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Original Article

Up-regulation of miR-200b by baicalin to inhibit migration of MCF-7 breast cancer cells

Yuzhe Gao, Qing Ni, Huajian Chen, Na Wei, Qi Jia

Department of Breast, Guizhou Provincial People's Hospital, Guiyang 550002, China

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Abstract: The incidence of breast cancer is increasing by years with the transition of life styles. The metastasis of cancer is one major challenge. MicroRNA (miR) plays crucial roles in the pathogenesis and progression of tumors. Previous study has shown that miR-200b could inhibit tumor cell migration. Baicalin is one flavonoids compound extracted from scutellaria of family Labiatae. It has multiple pharmaceutical activities including up-regulating miR expression in treating certain diseases. In pilot study, we found Baicalin could inhibit tumor cell migration but did not know its correlation with miR-200b. Breast cancer cell line MCF-7 was firstly tested under MTT assay to observe the effect of Baicalin on cell survival rate, followed by scratch assay targeting cell migration. Real-time qPCR was then used to detect the expression level of miR-200b, along with migrating related protein E-cadherin. Small molecule inhibition of miR was also employed to observe possible mechanisms. Baicalin (0~40 μM) had no significant inhibition on MCF-7 cell survival. Drugs at 80 μM had an inhibitory rate at 75.03% ± 3.06%. Baicalin can also up-regulate miR-200b level and E-cadherin protein expression, thus inhibiting MCF-7 cell migration. MiR-200b might be the target for the inhibition of cell migration by baicalin. This novel mechanism of tumor cell migration inhibition by baicalin provides new insights for developing novel treatment strategy in clinics.

Keywords: Baicalin, microRNA-200b, breast cancer cell MCF-7, tumor cell migration

Introduction

Baicalin is one flavonoids compound extracted from root and stem tissues of scutellaria of family Labiatae. It has been shown to have a wide anti-bacterial spectrum, along with pluripotent effects such as anti-inflammation, cholagogic, anti-allergy, anti-toxic, antipyretic, diuretic and antihypertensive functions [1]. Currently, baicalin preparation has been used to treat hepatitis with satisfactory efficacy decreasing alanine transaminase (ALT) during the active stage [2]. It can also be used in treating stroke, paralysis, decreasing cerebral vascular resistance, increasing cerebral blood flow, permeability of blood-brain-barrier, and antagonizing adenosine phosphate-induced platelet coagulation.

Recent study has revealed the importance of microRNA (miR) in the pathogenesis and progression of various diseases. MiR is one family of non-coding small single stranded RNA with 18~24 bp length. It can bind to 3'-untranslated region of target mRNA by complete or partial complementary base pairing manner. Under the direction of RNA exonuclease, mRNA can be selectively degraded to inhibit or activate downstream genes [3]. Therefore miR mainly achieves post-transcriptional regulation on protein synthesis under various physiological conditions. Abnormal regulation of miR was thus closely related with occurrence and process of various cancers such as thymic carcinoma, liver cancer and bile duct cancer [4, 5]. Early study showed important roles of miR-200b family in migration and invasion of pulmonary carcinoma cells [6]. Recently with the development of small molecule agonist and inhibitor of miR, the role of miR-200b in cancer treatment has also been illustrated gradually [7, 8]. The up-regulation of miR-200b could inhibit multiple cancers including the migration of pancreatic cancer cells [9] possibly via inhibiting Rho/ROCK signal pathway [10].

Our pilot study has illustrated that baicalin could up-regulate miR-200b expression level in breast cancer cells, and inhibit cell migration. Whether baicalin had any effects on the migra-

tion of tumor cells via miR-200b, however, is still unknown. This study thus investigated the effect of baicalin on miR-200b up-regulation, and the migration of breast cancer cells. MiR-200b inhibitor was subsequently utilized to observe any correlation between miR-200b up-regulation and the effect of baicalin on breast cancer cell migration.

Materials and methods

Drugs and reagents

Baicalin and MTT were purchased from Aladdin (Shanghai, China). MCF-7 cell was purchased from ATCC (MA, US). Real-time qPCR kit was purchased from TransGen (Beijing, China). Primers for miR-200b were purchased from Toyobo (Dalian, China). MiR-200b inhibitor was obtained from GenePharma (Shanghai, China). Anti-rabbit E-cadherin antibody was purchased from Abcam (Hong Kong, China). Goat anti-rabbit IgG (H+L) was purchased from Proteintech (Wuhan, China). Other reagents were all purchased from Sigma (LA, US).

Cell culture

All cells were cultured in DMEM medium containing 10% sterile fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin in a humidified chamber with 5% CO_2 at 37°C.

Real-time qPCR

All cells were rinsed in PBS for three times. 1 mL Trizol was added into each well, and was incubated on ice for 5 min. Cells were further rinsed by pipetting. Lysate was removed to new tubes, which were added with 200 μL chloroform for 15-sec vortex. After incubating under room temperature for 3 min, cell lysate was centrifuged at 12,000 g under 4°C for 15 min. Upper aqueous phase was saved and mixed with 500 μL isopropanol. After incubating under room temperature for 10 min, the mixture was centrifuged at 12,000 g under 4°C for 15 min. The supernatant was discarded. RNA pellet was rinsed in 1 mL ethanol, and was re-suspended in 20 μL DEPC H_2O.

Primers for miR-200b were designed as previously recorded [11]. Forward primer: 5'-UAAUA CUGCC UGGUA AUGAU GA-3'; Reverse primer: 5'-AUCAU UACCA GGCAG UAUAA AU-3'. PCR reaction was performed in 20 μL system under the following conditions: 94°C denature for 5 min, followed by 55°C annealing for 45 sec and 72°C elongation for 1 min in 45 cycles. Quantitative analysis was performed using $^{\Delta\Delta}$Ct method.

Cell survival assay

MTT assay was used to detect cell survival rate at 12 h. Cells at log-phase were cultured in 96-well plate. After reaching attachment growth, different concentrations of baicalin were added for 12 h incubation. MTT reagents were then added (20 μL each well), followed by triple reagents (100 μL each well) after 4 h. The cells were incubated for 12 h~15 h, and were tested for absorbance values at 450 nm using a microplate reader. The inhibitory effect of baicalin on breast cancer MCF-7 cells was quantified by comparing blank, control and treatment groups.

Scratch assay for cell migration ability

All cells were seeded into 6-well plate until reaching 90% confluence and full attachment. A scratch was made in the center of the plate using 200 μL pipette tips. Triple rinsing was performed using PBS to wash away detached cells. Different concentrations of baicalin or miR-200b inhibitors were given. An inverted microscope was employed to take images at 0 h and 12 h in the same field to observe cell migration.

Western blotting

Cells were treated under different drugs for 12 h. After removing culture medium, PBS was used to rinse cells. 100 μL lysis buffer (containing 100 mM PMSF) was added into each well. The plate was incubated on ice for 5~10 min. Cell debris were collected and transferred to new tubes. Under 4°C centrifugation for 5 min at 12,000 rpm, supernatants were collected as protein solutions.

Western blotting was performed as previously documented [12]. Total proteins were quantified by BCA approach and were adjusted to equal concentrations in loading buffer. After denaturing in boiled water for 5 min, proteins were loaded into 10% SDS-PAGE for electrophoresis until complete separation. Proteins were then transferred to PVDF membrane under 300 mA electrical field for 1 h. E-cadherin antibody (1:1000) was added for overnight incubation at 4°C. Using TTBS washing for three times, secondary antibody (1:1000) was added for 2 h incubation at 37°C. Chemilumi-

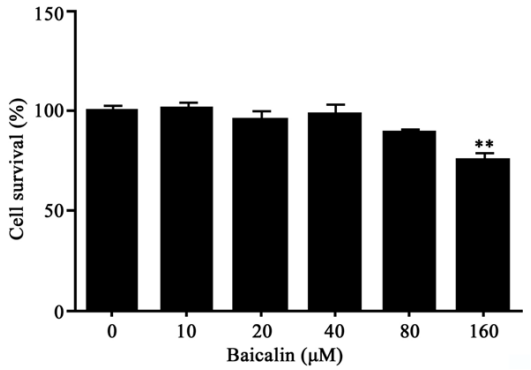

Figure 1. Effect of baicalin on MCF-7 cell survival. **, P<0.05 compared to control group.

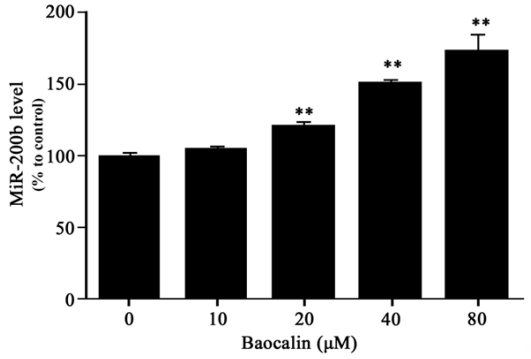

Figure 2. Baicalin increased miR-200b expression in a dose-dependent manner. **, P<0.05 compared to control group.

nescence method was employed to visualize protein bands.

Statistical analysis

SPSS15.0 software was used to analyze all collected data, which were presented as mean ± standard deviation (SD). Between-group-comparison was performed using student's t-test, while multiple-group-comparison was done by one-way analysis of variance (ANOVA). A statistical significance was defined when P<0.05.

Results

Baicalin effect on MCF-7 cell survival

To study the effect of baicalin on MCF-7 cell migration, we firstly selected appropriate drug concentration based on previous documents. MTT assay was employed to quantify the killing effect of drugs on MCF-7 cells after 12 h. As shown in **Figure 1**, 10, 20, 40 and 80 μM of baicalin (101.6 ± 6.2%, 95.6 ± 7.1%, 97.5 ± 8.6%, 91.5 ± 3.2%) had no significant killing effect on MCF-7 cells compared to 0 μM (100.5 ± 5.2%) (P>0.05). However, 160 μM of baicalin significantly decreased cell survival (76.8 ± 5.9%) (P<0.05).

Baicalin up-regulated miR-200b expression

12 h after giving different concentrations of baicalin, cells were collected for extracting total RNA and assayed for miR-200b level. As shown in **Figure 2**, baicalin significantly up-regulated miR-200b in a dose-dependent manner, as 20, 40 and 80 μM of baicalin (123.3 ± 6.3, 151.2 ± 7.9, 173.9 ± 15.6) significantly increased miR-200b compared to blank control group (100.2 ± 4.3) (P<0.05).

Baicalin inhibited MCF-7 cell migration

Using the "wound-healing" assay, images were taken at 0 h and 12 h after scratch making on the same field. As shown, in **Figure 3**, with increasing concentration of baicalin, the healing speed of cells was slower, indicating the dose-dependently inhibition of cell migration by baicalin.

Baicalin inhibited cell migration via up-regulating miR-200b

To investigate if baicalin affected cell migration via miR-200b, we replenished miR-200b specific inhibitor in a second round of wound healing assay. As shown in **Figure 4**, miR-200b inhibitor alone had no significant effect on cell migration. The combined use of inhibitor and baicalin, however, weakened the inhibition on cell migration as compared to baicalin with statistical significance, suggesting that baicalin could inhibit cell migration via up-regulating miR-200b.

Effects of baicalin on E-cadherin expression

As E-cadherin plays an important role during inducing MCF-7 cell migration [13], we observed the effect of baicalin on E-cadherin expression. As shown in **Figure 5**, 10 μM baicalin significantly increased E-cadherin level (1.28 ± 0.06) compared to blank control group (P<0.05). Higher concentrations of baicalin further evoked E-cadherin level in a dose-dependent manner (1.56 ± 0.18 for 20 μM, 1.96 ± 0.12 for 40 μM and 2.23 ± 0.13 for 80 μM).

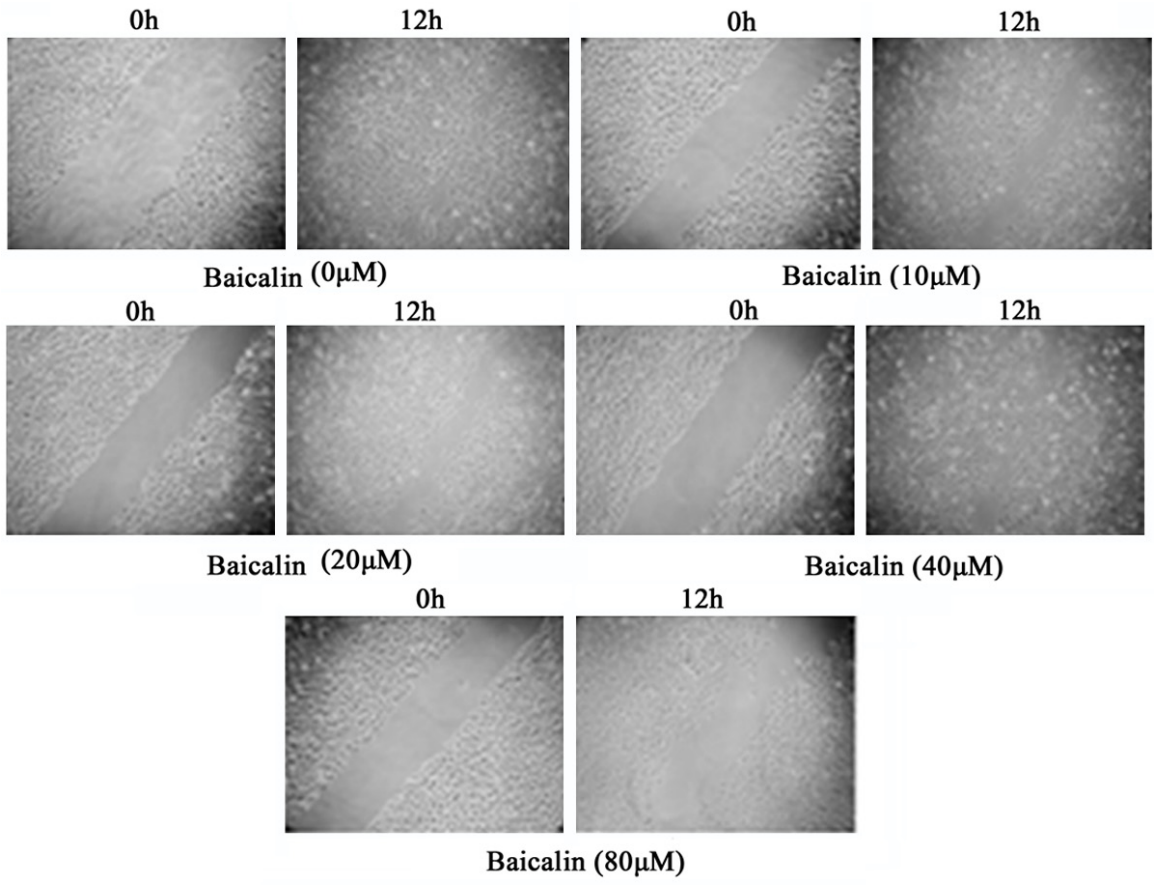

Figure 3. Baicalin inhibited MCF-7 cell migration in a dose-dependent manner.

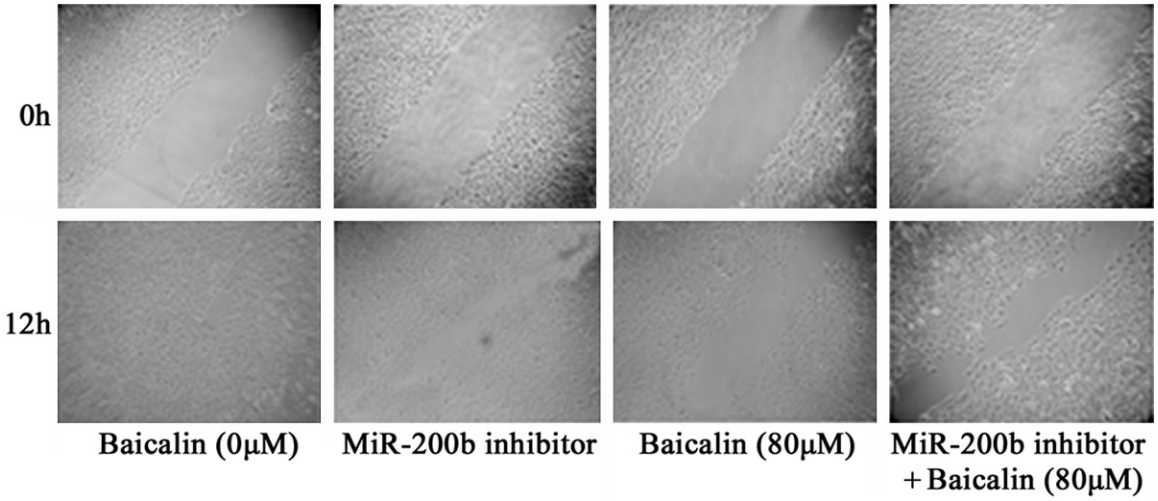

Figure 4. Baicalin inhibited MCF-7 cell migration via up-regulating miR-200b.

Baicalin affected E-cadherin expression via miR-200b

To further illustrate if the up-regulation of E-cadherin by baicalin was correlated with miR-200b, we tested the expression of E-cadherin after giving miR-200b inhibitor. As shown in **Figure 6**, the single application of miR-200b inhibitor had no significant effect on E-cadherin expression (0.88 ± 0.25) compared to control

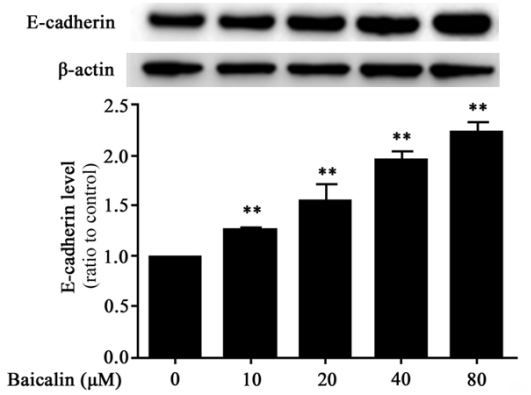

Figure 5. Effect of baicalin on E-cadherin. **, P<0.05 compared to control group.

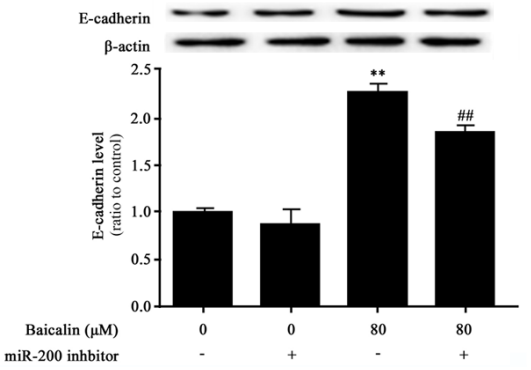

Figure 6. Baicalin increased E-cadherin expression via up-regulating miR-200b. **, P<0.05 compared to control group. ##, P<0.05 compared to Baicalin treatment group.

(1.00 ± 0.10) (P>0.05). The combined usage of baicalin (2.31 ± 0.15) and miR-200b inhibitor (1.81 ± 0.12), however, partially impaired the stimulatory effect of baicalin on E-cadherin expression, with statistical significance compared to control (P<0.05). These results suggested that baicalin-directed E-cadherin up-regulation might be achieved via miR-200b.

Discussion

Scutellaria (Scutellariabaicalensis Georgi), also named as camellia or tsuchikane, is one perennial herb in Genus Sutellaria, Family Labiatae [14]. Baicalin is major active component of scutellaria [15]. As one flavonoids compound, baicalin has multiple functions including antibacterial, diuretic, anti-inflammation, anti-allergy and anti-spasm [15], along with strong anti-tumor reaction potency [11], and has been widely applied in both pharmaceutical and cosmetic fields.

With the transition of lifestyles, the incidence of breast cancer is rapidly increased by years. Most of mortality are caused by secondary metastasis, whose occurrence depends on the acquirement of high metastatic ability of cells [16]. Therefore, the illustration of molecular mechanism regarding migration of breast cancer cell is of critical importance for studying tumor metastasis and treatment [17].

Our pilot study has shown that baicalin could increase miR-200b expression in a dose-dependent manner as one small molecule compound. MiR-200b, as one family member of miR-200, has potent contributions for regulating tumor invasion and migration as other members of the family [18]. The relationship between baicalin-induced cell migration of MCF-7 cells and its role in miR-200b up-regulation, is thus of interest for further study.

The inhibition of tumor cell migration is critical for cancer treatment [19]. In current basic study, the molecular marker for migration has no uniformed criteria across different tumor cell lines. E-cadherin is one calcium-dependent epithelial adhesion factor [20] and can form tight connection by homologous adhesion between epithelial cells. The down-regulation of E-cadherin can cause strengthening of epithelial cell migration, decreasing cell-to-cell polarity, enhancing invasiveness of cells towards peripheral tissues, thus playing important roles in progression and metastasis of tumors [21]. Previous study has shown that miR-200bb could target and inhibit zeb family expression, causing E-cadherin over-expression in a compensatory manner to inhibit cell migration [22]. We thus selected E-cadherin in "wound-healing" assay to investigate the effect of baicalin on cell migration.

In this study, MTT assay was firstly employed to observe the effect of baicalin on cell survival rate. Drugs within dosage range with no significant killing effects on cells were selected to rule out possible cytotoxicity effects. Different concentrations of drugs were subsequently applied on MCF-7 cells, whose expression level of miR-200b was measured by real-time qPCR. Via a dose-dependent measurement, we found strong correlation between baicalin dosage and miR-200b up-regulation. We then investigated the effect of baicalin of cell migration and E-cadherin expression, and respective effects after adding miR-200b inhibitor. Results showed that baicalin could increase miR-200b

and E-cadherin expressions in dose-dependent manner, thus inhibiting MCF-7 cell migration.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yuzhe Gao, Department of Breast, Guizhou Provincial People's Hospital, No. 83 of Zhongshan East Road, 550002, Guiyang, Guizhou, China. Tel: +86-0851-85273781; Fax: +86-0851-85273781; E-mail: changsongwangasd@163.com

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