ANXA1 Induces Oxaliplatin Resistance of Gastric Cancer through Autophagy by Targeting PI3K/AKT/mTOR

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Research

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Abstract

Background: Resistance to oxaliplatin (OXA) is a major cause of recurrence in gastric cancer (GC) patients. ANXA1 has been found to participate in the regulation of diverse cellular functions in a variety of cell types including anti-inflammatory processes. We aimed to investigate the role of ANXA1 in autophagy and chemoresistance of GC cells.

Methods: To identify the genes that regulate oxaliplatin resistance, we used RNA-seq to profile gene expression within oxaliplatin resistant GC and parental cells. Immunohistochemical and RT-qPCR was performed to detect ANXA1 expression in tissues of 2 cohorts of GC patients who received OXA-based chemotherapy. The chemoresistant effects of ANXA1 were assessed by cell viability, apoptosis, and autophagy assays. The effects of ANXA1 on autophagy were assessed by mRFP-GFP-LC3 and western blot. Gene set enrichment analysis (GSEA) and western blot was performed to detect the activity of PI3K/AKT/mTOR signaling under the regulation of ANXA1.

Results: Based on RNA-seq profiling, ANXA1 was selected as a candidate that was upregulated in oxaliplatin resistant GC cells. Furthermore, we discovered that ANXA1 is upregulated in chemo-resistant GC tissues. Knockdown of ANXA1, via inhibiting autophagy, enhanced the sensitivity of OXA-resistant GC cells to OXA in vitro and in vivo. Mechanically, we identified that PI3K/AKT/mTOR signaling pathway was activated in the ANXA1 stably knockdown AGS/OXA cells, which leads to the down-regulation of autophagy.

Conclusions: ANXA1 functions as a chemoresistant gene in GC cells by targeting the PI3K/AKT/mTOR signaling pathway and might be a prognostic predictor for GC patients who receive OXA-based chemotherapy.

1. Introduction

Gastric cancer ranks the sixth most common cancer and the fourth most common cause of cancer-related death worldwide(1). The majority of gastric cancer patients are diagnosed at an advanced stage because of vague and nonspecific clinical symptoms. Although adequate surgical resection or endoscopic resection combined with standard adjuvant chemotherapy strategies, such as FOLFOX (Folinic acid, fluorouracil, and oxaliplatin) have significantly improved disease-free survival (DFS) and overall survival (OS), the prognosis of patients with advanced GC is still disappointing(2). This is mainly because of the development of multi-drug resistance (MDR) of gastric cancer cells(3), however, the detailed mechanisms of MDR remain inconclusive. Therefore, it is of utmost importance to identify a marker to predict chemotherapy efficacy and study the molecular mechanisms underlying chemoresistance to improve the therapeutic effect, which is also meaningful for improving the prognosis of GC patients.

Both Genetic and epigenetic modifications can cause intrinsic or acquired chemoresistance(4). Accumulating evidence has demonstrated that autophagy may play a pivotal role in

cell survival that helps cancer cells overcome therapeutic stresses, which may play an important role in chemoresistance. Autophagy is a highly conserved intracellular process that involves the formation of the autophagosome, which subsequently fuses with the lysosome to generate the autolysosome, leading to the degradation of dysfunctional cellular organelles to provide biological components and energy for cellular homeostasis maintain. However, the role of autophagy in tumorigenesis is complex and controversial: on the one hand, autophagy always acts as a tumor suppressor in the early stage of cancer(5); on the other hand, in established cancers, growing evidence reveals that autophagy can promote tumor growth by providing cell with energy and vital compounds which can help the tumor cope with various stresses, such as hypoxia, nutrient deprivation, and cancer therapy(6, 7). Although autophagy acts as a double-edged sword in tumorigenesis, it is now commonly accepted that blocking autophagy could improve chemotherapy outcomes for cancer patients(8). Traditional cytotoxic chemotherapeutics and targeted therapies can not only induce cell apoptosis, but also activate autophagy through a number of signaling pathways including the DNA damage response, the ER stress response, PI3K/AKT/mTOR, AMPK signaling, and others(9, 10). Because of its most importance in tumorigenesis, the most notably inhibitor of autophagy is hydroxychloroquine (HCQ), which has been used in numerous clinical Phase I/II trials for the combined treatment of colon cancer(11), pancreatic cancer(12, 13), renal cell carcinoma(14), and glioblastoma(15). However, due to the low selection and penetration of HCQ in the acidic tumor microenvironment, the combined therapy of HCQ with chemotherapy compounds only showed limited activity in unselected populations. Thus, the mechanism of autophagy and autophagy-related chemoresistance remain a major focus in the field, which may lead to new combined therapeutic approaches for cancer treatment in the future.

In the current study, we established oxaliplatin-resistant gastric cancer cell lines AGS/OXA and HGC27/OXA and screened the expression profiles of Oxa-resistant GC cells and their parental cells during the generation of chemoresistance character, we focused on the up-regulated genes and identified that ANXA1 was implicated in the chemoresistance of gastric cancer cells. Moreover, we found that ANXA1 was significantly up-regulated in GC tissues, especially in chemo-resistant cancer tissues, also, GC patients with relative higher ANXA1 expression showed poorer survival rate. Functional research in vitro and in vivo were conducted to assess the biological role of ANXA1 in GC, we observed that ANXA1 can induce the chemoresistance of GC cells. Of note, ANXA1 was proved to be an autophagy activator, and ANXA1-mediated chemoresistance was autophagy dependent. Mechanically, Gene Set Enrichment Analysis (GSEA) analysis data showed that the PI3K/AKT/mTOR and Apoptosis signaling pathways were both activated in the ANXA1 stably knockdown AGS/OXA cells. Furthermore, we demonstrated that ANXA1 knockdown led to p-AKT up-regulation, while ANXA1 over-expression led to p-AKT suppression. ANXA1 could inactivate PI3K/AKT/mTOR pathway to promote the autophagic flux, which led to GC cell chemoresistance.

2. Materials And Methods

2.1. Cells and human tissues

Eight human gastric adenocarcinoma cell lines of SGC7901, BGC823, MGC803, HGC27, AGS, MKN45, NCI-N87, and SNU-1 as well as the normal gastric mucosal cell line GES-1 were used in this study, all above cell lines were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were routinely cultured in complete RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum, penicillin, and streptomycin (HyClone, Logan, UT, USA) at 37 °C in an atmosphere containing 5% CO₂. The OXA-resistant gastric cancer cells AGS/OXA and HGC27/OXA were established in a stepwise manner by continuous exposing the cells to increasing concentrations of OXA (oxaliplatin) from 0.25 µg/mL to 6 µg/mL and 2 µg/mL respectively in a period of eight months.

18 pairs of human gastric cancer tissues and their corresponding adjacent normal tissues were obtained from initially diagnosed gastric cancer patients who had received gastrectomy in the Department of Gastrointestinal, the Fifth Peoples' hospital of Shanghai (Fudan University) from June 2018 to January 2019. All patients were diagnosed with gastric cancer according to histopathological evaluation, and no any preoperative treatment was conducted. Written informed consents were obtained from all patients in accordance with the Declaration of Helsinki. This study was approved by the Human Ethics Committee of the Fifth Peoples' hospital of Shanghai.

2.2. RNA preparation and reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from tissues or cells using TRIzol reagent (Invitrogen, USA) and was reversed transcribed into cDNAs using the HiScriptQ Select RT SuperMix for qPCR (Vazyme, China) according to the manufacturer's protocol. The cDNAs were then amplified by real-time quantitative RT-PCR using the ChamQ™ Universal SYBR qPCR Master Mix (Vazyme, China). The real-time PCRs were performed in triplicate and the gene expression change was normalized to GAPDH expression and calculated by the 2- $\Delta\Delta C_t$ method. The primers used in this study were synthesized by Invitrogen (Shanghai, China) and are listed in Additional file 1: Table S1.

2.3. RNA interference, vector construction, and cell transfections

The duplex small interfering RNAs (siRNAs) that specifically target human ANXA1 were purchased from GenePharma (Shanghai, China). siRNA transfection was performed using Lipofectamine® 2000 (Invitrogen, USA) according to the manufacturer's protocol.

Flag (3×Flag)-tagged ANXA1 were cloned into pLVX plasmid, two shRNA-ANXA1 were synthesized by Invitrogen and cloned into lentivirus vector pLKO.1 HEK293T cells were used for lentivirus packaging. pLVX-ANXA1 and pLKO.1-ANXA1-shRNAs were then co-transfected with lentiviral packaging plasmids psPAX2 (Addgene plasmid, 12,260) and pMD2.G (Addgene plasmid, 12,259) using Lipofectamine® 2000

(Invitrogen, USA). Viruses were then harvested at 48h and 72h, respectively, after transfection. Cells that stably transfected with pLVX-ANXA1, pLKO.1-ANXA1-shRNAs, and negative control virus were selected by the treatment of puromycin (5µg/mL, Solarbio, China). The sequences of the siRNAs and shRNAs are listed in Additional file 1: Table S2.

2.4. Western blotting

Western blotting was performed according to the standard protocols. The total cellular or tissue protein was lysed by RIPA buffer supplemented with protease inhibitor and phosphatase inhibitor cocktail. Protein content was quantified by BCA Protein Assay Kit (Thermo Fisher Scientific, West Palm Beach, FL). Equal amounts (50 µg) of proteins were electrophoresed by 10% or 15% SDS polyacrylamide gels and were then transferred to NC membranes (Pall Corporation). The membranes were blocked with 5% skim milk for one hour and then incubated with the indicated primary antibodies at 4 °C overnight. After washing with TBST, the blots were labeled with HRP (Horseradish peroxidase)-conjugated secondary antibodies (Santa Cruz Biotechnology), and then detected using ECL kit (Pierce Biotech, Rockford, IL). The band intensities of the western blots were analyzed by ImageJ software. The antibodies used in this study were ANXA1 (#21990, 1:2000, Proteintech, China), LC3B (#2775, 1:1000, Cell Signaling Technology, USA), SQSTM1 (#18420, 1:2000, Proteintech, China). Tubulin (#SC8035, 1:1000, Santa Cruz, USA), p-AMPK (#2535, 1:1000, Cell Signaling Technology, USA), AKT (#4685, 1:1000, Cell Signaling Technology, USA), p-AKT (#4060, 1:2000, Cell Signaling Technology, USA).

2.5. Confocal Microscope

The adenoviral containing mRFP-GFP-LC3 vectors were purchased from HanBio Technology (Shanghai, China). AGS/OXA, HGC27/OXA cells and their parental non-resistant cells were plated in 6-well plates and allowed to reach 40% confluence at the time of infection. After that, mRFP-GFP-LC3 stable expression cells were selected by treatment of puromycin (5µg/mL). The stable cells were then transfected with pLVX-ANXA1, pLKO.1-ANXA1-sh1, or pLKO.1-ANXA1-sh2. After different treatments, the cells were fixed and stained with DAPI (Beyotime, Shanghai, China), and then analyzed using confocal microscopy (Olympus BX51, Japan).

2.6. Cell viability and colony formation assay

The effects of OXA on cell growth were measured by a cell viability assay. First, AGS/OXA, HGC27/OXA and their parental cells AGS, HGC27 were harvested and seeded into 96-well plates in triplicate for 24h of cultivation (1.5×10^3 cells/well). Then, OXA in different concentrations were added into designated wells (AGS, AGS/OXA: 1, 2, 4, 8, 12, 16, 24, 32 µg/mL; HGC27, HGC27/OXA: 0.1, 0.4, 0.8, 2, 4, 8, 12, 24 µg/mL). After 48h cultivation, cell viability were detected by MTT assays. The absorbance was measured by scanning with a microplate reader (Thermo

Fisher Scientific, Waltham, MA, USA) at 450nm. “Relative cell viability” = the viability of cells in OXA-containing medium/the viability of cells in OXA-free medium. Cell viabilities was further fitted to a dose-response curve to calculate the IC₅₀ (50% inhibition of growth) values for each group of cells.

Colony formation assay was used for long-term effects assessment of ANXA1 on cell viability with or without OXA. Each group of cells was plated into 6-well plates at a density of 1×10^3 cells per well and cultured with or without OXA, the culture medium was replaced every 3 days, and after 14 days cultivation, the colonies were fixed with 4% paraformaldehyde for 15 minutes, and then stained with 0.1% crystal violet for 30 minutes at room temperature, finally, the colonies were scanned and counted using ImageJ k 1.45 (<http://imagej.nih.gov/ij>).

2.7. Cell apoptosis assay

For apoptosis analysis, cells were seeded into 12-well plates (1.0×10^5 cells/well) and incubated for 12h. OXA-resistant cell lines AGS/OXA, HGC27/OXA and their parental non-resistant cell line AGS, HGC27 were treated with different concentrations of OXA for 12h. Meanwhile, pLVX-ANXA1 and pLVX-NC were transfected into AGS and HGC27 cells, while ANXA1-siRNA and NC-siRNA were transfected into AGS/OXA and HGC27/OXA cells. After 48h of transfection, each group was treated with OXA (1μg/mL) for 12h, then, cells were collected and stained with Annexin V-APC and 7-AAD for 30 minutes. The percentage of apoptotic cells was measured by flow cytometry (BD, USA).

2.8. Tissue micro array and immunohistochemical (IHC) staining

A human Tissue micro array (TMA) (cat no. TFstoma-01) containing 83 paired gastric tumor and normal tissue samples was purchased from Shanghai Tufei Biotech Co., Ltd. (Shanghai, China). The detailed clinicopathological information, including age, sex, tumor size, tumor location, differentiation grade, and TNM stage are shown in Table 1. All of the 83 patients enrolled were initially diagnosed GC with stage I-III after surgery resection and received postoperative adjuvant oxaliplatin or cisplatin-based chemotherapy: ECF regimen (Tegafur plus oxaliplatin), EOF regimen (Capecitabine plus oxaliplatin), or DCF (Cisplatin, fluorouracil and docetaxel). The median follow-up time after surgery was 56.98 months, and 43 patients experienced tumor recurrence or metastasis during the follow-up period, which was assigned as the chemo-resistant group, and the other 40 patients were enrolled as the relatively chemo-sensitive group.

IHC staining of TMA sections was performed according to the manufacturer's protocol. Slides were stained with a rabbit anti-ANXA1 polyclonal antibody (1:200 dilution; cat. no. 21990-1-AP; ProteinTech Group, Inc.). Two independent experienced pathologists evaluated the immunoreactive score (IRS) of each section in a double-blind manner. An IRS score system was used to semi-quantitate ANXA1 expression, as previously described[18]. Briefly, the intensity of staining (0, negative; 1, weak; 2, moderate;

and 3, strong) was multiplied by the percentage of positive cells in five gradations (0, negative; 1, < 10%; 2, 10%-50%; 3, 51%-80%; and 4, > 80%), each sample was measured in three different fields. The IRS score ranged from 0-12, with $IRS \leq 4$ indicating low ANXA1 expression and $IRS > 4$ indicating high ANXA1 expression.

2.9. In vivo chemo-sensitivity assay

For the in vivo chemo-sensitivity assay, fourteen 6-week-old female BALB/c nude mice were randomly divided into two groups ($n=7$ per group). AGS/OXA cells stably transfected with pLKO-ANXA1-SH2 or pLKO-NC (a total of 1.5×10^7 cells in 0.1 mL PBS mixed with 0.1 mL matrigel (Becton Dickinson, NJ, USA)) were subcutaneously injected into the right flank region of each nude mouse. Chemotherapy was established after 10 days of injection, when the tumors were measurable. Oxaliplatin (5mg/kg) were intraperitoneally injected into mice twice a week for 3 weeks. Xenografts were measured every 3 days with calipers, and tumor volumes were calculated using the formula $V = 0.5 \times \text{length} \times \text{width}^2$. On day 35, the animals were sacrificed and the tumors were excised, photographed, and weighed. The tumor growth curve was drawn based on tumor volume and the corresponding days after treatment. All experiments were performed in accordance with the guidelines of Shanghai JiaoTong University Animal Care Commission.

2.10. RNA-sequencing

Total RNA was extracted by TRIzol Reagent (Life Technologies, USA). After the total RNA sample is qualified, the polyA tailed mRNA is enriched with oligo (dT) magnetic beads, and the enriched mRNA is randomly interrupted with divalent cations in NEB Fragmentation Buffer. RNA-seq libraries were prepared using the NEB Next Ultra Directional RNA Library Prep Kit for Illumina (NEB, Beverly, MA, USA). Briefly, using fragmented mRNA as a template and random oligonucleotides as primers, the first strand of cDNA was synthesized in the M-MuLV reverse transcriptase system, and then the RNA strand was degraded with RNase H, and in the DNA polymerase I system, dNTPs were used to synthesize the second strand of cDNA as raw material. The purified double-stranded cDNA undergoes end-repair, A-tailing, and ligation of sequencing adapters and then uses AMPure XP beads to purify the PCR products to obtain a library. The cDNA was then sequenced in a HiSeq 2000 system (Illumina, San Diego, CA, USA). The gene expression level is estimated by counting the reads mapped to the genomic region or gene exon region. In addition to being directly proportional to the true expression level of the gene, the Reads count is also positively related to the length of the gene and the sequencing depth. To make the estimated gene expression levels of different genes and different experiments comparable, the concepts of RPKM (Reads Per Kilo base per million reads) and FPKM (Fragments Per Kilo base per million reads) have been introduced successively.

2.11. GSEA

GSEA analysis was performed by the GSEA v3.0 program provided by the Broad Institute (<http://www.broadinstitute.org/gsea/index.jsp>) to study the enrichment of genes in different pathways. Gastric cancer-related gene sets were obtained from the MSigDB database. The RNA-seq results of AGS/OXA-NC and AGS/OXA-ANXA1-SH2 were analyzed for GSEA. Briefly, the different expressed genes in the two groups were compared by Student's t-test and ranked. GSEA compares the ranked genes to a collection of pathway gene sets and assigns an enrichment score. The gene sets that significantly perform random-class permutations are considered significant as detailed previously.

2.12. Statistical analysis

Statistical analyses were performed using SPSS 20.0 (IBM SPSS, Chicago, IL, USA) and GraphPad Prism 7 software and the graphs were generated with GraphPad or R (ggplot). Paired T-test was used to analyze the differences of ANXA1 expression between GC tissues and corresponding noncancerous tissues. Independent samples t-test was used for comparing of ANXA1 expression differences between chemo-resistant and chemo-sensitive GC tissues. Chi-square test was performed to analyze the differences in group proportions. Survival rates between groups were analyzed by Kaplan-Meier method, and a Cox regression model was established to determine independent prognostic factors using variables that were significant in the univariate analysis. The power of differential diagnosis was evaluated by Receiver operating characteristic (ROC) curves using SPSS 20.0. All data were presented as standard deviation (SD), and a $P < 0.05$ was considered statistically significant.

3. Results

3.1. Screen and validation of genes related to GC oxaliplatin resistance

To investigate the molecular mechanisms that contribute to the oxaliplatin resistance of gastric cancer cells, firstly, we established the OXA-resistant gastric cancer cells and confirmed the IC_{50} values for OXA in AGS/OXA, HGC27/OXA cells and their parental cells (Fig. 1A). We found that the IC_{50} was fundamentally elevated in OXA-resistant GC cells compared with their parental cells (Fig. 1B), the IC_{50} for oxaliplatin was $2.02 \mu\text{mol/mL}$ and $0.95 \mu\text{mol/mL}$ in parental cells respectively, indicating more sensitive compared with that of OXA-resistant cells (AGS/OXA: $13.37 \mu\text{mol/mL}$ and HGC27/OXA: $5.28 \mu\text{mol/mL}$) (Table S3). Furthermore, the OXA-resistant cells showed lower cell apoptosis rate when treated with the same concentration of OXA ($1 \mu\text{g/mL}$), while the parental cells showed higher sensitivity to OXA induced apoptosis (Fig. 1C).

After the OXA-resistant GC cells were established and proved, we performed RNA-seq between AGS/OXA, HGC27/OXA and their parental cells AGS, HGC27 for two times. The first RNA-seq was performed at the time point of three months after the parental cells AGS, HGC27 have been cultured with escalating concentrations of OXA; The second RNA-seq was performed after the cells were treated with OXA for eight

months, and the OXA-resistant cells showed lower OXA-sensitivity when compared with the 3 months group. In total, we analyzed two RNA-seq data (4 groups), and found that 104 genes were up-regulated and 46 genes were down-regulated in all OXA-resistant groups (Fig. 1D). Furthermore, among the aberrantly over-expressed mRNAs in all four groups, we found 10 genes met the criteria (fold change > 2; Fig. 1E-F). Among these candidates, we observed that the fold change (FC) of ANXA1 elevated dramatically along with the augmented ability of OXA resistance. The FC of ANXA1 was up-regulated from 3.64 to 18.29 in AGS/OXA vs. AGS, while 4.40 to 5.02 in HGC27/OXA vs. HGC27.

Then we confirmed the expression level of ANXA1 in AGS/OXA, HGC27/OXA cells, and their parental cells AGS, HGC27 by qRT-PCR and WB. As expected, ANXA1 was significantly increased in both OXA-resistant GC cells compared to parental cells (Fig. 1G-H). Therefore, our results suggested that ANXA1 was up-regulated significantly in GC cells during the acquisition of OXA-resistance and may play an important role in chemoresistance.

3.2.Changes of ANXA1 expression in GC cell lines treated with different concentrations of oxaliplatin

To further study the effect of oxaliplatin on the expression of ANXA1 in GC cell lines, we used different concentration gradients of oxaliplatin (0, 2, 4, 8, 16 $\mu\text{g/mL}$) to treat the GC cell line AGS for 24 hours and then the expression level of ANXA1 was detected. The results of Western blotting showed that the expression level of ANXA1 gradually increased as the concentration of oxaliplatin increased, indicating that as the concentration of oxaliplatin increased, GC cells may produce corresponding protective measures, including the up-regulation of ANXA1 to cope with the cytotoxicity caused by oxaliplatin, indicating that ANXA1 plays a key regulatory role in the process of oxaliplatin resistance in GC (Fig. 1I).

3.3.Expression level of ANXA1 in GC tissues and its clinical significance

The expression levels of ANXA1 in 8 gastric cancer cell lines and a human normal gastric epithelial cell line (GES-1) were determined by qRT-PCR and Western blotting. The protein levels of ANXA1 were variable among gastric cancer cell lines, of note, the majority of GC cell lines showed higher levels of ANXA1 compared to GES-1 (Fig. 2A). The transcriptional level of ANXA1 showed the same tendency in these samples (Fig. 2B).

To further confirm our results in clinical samples, we detected ANXA1 expression in 18 GC tissues and the corresponding adjacent non-cancerous gastric tissues. As shown in Figs. 2C and 2D, the protein level of ANXA1 was elevated in 15 out of 18 GC tissues compared with the corresponding noncancerous gastric tissues. Furthermore, we investigated the expression of ANXA1 in a larger sample collection, immunohistochemical assays were performed to examine the ANXA1 protein levels in 83 cases of GC

with paired adjacent tissues. The IRS scores of ANXA1 in tumors were much higher than those in adjacent non-cancerous gastric tissues ($P = 0.00023$) (Fig. 2E-F). Moreover, 83 pairs of GC patients were further divided into two groups: clinical samples of patients who relapsed or recurred after oxaliplatin-based chemotherapy (OXA-resistant group; $n=43$); were compared with those of patients who did not (OXA-sensitive group; $n=40$). The results showed that ANXA1 expression was much higher in the OXA-resistant group than that of OXA-sensitive group, indicating that ANXA1 expression was positively associated with OXA-resistance in GC (Fig. 2F). When oxaliplatin sensitivities were included as the end point for detection compared to OXA-resistant, ANXA1 yielded an area under the ROC curve, $AUC = 0.7099$ ($95\%CI = 0.596-0.824$) (Fig. 2G).

3.4.High ANXA1 expression is associated with poor OS of patients with GC

Detailed clinical characteristics, including patient age, gender, tumor size, tumor location, histologic differentiation, number of lymph node metastasis, and TNM stage, are shown in Table 1. The correlation analysis of ANXA1 expression revealed a significant association between ANXA1 expression and TNM stage ($P = 0.008$; Table 1). And we further investigated the relationship between ANXA1 protein expression level and OS of GC patients and found that the OS was significantly lower among patients with high ANXA1 expression than those with low ANXA1 expression ($P = 0.0044$; Fig. 2H). In addition, a Cox univariate survival analysis revealed that ANXA1 expression, neural/vascular invasion, lymph node metastasis, and TNM stage were significant parameters affecting the survival time of patients with GC (Table 2). These four significant factors were subsequently substituted into the multivariate Cox survival analysis, which indicated that both neural/vascular invasion and TNM stage were independent predictors for unfavorable prognosis in patients with GC (all $P < 0.05$), while ANXA1 expression was not an independent predictor of GC overall survival (Table 3).

3.5. ANXA1 promotes GC cell OXA-resistance without influencing proliferation

To further confirm the role of ANXA1 in OXA-resistance of GC cells, we transfected ANXA1-siRNA to the HGC27/OXA and AGS/OXA cells that presented higher expression levels of ANXA1. pLVX-ANXA1 were transfected into AGS and HGC27 cells that presented lower expression levels of ANXA1 to up-regulate the expression of ANXA1. The down-regulation and over-expression efficiency was confirmed by Western blotting(Fig. 3A).

MTT assay demonstrated that knockdown of ANXA1 in OXA-resistant cells had a limited effect on the proliferation ability of OXA-resistant GC cells. Furthermore, ANXA1-overexpressed parental cells showed the same proliferation rate compared to their control cells(Fig. 3B). However, while the transfected cells were treated with OXA, as shown in Fig. 3C, ANXA1 knockdown significantly enhanced the sensitivity

of AGS/OXA and HGC27/OXA cells to OXA. While, ANXA1 overexpression heightened the IC₅₀ values of AGS and HGC27 cells to OXA.

The colony formation assay further verified that suppression of ANXA1 inhibited cell proliferation and promoted OXA sensitivity when the OXA-resistant gastric cancer cells were treated with OXA. In contrast, overexpression of ANXA1 in parental OXA-sensitive GC cells notably improved cell proliferation and enhanced OXA resistance. However, the data showed that neither suppression of ANXA1 in OXA-resistant cells nor overexpression of ANXA1 in parental OXA-sensitive cells affects the ability of colony formation (Fig. 3D-E), indicating the function of ANXA1 was restricted to the treatment of OXA in chemoresistant condition.

Flow cytometry analysis showed that OXA-induced apoptotic rate of GC cells was significantly decreased after transfected with pLVX-ANXA1. Whereas suppression of ANXA1 greatly increased the apoptotic rate of AGS and HGC27 cells treated with OXA (Fig. 4A-B).

3.6. Knockdown of ANXA1 improves the sensitivity of gastric cancer cells to OXA in vivo

To assess whether ANXA1 exerts OXA resistance function in vivo, we transfected AGS/OXA cells that presented relatively high expression levels of ANXA1 with two individual lenti-virus packaged shRNAs against ANXA1. Then we selected ANXA1-SH2 for the subsequent experiments for its higher efficiency of inhibition. AGS/OXA cells with stable transfection with Lv-ANXA1-SH2 or Lv-Control were subcutaneously injected into nude mice, to construct xenograft mouse models. OXA was intraperitoneally injected into mice twice a week. As shown in Fig. 4C, ANXA1 down-regulated group displayed smaller tumor size and weight than that of NC group (Fig. 4D-E). These results indicated that upregulation of ANXA1 in OXA-resistant GC cells was critical for developing the ability of chemo-resistance.

3.7. OXA-resistant GC cells exhibit enhanced autophagic activity

Autophagy, a conserved lysosome-mediated intracellular degradation system, could protect cancer cells during stress conditions, including cytotoxic stress(16). It has been reported that autophagy confers cancer cells the property of drug resistance(17). Thus, we hypothesized that OXA-resistant GC cells might exhibit increased autophagic flux. To our knowledge, LC3B was used as a marker of autophagy activation and the conversion of LC3-I to LC3-II is regarded as a measure of autophagic flux. The adapt protein p62 (SQSTM1), which interacts with LC3B-II, is also considered as a hallmark of autophagy(18). To reveal whether autophagy could contribute to the drug resistance of gastric cancer cells. As expected, parental cells presented a higher LC3-II to LC3-I ratio and increased p62 degradation compared to OXA-resistant cells (Fig. 5A-B). p-AMPK was thought to be the upstream activator of autophagy, so we also detected the

expression level of p-AMPK. Consistently, AGS/OXA and HGC27/OXA cells both presented an increased expression levels of p-AMPK compared with parental cells, indicating an activation of the autophagic flux of OXA-resistant cells (Fig. 5C). To further validate the autophagic flux level between OXA-resistant cells and parental cells, we established stably expressing tandem mRFP-GFP-LC3 gastric cancer OXA-resistant and parental cells using a lentiviral vector to localize and assess the autophagic flux. As GFP is sensitive to the PH change owing to the fusion of autophagosomes and lysosomes, we observed that the percentage of GFP positive cells was much higher in parental cells compared to OXA-resistant cells, indicating OXA-resistant cells harbored an enhanced autophagic flux process (Fig. 5D-E).

3.8. Enhanced autophagy contributes to the chemo-resistant properties of gastric cancer cells.

Next, we sought to explore whether autophagy could regulate the chemosensitivity of gastric cancer cells to oxaliplatin. We treated AGS/OXA and HGC27/OXA cells with chloroquine (CQ). As showed in Fig. 5F, increased expression levels of LC3II and p62 were observed. Moreover, MTT assays showed that CQ greatly enhanced the sensitivity of OXA-resistant cells to oxaliplatin as evidenced by the decreased IC₅₀ concentration (Fig. 5G). Meanwhile, we treated OXA-sensitive gastric cancer cells (AGS and HGC27) with rapamycin (RAPA), an autophagy activator, and found that the autophagy process was enhanced accompanied with increased IC₅₀ (Fig. 5F-G). Collectively, these data show that the OXA-resistance ability of GC cells was positively related to their enhanced autophagic activity, of note, inhibition of autophagy is capable to enhance the sensitivity of gastric cancer cells to oxaliplatin.

3.9. ANXA1 promotes autophagy process

Next, we addressed whether ANXA1 regulated the autophagy process in GC cells, we constructed ANXA1 stably over-expression cells with pLVX-ANXA1 and found that over-expression of ANXA1 greatly enhanced autophagy, as indicated by the elevated LC3-II to LC3-I ratio, attenuated p62 protein level (Fig. 6A). Consistently with the results above, we observed the enhanced autophagosomal-lysosomal fusion process in ANXA1 over-expression cells using a fluorescence microscope (Fig. 6B). We also constructed ANXA1 stably knockdown cells with an shRNA sequence targeting ANXA1. Knockdown of ANXA1 greatly inhibited autophagy, as indicated by the attenuated LC3-II to LC3-I ratio, elevated p62 protein levels. (Fig. 6C). The Western blotting analysis showed that the expression levels of LC3-II were elevated by CQ[10], an autophagy-lysosomal inhibitor. While the combination of sh-ANXA1 and CQ reduced the expression levels of LC3-II in OXA-resistant GC cells (AGS/OXA, HGC27/OXA) compared with the combination of sh-NC and CQ (Fig. 6D). Furthermore, we observed the suppressed autophagosomal-lysosomal fusion process in ANXA1 down-regulated cells using a fluorescence microscope (Fig. 6E).

3.10. The enhancement effect of ANXA1 on oxaliplatin resistance in gastric cancer depends on its promotion of autophagy

To explore whether the enhanced autophagy process played an important role in ANXA1-mediated oxaliplatin resistance in GC cells. By using the autophagy inhibitor, CQ, we found that the IC₅₀ of parental cells elevated by over-expression of ANXA1 were reduced by CQ, meanwhile, the IC₅₀ of chemo-resistant cells suppressed by ANXA1 knockdown were elevated by rapamycin (Fig. 6F). The rescue experiments indicated that autophagy activators or suppressors abrogated the effect of ANXA1 on the chemoresistance and autophagy of gastric cancer cells. These data suggest that ANXA1 can activate autophagy and promotes OXA-resistance, and its function in chemo-resistance is autophagy dependent.

3.11. ANXA1 activates autophagy via PI3K/AKT pathway

Next, we sought to identify the underlying mechanism by which ANXA1 promoted autophagy in gastric cancer cells. First, using qRT-PCR, we determined the knockdown effect of shRNA sequence targeting ANXA1, as shown in Fig. 7A, SH2 showed more efficiency than SH1; Second, we performed RNA-sequencing on ANXA1 stably knockdown cells and control cells, and analyzed the data by GSEA analysis, the results showed that PI3K/AKT/mTOR and Apoptosis pathways were both activated at different levels in ANXA1 stably knockdown cells (Fig. 7B). WB results indicated that over-expression of ANXA1 suppressed the expression of p-AKT, while the AKT level did not change (Fig. 7C); however, down-regulation of ANXA1 activated the expression of p-AKT, while did not affect the AKT level (Fig. 7D).

4. Discussion

At present, surgical resection remains the major therapy for advanced GC(19), and surgery supplemented by chemotherapy, radiotherapy, and targeted drug therapy can effectively prolong the overall survival(OS) rate of patients with advanced gastric cancer and significantly improve the quality of life(20). The drugs commonly used in gastric cancer chemotherapy include adriamycin(ADR), platinum drug, 5-FU, vincristine(VCR), paclitaxel(PTX), oxaliplatin used in this study is a second-generation platinum drug, which was recommended in both adjuvant and palliative GC chemotherapy, the main cytotoxic effect of which is DNA synthesis inhibition(21). However, intrinsic or acquired resistance to oxaliplatin leads to poor prognosis, the reasons of gastric cancer cells to form MDR include inhibition of apoptosis signals, cell cycle checkpoint disorders, enhancement of autophagic flow and cell proliferation, enhanced DNA damage repair, weakened drug endocytosis or enhanced exocytosis, EMT activation, and non-coding MicroRNAs, LncRNAs-mediated regulatory mechanisms, etc(22-24). Among those various mechanisms of MDR, the response and adaption to the stressful environment of cancer cells caused by anti-cancer agents may be a major cause. Autophagy is an evolutionarily conserved catabolic process, which is an important defense mechanism of tumor cells against chemotherapeutic drugs(25).

In the present study, we treated the parental GC cell lines AGS and HGC27 with oxaliplatin for 8 months, to acquire their OXA-resistant cell line, respectively. To our knowledge, we were the first to establish OXA-resistant gastric cancer cell lines for mechanism exploration. And during the acquisition of chemoresistance, we used RNA-seq to analyze the genes which were dysregulated dramatically. We identified that the expression levels of ANXA1 in the two batches of drug-resistant strains were higher than that of the parental strains, moreover, the fold change gradually increased during the enhancement of OXA resistance, indicating that ANXA1 may play a key role in the process of gastric cancer cells acquiring oxaliplatin resistance. Furthermore, we proved that ANXA1 was upregulated in gastric cancer tissues, especially in chemoresistant tissues, and high expression of ANXA1 is associated with advanced TNM stage.

Previous studies have demonstrated that ANXA1 is aberrantly up-regulated in GC tissues. In addition, we found that the high expression of ANXA1 in gastric cancer tissue was significantly related to the patient's TNM stage, hinting that high expression of ANXA1 might mediate the malignant differentiation of gastric cancer cells. The results of univariate survival analysis showed that the expression level of ANXA1, nerve/vascular infiltration, the number of lymph node metastases, and the TNM stage were all indicators that affect the prognosis of patients. Patients with higher ANXA1 expression have a poorer prognosis. Further multivariate Cox analysis showed that nerve/vascular infiltration and TNM staging were both independent prognostic factors for gastric cancer patients, while the expression level of ANXA1 was not. It is speculated that ANXA1 high expression group is more relative to nerve/vascular infiltration and late TNM staging, however, the latter two factors have been widely recognized as indicators for poor survival. The results of ROC curve according to the expression level of ANXA1 in the chemotherapy resistance and sensitive groups showed that ANXA1 can be used as a predictor of chemotherapy sensitivity. Although the above data indicated that ANXA1 could be a potential prognostic and chemo-sensitivity indicator for gastric cancer patients, a large cohort of multi-center samples should also be included to validate the results above.

Furthermore, functional research confirmed that ANXA1 induced chemoresistance of gastric cancer cells via augmentation of autophagy in vitro and in vivo by loss-and gain-of function assays. However, we found that ANXA1 has no obvious effect on GC cell proliferation, indicating that ANXA1 only acts specially in oxaliplatin induced chemoresistance. Mechanically, we identified that after knocking down ANXA1 in gastric cancer OXA-resistant cell lines, GSEA analysis showed that the apoptosis pathway and PI3K/AKT/mTOR signal were abnormally activated in gastric cancer OXA-resistant cell lines, therefore, we believe that ANXA1 can activate autophagy and enhance the resistance of gastric cancer cells to oxaliplatin by inhibiting the activity of PI3K/AKT/mTOR pathway.

It is well known that the PI3K/AKT/mTOR pathway is an important signaling pathway that regulates cell growth and proliferation. The abnormal activation of PI3K/AKT/mTOR is considered an important factor for many tumor cells to develop chemoresistance: Yu et al. reported that PTEN was suppressed in 28 cases of gastric cancer tissues, while the expression of AKT and p-AKT (Ser 473) was elevated, which could activate AKT activity after stimulation of etoposide and adriamycin to make the cells obtain drug

resistance, and PI3K inhibitors could effectively reverse the resistance of gastric cancer cells(26). The activation of p-AKT can inhibit the apoptosis caused by doxorubicin, increase the excretion and reduce its accumulation of doxorubicin in gastric cancer cells, furthermore, p-AKT can significantly up-regulate Bcl-2 and down-regulate the expression of Bax(27). The latest research by Zhang et al. showed that miR-567 is down-regulated in gastric cancer tissues and cell lines, functional experiments showed that miR-567 not only inhibited the proliferation of gastric cancer cells but also increased the sensitivity of cells to 5-FU and oxaliplatin, the mechanism by which miR-567 exerts its effect is to inhibit the activation of PI3K/AKT/c-Myc by directly inhibiting the expression of PIK3AP1, meanwhile c-Myc can in turn inhibit the expression of miR-567, thus forming a closed loop(28). In summary, the activation of PI3K/AKT signaling pathway in gastric cancer can lead to tumor cell proliferation and even drug resistance, but the regulation of cell physiological functions is a complex regulatory network. On the one hand, a large number of studies have shown that the activation of PI3K/AKT/mTOR signaling pathway can promote cell proliferation, resist apoptosis caused by drugs and other factors, leading to chemotherapy resistance, and inhibit cell death caused by excessive autophagy; on the other hand, inhibiting its activity can also increase the level of cell autophagy, which can help cells deal with external stimuli or cell death caused by drugs. Therefore, the inhibition of PI3K/AKT/mTOR signaling may also cause cell survival and proliferation by activating autophagy, and even lead to drug resistance.

In summary, our findings suggested that ANXA1 promotes gastric cancer oxaliplatin resistance by inducing autophagy, which depends on the inhibition of PI3K/AKT/mTOR signaling pathway. Therefore, our study provides evidence that ANXA1 acts as a chemoresistance gene and might be a promising prognostic predictor and therapeutic target for GC patients treated with OXA-based chemotherapy.

Declarations

Ethics approval and consent to participate

This study was approved by the Human Ethics Committee of the Fifth Peoples' hospital of Shanghai.

Consent for publication

The author agrees to publish the article.

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

The authors declare no potential conflicts of interest.

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Authors' contributions

RJ, H-ZQ, H-RQ, G-JW, and XJ performed the experiments. RJ and K-CW designed the study. N-GM analyzed the data. RJ wrote the manuscript.

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Tables

Table 1

Clinicopathological parameters	No. of patients	ANXA1 expression level		χ^2	P value
		Low	High		
Age(years)					
<65	41	17	24	0.098	0.754
≥65	42	16	26		
Gender					
Male	65	24	41	1.006	0.316
Female	18	9	9		
Tumor size(cm)					
<5	54	22	32	0.062	0.803
≥5	29	11	18		
Tumor location					
Upper	25	9	16	0.784	0.676
Middle	44	17	27		
Lower	14	7	7		
Differentiation grade					
Well/moderate	59	26	33	1.582	0.209
Poor	24	7	17		
Neural/vascular invasion					
No	74	31	43		0.306
Yes	9	2	7		
Lymph node metastasis (n)					
<3	50	23	27	2.045	0.153
≥3	33	10	23		
TNM stage					
I–II	22	14	8	7.125	0.008
III–IV	61	19	42		

Table 2

Clinicopathological parameters	Mean survival time(months)	95% CI	P value
Age(years)			
<65	58.279	54.072-62.487	0.632
≥65	59.353	54.836-63.870	
Gender			
Male	58.537	54.961-62.113	0.888
Female	60.111	54.324-65.898	
Tumor size(cm)			
<5	59.408	55.643-63.173	0.843
≥5	58.103	52.777-63.429	
Tumor location			
Upper	57.712	51.809-63.615	0.478
Middle	59.115	55.105-63.125	
Lower	60.214	51.739-68.690	
Differentiation grade			
Well/moderate	60.915	57.456-64.374	0.054
Poor	53.815	47.747-59.884	
Neural/vascular invasion			
No	61.091	58.190-63.992	0.000
Yes	40.667	32.176-49.157	
Lymph node metastasis (n)			
<3	62.408	58.670-66.145	0.001
≥3	53.606	48.759-58.453	
TNM stage			
I-II	69.132	66.557-71.707	0.000
III-IV	55.157	51.537-58.776	
ANXA1 expression			
Low	63.513	59.247-67.780	0.005

High	55.816	51.784-59.848
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Table3

Covariates	HR	95% CI for HR	P value
ANXA1 expression level (low vs. high)	1.802	0.911-3.565	0.091
Neural/vascular invasion (no vs. yes)	3.604	1.522-8.535	0.004
Lymph node metastasis (<3 vs. ≥3)	1.201	0.608-2.371	0.598
TNM stage (I–II vs. III–IV)	3.694	1.328-10.275	0.012

Figures

Figure 1

Screen and validation of genes related to GC oxaliplatin resistance. (A) After gastric cancer cell lines, AGS and HGC27 were treated in a stepwise manner by continuous exposing the cells to increasing concentrations of OXA from 0.25 $\mu\text{g/mL}$ to 6 $\mu\text{g/mL}$ and 2 $\mu\text{g/mL}$ respectively. The appearance of the AGS cell line becomes more slender, while the HGC27 cell line does not change much. (B) The sensitivities of OXA-resistant gastric cancer cells and their parental cells to oxaliplatin were determined by

MTT assay. (C) Oxaliplatin-induced apoptotic cell death was analysed by flow cytometry between OXA-resistant cells and parental cells. (D) The cluster heat map showed the differentially expressed genes in two pairs of cell lines for twice. Red colour indicates high expression level, and blue colour indicates low expression level. (E) Venn showed up-expressed genes over 2.0-fold change in all groups. (F) ANXA1 was among the ten upregulated genes. (G) Relative expression of ANXA1 in OXA-resistant gastric cancer cells and their parental cells was determined by qRT-PCR. (H) Relative expression of ANXA1 in OXA-resistant gastric cancer cells and their parental cells was determined by Western blotting. (I) After AGS cell line was treated with different concentrations of oxaliplatin, ANXA1 was detected by Western blotting. Data are presented as mean $\pm$ standard deviation from at least 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 2

UP-regulation of ANXA1 was associated with chemoresistance and poor prognosis. (A-B) The protein and mRNA level of ANXA1 expression in GC cell lines (SGC7901, BGC823, MGC803, HGC27, AGS, MKN45, NCI-N87 and SNU-1) were compared with that in the gastric mucosal epithelial cell (GES-1) by Western blotting and qRT-PCR respectively. (C) Western blotting analysis of the expression of ANXA1 protein in 18 GC tissues and their corresponding adjacent non-cancerous gastric specimens. N, non-cancerous gastric

tissue; T, tumor tissue. (D) The quantitation of western blot results in (C) using Image-J software. (E) Relative expression of ANXA1 in gastric cancer tissues and neighboring noncancerous tissues was detected by IHC ($P < 0.001$, $n = 83$). (F) The association of ANXA1 expression and OXA-resistance were measured by IHC in chemo-sensitive and resistant groups. ANXA1 expression was upregulated in the chemo-resistant group. (G) ROC curve for discriminating responders from non-responders based on ANXA1 expression; AUC = 0.7099 (95%CI = 0.596-0.824). (H) Kaplan-Meier survival curves for OS based on ANXA1 expression, Neural/vascular invasion, lymph node metastasis, and TNM stage in TMA samples ($n = 83$). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 3

ANXA1 promotes GC cell chemo-resistance without influencing proliferation. (A) Up: AGS/OXA and HGC27/OXA cells transfected with ANXA1-siRNA. Down: AGS and HGC27 cells transfected with PLVX-ANXA1. (B) The cell viability of AGS/OXA and HGC27/OXA and their parental cells under different transfection was determined by MTT assay. (C) The sensitivities of OXA-resistant gastric cancer cells under different transfections with oxaliplatin were determined by MTT assay. (D) The sensitivities of the

parental gastric cancer cells under different transfections with oxaliplatin were determined by MTT assay. (E) AGS and HGC27 cells weretransfected with PLVX-ANXA1 together with oxaliplatin treatment or not respectively. Colony formation assay were measured to show ANXA1 promoted OXA-resistance while the present of oxaliplatin. (F) AGS/OXA and HGC27/OXA cells were, respectively, transfected with ANXA1-shRNA together with oxaliplatin treatment or not. Colony formation assay were measured to show ANXA1 promoted OXA-resistance while the present of oxaliplatin. *P < 0.05, **P < 0.01 and ***P < 0.001.

Figure 4

Knockdown of ANXA1 promotes apoptosis and improves the sensitivity of gastric cancer cells to oxaliplatin in vivo. (A-B) The apoptotic rates of the indicated cells transfected with PLVX-ANXA1 or inhibitors, respectively, together with oxaliplatin treatment were detected by flow cytometry analysis. (C) Schematic outline of the treatment of AGS/OXA cells transfected with ANXA1-shRNA and normal control in a subcutaneous tumor model followed by intraperitoneal injection of oxaliplatin in nude mice. (D-E) Representative images of transplanted tumors removed from mice after sacrifice at the 35th day, tumor volume and tumor weight were measured. (F) Tumor growth curves of each group were calculated per week. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 5

Autophagy promotes the OXA-resistance of gastric cancer cells. (A-B) The protein levels of SQSTM1 and LC3B in OXA-resistant gastric cancer cells and their parental cells were determined by western blot assay. (C) The protein levels of p-AMPK in OXA-resistant gastric cancer cells and their parental cells were determined by western blot assay. (D-E) OXA-resistant gastric cancer cells and their parental cells that stably express the mRFP-GFP-LC3 fusion protein were established and observed by the fluorescence

microscope. (F) The protein levels of SQSTM1 and LC3B in OXA-resistant and parental gastric cancer cells under different transfection with CQ or rapamycin were determined by western blot assay. (G) The oxaliplatin sensitivities of the parental gastric cancer cells under rapamycin were determined by MTT assay. The oxaliplatin sensitivities of the OXA-resistant gastric cancer cells under CQ were determined by MTT assay. $**P < 0.01$ and $***P < 0.001$.

Figure 6

ANXA1 induces autophagy-associated OXA-resistance of gastric cancer cells in vitro. (A) The protein levels of SQSTM1 and LC3B in gastric cancer cells under transfection of PLVX-ANXA1 and normal control were determined by western blot assay. (B) Gastric cancer cells stably expressing mRFP-GFP-LC3 under different transfections were observed by the fluorescence microscope. (C) The protein levels of SQSTM1 and LC3B in OXA-resistant gastric cancer cells under transfections of ANXA1-shRNA and normal control were determined by western blot assay. (D) The protein levels of LC3 in OXA-resistant gastric cancer cells under different transfections of ANXA1-shRNA and normal control with or without CQ were determined by western blot assay. (E) OXA-resistant cancer cells stably expressing mRFP-GFP-LC3 under different transfections were observed by the fluorescence microscope. (F) The sensitivities of the parental gallbladder cancer cells under different transfection with or without CQ were determined by MTT assay. The sensitivities of the OXA-resistant gastric cancer cells under different transfections with or without rapamycin were determined by MTT assay.

Figure 7

ANXA1 activates autophagy via PI3K/AKT pathway. (A) Relative expression of ANXA1 in AGS/OXA cells under different transfection with ANXA1-shRNA and normal control was determined by qRT-PCR. (B) Gene set enrichment analysis (GSEA) plots showing the enrichment of genes upregulated in PI3K/AKT (left) and associated with Apoptosis (right) in the ANXA1-low group vs. the ANXA1-normal group of AGS/OXA cells. (C) Western blotting of AKT and p-AKT in AGS/OXA and HGC27/OXA indicated cells stably

expression sh-ANXA1 or sh-Con. (D) Western blotting of AKT and p-AKT in the indicated cells with or without over-expression of ANXA1-Flag.