Gemcitabine induces cell senescence in human pancreatic cancer cell lines

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Highlights:

1. Gemcitabine induced cell senescence but not apoptosis in human pancreatic cell lines which were resistance to gemcitabine.
2. Gemcitabine induced cell senescence partly through gemcitabine induced ROS generation in human pancreatic cancer cell lines.
3. Gemcitabine-induced senescence proceeded in vivo independently of CXCL8 expression.

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Abstract

Patients with pancreatic ductal adenocarcinoma (PDAC) commonly require chemotherapy because they frequently develop metastatic disease or locally advanced tumors. Gemcitabine, an analogue of cytosine arabinoside, is commonly used for PDAC treatment. We observed that gemcitabine induced senescence phenotypes characterized by enhanced senescence-associated β-galactosidase (SA β-Gal) staining and increased expression of senescence-associated molecules in two human pancreatic cancer cell lines, Miapaca-2 and Panc-1, which exhibit resistance to gemcitabine but not L3.pl cells with a high sensitivity to gemcitabine. Gemcitabine-induced cell senescence can be inhibited by reactive oxygen species inhibitor, N-acetyl cysteine. Although gemcitabine also enhanced CXCL8 expression, anti-CXCL8 antibody failed to reduce gemcitabine-induced increases in SA β-Gal-positive cell numbers. These observations would indicate that cell senescence can proceed independently of CXCL8 expression, a characteristic feature of senescence-associated secretion phenotype.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a solid tumor with in the exocrine compartment of pancreatic gland, and 5-year survival for all stages remains at less than 5 % over the last few decades [1]. Even at the time of diagnosis, about 80% of patients are subjected to chemotherapy, due to metastatic disease or locally invasive tumors. Moreover, the rest of the patients are eligible for potentially curative resection but frequently develop local recurrence or distant metastasis after surgery and as a consequence, need adjuvant therapy consisting of chemotherapy and/or irradiation.

Gemcitabine is an analogue of cytosine arabinoside (Ara-C) [2] and is used as a standard chemotherapeutic drug for PDAC treatment, alone or in combination with other chemotherapeutics[1, 3]. Like Ara-C, gemcitabine is a prodrug which requires cellular uptake and intracellular phosphorylation. It is transported across the cell membrane through multiple active nucleoside transporters located in the cell plasma membrane [4]. Inside the cell, gemcitabine is phosphorylated by deoxycytidine kinase to produce gemcitabine monophosphate (dFdCMP), which is further converted to active drug metabolites, gemcitabine diphosphate (dFdCDP) and gemcitabine triphophage (dFdCTP) [5]. dFdCTP directly inhibits DNA polymerase [6], cytidine triphosphate synthetase [7] and deoxycytidylate deaminase [8] , and is incorporated into DNA, thereby terminating chain elongation [9]. dFdCDP potently inhibits ribonucleotide reductase (RR), thereby decreasing deoxynucleotide pools necessary for DNA synthesis [10]， Despite its multiple intracellular targets, the resistance to gemcitabine often ensues [1] .

The term “cellular senescence” has been used to denote a stable and long-term loss of proliferative capacity, despite continued viability and metabolic activity [11]. Moreover, the overexpression of activated Ras proteins can induce a senescence-like phenotype, which is called oncogene-induced senescence (OIS) [12]. Cellular senescence

or OIS can induce the cells to undergo profound changes in their secretome, termed the senescence-associated secretion phenotype (SASP) [13, 14]. SASP is characterized by the overexpression of proinflammatory cytokines, interleukin (IL)-6 [15], and IL-8/CXCL8 [16]. OIS is presumed to serve as the first barrier of defense against cancer development [17], but can promote carcinogenesis by promoting inflammation arising from SASP [18,19].

We previously observed that human pancreatic cancer cell lines exhibited enhanced CXCL8 expression, which is reminiscent of SASP, in the presence of gemcitabine [20]. Because Aird and colleagues revealed that oncogene-induced repression of RR resulted in a decrease in deoxyribonucleotide triphosphate levels and subsequently caused OIS [21], these observations prompted us to explore whether gemcitabine with a capacity to inhibit RR, can induce senescence-like phenotypes in human pancreatic cancer cells. Indeed, gemcitabine induced senescence-like phenotypes in human pancreatic cancer cells.

Materials and Methods

Cell lines and reagents

Miapaca-2 and Panc-1 cells were obtained from ATCC and were maintained in RPMI-1640 medium while L3.6pl cells were obtained from Dr. Isiah J. Fidler (MD Anderson Cancer Center, Houston, TX) and were maintained in minimum essential medium. Both media were supplemented with 10% fetal bovine serum.

In vitro cell proliferation

Cell suspensions (2×10^3 cells /100 μl) were added to each well of 96-multi-well plates and incubated at 37^oC for 24 hr. Then, gemcitabine was added to the indicated

concentrations and the cells were incubated for the indicated time intervals. The cell viability was determined by using the cell counting kit-8 (Dojindo Co. Ltd., Kumamoto, Japan). The ratios of cell numbers were determined by comparing the cell numbers at day 0, and IC_{50} values for gemcitabine were calculated using logistic regression.

Senescence-associated β-galactosidase (SA β-gal) staining

Human pancreatic cancer cell lines were seeded at a density of 5 x 10^4 cells/well in 6-well plates. After gemcitabine treatment, the cells were fixed with 3% formaldehyde in PBS. In vivo experiment, frozen sections were cut at 5 μm and were fixed with 4% paraformaldehyde. Cells or frozen sections were stained overnight in X-gal staining solution consisting of 1 mg/ml X-gal, 40 mM citric acid/sodium phosphate buffer (pH 6.0), 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$ as previously described [22].

RNA isolation for RT-PCR

Total RNA was isolated and was reverse transcribed to cDNA as previously described [23]. Quantitative (q)RT-PCR was performed and mRNA amounts were normalized to the amounts of GAPDH as relative expression values as described previously [23]. The sequences of the used primers are listed in Table 1.

Cell apoptosis analysis

After treatment with various concentrations of gemcitabine for 48 hr, the cells were harvested. Phosphatidylserine exposure level was determined by staining the cells with the human Annexin V-FITC Kit (Bender MedSystem, Vienna，Austria). At least 30,000 stained cells were analyzed on a FACSCanto II system (BD Biosciences, San Jose, CA).

Animal experiments

Miapaca-2 cells were suspended in HBSS at a concentration of 3.5 x 10^7 cells/ml

and 200 μl cell suspensions were injected subcutaneously into the back of BALB/c nu/nu mice (SLC, Shizuoka, Japan). When the tumor size reached 100 mm^3, gemcitabine (30 mg/kg body weight) was administered to tumor-bearing mice together with anti-human CXCL8 monoclonal antibody or control antibody (100 μg/body) according to the schedule illustrated in Figure 3B. At the indicated time points after the injection, tumors were removed for SA β-gal staining. All animal experiments were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University.

Statistical analysis

Data was analyzed statistically using methods indicated in each figure legend. $p< 0.05$ was considered statistically significant.

Results

The cell proliferation of different human pancreatic cancer cells after gemcitabine treatment.

L3.6pl cells were highly sensitive to gemcitabine with IC_{50} of 0.002μM (Table 2). Consistent with the previous report [24], Miapaca-2 and Panc-1 cells were rather resistant to gemcitabine with IC_{50} of 0.7 and 5.5 μM, respectively (Table 2). Moreover, gemcitabine induced a marked apoptosis in L3.6pl cells as evidenced by enhanced annexin V staining, whereas only a marginal proportion of Miapaca-2 and Panc-1 cells became apoptotic upon exposure to gemcitabine (Figure 1). Thus, gemcitabine may inhibit cell proliferation by inducing apoptosis.

Gemcitabine-induced cell senescence in gemcitabine-resistant pancreatic cancer cells.

Given the potential of cell senescence to counteract irreversible cell death [25], we next examined the effects of gemcitabine on senescence. Gemcitabine efficiently induced

senescence in Miapaca-2 and Panc-1 cells in a dose-dependent manner (Figure 2A) but not in L3.6pl cells (data not shown), as evidenced by increased SA β-gal staining. Moreover, gemcitabine significantly increased the levels of senescence-associated molecules including P53, P21, P19, PML, and DCR2 in Miapaca-2 and Panc-1 cells, except P53 in Panc-1 cells (Figure 2B). Collectively, gemcitabine induced senescence but not apoptosis in Miapaca-2 and Panc-1 cells, the cell lines that are resistant to gemcitabine.

Involvement of reactive oxygen species (ROS) in gemcitabine-induced cell senescence.

We previously observed that gemcitabine induced ROS generation and that NAC with the capacity to scavenge ROS by interacting with $OH\cdot$ and H_2O_2 reduced gemcitabine-induced ROS generation [20]. Hence, we examined the effects of NAC on gemcitabine-induced senescence and observed that NAC decreased gemcitabine-induced increased SA β-gal-positive cell numbers (Figure 3A). Thus, gemcitabine induced cellular senescence partly via ROS generation.

Gemcitabine-induced cell senescence in human pancreatic cancer in vivo.

Gemcitabine treatment also increased SA β-gal-positive cell numbers when it was injected into a nude mouse bearing a tumor (Figure 3C). We previously observed that gemcitabine treatment enhanced intra-tumoral CXCL8 expression and that anti-CXCL8 antibody administration reduced tumor formation when it was given with the combination of gemcitabine [20]. On the contrary, anti-CXCL8 antibody failed to reduce gemcitabine-induced increases in SA β-gal-positive cell numbers (Figure 3C).

Discussion

Gemcitabine is widely used for the treatment of PDAC, but PDAC-derived cell lines exhibit a wide variation in responsiveness to gemcitabine. L3.6pl became mostly

apoptotic and extinguished in vitro in the presence of gemcitabine at doses lower than 0.01 μM. On the contrary, Miapaca-2 and Panc-1 cells survived 1 μM but these cells exhibited senescence-associated phenotypes instead of undergoing apoptosis. Senescence generally contributes to the elimination of damaged cells by phagocytic cells, and to the subsequent promotion of tissue remodelling, similarly as apoptosis does [26]. Moreover, senescence can be induced in pre-cancerous or cancer cells under various conditions such as oncogene activation, chemotherapy, or radiotherapy, and can be a barrier against cancer progression, particularly in the case of OIS [11, 12]. Senescence is initially considered as an irreversible state of cellular arrest, similar to apoptosis which causes irreversible chromosomal DNA cleavage. However, the suppression of P53 in senescent cells can lead to rapid re-entry into cellular proliferation with loss of senescence-associated molecule expression [25]. Thus, chemotherapy-induced senescent cells may be in a reversible cellular arrest to escape cell death. This may account for gemcitabine-induced senescence in two pancreatic cancer cell lines, which are highly resistant to gemcitabine.

We previously unraveled the involvement of ROS generation in gemcitabine-induced CXCL8 expression [20]. Likewise, NAC, an inhibitor of ROS, significantly reduced gemcitabine-induced increases in SA-β-gal-positive cell numbers, suggesting that gemcitabine induced cell senescence partly through gemcitabine-induced ROS generation, as previously observed [27].

SASP, a typical feature of cell senescence, is characterized by enhanced expression of IL-6 and/or CXCL8 and its related chemokines in senescent cells [15, 16]. SASP is proposed to be able to promote and advance carcinogenesis [17, 18]. However, it still remains elusive on the roles of SASP in cell senescence. We previously observed that Miapaca-2 and Panc-1 cells did not express receptors for CXCL8, CXCR1 and CXCR2, but that anti-CXCL8 antibody reduced *in vivo* tumor formation with the combined administration of gemcitabine [20]. We explored the possibility of the paracrine effects of CXCL8 in gemcitabine-induced cell senescence, by administering anti-CXCL8 antibody to tumor-bearing mice. However, the antibody failed to reduce

gemcitabine-induced increases in SA β-Gal-positive numbers. Thus, SASP may have few effects on gemcitabine-induced cell senescence.

References

[1]. Paulson AS, Cao TH, Tempero MA, Lowy, AM. Therapeutic advances in pancreatic cancer. Gastroenterol 2013; 144: 1316–26.

[2]. Hertel LW, Kroin JS, Misner JW, Tustin JM. Synthesis of 2-deoxy-2, 2-difluoro-D-ribose and 2-deoxy-2, 2'-difluoro-D-ribofuranosyl nucleosides. J Org Chem 1988; 53: 2406–09.

[3]. Burris HA 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol 1997; 15: 2403-13.

[4]. Mackey JR, Mani RS, Selner M, Mowles D, Young JD, Belt JA, et al. Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. Cancer Res 1998; 58: 4349-57.

[5]. Heinemann V, Hertel LW, Grindey GB, Plunkett W. Comparison of the cellular pharmacokinetics and toxicity of 2', 2'-difluorodeoxycytidine and 1-beta-D-arabinofuranosylcytosine. Cancer Res 1988; 48: 4024-31.

[6]. Hertel LW, Boder GB, Kroin JS, Rinzel SM, Poore GA, Todd GC, et al. Evaluation of the antitumor activity of gemcitabine(2',2'-difluoro-2'-deoxycytidine). Cancer Res 1990; 50: 4417-22.

[7]. Heinemann V, Schulz L, Issels RD, Plunkett W. Gemcitabine: a modulator of intracellular nucleotide and deoxynucleotide metabolism. Semin Oncol 1995; 22: 11-18.

[8]. Heinemann V, Xu YZ, Chubb S, Sen A, Hertel LW, Grindey GB, et al. Cellular elimination of 2', 2'-difluorodeoxycytidine 5'-triphosphate: a mechanism of self-potentiation. Cancer Res 1992; 52: 533-39.

[9]. Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W. Action of 2', 2'-difluorodeoxycytidine on DNA synthesis. Cancer Res 1991; 51: 6110-17.

[10]. Baker CH, Banzon J, Bollinger JM, Stubbe J, Samano V, Robins MJ, et al. 2'-Deoxy-2'-methylenecytidine and 2'-deoxy-2',2'-difluorocytidine 5'-diphosphates:

potent mechanism-based inhibitors of ribonucleotide reductase. J Med Chem 1991; 34: 1879-84.

[11]. Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. The essence of senescence.Genes. Develop. 2010; 24: 2463-79.

[12]. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 1997; 88: 593-602.

[13]. Coppé JP, Patil CK, Rodier F, Sun Y, Muñoz DP, Goldstein J, et al. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. Plos Biol 2008; 6: 2853-68.

[14]. Rodier F, Coppé JP, Patil CK, Hoeijmakers WA, Muñoz DP, Raza SR, et al. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. Nature Cell Biol 2009; 11: 973-979.

[15]. Kuilman T, Michaloglou C, Vredeveld LC, Douma S, van Doorn R, Desmet CJ, et al. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. Cell 2008; 133: 1019-1031.

[16]. Acosta JC, O'Loghlen A, Banito A, Guijarro MV, Augert A, Raguz S, et al. Chemokine signaling via the CXCR2 receptor reinforces senescence. Cell 2008; 133: 1006-18.

[17]. Campisi, J. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell 2005; 120: 513–22.

[18]. Pribluda A, Elyada E, Wiener Z, Hamza H, Goldstein RE, Biton M, et al. A senescence-inflammatory switch from cancer-inhibitory to cancer-promoting mechanism. Cancer Cell 2013; 24: 242-56.

[19]. Rielland M, Cantor DJ, Graveline R, Hajdu C, Mara L, Diaz Bde D, et al. Senescence-associated SIN3B promotes inflammation and pancreatic cancer progression. J Clin Invest 2014; 124: 2125-35.

[20]. Song Y, Baba T, Li YY, Furukawa K, Tanabe Y, Matsugo S,et al, Gemcitabine-induced CXCL8 expression counteracts its actions by inducing tumor

neovascularization. Biochem Biophys Res Commun. 2015,458:341-6.

[21]. Aird KM, Zhang G, Li H, Tu Z, Bitler BG, Garipov A, et al. Suppression of nucleotide metabolism underlies the establishment and maintenance of oncogene-induced senescence. Cell Reports 2013; 3: 1252-65.

[22]. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 1995; 92: 9363-67.

[23]. Sasaki S, Baba T, Shinagawa K, Matsushima K, Mukaida, N. Crucial involvement of the CCL3-CCR5 axis-mediated fibroblast accumulation in colitis-associated carcinogenesis in mice. Int J Cancer 2014; 135: 1297-306.

[24]. Pan X, Arumugam T, Yamamoto T et al. Nuclear factor-κB p65/relA silencing induces apoptosis and increases gemcitabine effectiveness in a subset of pancreatic cancer cells. Clin Cancer Res 2008; 14: 8143-51.

[25]. Dirac AM, Bernards R. Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. J Biol Chem 2003; 278: 11731-34

[26]. Muñoz ED, Serrano M. Cellular senescence: from physiology to pathology. Nature Rev Mol Cell Biol 2014; 15: 482-96.

[27]. Salvador Macip, Makoto Igarashi, Li Fang, Angus Chen, Zhen-Qiang Pan, Sam W. Lee, et al. Inhibition of p21 mediated ROS accumulation can rescue p21 induced senescence. The EMBO Journal (2002) 21, 2180-88.

Table 1. The list of the sequences of the primers used for qRT-PCR

Gene	sense	antisense
DCR2	5'-CCCAGAGGGATGGTCAAGGT-3'	5'-GTAGTGATAGGGAGAGGCAAGCA-3'
P21	5'-GGGACAGCAGAGGAAGACCAT-3'	5'- GGTCTGCCGCCGTTTTC-3'
PML	5'-GCCGACTTCTGGTGCTTTGA-3'	5'-GGGTTGGAGCAGAAGATGTTG-3'
P19	5'-ACCCAAGGGCAGAGCATTTA-3'	5'-CCTGAAGCAACGTGCACACT-3'
P53	5'-CCATCCTCACCATCATCACACT-3'	5'-CCCCTTTCTTGCGGAGATTC-3'
GAPDH	5'-GCCAAAAGGGTCATCATCTC-3'	5'-TGAGTCCTTCCACGATACCA-3'

Table 2. Effects of gemcitabine on the in vitro proliferation of human pancreatic cancer cell lines. IC_{50} values for gemcitabine were calculated using logistic regression.

Cell line	IC50 (μM)
Miapaca-2	0.7
Panc-1	5.5
L3.6pl	0.002

Legends to Figures

Figure 1. Gemcitabine-induced apoptosis in gemcitabine-resistant pancreatic cancer cells. Cells were stained with the human Annexin V-FITC Kit. Representative results from 3 independent experiments in the upper panels. Mean and 1 SE were calculated for 3 independent experiments, and are shown in the lower panels.

Figure 2. **A**. Cells were incubated in the presence of the indicated concentrations of gemcitabine for the indicated time intervals. Cells were then subjected to SA β-gal staining to determine positive cell numbers. Mean and 1 SE were calculated for 3 independent experiments and are shown in the lower panel. **, $p<0.01$, using one-way ANOVA followed by the Dunnett test, compared with untreated. **B**. Cells were then harvested and subjected to qRT-PCR to detect senescence-associated molecule mRNA. Mean and 1 SE were calculated for 3 independent experiments and are shown here. *, $p<0.05$; **, $p<0.01$ using Student's *t*-test.

Figure 3. Roles of ROS and CXCL8 in gemcitabine-induced cell senescence. **A.** The cells were subjected to SA β-Gal staining and the positive cell proportions were determined as described in Materials and Methods. Representative results from 3 independent experiments are shown in the left panel. Mean and 1 SE were calculated for 3 independent experiments and are shown here.*, $p<0.05$ using Student's *t*-test**.** B**.** Schematic representation of the procedures of subcutaneous injection of Miapaca-2 cells. **C.** At 14 days after the initiation of gemcitabine treatment, tumors were removed and subjected to SA β-Gal staining. Positive cell proportions were determined, and mean and 1 SE calculated for 3 independent experiments are shown here. ns., not significant. *, $p<0.05$ using one-way ANOVA followed by the Tukey-Kramer test.

Figure 1

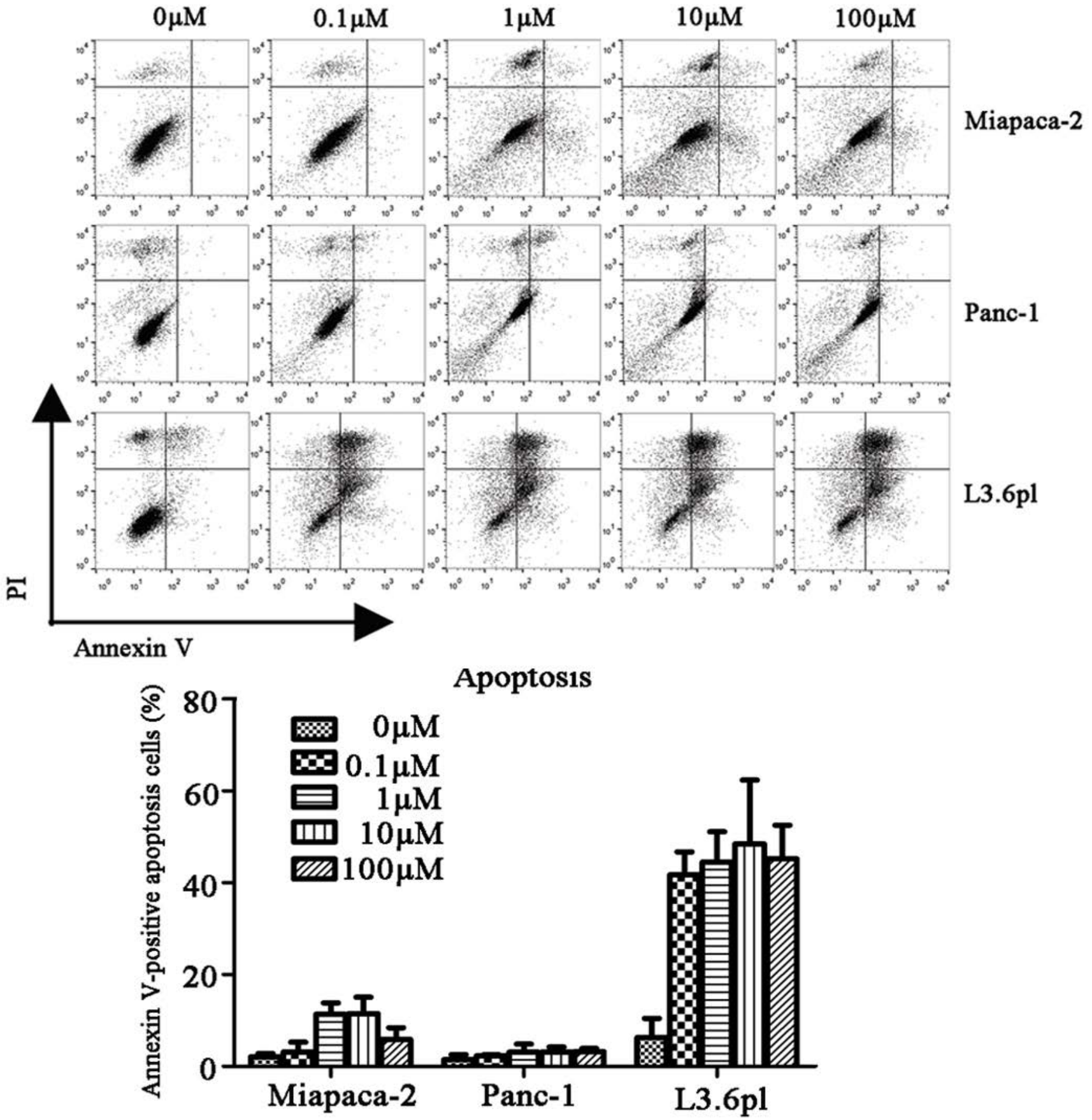

0μM
0.1μM
1μM
10μM
100μM
Miapaca-2
Panc-1
L3.6pl
PI
Annexin V
Apoptosis
Annexin V-positive apoptosis cells (%)
80
60
40
20
0
0μM
0.1μM
1μM
10μM
100μM
Miapaca-2
Panc-1
L3.6pl

Figure 2

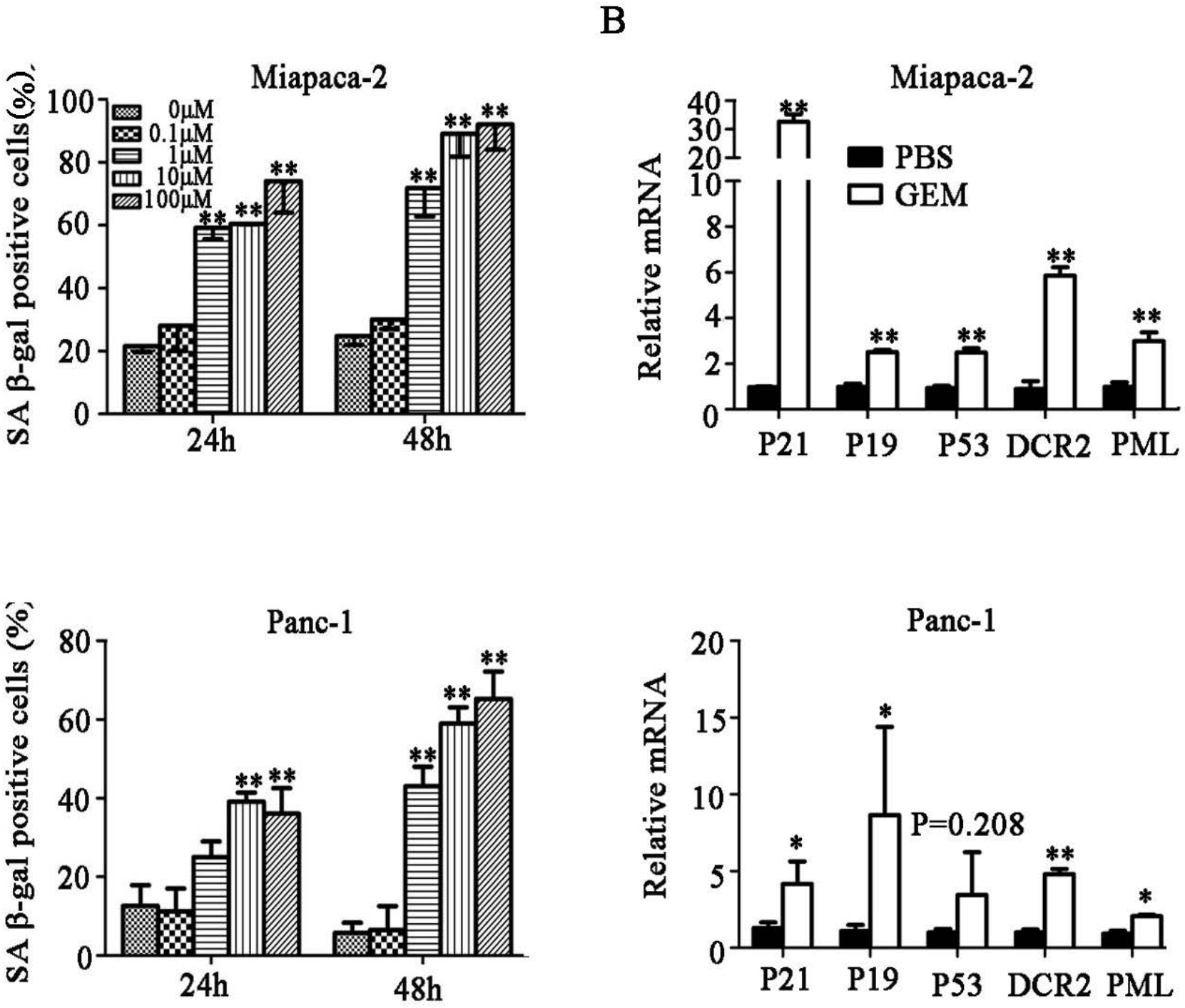
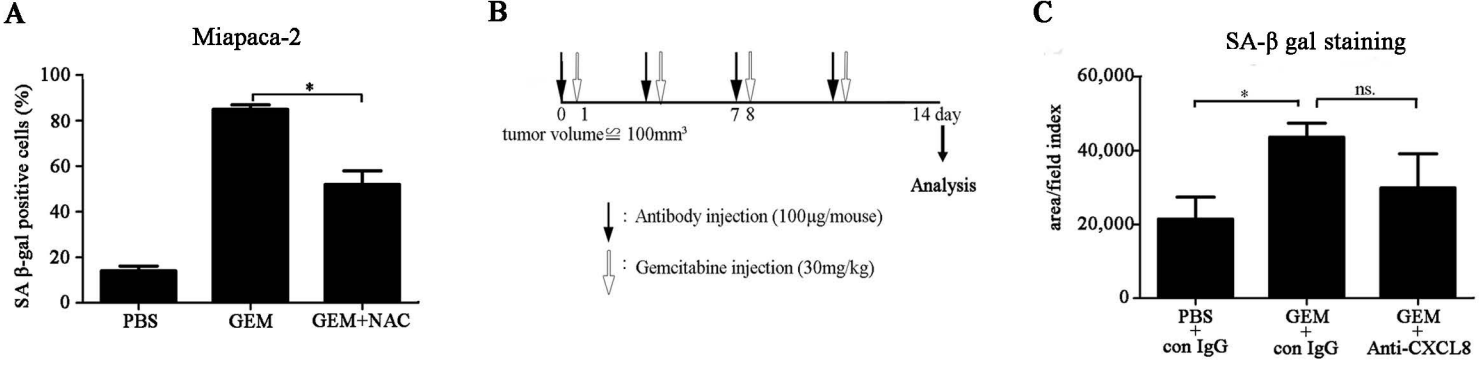

A
Miapaca-2
SA β-gal positive cells(%)
0μM
0.1μM
1μM
10μM
100μM
24h
48h
Panc-1
SA β-gal positive cells (%)
B
Miapaca-2
Relative mRNA
PBS
GEM
P21
P19
P53
DCR2
PML
Panc-1
P=0.208

Figure 3


A
Miapaca-2
SA β-gal positive cells (%)
100
80
60
40
20
0
*
PBS
GEM
GEM+NAC
B
0 1
7 8
14 day
tumor volume≧ 100mm³
Analysis
: Antibody injection (100μg/mouse)
: Gemcitabine injection (30mg/kg)
C
SA-β gal staining
area/field index
60,000
40,000
20,000
0
*
ns.
PBS + con IgG
GEM + con IgG
GEM + Anti-CXCL8