

All-*trans* retinoic acid enhances bystander effect of suicide gene therapy in the treatment of breast cancer

HENG KONG^{1,2}, XIA LIU³, LIUCHENG YANG⁵, KE QI¹, HAOYUN ZHANG¹,
JINGWEN ZHANG⁴, ZONGHAI HUANG⁵ and HONGXIAN WANG¹

¹Department of Thyroid and Breast Surgery, Shenzhen Key Laboratory for Endogenous Infection, Shenzhen Nanshan District People's Hospital (The Sixth People's Hospital of Shenzhen), Shenzhen, Guangdong 518052, P.R. China; ²Cancer Center, Georgia Regents University, Augusta, GA 30912, USA; ³Department of Human Resource, ⁴Clinical Laboratory, Shenzhen Nanshan District People's Hospital; ⁵Department of General Surgery, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong 510282, P.R. China

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Abstract. All-*trans* retinoic acid (ATRA) has been shown to enhance the expression of connexin 43 (Cx43) and the bystander effect (BSE) in suicide gene therapy. These in turn improve effects of suicide gene therapies for several tumor types. However, whether ATRA can improve BSE remains unclear in suicide gene therapy for breast cancer. In the present study, MCF-7, human breast cancer cells were treated with ATRA in combination with a VEGFP-TK/CD gene suicide system developed by our group. We found that this combination enhances the efficiency of cell killing and apoptosis of breast cancer by strengthening the BSE *in vitro*. ATRA also promotes gap junction intercellular communication (GJIC) in MCF-7 cells by upregulation of the connexin 43 mRNA and protein in MCF-7 cells. These results indicate that enhancement of GJIC by ATRA in suicide gene system might serve as an attractive and cost-effective strategy of therapy for breast cancer cells.

Introduction

Currently there is no effective treatment for patients with breast cancer in an aggressive phase. Breast cancer is caused by complex biological processes involving multi-gene mutations. Therefore any breakthrough in breast cancer treatment is likely to depend on improvement of gene therapy and development of new targeting drugs. Cytosine deaminase/5-fluorocytosine (CD/5-FC) and thymidine kinase/ganciclovir

(TK/GCV) are the most common suicide gene therapy treatment systems (1,2). Single suicide gene therapy often results in tumor relapse and combination gene therapy may potentially overcome the limitations of single gene therapy and improve the therapeutic efficacy (3,4). Our previous study confirmed that the double suicide gene system (CD/TK) driven by VEGF promoter can inhibit proliferation of human breast cancer cells and induce cancer cells apoptosis *in vitro* (5) yet even this strategy does not completely eradicate advanced tumors often seen in clinic.

It is well-known now that bystander effect (BSE) is very helpful in promoting the effectiveness of suicide gene system for anticancer therapy. Bystander effect elicits its effect mainly through gap junction-mediated intercellular communication (GJIC) (6). Gap junctions are an important gateway of information exchange by promoting transport of small (~1 kDa) molecules like ions or secondary messengers like cyclic AMP. A gap junction is composed of two connexons, also termed hemichannels, and each connexon is composed of six connexin sub-units (7,8). Three connexins (Cx43, Cx26 and Cx32) have been shown to be expressed in the human breast tissue in different temporal and spatial patterns. In normal human breast tissues, Cx26 and Cx32 are present in the mammary epithelium, whereas Cx43 is localized mainly to myoepithelial cells (9,10). It was reported that Cxs are often reduced or lost resulting in significantly decreased GJIC in tumor tissues, thus decreased gap junctional communication may be involved in oncogenesis (11). Studies have shown that human mammary tumor MCF-7 cell line has low expression of these connexins, particularly Cx43 (12). This suggests that MCF-7 cells may regulate GJIC by downregulation of gap junction proteins. Several chemicals [like ATRA, cAMP, and carotenoids (13)] are known for their induction of Cx43 expression and, subsequently, improvement of GJIC that consequently leads to an increased bystander effect seen in suicide gene therapy.

In this study we selected ATRA because it is strong inducer of GJIC in suicide gene therapy (14). ATRA is a derivative of vitamin A thus is non-toxic and well tolerated *in vivo*. We

Correspondence to: Dr Heng Kong, Department of Thyroid and Breast Surgery, Nan Shan District People's Hospital, 89 Taoyuan Road, Shenzhen, Guangdong 518052, P.R. China
E-mail: generaldoc@126.com

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observed that ATRA significantly improved bystander effect and cell apoptosis in suicide gene therapy for breast cancer MCF-7 cells due to upregulation of Cx43 expression and consequently GJIC. The purpose of our study was to find a more efficient strategy for improving the efficacy of suicide gene therapy for breast cancer.

Materials and methods

Human breast cancer cell lines. MCF-7 cells were obtained from the American Type Culture Collection (ATCC, VA, USA). They were maintained in a monolayer culture in DMEM (Dulbecco's modified Eagle's medium) with 10% fetal bovine serum and 0.5% penicillin/streptomycin. The cells were kept in humidified atmosphere of 5% CO₂ at 37°C.

Adenovirus vectors and viral infection. The adenovirus vectors (Ad-VEGFP-CD/TK) had previously been constructed by our group and successfully amplified by transient transfection of HEK-293 cells according to published protocols (15). The titer of Ad-VEGFP-CD/TK stock was 2.2×10^{11} pfu/ml analyzed by end-point dilution assay (16). MCF-7 cells at logarithmic growth phase were incubated in DMEM medium with 10% FBS containing the adenovirus vectors at multiplicities of infection (MOI) of 20, 40, 60, 80, 100 and 200 at 37°C for 24 h. The adenovirus vectors contained green fluorescent protein (GFP) gene as a marker of transduction. Infected cells were analyzed for percentage of GFP-positive cells by flow cytometry (FACS, Becton-Dickinson, San Jose, CA, USA). MCF-7 cells infected by the adenovirus vectors were termed as MCF-7/CD-TK.

Cell viability MTT assay. The MCF-7 cells and MCF-7/CD-TK cells were collected at logarithmic growth phase (1×10^5 /ml). MCF-7/CD-TK cells were trypsinized and mixed with uninfected MCF-7 cells at ratios of 0, 5, 10, 20, 40 and 80%. Then, the cells were seeded at a density of 1×10^4 cells/well in 96-well plates and incubated in DMEM at 37°C for 24 h, with or without 10^{-8} and 10^{-7} mol/l of ATRA. The following day, GCV (final concentration, 1 mg/l) and 5-FC (final concentration at 80 mg/l) were added in triplicate into the 96-well plates and further cultured for 2 days. Finally, the cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) assay. MTT was added at a final concentration of 0.5 mg/ml for 4 h at 37°C. After incubation, the supernatant was removed and replaced by DMSO. After the cell plates were shaken for 10 min, cell proliferation was measured in terms of optical absorbance per well by a microplate reader at a wavelength of 490 nm.

Cell apoptosis assay. To investigate the effect of ATRA on apoptosis in Ad-VEGFP-CD/TK suicide gene, we used JC-1 Assay kit (17) (Molecular Probes, Eugene, OR, USA) which measures the integrity of mitochondria. MCF-7/CD-TK cells were mixed with non-transduced parental cells at ratios of 2:3. Cells were seeded at a density of 1×10^6 cells in a 25-cm² culture bottle and incubated in DMEM at 37°C for 24 h, with or without 10^{-8} and 10^{-7} mol/l ATRA. The following day, GCV (1 mg/l) and 5-FC (80 mg/l) were added to cells for 2 days.

Pure MCF-7 cells in the control group were cultured in DMEM for 2 days. Then, the cells were collected and incubated with JC-1 working solution (JC-1 stock solution and assay buffer at a 1,100) at 37°C in the dark for 20 min. Cells were then collected by centrifugation at room temperature and washed twice with PBS. Apoptosis was measured a flow cytometry (Beckman Coulter, USA) to detect 5, 5', 6, 6'-tetrachlorol, 1', 3, 3'-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) staining, which emits green fluorescence (detected in FL1 channel) when it is in monomer (indication of cell apoptosis) and red (detected in FL2 channel) when it aggregates (which detects healthy cells). Apoptosis was indicated by an increase in the green/red fluorescence intensity ratio (18).

Flow cytometry analysis of GJIC in MCF-7 cells under ATRA treatment. GJIC was functionally evaluated by means of flow cytometry, as described by Peterson-Roth *et al.* (19). MCF-7 (3×10^5) cells per well were seeded into 6-well plates. The next day, ATRA was added to MCF-7 cells at 10^{-8} or 10^{-7} mol/l ATRA for the next 72 h. Cells without added ATRA served as a control. The donor cells were preloaded with fluorescent dyes calcein/AM (Molecular Probes) and 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes) for 20 min in a 5% CO₂ incubator at 37°C. After ATRA treatment for 72 h, the preloaded donor cells were added to the unlabeled recipient cells at a ratio of 1:50 and were co-cultured for 5 h in the presence of ATRA. After incubation, cells were collected by trypsinization, washed and resuspended in PBS and analyzed by flow cytometer. Data were analyzed by flow analysis software (FACSAria, FACSDiva Software, BD Biosciences, NJ, USA).

Real-time RT-PCR. MCF-7 cells were incubated in DMEM with 10^{-8} or 10^{-7} mol/l ATRA at 37°C for 72 h. The control group was devoid of ATRA. Total RNA from experimental and control group was prepared using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RT-PCR was performed on 0.5 µg total RNA using a QuantiTect Rev Transcription kit (Qiagen). Quantitative PCR was performed using an ABI 7500 Real-Time PCR system and a QuantiTect SYBR Green PCR kit (Qiagen); β-actin was used as an endogenous reference, and each sample was normalized to its β-actin content. All experiments were performed in duplicate and repeated twice. Primers for quantitative PCR are shown in Table I.

Flow cytometry analysis of Cx43 expression. MCF-7 cells were seeded at a density of 5×10^5 cells/well in a 50-ml cell culture bottle and incubated in DMEM at 37°C for 24 h. Cells were infected with Ad-VEGFP-CD/TK vector for 24 h following exposure to ATRA (0, 10^{-8} and 10^{-7} mol/l) for 72 h. Cells were then trypsinized, collected, and re-suspended in cold PBS (10^6 cells/ml). Polyclonal anti-Cx43 antibody (1:1,000, ProteinTech Group, Chicago, IL, USA) was added to cells for 30 min and cells were stained on ice. Cells were washed in FACS buffer (PBS+2% FBS) and FITC-labeled goat anti-rabbit IgG (1:500, Molecular Probes) was added or 30 min on ice. Cells were centrifuged and washed with PBS. The cells were harvested and analysis was performed using a Beckman Coulter Flow Cytometer (Beckman Coulter). The

Table I. Primer set used for real-time PCR.

Primer set	Sequence	Target gene
qCx43	Sense: 5'-AAATAGACAGGTCTGAGTGCCTGAA-3' Antisense: 5'-CCTCCAGCAGTTGAGTAGGCTTG-3'	<i>Homo sapiens</i> gap junction protein (NM_000165.4) Amplicon: 181 bp
q β -actin	Sense: 5'-TGGCACCCAGCACAATGAA-3' Antisense: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'	Actin, β (NM_001101) Amplicon: 186 bp

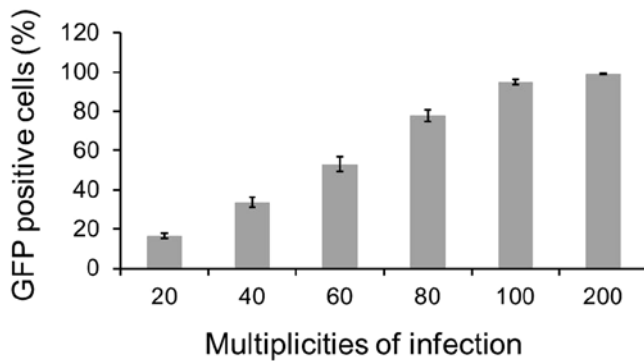


Figure 1. The efficiency of infection in MCF-7 cells by Ad-VEGFP-CD/TK with flow cytometry analysis.

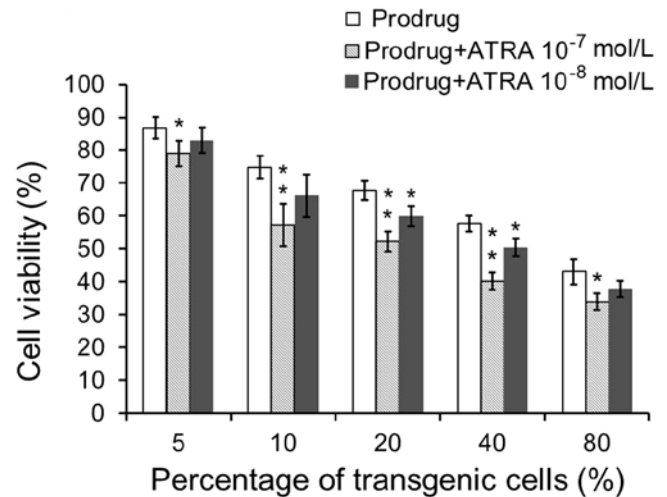
data were analyzed by Multicycle software (Phenix Flow Systems, USA).

Statistics. All data were expressed as means \pm SD and analyzed with SPSS version 16.0. A Dunnett's t-test and a one-way ANOVA were performed to assess the statistical significance between different groups. Significance for all tests was established at $p < 0.05$.

Results

Infection efficiency of Ad-VEGFP-CD/TK in MCF-7 breast cancer cells. As shown in Fig. 1, one day after infection, >16% of GFP-MCF-7 cells at a MOI of 20 were identified by flow cytometry. The infected GFP-positive cells were as high as 95.05% at MOI of 100. More than 99% of the infected cells expressed green fluorescence at an MOI of 200. The result indicated that the Ad-VEGFP-CD/TK vector was able to effectively infect MCF-7 cells *in vitro*.

Effect of ATRA on prodrug cytotoxicity. ATRA at a final concentration of 10^{-7} and 10^{-8} mol/l had no toxicity to MCF-7 cells. This concentration was established based on previous studies (20). We detected the ability of ATRA (10^{-7} and 10^{-8} mol/l) to enhance the bystander effect of our suicide gene system in mixed cultures of transgenic and non-transgenic cells at various ratios. As shown in Fig. 2, the MTT assays showed 79, 57, 52, 40 and 34% cell viability after prodrug and ATRA (10^{-7} mol/l) treatment for a ratio of 5, 10, 20, 40 and 80% infected cells, respectively, and 83, 66, 60, 50 and 38% cell viability after prodrug and ATRA (10^{-8} mol/l) treatment,

Figure 2. Augmented bystander effect of Ad-VEGFP-CD/TK suicide gene system combined with ATRA on different proportions of transgenic MCF-7 cells with MTT assay. * $p < 0.05$ and ** $p < 0.01$, compared with control group (prodrug).

respectively, and 87, 75, 68, 58 and 43% cell viability after prodrug alone treatment, respectively. The data showed that MCF-7 cell survival rate decreased along with the increasing ratios of CD/TK-transgenic cells. This result indicates that bystander effects exist in MCF-7 cells exposed to suicide gene system. In addition, the cell survival rate under treatment with the prodrug (5-FC and GCV) and ATRA (10^{-7} mol/l) was significantly lower than those of the cells treated with the prodrug alone ($p < 0.05$). In addition, the cell survival rate in the treatment with the prodrug and ATRA (10^{-8} mol/l) was significantly lower than the prodrug alone at ratios of 1:5 and 2:5, of the infected cells to the non-infected cells ($p < 0.05$). The result demonstrates ATRA can promote the bystander effects of the suicide gene system for breast cancer cells.

Effect of ATRA on apoptosis of breast cancer cells. When transgenic MCF-7 cells were mixed with non-transgenic MCF-7 cells at ratios of 40% and treated by prodrug and/or the ATRA, there was a significantly different apoptosis rate between the prodrug only group and ATRA plus the prodrug group (Fig. 3). In control group, MCF-7 cells showed a low rate of apoptosis of 5.45%. Under the treatment of 5-FC and GCV, cell apoptosis rate was 15.50%. Under the combined treatment of the prodrug and ATRA (the concentration of ATRA was

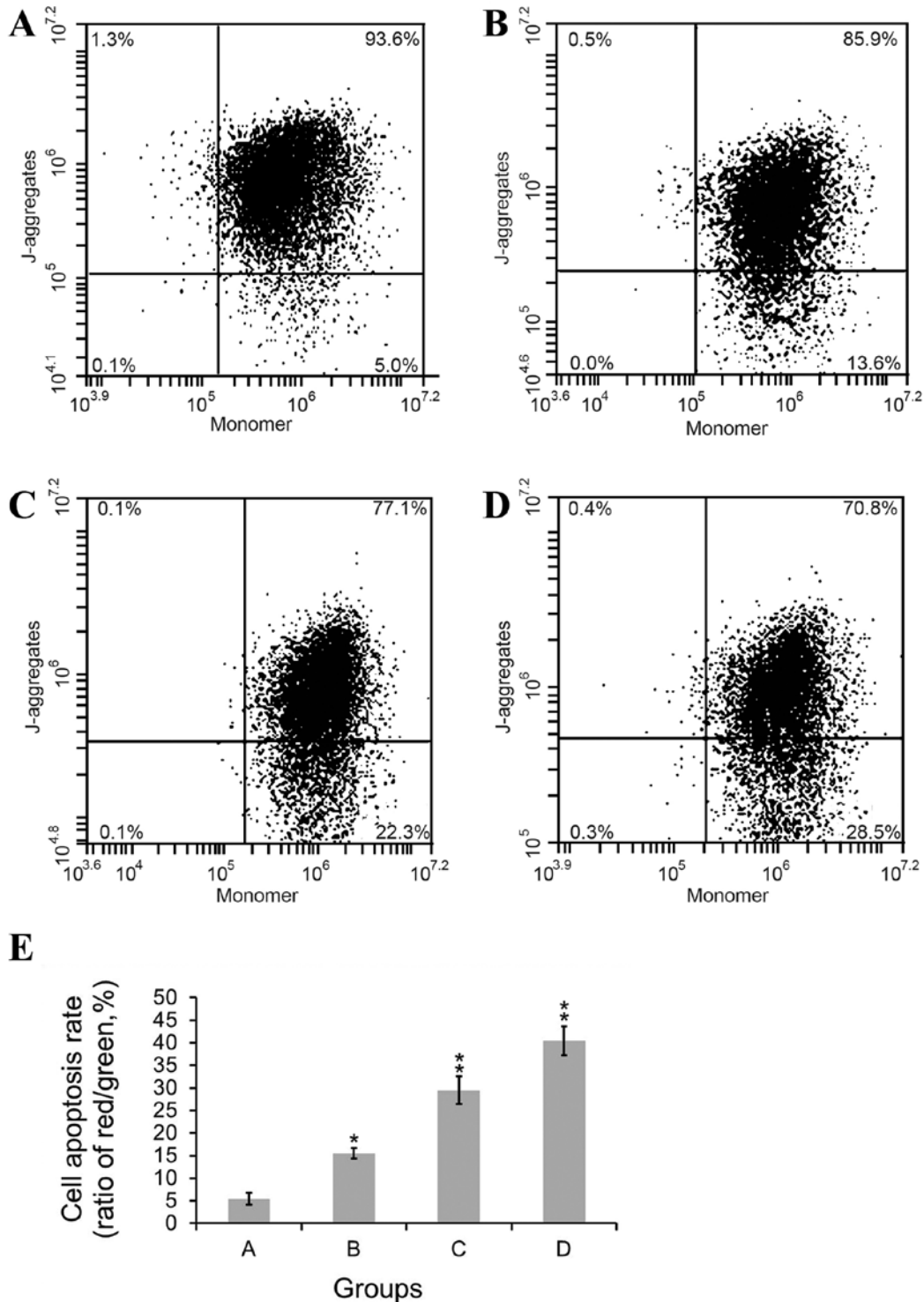


Figure 3. Flow cytometric percentage and histogram of MCF-7 cell apoptosis rates detected by JC-1 dye. (A) Control; (B) GCV (1 mg/l) and 5-FC (80 mg/l); (C) ATRA 10^{-8} mol/l + GCV (1 mg/l) and 5-FC (80 mg/l); (D) ATRA 10^{-7} mol/l + GCV (1 mg/l) and 5-FC (80 mg/l); (E) Histogram of MCF-7 cell apoptosis rates in (A), (B), (C) and (D) groups (* $p=0.001$, ** $p<0.001$).

10^{-8} mol/l or 10^{-7} mol/l), cell apoptosis rate was 29.48 and 40.46%, respectively. The results indicate that there is statistically significant difference among four groups ($F=125.277$). The data suggested that the prodrug only exerted some cell killing effects in mixed cells. The apoptotic rate was significantly increased with the addition of ATRA, especially when the concentration of ATRA increased from 10^{-8} mol/l

to 10^{-7} mol/l. The effect of ATRA showed a concentration-dependent manner. These results demonstrated that the drug plus ATRA therapy was superior to prodrug only.

Impact of ATRA on GJIC. To detect the change in GJIC caused by ATRA in MCF-7 cells, we used flow cytometry quantification of calcein/CM-DiI assays. DiI cannot pass through gap

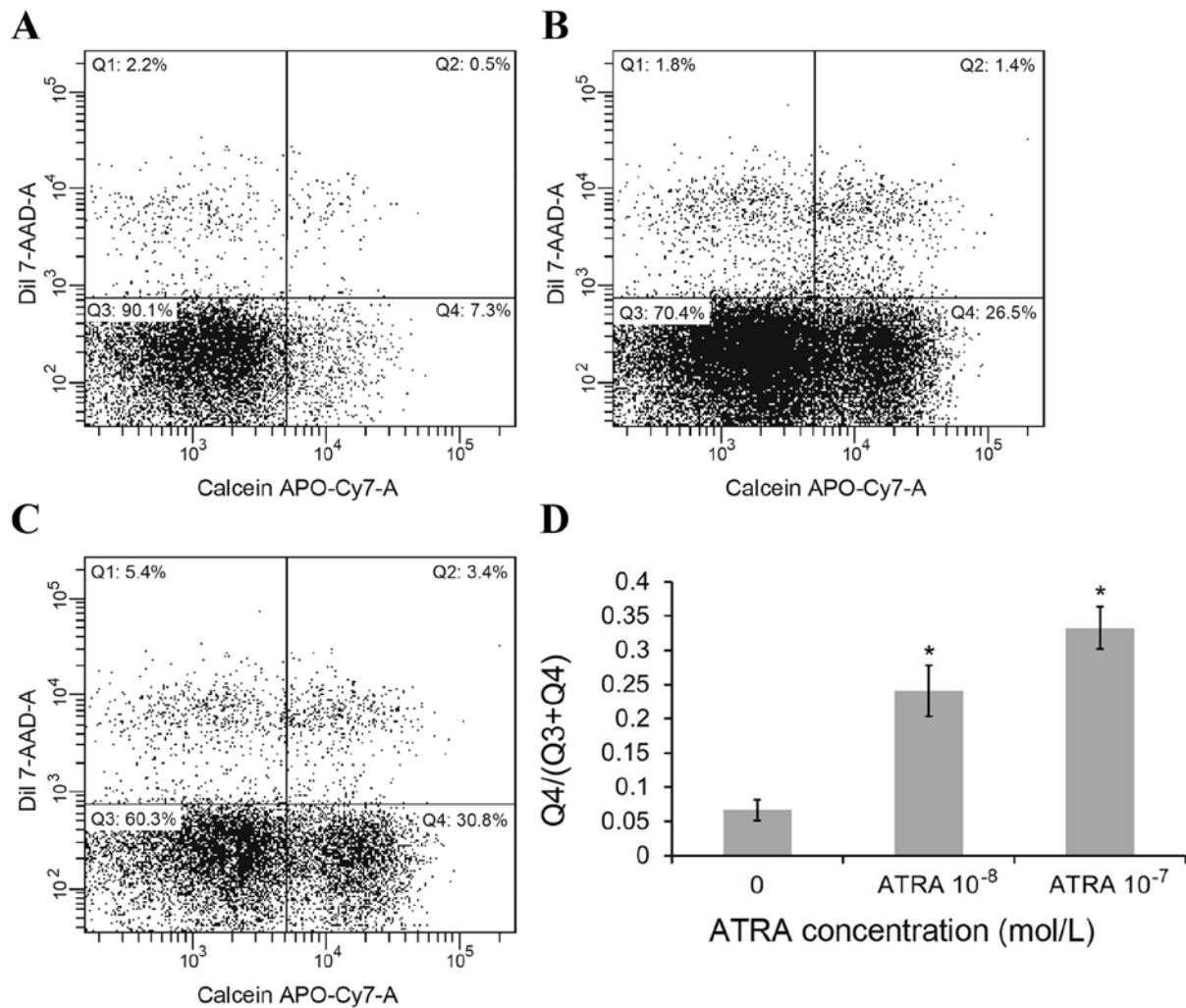


Figure 4. ATRA promotes GJIC of MCF-7 cells analyzed by flow cytometry. (A) Control; (B) ATRA 10⁻⁸ mol/l; (C) ATRA 10⁻⁷ mol/l; (D) Histogram of the ratio of Q4 to (Q3+Q4) in (A), (B) and (C) groups (*p<0.01, ATRA groups vs control group).

junctions, while calcein passes through gap junctions and transfer cells preloaded with calcein to a population of unloaded cells. In the FACS plot (Fig. 4), quadrant Q1 represents cell populations positive for DiI, and the cell population in quadrant Q2 represents preloaded cells which are positive for both dyes, and the population in quadrant Q4 represents positive cells for only calcein which are the unlabeled cells that received the calcein dye through GJIC. The population in quadrant Q3 represents the calcein-negative unlabeled cells. The ratio of MCF-7 cell numbers in quadrant Q4 to Q3 plus Q4 was used to evaluate the transfer of calcein as an indication of GJIC function. As seen in Fig. 4D, the ratio of Q4 to (Q3+Q4) was 0.07, 0.24 and 0.33 in the control group, 10⁻⁸ and 10⁻⁷ mol/l ATRA treatment group, respectively (p<0.01). The results show that ATRA significantly increased GJ transmission in MCF-7 cells in a concentration-dependent manner, which indicates GJIC function in the MCF-7 cells is significantly improved by ATRA treatment.

ATRA treatment upregulates connexin 43 gene expression. Our previous experiment indicated increased GJIC being an

effect of treatment with ATRA. It was crucial to delineate whether it is a sole enhancement in the intercellular communication or whether this is an effect on increased gap junctions resulting with increased expression of Cx43. Transgenic MCF-7 cells were treated with increasing concentrations of ATRA (0, 10⁻⁷ and 10⁻⁸ mol/l) for 72 h to determine the ATRA effect on the expression of Cx43 gene by real-time PCR. The upregulation of the Cx43 gene was pronounced, yielding a 1.5-fold increase (10⁻⁸ mol/l ATRA) and 3.6-fold (10⁻⁷ mol/l ATRA), respectively. Furthermore, the results indicate that the influence is dose-dependent. Compared with levels in control group, the mRNA expression levels of Cx43 in the transgenic MCF-7 cells treated with ATRA were increased significantly (p<0.01, Fig. 5).

Flow cytometry analysis of Cx43 protein expression in MCF-7 cells. To examine whether or not Cx43 protein expression in MCF-7-CD/TK cells changes under the influence of ATRA, different concentrations of ATRA (0, 10⁻⁷ and 10⁻⁸ mol/l) were added to cultures of MCF-7 cells. Flow cytometry analysis showed that positive rates of Cx43 protein expression in transgenic MCF-7 treated with the different concentrations

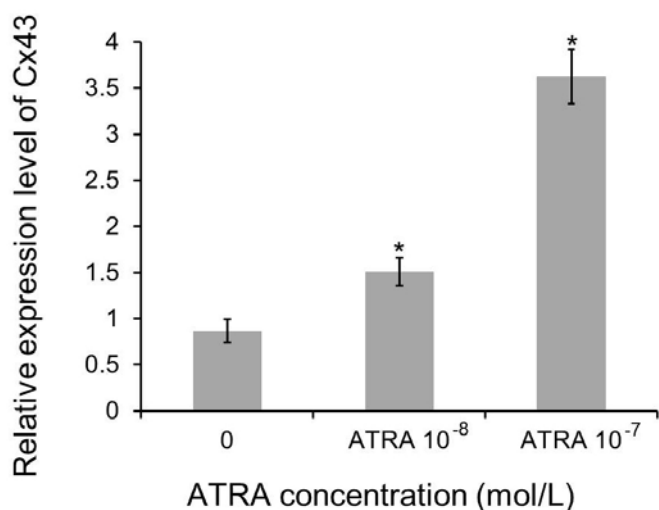


Figure 5. Relative expression of mRNA levels of Cx43 in MCF-7 cells treated by different concentration of ATRA (* $p < 0.01$, ATRA groups vs control group).

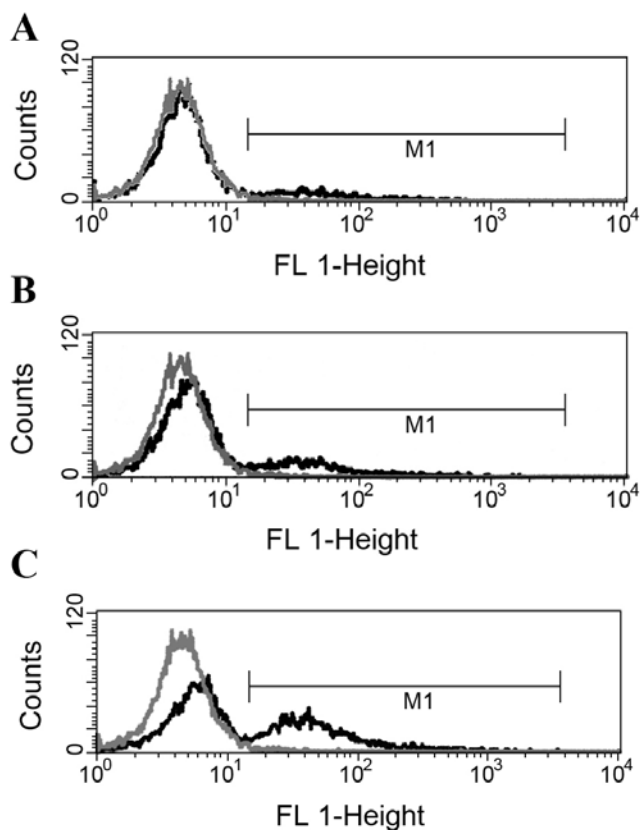


Figure 6. Flow cytometry analysis of Cx43 expression in transgenic MCF-7 cells under ATRA treatment. (A) Control group; (B) ATRA 10⁻⁸ mol/l; (C) ATRA 10⁻⁷ mol/l.

of 0, 10⁻⁸ or 10⁻⁷ mol/l ATRA were (6.27±1.75), (15.58±1.41) and (27.32±2.85)%, respectively. A significant difference was noted between the experimental group (10⁻⁸ or 10⁻⁷ mol/l ATRA) and the untreated control group. As shown in Fig. 6, at 72 h after ATRA exposure, the percentages of Cx43 protein detected using flow cytometry were also significantly higher

in cells of the experimental group than in the control cells both transfected with Ad-VEGFP-CD/TK vector.

Discussion

Suicide gene therapy is a potential way of targeting cancer, which is much less harmful to healthy cells. Many studies also have achieved good results in the application of suicide gene system in multiple malignancy tumors, such as glioblastoma, prostate cancer, and breast cancer (21-23). Moreover, some clinical trial of suicide gene therapy for patients with certain malignant tumors showed encouraging effects (24,25). However, the efficacy of the suicide gene therapy for the treatment of cancer is limited because of the low killing activity. The bystander effect in suicide gene therapy offers a way of promoting the effectiveness of suicide gene system for cancer. ATRA can enhance Cx43 expression and upregulate GJIC in various tumor cells. To enhance the antitumor activity, we determined whether suicide gene system combined with ATRA could potentiate the destruction of breast cancer.

Three connexin proteins have been identified in breast tissues, including Cx43, Cx26 and Cx32 (9). Expression of Cx26 and Cx32 are enhanced during pregnancy and further increased during lactation in normal breast tissues (10). Cx43 is the most widely studied of these connexin proteins because of its high expression in many cells (26) including breast cancer cells although human MCF-7 cells express low level of GJIC (12). In this study, QPCR and FACS methods detected upregulation of Cx43 gene and protein in MCF-7 cells under ATRA treatment. At the same time, ATRA was found to significantly increase GJ transmission in MCF-7 cells as a results of ATRA elevated Cx43 expression.

In the present study, we observed that the cell survival rate of transgenic MCF-7 cells treated with the prodrug and ATRA was significantly lower than those of the cells treated with the prodrug alone ($p < 0.05$). Our findings suggest that the therapeutic bystander effect of VEGFP-TK/CD system in MCF-7 cells was enhanced by combination with ATRA administration via overexpression of Cx43 and enhanced GJIC. We also found that compared with control group, the suicide gene system induced a marked increase in cell apoptosis rate in MCF-7 cells under the prodrug plus ATRA treatment group when MCF-7/CD-TK cells were mixed with non-transgenic MCF-7 cells at ratios of 40%. Intercellular communication through gap junctions plays an important role in maintaining tissue homeostasis by allowing transfer of ions and small molecules (molecular weight <1 kDa) by GJIC (27). So, the present study suggested that the transfer of apoptotic vesicles from dying cells to the surrounding non-transgenic tumor cells through gap junctions is one of the mechanistic actions of the suicide gene system on the BSE in cancer cells.

In conclusion, we demonstrated that ATRA can augment bystander effect and increase apoptosis in MCF-7 cells during suicide gene therapy against breast cancer. The effect was associated with enhanced GJIC via the upregulation of Cx43 expression driven by ATRA induction. Our study suggested the upregulation of gap junctional communication by ATRA may provide a novel pathway of chemical inducer for improving suicide gene system therapy for breast cancer.

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