European Review for Medical and Pharmacological Sciences 2013; 17: 1703-1708

Knockdown of PLA2G2A sensitizes gastric cancer cells to 5-FU *in vitro*

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Abstract. – BACKGROUND-AIM: Elevated expression of the PLA2G2A phospholipase in gastric cancer (GC) is associated with improved patient survival. PLA2G2A is also an important regulator of proliferation, invasion and metastasis in GC. However, no relation about PLA2G2A and chemosensitivity in GC cells was reported. 5-Fluorouracil (5-FU) is widely used for treatment of advanced gastric cancer. However, it is common for such patients to develop resistance to 5-FU, and this drug resistance becomes a critical problem for chemotherapy. The mechanisms underlying this resistance are largely unknown. In the present study, we investigated whether PLA2G2A could confer 5-FU resistance or sensitise in GC cells *in vitro*.

MATERIALS AND METHODS: The 5-FU sensitivity of GC cell lines SGC-7901, MKN-45, RF-48, N87, AGS, MKN-28, RF-1, MGC-803 were determined by MTT assays. PLA2G2A levels were determined by western blot assays. The effects of 5-FU on PLA2G2A expression were determined *in vitro*. PLA2G2A was inhibited by silencing of the PLA2G2A using small interfering RNA *in vitro*. PLA2G2A was overexpressed by transfection of full-long PLA2G2A cDNA *in vitro*, and the effects were evaluated on 5-FU sensitivity.

RESULTS: The cell lines SGC-7901, MKN-45, RF-48 and N87 were sensitive, whereas AGS, MKN-28, RF-1 and MGC-803 were resistant to 5-FU. Significant correlation was observed between basal PLA2G2A and 5-FU sensitivity. Silencing of PLA2G2A increased 5-FU killing in 5-FU-treated cells, and overexpression of PLA2G2A decreased 5-FU killing in 5-FU-treated cells.

CONCLUSIONS: PLA2G2A was correlated with sensitivity to 5-FU. Silencing of PLA2G2A was sensitive to 5-FU treatment. Thus, PLA2G2A may be a useful therapeutic target for a subset of gastric cancers.

Key Words:
Gastric cancer, Chemosensitivity, 5-FU, PLA2G2A.

Abbreviations

PLA2G2A = Phospholipase A2 group IIA;
GC = Gastric cancer;
FITC = Annexin V-fluorescein isothiocyanate;
MTT = 4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;
PBS = phosphate-buffered saline;
5-FU = 5-Fluorouracil;
EGTA = ethylene glycol tetraacetic acid;
AEBSF = 4(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride;
SDS-PAGE = Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis;
ECL = Enhanced Chemiluminescence;
MAGIC Trial = The Medical Research Council Adjuvant Gastric Infusional Chemotherapy Trial.

Introduction

Gastric cancer is the second most common cause of cancer death worldwide. Advanced gastric cancer is incurable[1]. Chemotherapy for gastric cancer patients, however, is not effective. The most widely investigated single-agent chemotherapy is 5-fluorouracil (5-FU), with partial response rates up to 20%[2]. Although combination chemotherapy improves survival compared to single-agent 5-FU, but the effect size is much smaller[3-4]. A common critical issue responding to 5-FU is the development of resistance to the drug, which has become a major obstacle in chemotherapy[5-6]. A significant obstacle for the successful management of patients with cancer is intrinsic drug resistance or, in patients who respond to chemotherapy, acquired drug resistance[7]. Thus, evaluation of the chemosensitivity of gastric cancer cells to anticancer agents based on the phenotype difference of individual cell lines will provide more useful information for choosing correct drugs for gastric cancer patients. Furthermore, understanding 5-FU resistance mechanisms at a molecular level seems essential to design strategies to overcome this resistance.

Phospholipase A2 (PLA2) catalyzes hydrolysis of the sn-2 fatty acyl ester bond of phosphoglycerides, releasing free fatty acids and

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lysophospholipids[8]. PLA2 group IIA (PLA2G2A) is a secreted PLA2.PLA2G2A belongs to the subfamily of group II sPLA2s, comprising PLA2 group IIA, PLA2 group IIC, PLA2 group IID, PLA2 group IIE, PLA2 group IIF, and PLA2 group V. All of these enzymes are encoded by a cluster of highly homologous genes located within a ~250 kb genomic segment on human chromosome 1p35, and on its homologous region on mouse distal chromosome[8].

PLA2G2A has been reported to be expressed in many human cancers.In pancreatic cancer and esophageal squamous cell carcinoma(ESCC), upregulation of PLA2G2A is associated with better prognosis[9-10]. In prostate cancer expression of PLA2G2A is upregulated at all stages of prostate cancer, and its upregulation may be androgen inducible[11]. In the lung cancer, PLA2G2A inhibition could reduce lung cancer growth in vitro and *in vivo*[12]. PLA2G2A expression is also positively associated with resistance to human gastric cancer progression[13]. Furthermore, PLA2G2A is a direct target of Wnt/beta catenin signaling in human gastric cancer. Its expression is silenced by hypermethylation of promoter elements that contain beta catenin target sites. Expression of PLA2G2A suppressed gastric cancer migration and invasion in cell culture and this phenotype was mediated by the inhibition of S100A4 and NEDD9[14]. It has found, PLA2G2A could confer to chemotherapy resistance in pediatric osteosarcoma and prostate cancer cells[15-16]. It is not clear whether PLA2G2A could confer to chemotherapy resistance in gastric cancer.

In the present study,we test the hypotheses that (a) levels of PLA2G2A could distinguish sensitive from resistant gastric cancer cells; and (b) that inhibition of PLA2G2A by small interfering RNA (siRNA) would increase the sensitivity of gastric cancer cell lines to 5-FU mediated apoptosis; and (c) that overexpression of PLA2G2A by transfection of full long PLA2G2A cDNA would decrease the sensitivity of gastric cancer cell lines to 5-FU mediated apoptosis.

Materials and Methods

Cell Lines and Animals

Gastric cancer (GC) cell lines SGC-7901, MKN-45, RF-48, N87, AGS, MKN-28, RF-1, MGC-803 were obtained from American Type Culture Collection (ATCC, Shanghai, China). They were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) (Life Technologies, Inc., Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mg/ml sodium bicarbonate, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.) at 37°C in 5% CO_2.

Drug Exposure

5-FU was purchased from Sigma Chemical Co. (St Louis, MO, USA). It was diluted in 0.9% saline solution and stored at 25 C and freshly diluted in culture medium before each experiment. To examine the response to 5-FU, the GC cells were cultured in a 96-well plate overnight and stimulated with various concentrations of 5-FU for 48 h.

Construction and Transfection of the PLA2G2A Expression Vector

Human PLA2G2A cDNA was from Proteintech Group, Shanghai. China. pcDNA3 (mutant connexim 43 plasmid) was purchased from Geneland, Shanghai, China. pcDNA3-PLA2G2A cDNA plasmid was constructed as the manufacture's instruction. To establish cell lines overexpressing PLA2G2A, we transfected SGC-7901, MKN-45, RF-48, N87 cells with pcDNA3-PLA2G2A or control pcDNA3 constructs to examine the effect of increased PLA2G2A on the chemoresistance. This construct (0.4 μg) or empty pcDNA3 vector (0.4 μg) was transfected into 10^5 cells/well in 24-well plates using Lipofectamine Reagent according to the manufacturer's recommendations, and the transfected cells were treated with 25 μg/L 5-FU for 36 h to determine the drug resistance.

Silencing of PLA2G2A by Small Interfering RNA Transfection

For PLA2G2A silencing, siRNA duplexes were obtained from Dharmacon (Lafayette, CO, USA). The procedure of transfection of siRNA into AGS, MKN-28, RF-1, MGC-803 cells was as the manufacture's instruction. In brief, AGS, MKN-28, RF-1, MGC-803 cells in exponential growth phase were plated in six-well plates at 8×10^5 cells per well, grown for 24 h, then transfected with PLA2G2A siRNA duplex using cationic lipid oligofectamine (Invitrogen, Carlsbad, CA, USA), as described by the manufacturer's instructions. Silencing was examined 48 h after transfection with siRNA-oligofectamine complexes. In addition, a control siRNA duplex was

obtained from Dharmacon. The transfected cells were treated with 25 μg/L 5-FU for 72 h to determine the drug resistance.

MTT Assay

The MTT assay was done as previously described[17]. Briefly, cells (5×10^4 cells per well) in different groups at different time point was incubated with MTT solution, isopropanol was added to dissolve the formazan crystals. Absorbance was measured using a Vmax Microplate Reader (Molecular Devices, Silicon Valley, CA, USA) at 590 nm. Survival was calculated from the mean of pooled data from three separate experiments with five wells.

Apoptosis Analysis

The cells were washed twice with cold 10 mM 1× PBS and resuspended in 1× binding buffer (BD Biosciences, San Jose, CA, USA). Apoptosis in GC cells was quantified by staining with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) [annexin V-Phycoerythrin (PE) and 7-amino-actinomycin D (7-AAD) for apoptosis analysis for cells. The samples were analyzed using flow cytometry (FACSCalibur, BDBiosciences, San Jose, CA, USA).

Western Blot Analysis

Cells were washed in ice-cold phosphate buffered saline (PBS), lysed in whole cell extraction buffer (250 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride and protease inhibitors cocktail containing 500 lM AEBSF, 1 μg/ml aprotinin, 1 lM E-64, 500 lM EDTA and 1 lM leupetptin), and centrifuged at 14,000 g for 20 min. About 40 ug of the proteins were separated by 10-12% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. Membranes were blocked with 5% skim milk and then probed with antibodies specific for human PLA2G2A and β-actin (Chemicon, Temecula, CA, USA; final dilution: 1/5,000). After being probed with an appropriate secondary antibody, immunodetected proteins were visualized by an ECL system according to the manufacturer's instructions (Amersham Pharmacia Biotech, Uppsala, Sweden).

Statistical Analysis

Statistical analyses were performed using Student's *t* test. The data were presented as the mean ± SD, and $p < 0.05$ was considered significant.

Results

Gastric Cancer Cell Lines Vary in Resistance to 5-FU

We examined the relative sensitivity of six commonly used gastric cell lines (SGC-7901, MKN-45, RF-48, N87, AGS, MKN-28, RF-1 and MGC-803) to 5-FU *in vitro*. Cells were treated with different concentrations of 5-FU for 72 hours and the number of surviving cells was analyzed. Whereas the 5-FU LD50 >100 mg/L was for the AGS, MKN-28, RF-1, MGC-803 cells, the LD50 was around 25 mg/L for SGC-7901, MKN-45, RF-48 and N87 cells (Figure 1A). The same sensitivities were obtained when the effects of 5-FU were analyzed on apoptosis using flow cytometry (Figure 1B). These data supported the classification of the AGS, MKN-28, RF-1 and MGC-803 cell lines as 5-FU-resistant and the SGC-7901, MKN-45, RF-48 and N87 cell lines as 5-FU-sensitive.

PLA2G2A Levels in Gastric Cancer Cell Lines

To test the hypothesis that PLA2G2A basal levels could predict 5-FU sensitivity, we initially evaluated PLA2G2A protein level by western blot assay. Highest levels of PLA2G2A were observed in the AGS, MKN-28, RF-1 and MGC-803 cells, and lowest PLA2G2A was observed in the SGC-7901, MKN-45, RF-48 and N87 cells (Figure 2). Thus, gastric cancer cells exhibited elevated PLA2G2A were resistant to 5-FU treatment,and gastric cancer cells exhibited low PLA2G2A were sensitive to 5-FU treatment. PLA2G2A basal levels could predict sensitivity to 5-FU.

Down-Regulation of PLA2G2A Enhances 5-FU-induced Cell Death

To investigate the role of PLA2G2A on the 5-FU mediated cellular response, we attempted to down-regulate its expression by employing siRNAs targeting the PLA2G2A in the 5-FU-resistant cell line AGS, MKN-28, RF-1 and MGC-803 cells. Protein expression analysis indicated that down-regulation of the PLA2G2A was visible after 24 h from the initial siRNA transfection,and completely inhibited after 72 hours (Figure 3A). Whereas 5-FU treatment did not affect the PLA2G2A levels (data not shown). Next, flow cytometry analysis was conducted to measure cell death in response to 5-FU (25 ug/L) treatment in PLA2G2A knockdown cells (Figure 3B). AGS, MKN-28, RF-1 and MGC-803 cells was transfected with PLA2G2A siRNA for 72 hours, then treated with 25 ug/L 5-FU for 72 hours,

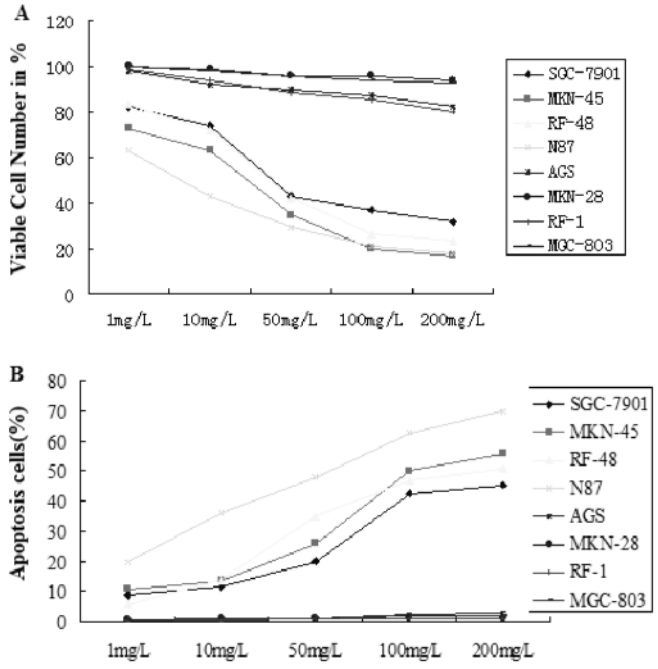

Figure 1. Gastric cancer cells have differing levels of native resistance to 5-FU. Human gastric cancer cell lines SGC-7901, MKN-45, RF-48, N87, AGS, MKN-28, RF-1 and MGC-803 were treated with increasing concentrations of 5-FU (0-200 mg/L) for 72 h. ***A***, The viabilities indicated on the y axis were determined by MTT assays and normalized to control. ***B***, The apoptosis cells indicated on the y axis were determined by MTT assays and normalized to control. Data shown are means ± SE for n = 3 independent experiments.

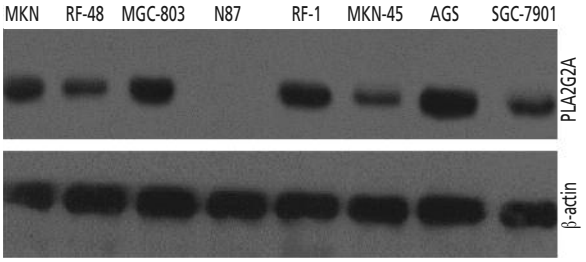

Figure 2. Basal levels of PLA2G2A correlate with 5-FU sensitivity. PLA2G2A was analyzed in human gastric cancer cell lines by western blot assay.

led to 39, 51, 46 and 42% cell death, respectively (Figure 3B), and PLA2G2A siRNA transfection alone did significantly kill cells (Figure 3B). MTT assay has the same results (data not shown).

Up-regulation of PLA2G2A Decreased 5-FU-induced Cell Death

SGC-7901, MKN-45, RF-48 and N87 cells were transfected with PLA2G2A cDNA for72 hours. Protein expression analysis indicated significant PLA2G2A increase was found (data not shown). After the cells was transfected with PLA2G2A cDNA for 72 hours, they were then

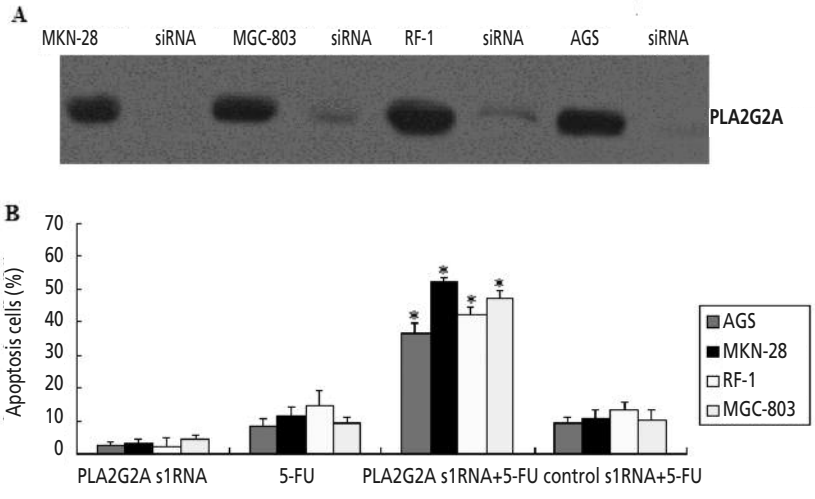

Figure 3. Silencing of PLA2G2A is effective against 5-FU-resistant cells. ***A***, Effects of PLA2G2A siRNA transfection on PLA2G2A in AGS, MKN-28, RF-1 and MGC-803 cells. ***B***, Effects of PLA2G2A siRNA transfection on cell apoptosis of AGS, MKN-28, RF-1 and MGC-803 cell lines after silencing of PLA2G2A. Human gastric cancer cell lines AGS, MKN-28, RF-1 and MGC-803 cells were transfected with PLA2G2A siRNA for 48 hours,then treated with 25 mg/L 5-FU for 72 h. Cell death assay for measuring apoptosis induced by PLA2G2A siRNA, 5-FU, and combination. Data are means ±SE for n = 3 experiments (vs control, $*p < 0.05$).

treated with 25 mg/L 5-FU for 72 hours, only to find less cell death, respectively (Figure 4), and PLA2G2A cDNA transfection alone did significantly kill cells (Figure 4). MTT assay has the same results (data not shown).

Discussion

Although it is thought that adjuvant chemotherapy following surgery for the treatment of GC may protect against the development of metastases or recurrent disease, a definite benefit for adjuvant chemotherapy after surgery has yet to be confirmed in gastric cancer[18]. Indeed, there is a high prevalence of local or distant recurrence after histologically curative surgery for GC[19]. While an effective chemotherapy may control GC recurrence after removal of the main tumor, a standard regimen with good efficacy for most GC cases remains to be established. As a result, GC patients may suffer from drug toxicity from chemotherapies that are ineffective against the tumor and, therefore, do not improve the survival or quality of life (QOL) of GC patients after surgery.

5-FU has been shown to be the most effective treatment option after removal of the main gastric tumor. Validation of postoperative chemotherapy and surgery alone has demonstrated 3-year overall survival (OS) rates of 80.1% and 70.1%, respectively, in Japan[20]. Postoperative chemoradiotherapy (CRT) has been conducted in the United States for advanced gastric cancer, and the OS after CRT has been reported to be 50%, while that of surgery alone was 41%[21]. In Europe, the MAGIC trial revealed the efficacy of perioperative chemotherapy and demonstrated that the 5-year OS was 36.3% and 23.0% in the perioperative chemotherapy and surgery alone groups, respectively[22].

With a standard of care established, we now face the issue of how to treat patients who fail these standard therapies. In fact, 30-40% of patients treated with the standard adjuvant chemotherapy experience recurrence after surgery within five years in advanced gastric and colon carcinomas[23]. Therefore, it has been an important goal to improve treatment regimens for the subset population where standard therapy may be ineffective.

It has found, PLA2G2A could confer to chemotherapy resistance in pediatric osteosarcoma and prostate cancer cells[15-16]. In the present study, we found gastric cancer cells exhibited elevated PLA2G2A was resistant to 5-FU treatment,and gastric cancer cells exhibited low PLA2G2A was sensitive to 5-FU treatment. Therefore, PLA2G2A levels would predict chemoresistance to 5-FU in gastric cancer cells *in vitro*. Of particular significance, our results on the effects of PLA2G2A inhibition on sensitivity to 5-FU indicated that resistant cells were affected.

Furthermore, in 5-FU sensitive cells, after the cells was transfected with PLA2G2A cDNA,the cells were resistant to 5-FU treatment.

Our data indicate that the benefits of inhibiting PLA2G2A are limited to cell lines that are natively 5-FU-resistant. Resistant gastric cancer cells rely on PLA2G2A for survival under basal conditions and during treatment with 5-FU. Thus, the sensitivity of gastric cancer cells to 5-FU and to PLA2G2A silencing was correlated. The mechanisms responsible for 5-FU resistance need further investigation.

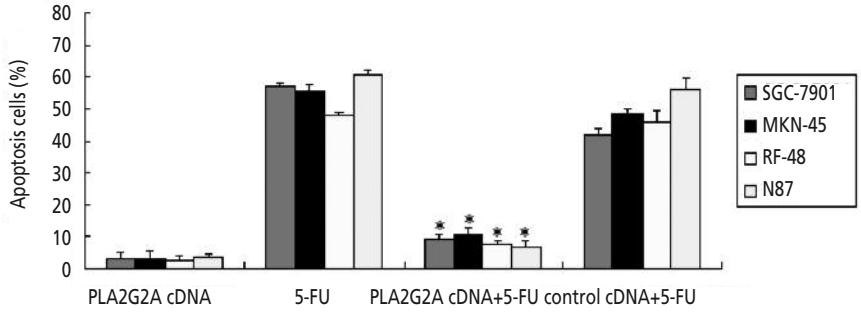

Figure. 4. Overexpression of PLA2G2A is effective against 5-FU-sensitive cells. Effects of PLA2G2A cDNA transfection on cell apoptosis of SGC-7901, MKN-45, RF-48 and N87 lines after overexpression of PLA2G2A. Human gastric cancer cell lines SGC-7901, MKN-45, RF-48 and N87 cells were transfected with PLA2G2A cDNA for 72 hours, then treated with 25 mg/L 5-FU for 72 h. Cell death assay for measuring apoptosis induced by PLA2G2A cDNA, 5-FU, and combination. Data are means ±SE for n = 3 experiments (vs control,*$p < 0.05$).

Conclusions

Inhibition of PLA2G2A seems to be a useful treatment in gastric cancer cell lines that are natively resistant to 5-FU. In such cells, silencing of PLA2G2A improved the effectiveness of 5-FU. It is possible that PLA2G2A could predict chemoresistance to 5-FU in gastric cancer cells *in vitro*.

Conflict of Interest

None.

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