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The Zuo Jin Wan Formula increases chemosensitivity of human primary gastric cancer cells by AKT mediated mitochondrial translocation of cofilin-1

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[ABSTRACT] Resistance to cisplatin (DDP)- based chemotherapy is a major cause of treatment failure in human gastric cancer (GC). It is necessary to identify the drugs to re-sensitize GC cells to DDP. In our previous research, Zuo Jin Wan Formula (ZJW) has been proved could increase the mitochondrial apoptosis via cofilin-1 in a immortalized cell line, SGC-7901/DDP. Due to the immortalized cells may still difficult highly recapitulate the important molecular events in *vivo*, primary GC cells model derived from clinical patient was constructed in the present study to further evaluate the effect of ZJW and the underlying molecular mechanism. Immunofluorescent staining was used to indentify primary cultured human GC cells. Western blotting was carried out to detect the protein expression. Cell Counting Kit-8 (CCK-8) was used to evaluate cell proliferation. Flow cytometry analysis was performed to assess cell apoptosis. ZJW inhibited proliferation and induced apoptosis in primary DDP-resistant GC cells. Notably, the apoptosis in GC cells was mediated by inducing cofilin-1 mitochondrial translocation, down-regulating Bcl-2 and up-regulating Bax expression. Surprisingly, the level of p-AKT protein was higher in DDP-resistant GC cells than that of the DDP-sensitive GC cells, and the activation of AKT could attenuate ZJW-induced sensitivity to DDP. These data revealed that ZJW can increase the chemosensitivity in DDP-resistant primary GC cells by inducing mitochondrial apoptosis and AKT inactivation. The combining chemotherapy with ZJW may be an effective therapeutic strategy for GC chemoresistance patients.

[KEY WORDS] Primary GC cells; ZJW; AKT; Chemoresistance

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Introduction

Gastric cancer (GC) which is characterized by high morbidity and mortality, is one of the most common digestive malignancies, with nearly 700 000 death tolls reported annually in the world [1]. Despite enormous efforts have been made to develop various strategies for GC therapies, the outcomes of treatment frequently disappointing, particularly for advanced stages of GC patients [2-3]. It is worth noting that the intrinsic or acquired drug resistance is the major barrier limiting effective treatment of GC [4]. Results from these studies have prompted an urgent need for the identify the drugs to re-sensitize GC cells to DDP.

Zuo Jin Wan Formula (ZJW), a traditional Chinese medicine formula, has been widely used in gastrointestinal diseases in China [5]. Accumulating evidence suggests that ZJW possesses anti-cancer properties, and its use is associated with favorable outcomes in several cancers. ZJW has been reported have significant cytotoxic activities in eight kinds of human cancer cell lines by inducing mitochondrial apoptosis [6]. Be-

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sides, ZJW extracts could suppress CRC cell growth and invasion by Wnt/*β*-catenin signaling [7]. Administration of ZJW has shown lower tumor growth in HepG2 xenograft-bearing immunocompetent mice as well as breast cancer ZHENG model [8-9]. Moreover, ZJW extracts could increase the sensitivity of chemotherapy to CRC cell lines by reverses the P-gp-mediated MDR [10].

Notably, ZJW has shown promising effects in GC treatments. Our previous studies indicated that ZJW inhibited GC cell growth and increased sensitivity to DDP in a immortalized cell line, SGC-7901/DDP [11-12]. Actually, ZJW could induce the initiation of PP1 and PP2A-mediated cofilin-1 mitochondrial translocation, which led to the increased apoptosis rates in DDP-resistant GC cell lines [11-12]. It is well established that drug resistance in GC cells is closely associated with the activation of the PI3K/AKT signaling pathway. Especially, AKT, also known as protein kinase B (PKB), has been considered as one of the key multidrug resistance genes [13]. For instance, the protein level of p-AKT significantly increases in ovarian cancer tissues of chemo-resistance [14]. Activated AKT contributes to DDP chemoresistance in human gynecological carcinoma cells by modulating PPM1D stability [15], whereas inhibition of AKT sensitizes chemo-resistant cells to DDP-induced apoptosis [16]. In our previous study, activation of PP1 and PP2A induced the apoptosis of DDP-resistant GC cell lines. In addition, PP1 and PP2A are negatively regulated by PI3K/AKT pathway [17]. As such, we hypothesized that the effect of ZJW on GC resistance to DDP may associate with AKT.

The aforementioned concerns encouraged us to further reveal whether the AKT would contribute to the effect of ZJW on GC resistance to DDP and to investigate the underlying molecular mechanism. Basic research on GC has traditionally been conducted in immortalized cell lines, which has provided a molecular basis for anti-tumor effect of ZJW. However, cell lines has recently been found to be deficient in mimicking the in vivo the important molecular events more closely which may provide inaccurate information predict drug response [18]. While primary tumor cells could closely mimic the physiological and pathological state of cells in vivo and generate more relevant data representing personal living systems [19]. Hence, the present study were conducted to determine whether ZJW would have similar effects in primary GC cells and the substantial intracellular molecular events.

Material and Methods

Patient samples

Gastric tissues (n = 15) were collected from patients (age, 35–85) undergoing surgical gastrectomy. All gastric tissue samples were obtained from Putuo Hospital of Shanghai University of Traditional Chinese Medicine between July 2017 and July 2018. Detail information were listed in Table 1. The specimens were put into ice-cold PBS supplemented with 100 U·mL^{-1} penicillin G and 100 μg·mL^{-1} streptomycin after surgical gastrectomy and transported to the laboratory.

Table 1 The basic information of the patients involved

Patients' No	Gender	Age	Pathological Types
1	female	85	Adenocarcinomas
2	male	41	Adenocarcinomas
3	male	64	Adenocarcinomas
4	male	60	Adenocarcinomas
5	male	61	Adenocarcinomas
6	female	64	Adenocarcinomas
7	male	68	Adenocarcinomas
8	male	84	Adenocarcinomas
9	male	69	Adenocarcinomas
10	male	84	Adenocarcinomas

This study was approved by the Medical Ethics Committee of Putuo Hospital of Shanghai University of Traditional Chinese Medicine (No. PTEC-A-2015-5-3). Approval date: 2017.4.23. All patients included in this research were required to offer written informed consent.

Primary GC cell isolation and culture

Gastric tissues were washed three times with PBS and cut into small fragments in a size of about 1 mm^3. Then, the fragments were incubated with 1% collagenase IV (Biosharp, China) for 1–2 h at 37 °C, followed centrifugation (500 r·min^{-1}, 5 min) to remove the undigested tissue. After passed through a 40 μm filter, the resulting cell suspension was washed twice and centrifugated 10 min at a speed of 3000 r·min^{-1}. Then the pellet was incubated in Roswell Park Memorial Institute (RPMI) 1640 (Hyclone, USA) containing 10% FBS (Gibco, Grand Island, NY, USA), 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin, in 37 °C with 5% CO_2. Culture medium was re-freshed after 24 h, the first medium change was performed. GC cells were identified using pan-Cytokeratin and Calretinin markers.

Immunofluorescent staining

Cells were harvested at indicated time points. Slides were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 5% bovine serum albumin (BSA) (Gibco, Grand Island, NY, USA) for 1 hour at room temperature. Cells were probed with Primary antibodies anti-pan-Cytokeratin (Santa Cruz, sc-81714) or anti-Calretinin (Abcam, ab92341) antibodies at 4 °C overnight. The sections were rinsed 3 times in PBST and then incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG or Alexa Fluor 488-conjugated goat anti-mouse IgG (Beyotime Inc, Shanghai, China) in the dark for 1 h at room temperature. The nuclei were stained with DAPI. Images were captured with a fluorescence microscope (Leica, Wetzlar, Germany).

Preparation of the ZJW extracts

ZJW was formulated by *Rhizoma Coptidis* and *Fructus evodiae* (in a ratio of 6 : 1). Two herbs used in the present study (*Rhizoma Coptidis* and *Fructus evodiae*) were from

TCM pharmacy of Putuo Hospital, Shanghai University of Traditional Chinese Medicine (Shanghai, China). ZJW extracts were prepared as previously described [12]. ZJW was extracted through two 1-hour reflux procedures in ethanol (1 : 8, *V*/*V*). The fltrates were concentrated and dried in vacuum at 60 °C. The concentrated extract was then dried by lyophilization to obtain the ZJW extract at a yield of dried powder of 24.4%. The extract was stored at 4 °C, and its preparations were standardized and quality controlled according to the guidelines defined by Chinese State Food and Drug Administration (SFDA).

Chemical analysis of ZJW extract

Sample Preparation: The ZJW powder (10.2 mg) was extracted with 5 mL of methanol under sonication for 30 min before centrifugation at 20 000 × *g* for 20 min at 4 °C. The supernatant was diluted 20 fold by adding methanol and filtered through a syringe filter (0.22 μm) prior to analysis.

LC-HRMS Analysis: Component identification were performed on a Agilent 1290 UPLC coupled with Sciex TripleTOF 4600 quadrupole-time of flight mass spectrometer equipped with a DuoSpray source (Sciex). Separation was achieved on an Agilent SB-Aq column (2.1 mm × 100 mm i.d., 1.8 m; Agilent). 0.2% formic acid (A) and acetonitrile (B) were used as the mobile phase at a flow rate of 0.3 mL·min^{-1}. For all samples, the following gradient condition was used: 0.0–20.0 min 5% B–45% B; 20.0–30.0 min 45% B–95% B; 30.0–35.0 min, 95% B; 35.1 min balance to 5% B. The injection volumes for all samples were 5 μL. Column oven temperatures were 30 ºC. Ionization was conducted using an electrospray ionization (ESI) source. Data were collected both in the positive and negative ion mode with Analyst TF software (Sciex). The mass spectrometer was operated in full-scan TOF-MS at *m/z* 100–1000 and information-dependent acquisition (IDA) MS/MS modes, the collision energy was 40/–40 ± 20 eV. Both ion source gas 1 and 2 were set 50 psi. Curtain gas was 35 psi. The temperature and ionspray voltage floating were 500 °C and 5500/–4500 V, respectively. Representative chromatographs are shown in Fig. 1. The details of 27 compounds identified in ZJW, including retention times, experimental and calculated mass *m/z*, ppm errors, common fragment characteristic ions and identified name are summarized in Table 2.

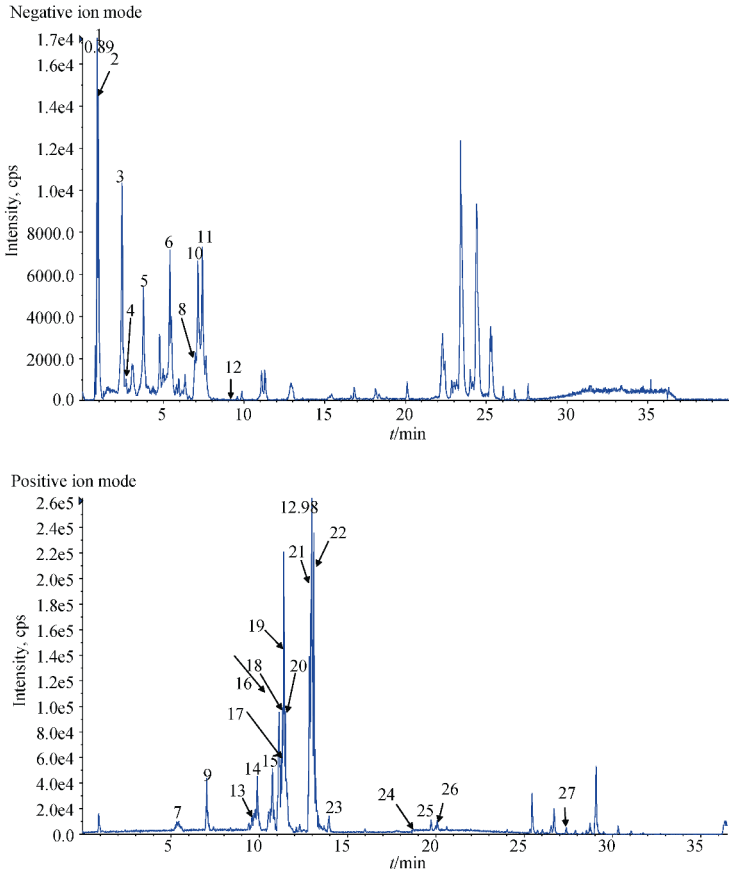

Fig. 1 LC-HRMS of ZJW. Peak 1: Quinic acid; 2: Glucosyringic acid or isomer; 3: Glucosyringic acid or isomer; 4: Gentisic acid 5-*O*-*β*-D-glucopyranoside or isomer; 5: Neochlorogenic acid; 6: Chlorogenic acid; 7: magnoflorine; 8: 5/4/3-*O*-feruloylquinic acid or isomer; 9: magnoflorine isomer; 10: 5/4/3-*O*-feruloylquinic acid or isomer; 11: 5/4/3-*O*-feruloylquinic acid or isomer; 12: Hyperoside; 13: 2-hydroxyjatrorrhizine;14: berberrubine; 15: dehydroevodiamine; 16: columbamine; 17: groenlandicine; 18: Jateorhizine; 19: berberine; 20: coptisine; 21: palmatine; 22: epiberberine; 23: 13-methylberberine; 24: limonin; 25: evodiamine; 26: rutaecarpine; 27: Evocarpine

Table 2 LC-HRMS /MS data of 27 components identified in ZJW

No.	t_R/min	*m/z*	*Calc m/z*	ppm	Identification	Fragement ions
1	0.93	191.0564	191.0561	1.5	quinic acid	191.0568; 127.0392; 93.0336; 85.0293; 59.0128
2	0.96	359.0990	359.0984	1.8	glucosyringic acid or isomer	359.0984; 197.0454; 179.0345; 135.0447; 123.0448; 72.9927
3	2.43	359.0989	359.0984	0.8	glucosyringic acid or isomer	359.0966; 197.0465; 179.0353; 135.0457; 123.0451; 72.9927
4	2.69	315.0723	315.0722	0.5	gentisic acid 5-O-β-D-glucopyranoside or isomer	315.0683; 152.0110; 108.0222; 59.0149
5	3.76	353.0881	353.0878	0.8	neochlorogenic acid	353.0870; 191.0562; 179.0351; 135.0443; 85.0288
6	5.39	353.0882	353.0878	1.1	chlorogenic acid	191.0555; 179.0344; 173.0449; 135.0441; 85.0287
7	5.43	342.1693	342.1700	2.0	magnoflorine	342.1745; 297.1174; 265.0899; 237.0937; 219.0842; 165.0724
8	6.98	367.1036	367.1035	0.4	5/4/3-O-feruloylquinic acid or isomer	193.0496; 173.0442; 155.0362; 134.0371; 93.0342; 67.0186
9	7.02	342.1698	342.1700	–0.5	magnoflorine isomer	342.1717; 297.1135; 265.0875; 237.0922; 191.0871; 165.0729
10	7.14	367.1036	367.1035	1.5	5/4/3-O-feruloylquinic acid or isomer	193.0509; 173.0455; 134.0376; 93.0338
11	7.40	367.1039	367.1035	1.2	5/4/3-O-feruloylquinic acid or isomer	191.0555; 173.0458; 134.0369; 93.0350; 67.0188
12	9.05	463.0887	463.0882	1.1	Hyperoside	463.0862; 301.0313; 271.0212
13	9.59	324.1224	324.1230	–2.0	2-hydroxyjatrorrhizine	324.1269; 308.0963; 280.1000; 266.0842
14	9.88	322.1071	322.1074	–0.9	berberrubine	322.1114; 307.0880; 279.0929; 250.0898; 222.0952
15	10.74	302.1289	302.1288	0.4	dehydroevodiamine	286.1015; 258.1052; 167.0620
16	11.12	338.1383	338.1387	–1.1	columbamine	338.1417; 322.1114; 294.1165; 279.0931; 265.0778; 250.0899
17	11.25	322.1070	322.1074	–1.2	groenlandicine	322.1113; 307.0866; 279.0915; 251.0966; 208.9067
18	11.34	338.1381	338.1387	–1.7	Jateorhizine	338.1426; 322.1113; 294.1161; 279.0922; 265.0763; 251.0969
19	11.38	336.1228	336.1230	–0.7	berberine	336.1228; 320.0954; 292.1002; 234.0943
20	11.45	320.0918	320.0917	0.2	coptisine	320.0950; 292.0996; 262.0887; 249.0870; 204.0829
21	12.85	352.1548	352.1543	1.3	palmatine	352.1579; 336.1272; 308.1307; 294.1149; 278.0843; 250.0910
22	12.96	336.1238	336.123	0.8	epiberberine	336.1277; 320.0963; 292.1010; 278.0856; 275.0984
23	13.94	350.1386	350.1387	–0.2	13-methylberberine	350.1411; 306.1151; 292.0989; 262.9492; 236.8979
24	18.57	471.2007	471.2013	–1.4	limonin	471.2042; 425.2070; 213.0934; 161.0628; 133.0671
25	19.98	304.1441	304.1444	–1.1	evodiamine	304.1486; 275.1257; 171.0947; 161.0732; 134.0626; 116.0507
26	20.09	288.1129	288.1131	–0.8	rutaecarpine	288.1160; 273.0936; 244.0887; 169.0783; 142.0671
27	27.17	312.2317	312.2322	–1.6	evocarpine	312.2338; 186.0944; 173.0854; 158.0587; 130.0680

To ensure the quality of ZJW solution, we used Ultra Performance Liquid Chromatography (UPLC) to confirm the final concentration of this solution. Various components in ZJW extracts were determined by referring to the calibration curve established by the running standard at varying concentrations under the same conditions. Quantitative results was shown in Table 3, quantitative results of coptisine, epiberberin, berberine, and palmatine in *Rhizoma Coptidis* were 2.72%, 1.35%, 11.91% and 2.82%, respectively. Quantitative results of evodiamine and rutaecarpine in Evodia were 0.175% and 0.231%, respectively. Quantitative results of coptisine, epiberberin, berberine, palmatine, evodiamine and rutaecarpine in ZJW extract were 7.12%, 4.15%, 31.4%, 8.4%, 0.053%, 0.051% respectively.

Cell viability analysis

Cells were seeded in 96-well plates at a density of 5000 cells per well, incubated overnight. The cell proliferation was analyzed using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Japan) according to the manufacturer's recommendations. Control cells were treated with cultured media. Experiments were repeated three times. The dose of ZJW and DDP used in our present study was based on clinical needs.

Cell apoptosis assays

Cells were treated as indicated. After treated for 48 hours, cells were collected and stained with Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, San Jose, CA, USA). Proportions of apoptotic cells were analyzed by flow cytometry (BD Biosciences).

Western blot analysis

Cells were harvested after treatment for indicated time. Total cell proteins and cytoplasmic and mitochondrial protein fractions were extracted as previously described [12]. The protein concentrations were analyzed by BCA protein Assay Reagent (Sangon Biotech, Shanghai, China). Soluble lysates containing about 20 μg proteins per sample were resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% SDS-PAGE) and then transferred onto polyvinylidene fluoride membranes. Membranes were blocked (room temperature, 1 hr) with 5% BSA, then incubated overnight with primary antibodies [p-AKT, AKT, cofilin-1, p-cofilin-1, Bax, Bcl-2, GAPDH, COX IV (Cell Signaling Technology, Danvers, MA, USA)]. Following washing with TBST, the membranes were incubated with the horse-radish peroxidase (HRP)-labeled secondary antibody (Beyotime Inc, Shanghai, China) at room temperature for 1–2 h. The membrane signals were detected using an Enhanced Chemiluminescent Western Blotting Detection System (Millipore, Billerica, MA, USA) in accordance with the manufacturer's instruction. The proteins were quantified using the Image J software (the National Institutes of Health, US).

Statistical analysis

Data are presented as the mean ± SD. Group means were compared using student's t-test or one-way ANOVA followed by Dunnett's multiple-comparison test with GraphPad Prism version 5.01. $P < 0.05$ was considered as statistically significant.

Results

Identification of primary cultured human GC cells in vitro

The cells were cultured by the removal of fibroblast. The contaminating fibroblasts were removed after trypsin enzymic digestion and differential attachment repeatedly until no fibroblast cells were detected. Cells were cultured for numerous months to maintain the cell morphology and growth rate (Fig. 2). After seeding on the plate for 7 days, the number of adherent cells could be easily observed and the cell morphologies were mixed: round, spindle etc. With longer time in culture, the contaminated cells were gradually eliminated with the repeated passage. The cells were in the shape of round approximately and had a strong proliferative ability.

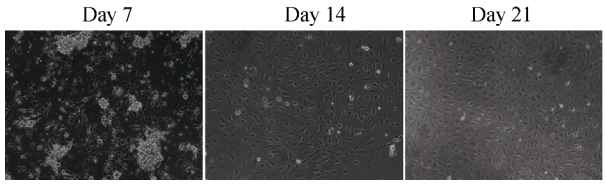

Fig. 2 Morphology of primary cultured GC cells derived from surgical specimens were observed under the inverted microscope on day 7, 14 and 21 respectively (magnification, 100 ×)

In addition to our observations under phase contrast microscopy, immunofluorescent staining revealed the expression pattern of biomakers for epithelial-derived tissue and GC. As shown in Fig. 3, pan-Cytokeratin was positively expressed while Calretinin was negatively expressed. These above data suggested primary cultured human GC cells were established *in vitro*, which could be used in further study [20–21].

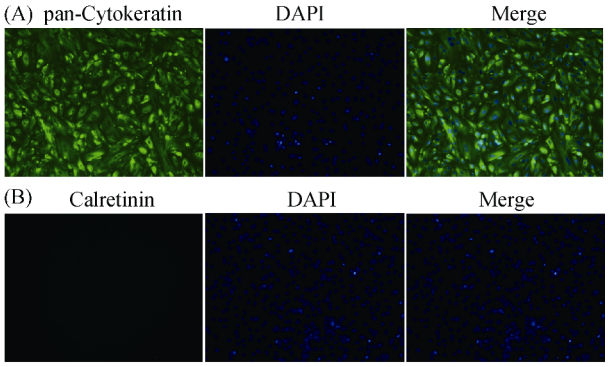

Fig. 3 Immunofluorescent staining analysis for human primary GC cells in vitro in order to confirm epithelial and gastric cancer status. DAPI (blue) was used to visualize nuclei. (A) pan-Cytokeratin staining (100 ×); (B) Calretinin staining (100 ×)

Identification of DDP-resistant primary human GC cells

Out of the fifteen patient samples collected, only ten primary cultures of GC cells could be established. CCK-8 cell viability assay was used to detect the inhibitory effects of DDP on these primary GC cells for 48 h, respectively. As shown in Fig.

4, the three most sensitive cell lines were No. 4 (B_1) (IC_{50} 3.706 μg·mL^{-1}), No. 5 (B_2) (IC_{50} 4.26 μg·mL^{-1}), No. 9 (B_3) (IC_{50} 2.817 μg·mL^{-1}), and the most resistant were No. 2 (A_1) (IC_{50} 24.25 μg·mL^{-1}), No. 6 (A_2) (IC_{50} 17.2 μg·mL^{-1}), No.7 (A_3) (IC_{50} 16.15 μg·mL^{-1}). Since the IC_{50} of drug-resistant cells (A_1, A_2, A_3) differed significantly from that of sensitive cells (B_1, B_2, B_3) [22], these six cell lines (A_1, A_2, A_3, B_1, B_2, B_3) were selected for follow-up experiments.

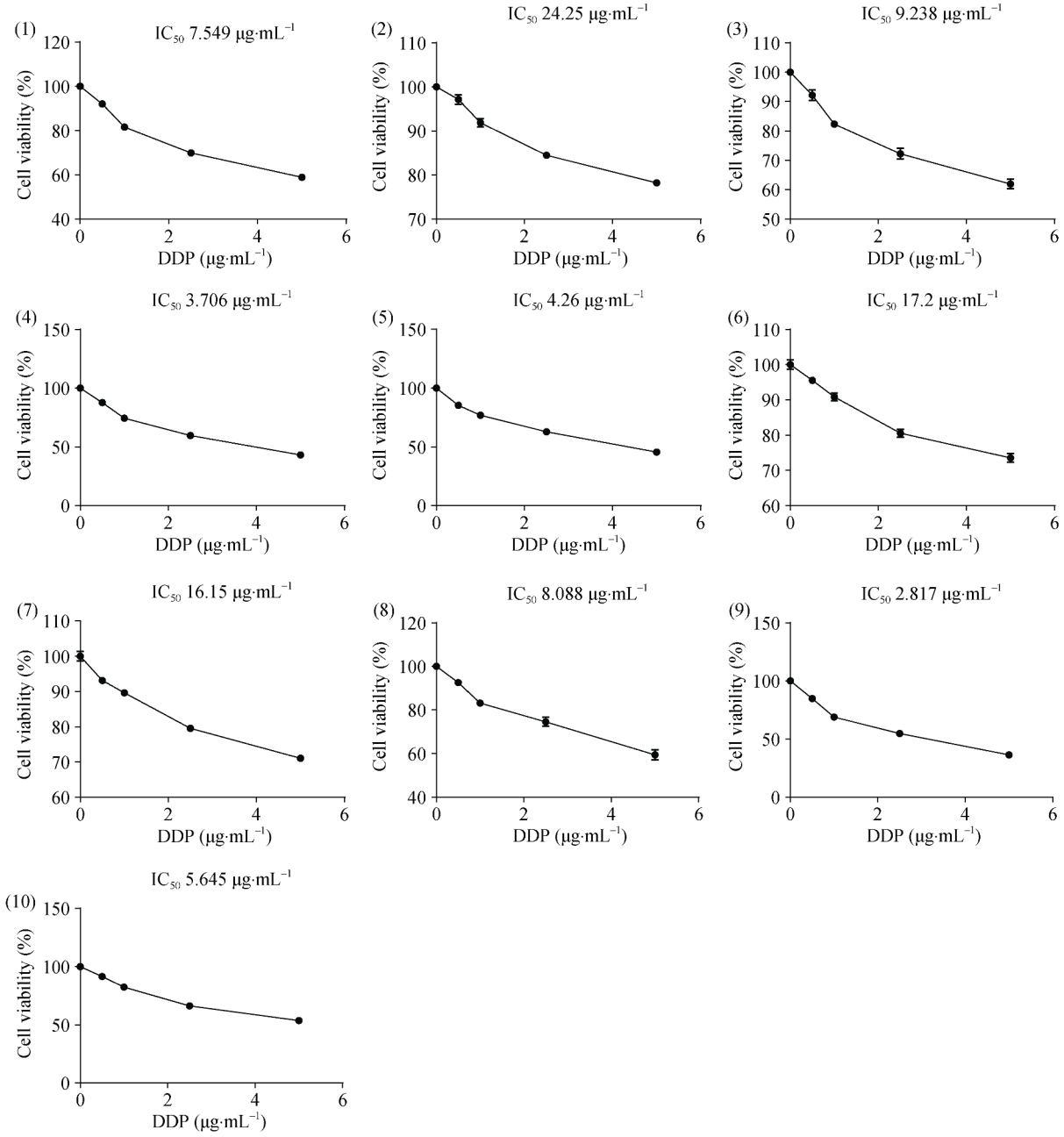

Fig. 4 Identification of DDP-resistant primary human GC cells. The viabilities (mean±SD) of 10 primary GC cells were determined by the CCK-8 assay with various doses of DDP for 48 h. IC_{50} values were calculated. All data are represented as the mean ± SD (n = 3) from three independent experiments

ZJW treatment increased chemosensitivity in DDP-resistant primary human GC cells

To examine the effects of ZJW on cell chemosensitivity, DDP-resistant primary GC cells (A_1, A_2, A_3) were treated with DDP and ZJW. Growth inhibitory effect of ZJW on DDP- resistant primary GC cells (A_1, A_2, A_3) was observed as evidenced by CCK-8 assay. As shown in Fig. 5, ZJW (20, 50, 100 μg·mL^{-1}) combined DDP significantly inhibited cell viability in a dose-dependent manner ($P < 0.001$), which was consistent with our previous studies in immortalized cells [12]. These results suggested that ZJW could increase the chemosensitivity of DDP-resistant primary human GC cells.

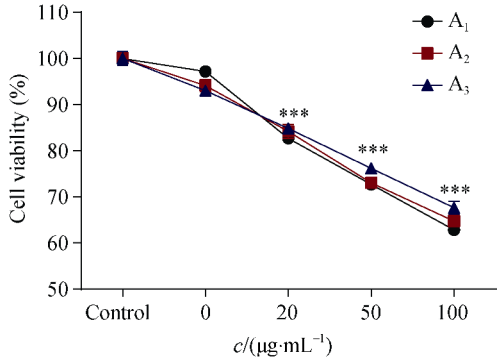

Fig. 5 ZJW increases the sensitivity to DDP in DDP-resistant primary GC cells. The three most resistant cells (A_1, A_2, A_3) were cultured without (control) and with DDP (1 μg·mL^{-1}) and several concentrations of ZJW (0, 20, 50, 100 μg·mL^{-1}) for 48 h, the cell viability were measured by CCK-8 assay. $^{***}P < 0.001$ *vs* control

Expression levels of cofilin-1 and AKT in DDP sensitive and DDP resistant primary GC cells.

As previous reported, mitochondrial translocation of cofilin-1 plays an important role in DDP-resistance in GC cells. We further extracted total cell proteins, cytoplasmic and mitochondrial protein fractions, attempting to elucidate protein expression of the cofilin-1 and AKT in the six primary GC cell lines. We observed the DDP-resistant primary GC cells exhibited significantly higher p-cofilin-1, cofilin-1 (M) and p-AKT expression levels, relative to DDP-sensitive cells (B_1, B_2, B_3) (Fig. 6). In contrast, the levels of cofilin-1(C) in the DDP-resistant cells noticeably lower than DDP-sensitive cells. The expression of cofilin-1(W) and AKT was also measured, and there was no significant change of them in all the groups. These results suggest that the expression level of cofilin-1 and activated AKT correlated with DDP-resistance in primary GC cells.

AKT mediates ZJW induced Translocation of cofilin-1 and Mitochondria apoptosis.

Previous studies demonstrated that ZJW treatment could induce the cofilin-1 in the mitochondria and lead to the initiation of mitochondrial apoptosis [12]. Accordingly, we examined the apoptosis rate in response to ZJW in DDP-resistant primary GC cells. As shown in Fig. 7 (A, C), ZJW and DDP combination treatment had a more pro-apoptosis ability in DDP-resistant primary GC cells, as compared with DDP or ZJW alone. Expressions of cofilin-1 and apoptosis related proteins were also measured in each group. ZJW induced Bax expression and cofilin-1(M) accumulation, whereas the expression of apoptosis inhibitor Bcl-2 was decreased. These results further confirmed pro-apoptosis effects of ZJW on DDP-resistant GC cells.

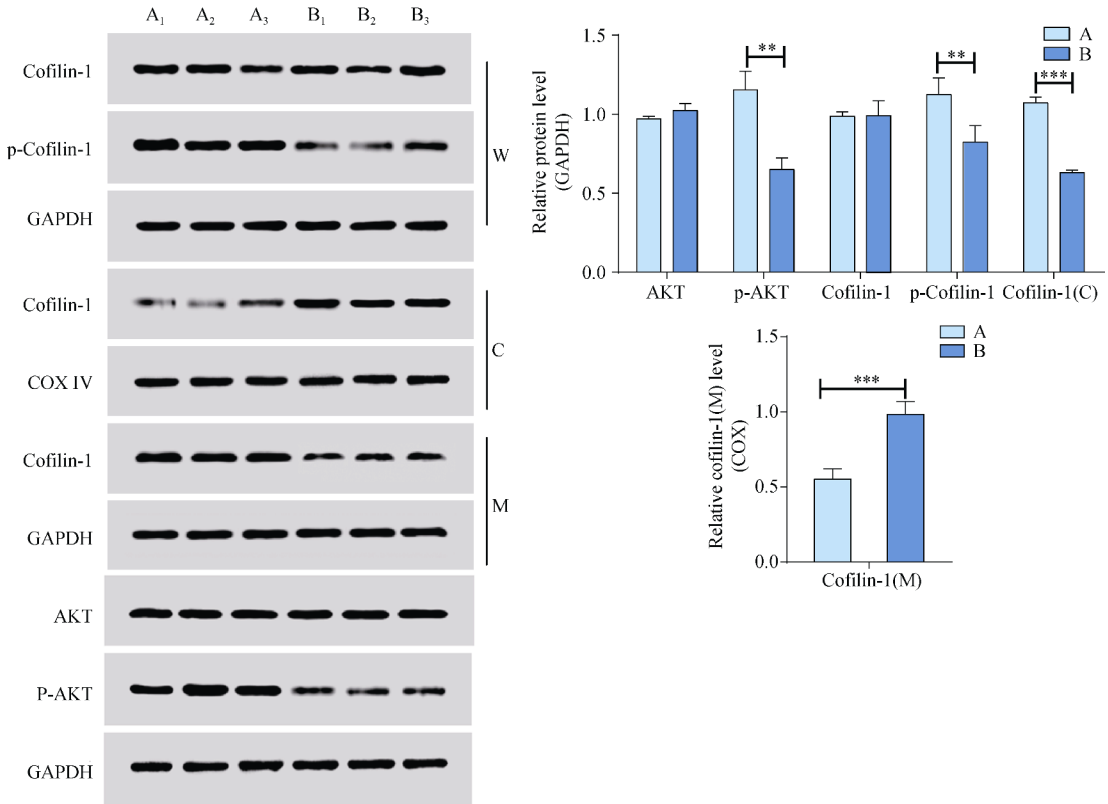

Fig. 6 Expression levels of cofilin-1 and AKT in DDP sensitive (B_1, B_2, B_3) and resistant (A_1, A_2, A_3) primary GC cells. Whole cell lysates, mitochondrial and cytosolic proteins were prepared for the detection of p-coflin-1, coflin-1, AKT and p-AKT. The level of each protein was normalized against those of GAPDH (total and cytosolic proteins) or COX IV (mitochondrial proteins). $^{**}P < 0.01$, $^{***}P < 0.001$

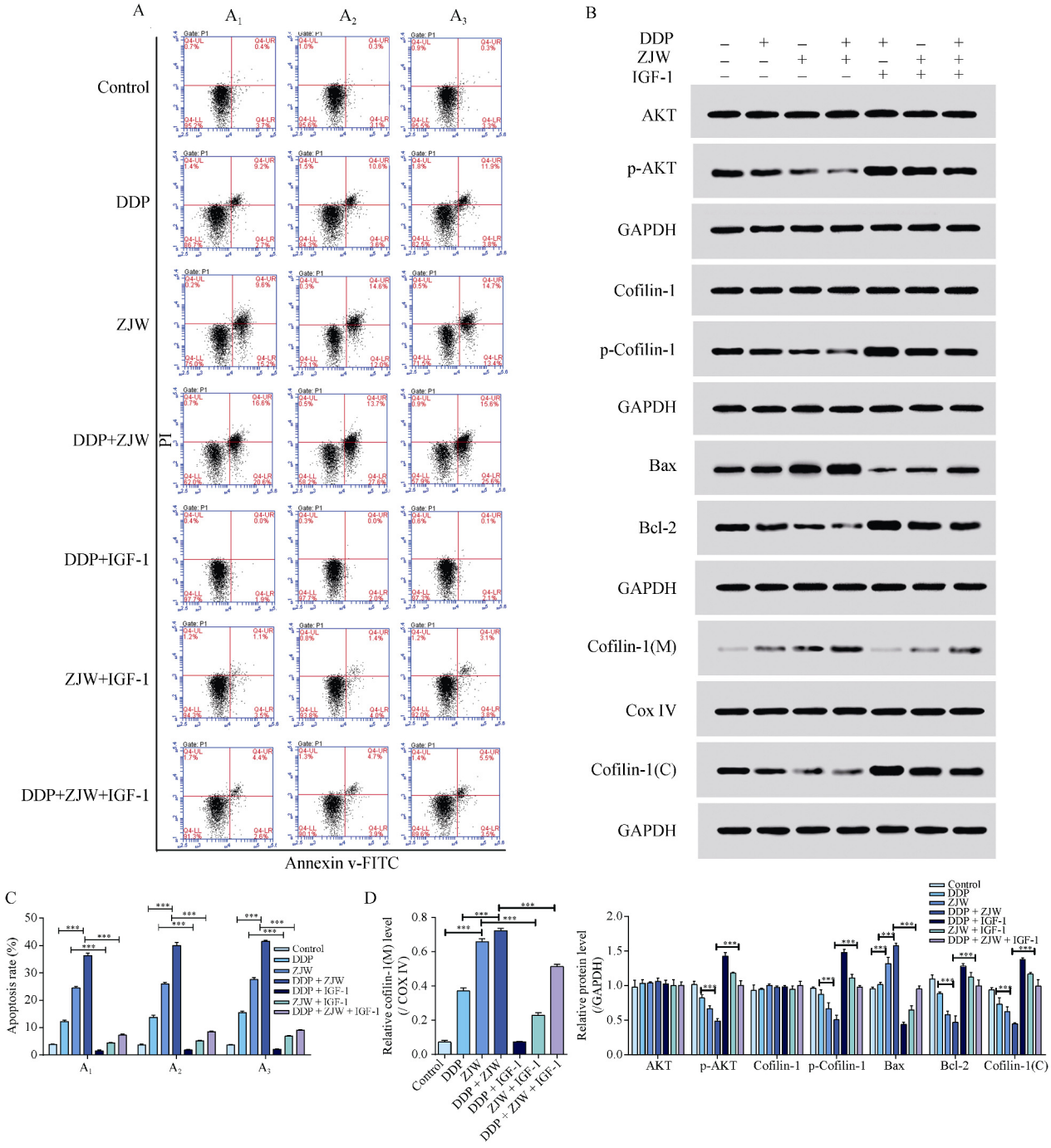

Fig. 7 AKT mediates ZJW induced Translocation of cofilin-1 and Mitochondria apoptosis. Cells were randomly divided into seven groups: a negative control group, a DDP group (1 μg·mL^{-1}), a ZJW group (50 μg·mL^{-1}), a DDP plus ZJW group, a DDP plus 100 ng·mL^{-1} IGF-1group, a ZJW plus 100 ng·mL^{-1} IGF-1 group, and a DDP plus ZJW plus 100 ng·mL^{-1} IGF-1group. (A) Cells were stained with Annexin V-FITC/PI and apoptotic rates were analyzed by using flow cytometry. Experiments were performed three times independently. (B) WB measuring the expression levels of AKT, cofilin-1 and apoptosis related proteins in each group. (C) Summary of the percentages of apoptotic cells. (D) quantification of protein levels. $^{***}P < 0.001$

Given that AKT signaling has been established as a major determinant of tumor cell chemoresistance and DDP-resistant cells showed higher AKT activation. Moreover, ZJW inhibited the protein level of p-AKT. Therefore, AKT activator IGF-1 was used to explore the role of AKT in the ability of ZJW to modulate DDP resistance. The apoptosis rate was significantly lower in treated with IGF-1 in combination with ZJW compared with those treated with ZJW alone. In addition, IGF-1 also inhibits DDP and ZJW combination induced apoptosis, indicating the inhibited effect of AKT in the proc-

ess of ZJW-induced apoptosis. Finally, cofilin-1 and proteins associated with mitochondrial apoptosis were detected. As shown in Fig. 7 (B, D), IGF-1 addition reduced Bax/Bcl-2 ratio and cofilin-1 mitochondrial translocation.

Discussion

For patients with advanced or metastatic GC, the response rate to multiagent chemotherapy out numbers 50%, but nearly all patients develop chemotherapy resistance [23-24]. Despite the significant advances in tumor therapies, the prognosis of GC remains unsatisfactory and potential mechanisms of resistance to chemotherapy are still not fully understood [25]. Consequently, it is urgent to develop new treatment options to improve clinical outcomes. Basic studies with cancer cell lines is important to help us better understand cancer progression and discover new candidate medications. However, analysis of cancer cell lines may not really represent clinical scenarios in patients with GC due to immortalized cell line may lost key molecular event and are inadequate for assessing drug response. Therefore, the establishment of human primary GC lines exhibiting a more clinical related physiological state is an important step in the study of GC. Primary tumor cell lines derived from clinical patient are essential requisite for assessing the effect of adjuvant therapy.

ZJW has shown anticancer effects in many types of cancers both *in vitro* and *in vivo*. Our previous studies also demonstrated the promoting apoptosis of ZJW in SGC-7901/DDP cells, which provides the rationale to evaluate ZJW in cancer clinical trials. Encouraged by these above results, we further extracted primary human primary GC cells from surgical tissues and evaluated the effects of ZJW on cell chemosensitivity to DDP in the present study. First, experimentation was done involving the primary culture ex vivo to identify sensitive and resistant phenotypes. IC_{50} values for DDP from patient No. 4, 5 and 9 was significantly lower than patients No. 2, 6 and 7 [26]. Thus, six primary human GC cell lines have been divided into naturally sensitive or naturally resistant categories according to their responses to DDP. Then, CCK-8 assay results revealed that ZJW significantly increased the sensitivity to DDP in DDP-resistant primary gastric cell lines (No. 2, 6 and 7). Increasing studies have shown that cofilin-1 plays a powerful role in multidrug resistance in many types of cancers [25, 27], which are in agreement with the findings presented here. We also found that p-cofilin-1 and cofilin-1(M) protein expression in DDP-resistant primary gastric cells were up-regulated compared to DDP sensitive cells. The treatment with ZJW extracts also resulted in increased apoptosis in DDP-resistant primary gastric cells. Expression of cofilin-1 and mitochondrial apoptosis-related protein play an important role in the regulation of apoptosis in the present study. In SGC-7901, ursolic acid promotes apoptosis through inducing mitochondrial translocation of cofilin-1 and expression of mitochondrial apoptosis-related proteins [28]. Similarly, the levels of the pro-apoptotic Bcl-2 and cofilin-1(M) were increased remarkably under the intervention of ZJW.

We also explored the potential mechanisms by which ZJW increased the sensitivity to DDP. Improper activation of AKT has been associated with chemoresistance in several tumor types [29-32]. In the present study, we have observed up-regulation of active AKT in the tissues of chemoresistance GC, and indicated that AKT might play an important role in the regulation of GC chemoresistance. AKT activation reduces the DDP-induced or ZJW-induced apoptosis in DDP-resistant primary gastric cells and promotes chemoresistance, suggesting that inhibition of AKT is required for ZJW-increased chemosensitivity of human primary GC cells. DDP-based chemotherapy exerts anticancer effects mainly by activating DNA damage response and inducing mitochondrial apoptosis [33]. In fact, AKT signaling pathway plays a major role in regulating cellular processes such as cell proliferation, survival or migration, which is frequently up-regulated in cancer and accordingly represents an important anticancer target. A main function of AKT is the inhibition of both the DNA damage and apoptotic responses following a DNA-damaging insult [34]. Importantly, we have shown ZJW could significantly inhibit AKT activation, which conferred ZJW-increased chemosensitivity to DDP.

Zhao *et al* [35] have revealed that the chemosensitivity of BTG1 transfectants to paclitaxel, cisplatin, MG132 or SAHA was positively correlated with its apoptotic induction. The apoptotic response was due to the loss of mitochondrial membrane potential. Specifically, it has been proved that arctigenin induces mitochondrial apoptosis of rheumatoid arthritis fibroblast-like synoviocytes and activates mitochondrial pathway, attenuating the phosphorylation of AKT [36-37]. Similarly, ZJW also induced the expression of mitochondrial apoptosis related proteins while reduced the activation of AKT which was consistent with previous results.

In the present study, the anti-tumor effects of ZJW in primary GC cells and the underlying molecular have been evaluated. ZJW has been prove to increase the chemosensitivity in DDP-resistant primary GC cells by inducing mitochondrial apoptosis and AKT inactivation, which provided novel therapeutic strategy for GC chemoresistance patients. Notably, the fresh human GC living tissues were used to perform drug sensitivity test, which could detect the drug sensitivity of tumor cells before chemotherapy. The strategy used in the present study can predict the *in vivo* therapeutic effects more accurately, as well as screen relatively effective chemotherapy drug, which provide scientific basis for clinicians to determine the chemotherapy plan and carry out individualized treatment. However, a limitation of this current study is that we did not examined the expression of well-known genes involved in multidrug resistant, such as MDR1 and mTOR [38], and further investigation is needed.

Conclusion

In conclusion, in this study we explored the underlying

mechanism of apoptotic potential of ZJW on primary DDP-resistant GC. We have demonstrated for the first time that AKT is differentially expressed between primary sensitive and resistant GC cell lines. Furthermore, AKT may regulate ZJW-induced DDP sensitivity in primary GC cells. Our data also support further clinical studies to validate the benefit of ZJW combined with traditional chemotherapy in the management of GC patients with DDP resistance.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article. Additional datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Inclusion of human participants, and use of human data and human tissue in this study were approved by the Ethics Committee of Putuo Hospital, Shanghai University of Traditional Chinese Medicine (No. PTEC-A-2015-5-3). Approval date: 2017.4.23.

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