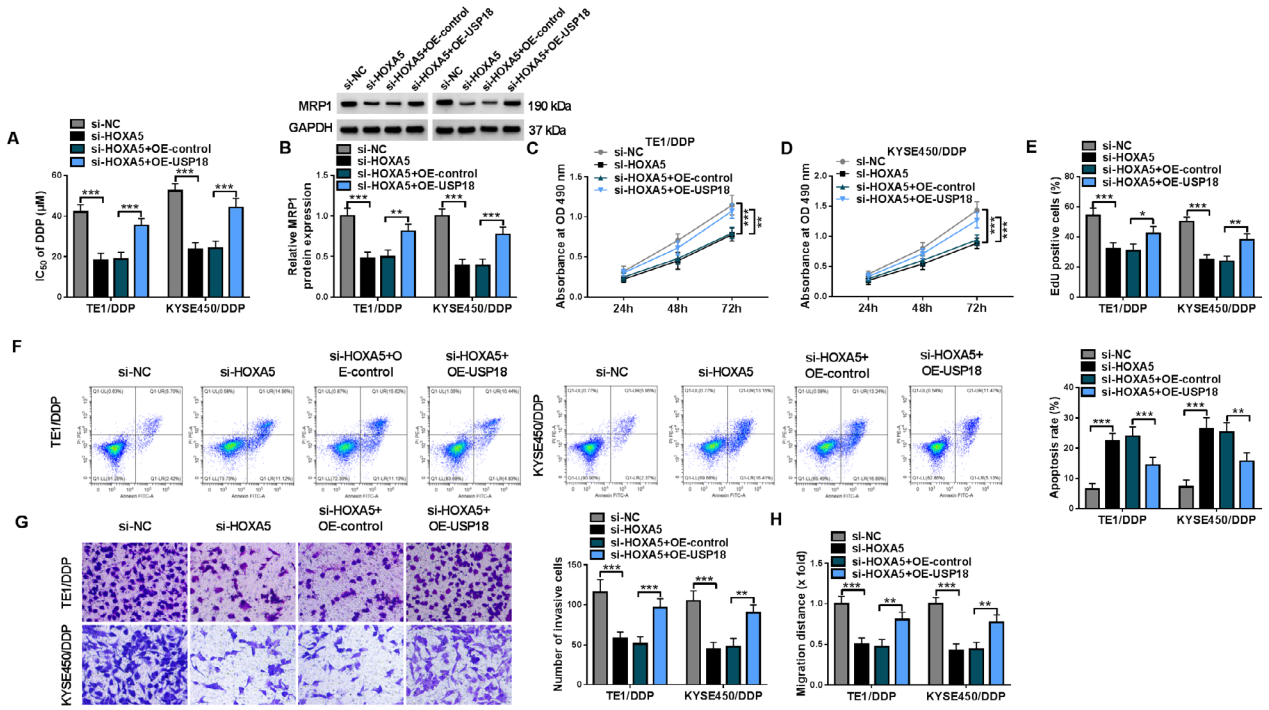

FIGURE 6 | HOXA5 inhibition could impede cell development and DDP resistance in DDP-resistant ESCC cells by targeting USP18. TE1/DDP and KYSE450/DDP cells were transfected with si-NC, si-HOXA5, si-HOXA5 + OE-control, or si-HOXA5 + OE-USP18. (A) DDP resistance was assessed using IC_{50} value of DDP was measured by MTT assay in transfected TE1/DDP and KYSE450/DDP cells. (B) Western blot analysis of MRP1 protein level. (C and D) Cell viability was monitored by MTT assay. (E) EdU-positive cells were tested using EdU assay. (F) Cell apoptosis rate was assessed using flow cytometry. (G and H) Cell invasion and migration were determined using Transwell and wound healing assays. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

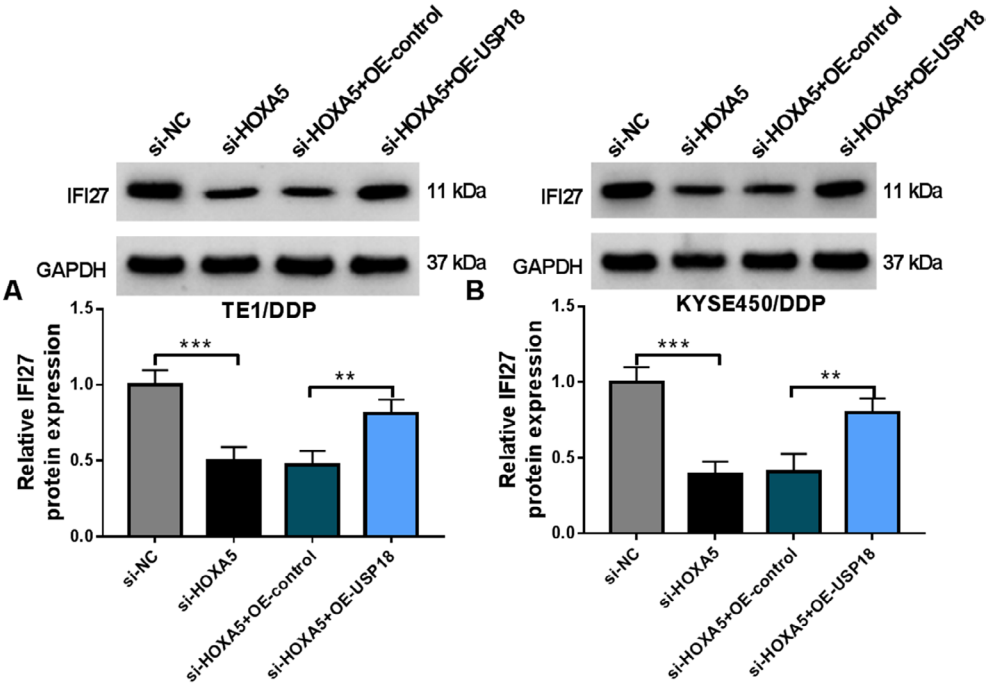

FIGURE 7 | IFI27 expression was regulated by HOXA5/USP18. (A and B) IFI27 protein level was determined in TE1/DDP and KYSE450/DDP cells were transfected with si-NC, si-HOXA5, si-HOXA5 + OE-control, or si-HOXA5 + OE-USP18 using western blot. $^{**}p < 0.01$; $^{***}p < 0.001$.

the clinical utility of cancer patients [35, 36]. Therefore, it is important to identify the pivotal elements driving drug resistance, particularly epigenetic modifications, which could offer critical insights into conquering DDP resistance in ESCC. Herein, a public Gene Expression Omnibus (GEO) dataset (GSE169337) was applied to validate differentially expressed genes in ESCC

implicated in DDP resistance. Following detection candidate upregulated genes in KYSE450/DDP cells relative to parental ESCC cells (KYSE450), we finally focused on IFI27, a member of the interferon-stimulated gene (ISG) family. Furthermore, IFI27 is a multi-pass membrane protein and is widely involved in the regulation of cell apoptosis. It has been reported that IFI27 could enhance cell sensitivity to apoptosis induced by DNA damage [16, 37]. Currently, the DDP resistance mechanism inside tumor cells has gained increasing attention, including decreased drug accumulation, enhanced detoxification activity, promotion of DNA repair capacity, and inactivated cell death signaling [38]. Furthermore, some studies have indicated that IFI27 served as an oncogene in many human cancers, containing ESCC [24]. Beyond that, IFI27 was reported to be closely associated with drug resistance in different tumors [39, 40]. Therefore, we hypothesized that IFI27 could be implicated in the regulation of cisplatin resistance in ESCC. Herein, our data identified that IFI27 content was increased in DDP-resistant ESCC subjects and cells, and its deficiency could block drug resistance, cell growth, and metastasis in DDP-resistant ESCC cells. These findings presented the key role of IFI27 in DDP resistance in ESCC for the first time.

Regarding molecular mechanism, growing evidence has suggested the essential role of ubiquitination in maintaining cellular protein homeostasis, containing tumor chemoresistance-related proteins [41, 42]. In recent years, DUBs have been reported as promising targets for drug resistance in human cancers [42]. As an important member of the DUBs, USP18 has been reported to decrease paclitaxel sensitivity of triple-negative breast cancer by regulating autophagy [43]. Beyond that, elevated USP18 could promote colorectal cancer cell survival after treatment with DDP [31]. It has been reported that DUBs function to remove ubiquitin from post-translationally modified protein substrates, thereby regulating various cellular processes. Furthermore, overexpressing USP18 could enhance the protein stability of ZEB1 by decreasing its ubiquitination, thus boosting ESCC cell growth and metastasis [32]. Herein, our data confirmed that USP18 physically interacted with IFI27 to enhance the protein stability of the latter by decreasing its ubiquitination. Furthermore, a series of rescue experiments demonstrated that IFI27 upregulation partially reversed the repression of USP18 deficiency on DDP resistance, cell proliferation, and metastasis in DDP-resistant ESCC cells. As a crucial transcriptional regulator, HOXA5 (also known as HOX1C) has been reported to partake in tumor development by interacting with many target genes [44]. For example, HOXA5 could directly bind to the promoter region of PTPRZ1 for its transcriptional activation, thereby promoting glioblastoma progression [45]. Meanwhile, HOXA5 deletion could hinder ESCC cell growth and metastasis by interfering with the Wnt/β-catenin pathway [46]. In addition, HOXA5 confer tamoxifen resistance by PI3K/AKT signaling in breast cancer [47]. Herein, our data identified that HOXA5 could transcriptionally activate USP18 by directly binding to its promoter region, supporting USP18 as a novel HOXA5-target gene. Moreover, HOXA5 knockdown-mediated DDP resistance, cell proliferation, and metastasis inhibition were reversed by USP18 overexpression in DDP-resistant ESCC cells. As expected, USP18 upregulation could partly abolish the suppressive role of HOXA5 downregulation on IFI27 expression, further supporting the regulatory mechanism of HOXA5/USP18/IFI27 in ESCC.

5 | Conclusion

Collectively, our study manifested that HOXA5 could enhance USP18 transcription by directly binding to its promoter region. Furthermore, HOXA5/USP18 could contribute to ESCC progression and DDP resistance by increasing IFI27 expression via deubiquitination. These findings provided an underlying biomarker for predicting the prognosis of chemotherapy and a potential novel therapeutic target for enhancing DDP sensitivity of ESCC.

Author Contributions

Shiheng Ren and Zheng Pan designed and performed the research. Yuxin Chen and Xiangyan Liu analyzed the data. Qiang Wang wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors have nothing to report.

Ethics Statement

Written informed consents were obtained from all participants and this study was permitted by the Ethics Committee of Shandong Provincial Hospital.

Consent

Written informed consents were obtained from all participants.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.