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Original Article

Assessing the interactions between p53 gene and three types of flavonoid therapeutic compound in ovarian cancer cell line

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Abstract: The search of new anti-cancer drugs have always been an attractive but challenging topic. Flavonoid family draws considerable attentions in anti-cancer studies due to their anti-tumor functions. In this work, we studied the interactions between three types of flavonoid compounds (i.e. wogonin, apigenin and baicalein) and p53 genes in an ovarian cancer cell line. The flavonoid compounds were loaded in biodegradable nanoparticles to improve their bio-availability. P53 gene functions in the cells were regulated by transfecting the cells with HPV16 E6 cloned into a pCMV plasmid. *In vitro*assays such as cell viability, growth curve and cell cycles were employed to exhibit how flavonoid influence cell functions. Among the three flavonoids, wogonin was the most potent in inhibiting cancer cell viability, growth rate. Additionally, wogonin enhanced the arrest of HPV16 E6 transfected cells in G0/G1 phases. This study revealed that the interactions between p53 genes and flavonoid members, indicating that wogonin could be a potential choice for cancer treatments.

Keywords: Flavonoid, wogonin, p53, nanoparticles, viability, growth, cell cycle

Introduction

Ovarian cancer is a challenging disease that caused hundreds of thousands deaths all over the world each year. There were more than two hundred thousand cases of ovarian cancer were diagnosed each year. In most cases, over 75% of female ovarian cancer patients was not diagnosed until the III and IV stages, where the cancer cells have spread to all over the ovary tissue [1]. Current ovarian cancer treatment mainly relies on the integration of cyto-reductive surgery and chemotherapy. Platinum based therapeutic agents including paclitaxel and carboplatin are the first line of these chemotherapies [2]. While these chemotherapeutic agents are effective in a variety of patients, chemo-resistance develops in a significant amount of patients as well. When chemo-resistance develops, the only applicable strategy for treating this disease is to improve patient's quality of life by reducing the pain, where the mean survival time for patients is around 15 months [3-6]. Thus it is urgent to explore new therapies for ovarian cancer.

Similar to most cancer diseases, while studies are revealing the cancer development mechanism, the reasons of why the disease re-occur and progress of the disease are poorly understood [7-9]. In some studies, ovarian cancer was linked with inflammation of the ovary tissues [10-12]. These studies illustrated that the excessive inflammation was associated with ovarian cancer development. Interestingly, the reduction of ovulation by pregnancy, breast-feeding or other activities such as oral contraction can lower down the risk of this disease. Based on these findings, multiple regulation pathways, such as NF-kB-a key switch for inflammatory regulation, have been employed to modulate ovarian cancer treatment [11, 12].

In recent years, therapeutic compounds such as wogonin, apigenin and curcumin have been

explored for multiple different therapeutic purposes [13-17]. Among these compounds, flavonoid family draws considerable attentions in anti-cancer and anti-inflammation studies [16, 18]. For example, wogonin, a key member of flavonoid family can induce the apoptosis in several different cancer cell lines (e.g. human ovarian cancer, and carcinoma cancer) [8, 9, 19]. Additionally, the flavonoid members can also regulate inflammatory responses by inhibiting nitric oxide generation or reducing the production of radical scavenging generations [7, 20, 21]. These studies indicate that flavonoid family could be a potential choice for the treatment of ovarian cancer. P53 gene is one of the major oncogenes that regulate cancer occurrence and progression [22-24]. Under genotoxic stresses, p53 is activated and mutated, inducing the activation of downstream genes such as BAX, GADD-45 and p21WAF1/CIP1 [18, 25, 26]; the downstream genes in turn yield mutations in cell apoptosis and cell cycles [5, 27-29]. Therefore, it is necessary to study the interactions of p53 genes and therapeutic cargos, especially flavonoid family.

In this work, three flavonoid family members (i.e. wogonin, apigenin and baicalein) were selected to test their interactions with p53 genes in ovarian cancer cell. P53 genes in the ovarian cancer cells were shut down using a therapeutic cargo, or it remains its original form and functions as a control. The flavonoid cargos were loaded into a well-characterized polymeric carrier for controlled release. *In vitro* assays such as proliferation, viability and cell cycle were employed to test the impact of the flavonoid cargos on ovarian cancer cells. Our study identified that wogonin and apigenin, instead of baicalein were effective in inhibiting ovarian cancer cell development.

Material and method

Materials

Polyvinyl alcohol (PVA, 87.0-89.0% Partially Hydrolyzed), wogonin, baicalein and apigenin were obtained from Sigma. Dimethyl sulfoxide (DMSO), Triton X-100 and Methylthiazoltetrazolium (MTT) were from VWR. Wogonin, baicalein and apigenin were stored DMSO at -20°C before use. Propidium Iodide (PI) and 4, 6-diamidino-2-phenylindole (DAPI) were from Invitrogen. Annexin V-fluorescein isothiocyanate Apoptosis Detection Kit and Cell Cycle Detection Kit were obtained from KeyGen Biotech Co. Ltd.

Preparation and characterization of PLGA nanoparticles

Poly (lactic-co-glycolic acid) (PLGA) nanoparticles were prepared by precipitation method. In brief, 5 mg PLGA in 3 mL dichloromethane was used as organic phase. 3% (wt/wt) PVA in 12 mL deionized water was employed as aqueous solution. The organic phase and aqueous solution were mixed together and sonicated for 30 min. After sonication, the mixtures were reacted for another 45 min to generate the particles. The nanoparticles were then washed with PBS for 2 times and collected by centrifugation (8000 G, 20 min). To generate flavonoid (wogonin, apigenin or baicalein) loaded nanoparticles, 3 mg flavonoid compound was added into the organic phase during the reaction. The loading of flavonoid compound in the nanoparticles were assessed by incubating 1 mg of nanoparticles in DMSO, followed by using UV-Vis spectrophotometer or fluorimeter to assess the measure the absorbance of the DMSO to confirm the flavonoid loading. In this measurement, a same amount of PLGA empty particles dissolved in DMSO was employed as background control. For all three types of flavonoid compound, standard curves (concentration vs. absorbance) were established to quantify the loading. Dynamic light scattering (Malvern Instruments Zetasizer Nano-S) was employed to measure the size and zeta potential of the particles. The measurement was repeated 3 to 5 times to obtain an average value. Flavonoid releasing profile was obtained as follows: 1 mg of nanoparticles loaded with flavonoid was incubated in PBS (1×) at 37°C; the release of each flavonoid compound was measured by assessing PBS absorbance via fluorimeter.

Cell culture

Ovarian cancer cell line (OVCAR4 cell line) was obtained from 301 Hospital (Beijing, China). Eagle's MEM cell culture medium supplemented with fetal bovine serum (10%), penicillin (100 U/mL), and streptomycin (100 μg/mL) were used for culture the cells. The cells were

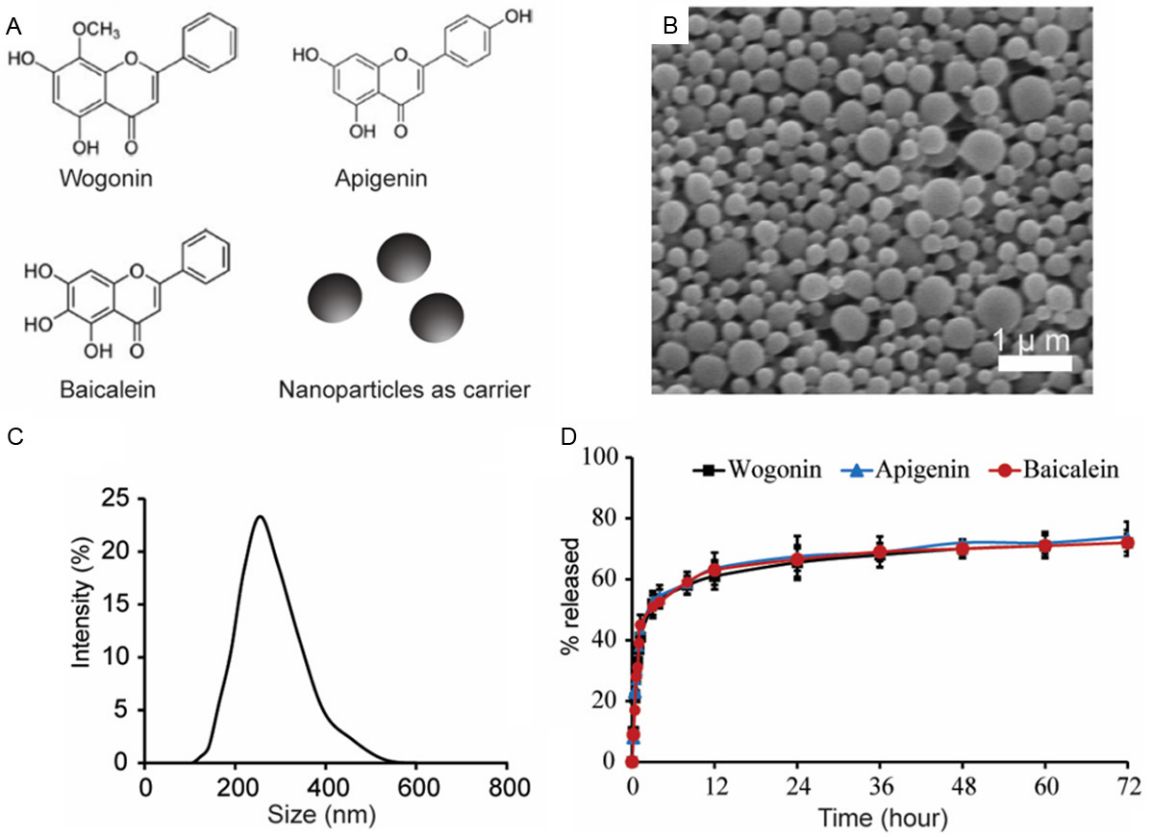

Figure 1. Characterization of three types of flavonoids (i.e. wogonin, apigenin, and baicalein) loading into polymeric nanoparticles. A: Chemical structure of three types of three types of flavonoids and the nanoparticles. B: Scanning electron microscope images of PLGA nanoparticles used for loading the flaconoids. C: Dynamic light scattering characterization of the nanoparticles. The nanoparticles had an average diameter of 243 nm. D: Release kinetics of three types of flavonoids from the nanoparticles. The nanoparticles were incubated in PBS buffer and release of cargos from the nanoparticles were measured at certain time point.

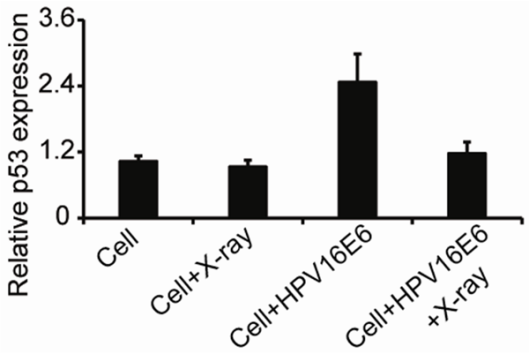

Figure 2. RT-PCR characterization of relative p53 gene expression from cells with different treatments. In cells with no transfection, the cells have enhanced expression of p53 when exposed to X-ray. As a contrast, in cells transfected with HPV16E6, there was no significant increase in the production of p53 when exposed to X-ray.

raised at 37°C 5% CO_2 with well controlled humidity.

Transfection of HPV16E6 to cells

HPV16E6 gene cloned in a pCMV plasmid was used for the transfection [30, 31]. Briefly, 2×10^6 cancer cells raised in 6 well plates were incubated with the plasmid using Lipofecamine (Life Technologies). After 12 to 15 days, G418-resistant colonies were selected and expanded. HPV16 E6 protein can degrade p53 functions via the ubiquitin dependent proteolytic pathway.

X-ray radiation of cancer cells

5-Gy dose of X-ray was used to irradiate the cells. G0/G1 arrest of cells was assessed at certain time point for at least 96 hours.

Cell viability assay

Viability of cells wasassessed with a viability kit by following the producer's instructions. Briefly,

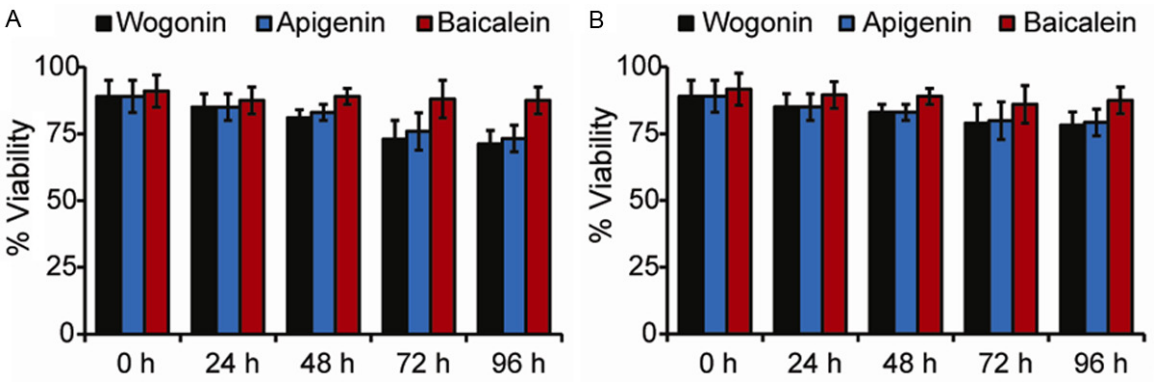

Figure 3. Viability of (A) cells with HPV16E6 transfection and (B) cells with no transfection after treating with different flavonoid (i.e. wogonin, apigenin or baicalein) loaded in nanoparticles. The flavonoid was loaded in PLGA nanoparticles and were employed to treat the cells for 48 hours, followed by viability test.

cells in a 96-well plates (10^5 cells per well) were treated with particles loaded with wogonin, apigenin or baicalein for 48 hours. After the treatment, cells were then washed with PBS and added with dual fluorescence calcein AM/EthD-1 assay reagent (125 μl). After incubating in dark for 25 min, the cell culture plates were read with a microplate reader (Synergy HT multimode microplatereader, Winooski, VT) at 530 and 630 nm, respectively.

Flow cytometry assay

Flow cytometry was employed to assess the cell cycle. Briefly, cells were treated with flavonoid compound loaded nanoparticles or flavonoid compound in free form for 48 hours. The cells were cell collected and washed with PBS for 2 times, followed by staining with propidium iodide (PI) and Annexin V. The cells were then analyzed with flow cytometry (BD FACSCanto II Analyzer, BD Biosciense) to analyze the cell cycle.

Result and discussion

The study started by loading three types of flavonoid compounds into a biodegradable carrier (PLGA). All three types of flavonoid compounds (i.e. wogonin, apigenin and baicalein have similar molecular structures (**Figure 1A**). The PLGA nanoparticles were imaged under scanning electron microscope-the particles had a spherical shape (**Figure 1B**). Size of the PLGA nanoparticles were assessed with dynamic light scanning (**Figure 1C**). The nanoparticles loaded with the flavonoid compounds were incubated in PBS buffer to assess the release kinetics of the compounds. The release profiles of all three flavonoid compounds were similar probably because all these cargos had similar chemical structure. 60% of the cargos were released within the first 12 hours of incubation. The loading level of these compounds were similar as well, there were around 73, 75 and 77 μg of wogonin, apigenin and baicalein per mg of PLGA, respectively. These were cargo when released could therefore be employed to interact with the cancer cells.

After setting up the platform for controlled release of cargos, we next used gene transfection to manipulate p53 expression within the cells. Briefly, The HPV16 E6 gene was cloned into a pCMV plasmid. The protein produced by this gene can facilitate p53 degradation through the ubiquitin dependent proteolytic pathway, reducing p53 gene production. Following the HPV16 E6 transfection, the cells were exposed to 5 Gy of X-ray. These cells with no transfection were employed as control. Upon X-ray radiation, DNA within the cells were damaged, stimulating the expression of p53 genes. Using RT-PCR, we assessed the expression of p53 and p21 genes within the cells (**Figure 2A** and **2B**). Briefly, the cells with HPV16 E6 transfection had no significant increase in p53 expression, indicating the transfections hindered p53 production. As a contrast, the cells with no HPV16 E6 transfection had a high level of p53 expression upon exposing to X-ray. This is because the cells were responding to DNA damage by enhancing p53 expression (**Figure 2A**). Similar trend was observed in the expression of p21-a downstream gene of p53. The cells transfected with HPV16 E6 gene had no

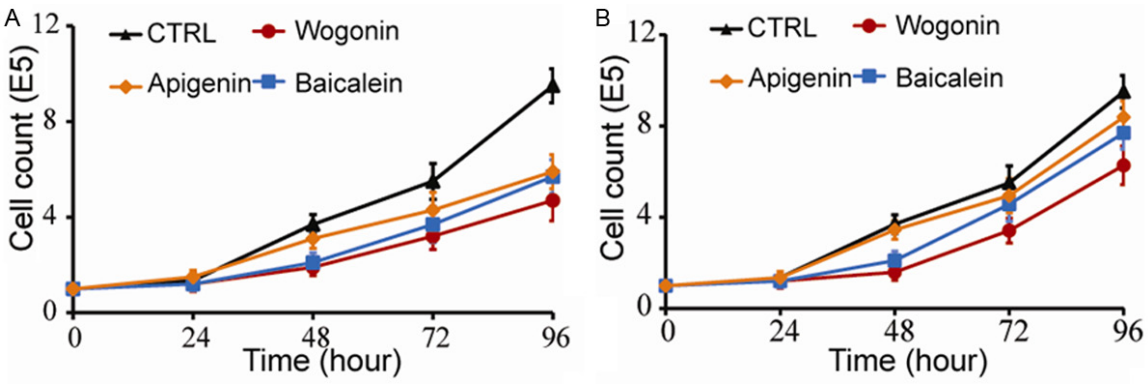

Figure 4. Growth curve of (A) cells with HPV16E6 transfection and (B) cells with no transfection after treating with different flavonoid (i.e. wogonin, apigenin or baicalein) loaded in nanoparticles. The flavonoid was loaded in PLGA nanoparticles and were employed to treat the cells. Number of cells were assessed at different time point.

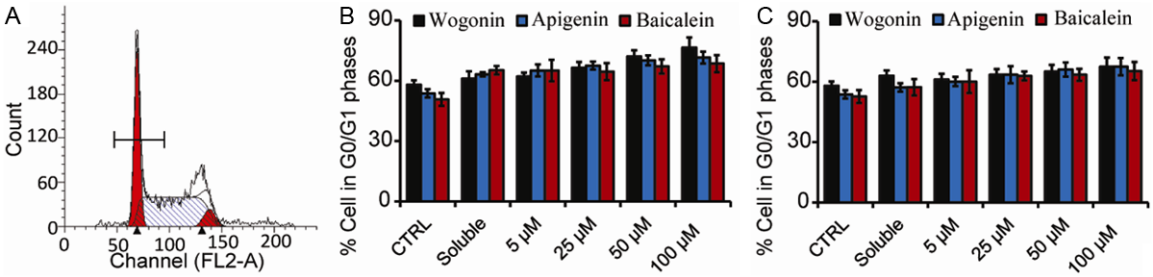

Figure 5. Ratio of cells arrested in G0/G1 phases after different treatment. (A) Flow cytometry analysis of cells in G0/G1 phases. The cells have no treatment. Ratio of G0/G1 phases in cells (B) transfected with HPV16E6 or (C) with no transfection after treating with different flavonoid (i.e. wogonin, apigenin or baicalein) loaded in nanoparticles. The flavonoid was loaded in PLGA nanoparticles and were employed to treat the cells. Number of cells were assessed at different time point. Soluble wogonin, apigenin or baicalein at a concentration of 50 μM in soluble form was employed as control.

significant p21 production upon exposing to x-ray; as a contrast, cells without HPV16 E6 gene transfection had a relatively higher level of p21 production (**Figure 2B**). These cells received no x-ray had a similar level of p53 and p21 production (**Figure 2A** and **2B**). These data confirmed that HPV16 E6 transfection can inhibit p53 gene functions in the cells. Using this cellular platform, we tested interactions between the three types of flavonoid member and the cancer cells.

We first tested the impact of wogonin, apigenin and baicalein on cell viability. For cells with p53 transfection, wogonin and apigenin loaded in nanoparticles were effective in reducing cancer cell viability; baicalein loaded in nanoparticles was not very effective in killing the cancer cell in the 96 hours observation (**Figure 3A**). As a contrast, wogonin and apigenin loaded in nanoparticles slightly reduced the viability of cells with HPV16 E6 transfection. Baicalein loaded in nanoparticles did not affect the viability of the cells (**Figure 3B**). These data indicated that wogonin and apigenin were more effective in inhibiting cancer cell viability. Importantly, this inhibition were more dramatic in p53 dysfunctional cells, suggesting the inhibition were associated with p53 gene functions. On the other hand, wogonin and apigenin also slightly inhibited the viability of cells with no gene transfection, indicating other genetic pathways were involved in the cell viability inhibition.

We next assessed the impact of these flavonoids on cell growth. Wogonin loaded in nanoparticles reduced the growth of cells with HPV16 E6 transfection, compared to the other two candidates (**Figure 4A**). Apigenin and baicalein also slightly inhibited cell growth in all cells, although the inhibition was not as dramatic as wogonin (**Figure 4A**). In cells with no gene transfection, wogonin still slightly inhibited the cell

growth more effectively than the other two flavonoid members (**Figure 4B**). Apigenin and baicalein slightly inhibited cell growth (**Figure 4B**). The cell inhibition study showed that wogonin was the most effective drug in inhibiting HPV16 E6 transfected cells growth. Considering the role of HPV16 E6 transfection-degrading p53 functions -the study illustrated that wogonin inhibit cancer growth through interacting with p53 gene. Of note was that wogonin in nanoparticles also inhibited the growth of cells without HPV16 E6 transfection, indicating other cellular pathway was involved in the interactions.

Followed by studying the cell growth, we assessed the impact of these flavonoid members on cell cycle. Briefly, the cells were treated with flavonoid cargos for 48 hours, followed by assessing with cell cycles with flow cytometry. **Figure 5A** shows the G0/G1 ratio of cells with no treatment. In this study, a series of flavonoid cargo concentrations were tested (i.e., 5, 25, 50 and 100 μM). Wogonin, apigenin and baicalein in soluble form was also employed for the cell cycle test as a control. For cells with HPV16 E6 transfection, all three flavonoids arrested cancer cell in G0/G1 phases in a dose dependent manner (**Figure 5B**). Wogonin in nanoparticles were more dramatic in arresting cancer cell in G0/G1 phases than the other two types of flavonoid compounds (**Figure 5B**). In HPV16 E6 transfected cells with no X-ray treatment, flavonoid compounds in soluble form (50 μM) can also slightly arrest cells in G0/G1 phases (**Figure 5B**). For the cells with no gene transfection, we observed that these compounds could also slightly enhance the arrest of cells in G0/G1 phasesprobably due the influence of X-ray damage (**Figure 5C**). Among three flavonoid compounds, wogonin was still most effective in arresting cells in G0/G1 phases. In all studies, flavonoids in soluble form was not as effective as those loaded in nanoparticles in arresting the cell cycle (**Figure 5B** and **5C**). This was because flavonoid compounds have a low solubility in cell culture medium, which influence their interactions with cells. This is one of major reasons why biodegradable particles had been widely employed for delivering these compounds.

Conclusion

This work studied the interaction between p53 gene function and three flavonoid compounds. The flavonoid compounds were loaded in biodegradable nanoparticles to improve the release and bio-availability. The cancer cell was transfected with HPV16 E6 to regulate p53 gene functions. The flavonoid members, wogonin was the most effective in inhibiting cell viability, growth, and arresting cell cycle. In addition, the flavonoid members also inhibited the viability, growth and cell cycles in cells with no HPV16E6 transfection, indicating other cellular pathways also joined the regulation. This study revealed p53 gene is involved flavonoid anti-cancer process. Future study will include exploring other cellular pathways that might be involved in the interactions between flavonoids and cancer.

Disclosure of conflict of interest

None.

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