RESEARCH ARTICLE

Antitumor Activity of Chloroquine in Combination with Cisplatin in Human Gastric Cancer Xenografts

Hui-Qing Zhang¹, Nian Fang^{2*}, Xiao-Mei Liu¹, Shu-Ping Xiong¹, Yu-Qian Liao¹, Wen-Jian Jin³, Rong-Feng Song¹, Yi-Ye Wan¹

Abstract

<u>Purpose</u>: To investigate the antitumor activity and mechanism of chloroquine (CQ) in combination with cisplatin (DDP) in nude mice xenografted with gastric cancer SGC7901 cells. <u>Materials and Methods</u>: 35 cases of gastric cancer patients with malignant ascites were enrolled and intraperitoneal cisplatin injection was performed. Ascites were collected before and 5 days after perfusion for assessment of autophagy levels in cancer cells. In addition, 24 tumor-bearing mice were randomly divided into control, DDP, CQ and CQ + DDP groups. <u>Results</u>: In 54.3% (19/35) of patients the treatment was therapeutically effective (OR), 5 days after peritoneal chemotherapy, 13 patients had the decreased ascites Beclin-1 mRNA levels. In 16 patients who had NR, only 2 cases had decreased Beclin-1 (P=0.001). Compared with the control group, the xenograft growth in nude mice in the DDP group was low, and the inhibition rate was 47.6%. In combination with chloroquine, the inhibition rate increased to 84.7% (P<0.01). The LC3-II/I ratio, and Beclin1 and MDR1/P-gp expression were decreased, while caspase 3 protein levels increased (P<0.05). <u>Conclusions</u>: Antitumor ability of cisplatin was associated with autophagy activity and chloroquine can enhance chemosensitivity to cisplatin in gastric cancer xenografts nude mice.

Keywords: Cisplatin - chloroquine - gastric cancer - multi-drug resistance - xenografts

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Introduction

Gastric cancer is the fourth most common malignancy and the second leading cause of cancer deaths worldwide. A total of 989,600 new stomach cancer cases and 738,000 deaths are estimated to have occurred in 2008, accounting for 8% of the total cases and 10% of total deaths. Over 70% of new cases and deaths occur in developing countries, with the majority in China (Jemal et al., 2011). Chemotherapy is the primary treatment for patients with advanced gastric cancer and plays an important role in preoperative and postoperative adjuvant therapy (Ashraf et al., 2013; GASTRIC Group, 2013). As such, resistance to chemotherapy is a major impediment of successful systemic treatment of gastric cancer.

Autophagy is also known as type II programmed cell death, it is a metabolism process of intracellular substance degradation of eukaryotic cells via the lysosomal pathway. The most common subtype is giant autophagy which is referred to as autophagy (Choi et al., 2013). Autophagy is often regarded as the survival and protective mechanism under stress conditions in the body. It can maintain the integrity of the cell by regenerating metabolic precursors and removing subcellular fragments (Levine

& Kroemer, 2008). A number of studies confirmed that the chemotherapy drugs can induce tumor cells to develop autophagy, it will result in the decreased sensitivity to chemotherapy (Huang et al., 2012; Pan et al., 2013; Selvakumaran et al., 2013; Zhang et al., 2013). Our previous in vitro experiments found that cisplatin induced apoptosis of gastric cancer SGC7901cells, which can also cause autophagy. After chloroquine was used to inhibit autophagy, the anti-cancer ability of cisplatin significantly was enhanced (Zhang et al., 2013). Based on this, this study was designed to study the effect of chloroquine on cisplatin capabilities against gastric cancer cells and to explore its mechanism.

Materials and Methods

Reagents

Cisplatin and chloroquine were purchased from Sigma-Aldrich Company of the United States. Caspase-3 antibody, P-gp antibody, Beclin 1 antibody and LC3 antibody were purchased in CST Company of United States. P-gp antibody was purchased from Abcam UK Company. MDR1 and Beclin 1 gene amplification primers were synthesized by Shanghai Sangon company.

¹The Third Department of Medical Oncology, ³Department of Geriatric Oncology, Jiangxi Provincial Cancer Hospital, ²Gastrointestinal Department of Internal Medicine, The Fourth Affiliated Hospital of Nanchang University, Nanchang, China *For correspondence: fnian3893@126.com

Hui-Qing Zhang et al Clinical study

<u>Subjects</u>: From January 2012 to June 2013, 35 cases of gastric cancer patients with malignant ascites were enrolled in our hospital, 22 cases were males and 13 were females, the median age was 53 years (24-71). All patients were diagnosed histologically as gastric adenocarcinoma, the cancer ascites cytology showed no cancer cells. ECOG score \geq 3 points, the expected survival time> 1 month. Patients did not receive chemotherapy within 1 month, they did not have intraperitoneal chemotherapy contradiction. All patients signed the informed consents of chemotherapy.

Intraperitoneal chemotherapy

The ascites were located with ultrasound in patients, the routine peritoneal cavity catheter was used. After drainage of ascites, the intraperitoneal injection of 250 ml of saline solution+60 mg of cisplatin+5 mg of 5% dexamethasone was performed. The position in patients was changed in every 15 min. The administration should be repeated once a week if necessary.

Assessment of intraperitoneal chemotherapy efficacy

The assessment was according to the WHO evaluation criteria: Complete remission (CR): complete disappearance of ascites, it lasted for more than four weeks. Partial remission (PR): $\geq 50\%$ reduction in malignant ascites, it lasted more than four weeks. Stable disease (SD): ascites decrease<50% or remained unchanged. Progress disease (PD): ascites restituted or increased within a month of treatment. The patients were divided into objective response group (OR) and no response group (NR) according to the efficacy, OR=CR+PR, NR=SD+PD.

Isolated cancer cells isolation in ascites and RT-PCR

20mL of ascites were collected in patients before chemotherapy and 5 days after peritoneal chemotherapy. The cancer cells were isolated from ascites according to methods by Wang et al (Wang et al., 2011). The total RNA was extracted for RT-PCR, Beclin-1 mRNA was detected. Beclin-1 primer sequence: 5'-ATCCTGGACCGTGT CACCA TCCAGG-3 ' (upstream), 5'-GTTGAGCTGAGTGTCCAGCTGG-3' (down- stream).

Animal experiments

Experimental cell lines and animals: The human gastric cancer cell line SGC7901 was routinely subcultured. 24 of 8-week-old BALB/C female mice were purchased from Experimental Animal Center of Sun Yat-sen University, they were kept in clean laminar rack of SPF barrier system.

Animal grouping and drug treatment: SGC7901 cells in logarithmic growth phase were inoculated subcutaneously into lower right side of back in nude mice, about 5×10^6 cells were inoculated in each mouse. 6 days after inoculation, a random number table was used to randomly divide 24 mice into 4 groups: control group (Con), chloroquine group (CQ), cisplatin (DDP) and combination group (CQ+DDP), n=6 in each group. PBS was given in control group, 45mg/kg of CQ was given in

chloroquine group, 5mg/kg of DDP was given in cisplatin group, 45mg/kg of CQ and 5mg/kg of DDP was given in combination group. The mice were administered once every three days and a total of 10 times, long and short diameters of tumors were measured every three days. 3 days after final injection, the animals were killed, the tumors tissues were cut out and weighed. Tumor volume formula: Volume=long diameter × short diameter $^2/2$.

<u>RT-PCR</u>: 50-100 mg of frozen samples were weighed and placed in liquid nitrogen-cooled mortar for grinding. After grinding, 1 mL Triol agents were added in the sample for continuous grinding. The well grinded tissues were moved into 1.5 mL of enzyme free EP tube, RNA was extracted for RT-PCR according to instructions. The primer sequences for the MDR1 gene were 5'-CCCATCATTGCAATAGCAGG and 3'-GTTCAAACTTCTGCTCCTGA-5 '. The products were used for gel imaging. Gel-Pro3.2 PCR software was used for electrophoresis image analysis.

Western Blot: 100 mg of tumor tissues were added to 300µL RIPA lysis buffer, EP tube was used for collection, then the ultrasound was performed. After the ultrasound, 14000r/min centrifugal was performed for 30min, the supernatant was obtained, the protein quantitation was performed according to Bradford method. After boiling for 5 min, the samples were added and cooled on ice, after 10000 r / min centrifugation for 30 s, the supernatant was taken for SDS-PAGE (200V, 45 min). After 100V film transfer for 1h, PVDF membrane was sealed with 0.5% (W / V) of skim milk at room temperature for 1h. First antibodies were added and incubated overnight at 4°C, the membrane was washed twice with 0.1% (V / V) TBST, secondary antibodies were added and incubated at room temperature for 1h, the membranes were washed 3 times, ECL method was used for coloring.

Immunohistochemistry: Two-step methods were used to detect the autophagy and apoptosis-related protein Beclin 1, LC3-I II and Caspase 3 expression of tumor tissues. The fixed tissues were used to make the paraffin sections $(4 \sim 6 \text{ um})$, after dewaxing, hydration, microwave antigen retrieval. They were incubated with 3% H₂O₂ at 37°C for 10 min, 10% goat serum was added to seal at room temperature for 10 min, first antibodies were added and incubated overnight at 4°C. The phosphate buffer solution was used for washing, horseradish peroxidaselabeled secondary antibody was added for incubation at room temperature for 30 min. Diaminobenzidine was used for color rendering for $3 \sim 5 \min$, after hematoxylin resin color rendering, the plates sealed for microscopic examination. Photos were analyzed using Image-Pro Plus 6.0 image processing software.

Statistical methods

Experimental data were expressed as \pm s, and SPSS 13.0 statistical software was used for statistical analysis. Continuous data were compared with t test between the two groups, and categorical data were compared using the chi-square test. Between experimental group and the control group, *P*<0.05 was considered statistically significant.

Results

Beclin-1 mRNA changes in isolated cells from ascites and chemotherapy efficacy

In all 35 patients, the median times to complete intraperitoneal cisplatin chemotherapy were 3 times (2-5), 2 cases had complete remission, 17 cases had partial remission, 10 cases had stable disease, 6 cases had progression disease, the response rate was 54.3% (19/35). In 19 patients with effective treatment (OR), RT-PCR technology was used for detection: Compared with that before treatment, 13 cases had decreased Beclin-1 mRNA levels in abdominal ascites 5 days after chemotherapy, 6 cases had increased Beclin-1 mRNA levels in abdominal ascites 5 days after chemotherapy. In 16 NR patients, only 2 cases had decreased Beclin-1 mRNA levels, 14 cases had increased Beclin-1 mRNA levels, 14 cases had increased Beclin-1 mRNA levels. The chi-square test value was χ^2 =11.091, *P*=0.001, the difference was statistically significant (Figure 1, Table 1).



Figure 1. Expression of Beclin-1 and BCL-2 mRNA in Exfoliative Cancer Cells Before and After IC. 1: OR, 2: NR, A: before IC, B: 5 days after IC. IC, Introperitoneal chemotherapy

Table 1. Changes of Beclin-1 mRNA in ExfoliativeCancer Cells between two Groups

Effect	Beclin-1 Reduction	Beclin-1 No Reduction	Р
OR (n=19)	13	6	0.001
NR (n=16)	2	14	



Figure 2. Xenograft Tumor Growth Conditions with Gastric Cancer SGC7901 Cell Lines in Each Group. **P*<0.05 vs Con group; **P*<0.01 vs DDP group; **P*<0.05 vs CQ groups

Growth state of xenograft tumor

Nude body weight difference between groups was not statistically significant before administration (P>0.05). After administration, rapid tumor growth was noticed in Con group, the growth was relatively slow in DDP group and CQ+DDP group. In the process of observation, compared with Con group, the xenograft tumor volume was smaller in CQ group (P < 0.05), tumor volume showed significant difference between DDP group and CQ+DDP group (P < 0.05). 36 days after administration, the mice were killed, the tumor weights were (0.96±0.21g) and (0.28±0.08g) in DDP group and CQ+DDP group which were lower than that in Con group $(1.83\pm0.41g)$ (all P < 0.05), the inhibition rates were 47.6% and 84.7%, respectively. Compared with DDP group, the xenograft tumor weight was significantly lighter in CQ +DDP group (*P*<0.01) (Figure 2).

Enhanced xenograft tumor autophagy with SGC7901 gastric cancer cells induced by cisplatin

Western blot results showed that compared with the con, tumor tissues Beclin-1 expression increased in nude mice of DDP group, LC3-II/I ratio increased significantly (P<0.01). Compared with DDP group, LC3-II/I ratio significantly decreased in DDP+CQ combination group (Figure 3).

Western Blot detection of P-gp and caspase3

In the Con group, P-gp expression was high in xenograft tumor cells, caspase3 was low. Compared with Con, caspase3 levels in DDP group and CQ+DDP group increased, P-gp levels decreased, changes were



Figure 3. Gastric Cancer Xenograft Tumor Cells Associated Protein Detected with Western Blot



Figure 4. RT-PCR Detection of Multidrug Resistance Gene MDR1 in Xenograft Tumor of Nude Mice



Figure 5. Apoptosis and Autophagy-related Proteins Expression in Xenograft Tumor of Gastric Cancer Nude Mice via Immunohistochemical Detection

more obvious especially in CQ+DDP group (all *P*<0.05) (Figure 3).

RT-PCR detection of multidrug resistance gene MDR1

After 10 times of intraperitoneal injection, compared with the control group (con), MDR1mRNA expression in chloroquine group (CQ) showed no significant difference (P>0.05). Compared with cisplatin (DDP) group, MDR1mRNA level in combination group (CQ+DDP) decreased, the difference was statistically significant (P<0.05) (Figure 4).

Immunohistochemical staining results

Immunohistochemical staining results showed compared with the Con group, Beclin-1 and LC3-I/II expression increased in nude mice tumor tissues of DDP group (both P<0.01). Compared with DDP group, Beclin-1 and LC3-I/II expression decreased in DDP+CQ combination group with increase in the caspase 3 expression (both P<0.01) (Figure 5).

Discussion

In 2008, there is 738,000 patients died of gastric cancer worldwide, the rate came in second place in all cancer-related deaths (Jemal et al., 2011). Surgery is the only curative treatment of gastric cancer. However, more than 2/3 of patients had advanced gastric cancer when diagnosed. The radical resection is not possible. In RO resection patients, more than 25% had recurrence or metastasis (Kim et al., 2013). Chemotherapy is the main method for the treatment of advanced gastric cancer. It can prolong the survival and improve the quality of life to some extent, program containing cisplatin will better benefit advanced gastric cancer patients undergoing chemotherapy (*P*asini et al., 2011). Currently, the effective

rate of chemotherapy for advanced gastric cancer was 34.5%-47.3%. The median survival time is between 9.2 months to 13.8 months, especially in gastric cancer patients with malignant ascites, the mOS was less than six months (Van et al., 2006; Bang et al., 2010; Fang et al., 2013).

Recent studies showed that the combination of chemotherapy drugs and autophagy modulators iss expected to improve the prognosis of cancer patients and reverse drug resistance (Chen et al., 2010; Levy et al., 2011). A randomized PhaseII clinical trials conducted by Sotelo et al (Sotelo et al., 2006) showed that compared with placebo, on the basis of the conventional radiation, 150mg oral chloroquine daily was given to glioblastoma patients for 12 months, it prolonged median survival of patients to some extent.

Chloroquine is an autophagy inhibitor, it has functions of weakening the acidic environment of lysosomes and stabilize lysosomal membrane. It can enhance the killing effect of many chemotherapeutic agents to tumor cells (Levy & Thorburn, 2011; Kimura et al., 2013). Our previous studies have shown that when 5mg/L of cisplatin was used in gastric cancer SGC7901 cells, apoptosis was noticed, autophagy vesicles was also noticed to increase. Beclin-1 and LC3-II protein expression were up-regulated to induce autophagy. After the combined use with chloroquine, the autophagic activity significantly decreased, apoptosis significantly increased. Therefore, we inferred that cisplatin-induced autophagy was the protective autophagy, it partially antagonized the cisplatininduced apoptosis of gastric cancer cells and led to decreased sensitivity to chemotherapy (Zhang et al., 2013).

We first reported the relationship between the autophagy levels and chemotherapy efficacy in isolated ascites cancer cells in the human body. We enrolled the gastric cancer patients with malignant ascites as the research subjects, they accepted to receive the intraperitoneal cisplatin. WHO criteria was used to evaluate the efficacy of chemotherapy, the cancer cells were isolated from ascites and collected before and after chemotherapy. Beclin-1 mRNA levels can be detected. The results showed that there were significant differences in Beclin-1 mRNA change trend between OR group and NR group (P=0.001). In the OR group, 68.4% of the patients had the decreased Beclin-1 mRNA levels 5 days after chemotherapy. In the NR group, 87.5% of the patients had stable or increased Beclin-1 mRNA levels suggesting that the autophagic activity increased. These results above suggested that anti-cancer ability of cisplatin may be related to the level of cellular autophagy, the activation of autophagy activity reflected the resistance to chemotherapy, while the lower autophagic activity was manifested as higher sensitive to chemotherapy. The results were consistent with in vitro results by O'Donovan et al (O'Donovan et al., 2011), they cultured 4 esophageal cancer cell lines, 5-FU and DDP were added, drug sensitive cell lines and resistant cell lines were separated. Apoptosis was mainly observed in sensitive strains and autophagy was mainly observed in resistant strains. Autophagy silencing gene Beclin-1 and AGT7 were used to interfere RNA which significantly increased the chemosensitivity to 5-FU/DDP.

In addition, we built gastric cancer SGC7901 cells in xenograft tumor of nude mice model, we found that compared with cisplatin treatment group, the tumor growth rate slowed down in cisplatin and chloroquine group, tumor volume was significantly reduced. Western Blot and immunohistochemistry results were consistent, Beclin-1 and LC3-IIexpression levels decreased in combined treatment group, the caspase 3 levels increased suggesting that intraperitoneal injection of chloroquine may inhibit autophagy activity in nude mice, it can thereby inhibit the tumor growth and increase the anticancer effects of cisplatin. With changes in MDR1 mRNA and P-gp protein levels, we speculated that chloroquine may enhance chemosensitivity to cisplatin by inhibiting the expression of multidrug resistance gene. This was consistent with the studies in the human hepatoma cell lines by Mazzanti R, etc (Mazzanti et al., 2009). They found that when celecoxib acted on the hepatoma cells with high expression of MDR, autophagy and cell cycle stationary will occur. This role was associated with downregulation of P-gpmediated HGF /MET autocrine loop.

Generally, the chloroquine can enhance the efficacy of anti-cancer drugs by inhibiting autophagy activity. In fact, academic world had different point of view and conclusions. Maycotte et al (Maycotte et al., 2012) used breast cancer cells of 67NR and 4T1 mouse as the subjects, they found that cisplatin can induce autophagy while killig cells. After the combination with chloroquine, autophagy activity was inhibited, cytotoxicity of cisplatin was enhanced. But this sensitizing effect was not achieved by knocking out Agt12 and Beclin 1 gene expression. Zinn et al (Zinn et al., 2013) used the Bcl-2 inhibitor ABT-737 in small-cell lung cancer, autophagy activity was detected to be activated, after the combination with chloroquine, the cell viability was significantly inhibited, the apoptotic protein Caspase 3 activity increased. However, after interference of autophagy gene Beclin 1 expression, the apoptosis promotion effects of ABT-737 decreased. One explanation for this phenomenon was that the anticancer effects of chloroquine may not be associated with specific autophagy pathway. In addition, studies also reported that chloroquine had the cell cycle regulation and differentiation effects (Zhou et al., 2000; Solomon& Lee., 2009). In our study, we also observed that compared with the control group, the chloroquine monotherapy inhibited the tumor growth when acting on gastric cancer SGC7901 cells of xenografts tumor in nude mice

In summary, we demonstrated that the cisplatin anticancer activity was associated with autophagy ability in vivo experiments. The combination of chloroquine and cisplatin can enhance the cell killing effect in gastric cancer xenografts in nude mice and chemosensitivity. The reason may be associated with autophagy activity inhibition and the down regulated expression of multidrug resistance gene MDR1/P-gp. This study provided the theoretical basis for clinical researches on chloroquine adjuvant treatment of gastric cancer. The appropriate patients selection and autophagic activity monitor in vivo may be the future focus and direction of research.

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