Original Article

NF-κB/Twist axis is involved in chrysin inhibition of ovarian cancer stem cell features induced by co-treatment of TNF-α and TGF-β

Huazhen Li¹, A Chen², Qiaoqi Yuan¹, Weifeng Chen¹, Huimin Zhong¹, Mo Teng¹, Chang Xu², Yebei Qiu², Jianguo Cao²

¹Department of Gynecology and Obstetrics, The Second Affiliated Hospital of Guangzhou Medical University, 511447, Guangdong, China; ²Department of Pharmaceutical Science, Medical College, Hunan Normal University, Key Laboratory of Study and Discovery of Small Targeted Molecules of Hunan Province, Changsha 410013, China

Received September 8, 2018; Accepted November 6, 2018; Epub January 1, 2019; Published January 15, 2019

Abstract: Chrysin (ChR) inhibits various cancer cells and possesses anti-inflammatory activities. NF-κB has been shown to regulate the expression of genes involved in epithelial-mesenchymal transformation (EMT) by upregulation of TWIST1. This study aimed to assess whether ChR can inhibit EMT phenotype and cancer stem-like cell (CSLC) features in ovarian cancer cells co-treated with TNF-α and TGF-β. Here, OVCAR-3 cells were co-treated with TNF-α and TGF-β in the presence or absence of ChR. Then, the expression levels of E-cadherin, N-cadherin, CD133, CD44, NF-κBp65, and TWIST1 were analyzed by western blotting. Wound healing and tumor sphere formation assays were performed to assess the migration and sphere-forming capabilities of cells, respectively. Overexpression and/or knockdown of NF-κBp65 and/or TWIST1 were used to explore the molecular mechanisms. We showed that ChR inhibited EMT and CSLC properties in ovarian cancer cells administered TNF-α after prolonged TGF-β treatment, in a dose-dependent manner. Also, knockdown of NF-κBp65 and ChR cooperatively enhanced the inhibition of NF-κBp65 and TWIST1 expression, EMT phenotype, and CSLC properties. Conversely, overexpression of NF-κBp65 antagonized the above-mentioned activities of ChR. Furthermore, TWIST1 silencing or overexpression did not affect the ChR treatment effect on NF-κBp65 levels, but it reduced or enhanced EMT and CSLC properties. In conclusion, ChR can inhibit a proinflammatory cytokine to induce EMT and CSLC characteristics in OVCAR-3 cells, which may be involved in blocking the NF-κB/Twist axis.

Keywords: Ovarian cancer, cancer stem cells, epithelial-mesenchymal transition, chrysin, NF-κB, TWIST1

Introduction

Ovarian cancer is the most frequent cause of death among gynecologic malignancies due to the absence of an early effective diagnostic approach [1-3]. Although the majority of patients typically respond well to the first line of chemotherapy based on platinum compounds and taxanes, the disease mainly enhances recurrence and chemoresistance [4]. Reliable cures are lacking. Therefore, finding new drugs to prevent and treat ovarian cancer is important.

In recent years, cancer stem cells (CSCs) were so called because of their self-renewal capabilities; in addition, they can differentiate into multiple cell lineages and form heterogeneous tumors [5, 6]. The existence of ovarian cancer stem cells (OCSCs) is one of the fundamental drivers of occurrence and recurrence in ovarian cancer [7-9]. Epithelial-mesenchymal transition (EMT), referring to changes in the cell phenotype from an epithelial morphology to a mesenchymal morphology, is also tightly associated with the gain of CSC nature [10]. A study conducted by Gao et al. revealed that transforming growth factor β (TGF-β) isoforms induce EMT-independent migration of ovarian cancer cells [11]. The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) has been shown to alter the expression of genes involved in EMT by upregulating TWIST1 [12]. Research performed in our laboratory demonstrated that
ChR inhibits OCSLC properties by co-treatment with TNF-α and TGF-β

HeLa cells exposure to TNF-α combined with TGF-β could induce EMT and cancer stem cell-like (CSCL) properties in vitro through the NF-κB/Twist axis [13]. However, whether OVCAR-3 cell exposure to TNF-α combined with TGF-β can induce EMT and CSCL properties has still remained unclear.

Chrysin (ChR) is a bioactive flavonoid that is extensively used [14, 15]. Previous studies have shown that ChR exerts strong anti-inflammatory, antioxidative, and anticancer effects [16, 17], such as inducing apoptosis of human ovarian cancer cells [18, 19]. Recent studies have reported that ChR and its analogues can inhibit the self-renewal capabilities of human liver CSCs [20] and cervical CSLCs [21, 22]. However, there is lack of literature on regulation of NF-kB/TWIST1 axis in OCSCs by ChR treatment. In the present study, we attempted to investigate whether ChR can inhibit EMT and CSLC features by regulating the NF-kB/Twist axis in an inflammatory microenvironment.

**Materials and methods**

**Cell culture and treatment**

The OVCAR-3 cells, obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China), were cultured as previously described [22]. Cells (2 × 10^6) were treated with TNF-α (10.0 ng/mL, Sino Biological, Beijing, China) combined with TGF-β (5.0 ng/mL, Sino Biological, Beijing, China) for 24 h, followed by TGF-β (5.0 ng/mL) administered for 12 days to induce EMT and CSLC features. Aforementioned cells were treated with ChR (5.0, 10.0, and 20.0 μmol/L, Sigma-Aldrich, St. Louis, MO, USA) for 24 h. To explore the underlying mechanisms, OVCAR-3 cells expressing NF-κBp65 siRNA or NF-KBP65, Twist siRNA, or Twist were treated with or without ChR (5.0 μmol/L) for 24 h.

**Sphere formation assay**

Single cells in serum-free DMEM/F12 (Gibco, Grand Island, NY, USA) containing 100 IU/ml penicillin (Gibco, Grand Island, NY, USA), 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA), 20 ng/ml hrEGF (PeproTech Inc., Rocky Hill, NJ, USA), 20 ng/ml hbFGF (PeproTech Inc., Rocky Hill, NJ, USA), 0.2% B27 (Invitrogen, Carlsbad, CA, USA), 0.4% bovine serum albumin (BSA; Invitrogen, Carlsbad, CA, USA), and 4 μg/ml insulin (Invitrogen, Carlsbad, CA, USA) at 10^3 cells/ml were seeded into ultra-low attachment 24-well plates (Corning Inc., Corning, NY, USA). After 8 days of incubation, spheroids were counted in order to calculate the sphere-formation rate by dividing the total number of spheres obtained by the live cells seeded, then multiplying by one-hundred.

**Wound-healing assay**

OVCAR-3 cells were cultured until achieving optimal confluency (80-90%). Then, the monolayer was gently and slowly scratched with a new 100 μL pipette tip across the center of the well. The cells were washed twice with phosphate buffered saline (PBS), and imaged in the same field for analysis at 0 and 24 h, respectively. The cells untreated with combination of TNF-α and TGF-β or treated with vehicle were used for standardizing the number of migrated cells.

**Western blot analysis**

Western blot analysis was carried out as previously described [17]. The primary antibodies were used for incubation of membrane with targeted E-cadherin (Cell Signaling Technology, Danvers, MA, USA), N-cadherin (Cell Signaling Technology, Danvers, MA, USA), CD133 (Abcam, Cambridge, UK), CD44 (Abcam, Cambridge, UK), NF-KBP65 (Abcam, Cambridge, UK), and TWIST1 (Abcam, Cambridge, UK). After blocking with 5% (w/v) skim milk for 2 h, the membranes were further incubated with appropriate HRP-conjugated secondary antibodies (Beyotime Institute of Biotechnology, Shanghai, China) at room temperature for 1 h. Besides, β-actin (Santa Cruz Biotechnology, Dallas, TX, USA) was used for an internal reference. Immunoreactive bands were revealed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The results were visualized by using an enhanced chemiluminescence detection system (Ranon GIS-2008, Tanon Science & Technology Co., Ltd., Shanghai, China). The results of western blot analysis were scanned and semi-quantitated using Image Pro-Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

**Plasmids and transfections**

Here, NF-κBp65 siRNA, Twsit1 siRNA, and Control siRNA were supplied by Invitrogen.
ChR inhibits OCSLC properties by co-treatment with TNF-α and TGF-β.

Figure 1. Co-treatment of TGF-β and TNF-α contributes to EMT and CSLC features in OVCAR-3 cells. OVCAR-3 cells co-treated with TNF-α and TGF-β displayed mesenchymal morphology (A, magnification of 20 ×), enhanced self-renewal (B), and migration (C) capabilities, reduced E-cadherin, and elevated N-cadherin levels (D), upregulated CD133 and CD44 (E), as well as upregulated NF-κBp65 and TWIST1 (F). *P < 0.05, vs. untreated cells; #P < 0.05, vs. treated cells with TGF-β alone.
ChR inhibits OCSLC properties by co-treatment with TNF-α and TGF-β

To assess the effect of ChR on EMT and CSLC features in OVCAR-3 cells, the OVCAR-3 cells co-treated with TNF-α and TGF-β were exposed to ChR. As shown in Figure 2A, ChR could reverse the morphological changes of EMT. The sphere-forming and wound-healing assays revealed that ChR suppressed the self-renewal and migration abilities of OVCAR-3 cells co-treated with TNF-α and TGF-β, in a dose-dependent manner (Figure 2B and 2C). In addition, ChR upregulated the expression of E-cadherin and downregulated the expression of N-cadherin, CD133, and CD44 at the protein level in a dose-dependent manner (Figure 2D and 2E). Furthermore, compared with untreated cells, ChR significantly decreased the increase of NF-κBp65 and Twist protein levels (Figure 2F) after co-administration of TNF-α with TGF-β. These results indicated that ChR could inhibit EMT and CSLC features in OVCAR-3 cells co-treated with TNF-α and TGF-β.

Knockdown of NF-KBp65 cooperatively increased the inhibitory effects of ChR on EMT and CSLC features in OVCAR-3 cells co-treated with TNF-α and TGF-β

To assess the role of NF-KBp65 expression in ChR on inhibiting EMT and CSLC features, OVCAR-3 cells expressing NF-κBp65 siRNA exposed to combination of TNF-α and TGF-β were treated with ChR. Figure 3A shows that knockdown of NF-KBp65 and ChR cooperatively reversed the morphological changes of EMT. Furthermore, sphere-formation and wound-healing assays revealed that knockdown of NF-KBp65 combined with ChR cooperatively decreased the self-renewal and migration abilities (Figure 3B and 3C). Figure 3D-F shows that knockdown of NF-KBp65 and ChR cooperatively upregulated E-cadherin and downregulated N-cadherin, CD133, CD44, NF-κBp65, and Twist at the protein level. These results suggested that ChR could inhibit EMT and CSLC features in OVCAR-3 cells co-treated with TNF-α and TGF-β, likely by downregulation of NF-κBp65 expression.

Knockdown of TWIST1 enhanced the inhibitory effects of ChR on EMT and CSLC features in OVCAR-3 cells co-treated with TNF-α and TGF-β

OVCAR-3 cells expressing TWIST1 siRNA were treated with combination of TGF-β and TNF-α
ChR inhibits CSC-LC properties by co-treatment with TNF-α and TGF-β

Figure 2. Effect of ChR on EMT and CSC-LC features in OVCAR-3 cells induced by TNF-α combined with TGF-β. OVCAR-3 cells co-treated with TNF-α and TGF-β were also treated with or without ChR. Reversed mesenchymal morphology (A, magnification of 20 ×), inhibited cell self-renewal (B) and migration (C) capabilities, regulated E-cadherin and N-cadherin (D), downregulated CD133, CD44 (E), and also downregulated NF-κBp65 and TWIST1 (F) at the protein level are shown. *P < 0.05, vs. 0.1% DMSO treatment; †P < 0.05, vs. ChR (5.0 μmol/L) treatment.
ChR inhibits OCSLC properties by co-treatment with TNF-α and TGF-β

Figure 3. Effects of knockdown of NF-κBp65 on ChR associated with inhibition of EMT and CSLC features in OVCAR-3 cells induced by co-treatment of TGF-β and TNF-α. OVCAR-3 cells expressing NF-κBp65 siRNA with co-treatment of TNF-α and TGF-β were incubated with or without ChR (5.0 μmol/L). Then, cell morphology (A, magnification of 20 ×), self-renewal (B), and migration (C) capabilities, E-cadherin and N-cadherin protein levels (D), and the protein expression levels of CD133 and CD44 (E), as well as NF-κBp65 and TWIST1 protein levels (F) were assessed. Control siRNA represents the cells transfected with the scrambled siRNA, and NF-κBp65 siRNA demonstrates the cells transfected with NF-κBp65 siRNA. *P < 0.05 vs. control siRNA; #P < 0.05 vs. control siRNA with ChR (5.0 μmol/L) treatment.
ChR inhibits OCSLC properties by co-treatment with TNF-α and TGF-β

Figure 4. Effects of knockdown of TWIST1 on ChR associated with inhibition of EMT and CSLC features in OVCAR-3 cells induced by co-treatment of TGF-β and TNF-α. OVCAR-3 cells expressing TWIST1 siRNA co-treated with TNF-α and TGF-β were incubated with or without ChR (5.0 μmol/L). Then, cell morphology (A, magnification of 20 ×), self-renewal (B) and migration (C) capabilities, E-cadherin and N-cadherin protein levels (D), and the protein expression levels of CD133 and CD44 (E), as well as NF-κBp65 and TWIST1 protein levels (F) were evaluated. Control siRNA represents the cells transfected with scrambled siRNA, and TWIST1 siRNA demonstrates the cells transfected with TWIST1 siRNA. *P < 0.05 vs. control siRNA; #P < 0.05 vs. control siRNA with ChR (5.0 μmol/L) treatment.
ChR inhibits OCSLC properties by co-treatment with TNF-α and TGF-β

Figure 5. Effects of NF-κBp65 overexpression on ChR associated with inhibition of EMT and CSLC features in OVCAR-3 cells induced by co-treatment of TGF-β and TNF-α. OVCAR-3 cells expressing NF-κBp65 co-treated with TNF-α and TGF-β were incubated with or without ChR (5.0 μmol/L). Then, cell morphology (A, magnification of 20 ×), self-renewal (B) and migration (C) capabilities, E-cadherin and N-cadherin protein levels (D), and the protein expression levels of CD133 and CD44 (E), as well as NF-κBp65 and TWIST1 protein levels (F) were investigated. pcDNA3.1-LacZ represents the cells transfected with pcDNA3.1-LacZ plasmids, and pcDNA3.1-NF-κB demonstrates the cells transfected with pcDNA3.1-NF-κB plasmids. *P < 0.05 vs. pcDNA3.1-LacZ; †P < 0.05 vs. pcDNA3.1-LacZ with ChR (5.0 μmol/L) treatment.
ChR inhibits OCSLC properties by co-treatment with TNF-α and TGF-β

with or without ChR to explore whether the inhibitory effects of ChR on EMT and CSLC features are involved in NF-κB/Twist signaling. Figure 4A shows that knockdown of TWIST1 and ChR reversed the morphological changes of EMT. Sphere-formation and wound-healing assays revealed that knockdown of TWIST1 and ChR cooperatively decreased the sphere-forming and migration abilities (Figure 4B and 4C). Figure 4D-F shows that knockdown of TWIST1 cooperated with ChR to upregulate E-cadherin and downregulate N-cadherin, CD133, CD44, and TWIST1 at the protein level. However, it did not affect the expression of NF-κBp65. These results suggest that inhibitory effects of ChR on EMT and CSLC features in OVCAR-3 cells co-treated with TNF-α and TGF-β may be mediated by the NF-κB/TWIST1 signaling axis.

Overexpression of NF-κBp65 attenuates the inhibitory effects of ChR on EMT and CSLC features in OVCAR-3 cells co-treated with TNF-α and TGF-β

To further confirm that the inhibitory effects of ChR on EMT and CSLC features may require downregulation of NF-κBp65 expression, OVCAR-3 cells overexpressing NF-κBp65 co-treated with TNF-α and TGF-β were also treated with ChR. As shown in Figure 5A, overexpression of NF-κBp65 attenuated ChR-associated reversal of morphologic changes of EMT. Meanwhile, sphere-formation and wound-healing assays demonstrated that overexpression of NF-κBp65 diminished ChR-associated reduction of sphere-forming and migration abilities (Figure 5B and 5C). Figure 5D-F shows that overexpression of NF-κBp65 reduced ChR associated with upregulation of E-cadherin as well as downregulation of N-cadherin, CD133, CD44, and TWIST1 at the protein level. However, expression of NF-κBp65 was not affected. These results indicated that ChR-associated inhibition of EMT and CSLC features in OVCAR-3 cells co-treated with TNF-α and TGF-β may involve the NF-κB/TWIST1 signaling axis.

TWIST1 overexpression antagonized the effects of ChR on the EMT and CSLC features in OVCAR-3 cells co-treated with TNF-α and TGF-β

To further assess whether the inhibitory effects of ChR on EMT and CSLC features may involve NF-κB/TWIST1 signaling, OVCAR-3 cells expressing TWIST1 co-treated with TNF-α and TGF-β were incubated with ChR. Figure 6A shows that overexpression of TWIST1 attenuated ChR-associated reversal of morphologic changes of EMT. Sphere-formation and wound-healing assays demonstrated that TWIST1 overexpression diminished ChR-associated inhibition of sphere-forming and migration abilities (Figure 6B and 6C). Figure 6D-F shows that overexpression of TWIST1 reduced ChR associated with upregulation of E-cadherin as well as downregulation of N-cadherin, CD133, CD44, and TWIST1 at the protein level. However, expression of NF-κBp65 was not affected. These results suggested that ChR-associated inhibition of EMT and CSLC features in OVCAR-3 cells co-treated with TNF-α and TGF-β may involve the NF-κB/TWIST1 signaling axis.

Discussion

We recently reported that TNF-α in combination with TGF-β could promote EMT and CSLC properties of HeLa cells through NF-κB/Twist signaling [13]. In the present study, a chronic inflammation model for co-administration of TNF-α with prolonged exposure to TGF-β conferred the EMT phenotype and CSLC features by up-regulation of NF-κBp65 and TWIST1 expression in OVCAR-3 cells. The results prove that chronic inflammation is critical for tumor progression in several malignancies, including ovarian cancer. Furthermore, we first indicated that ChR could inhibit EMT and CSLC characteristics of ovarian cancer cells, which is involved in blocking the NF-κB/Twist axis, demonstrating the potential of ChR on chemoprevention and treatment of human ovarian cancer in an inflammatory microenvironment.

Several studies revealed the metastatic nature and carcinogenesis of tumors induced by proinflammatory cytokines [23, 24]. EMT is an important step in the invasion and metastasis of cancer. During the EMT process, cancer cells with epithelial features transform into malignant cells with mesenchymal features through the alternation of cellular polarity and adhesion [25]. Our results confirmed that co-administration of TNF-α with prolonged exposure to TGF-β synchronously led to the acquisition of EMT phenotype and OCSLC properties in OVCAR-3 cells.

ChR has extensive biological activities, such as antioxidant, anti-inflammatory, antimicrobi-
Figure 6. Effects of TWIST1 overexpression on ChR associated with inhibition of EMT and CSLC features in OVCAR-3 cells. Cells were induced by co-treatment of TGF-β and TNF-α. OVCAR-3 cells expressing TWIST1 co-treated with TNF-α and TGF-β were incubated with or without ChR (5.0 μmol/L). Then, cell morphology (A, magnification of 20 ×), self-renewal (B) and migration (C) capabilities, E-cadherin and N-cadherin protein levels (D), and the protein expression levels of CD133 and CD44 (E), as well as NF-κBp65 and TWIST1 protein levels (F) were assessed. In addition, pcDNA3.1-LacZ demonstrates the cells transfected with pcDNA3.1-LacZ plasmids, and pcDNA3.1-TWIST1 represents the cells transfected with pcDNA3.1-TWIST1 plasmids. *P < 0.05 vs. pcDNA3.1-LacZ; #P < 0.05 vs. pcDNA3.1-LacZ with ChR (5.0 μmol/L) treatment.
ChR inhibits OCSLC properties by co-treatment with TNF-α and TGF-β

al, anti-allergic, and anti-tumor effects [15, 26, 27]. In this study, we showed that ChR dose-dependently suppressed EMT and CSLC features, and downregulated NF-κBp65 and TWIST1 in OVCAR-3 cells co-treated with TNF-α and TGF-β. These findings suggest that the multiple biological activities of ChR in inflammation-associated ovarian cancer may be involved in regulating the NF-κB/Twist axis, raising the rationale for chemopreventive activities of ChR in inflammation-associated cancers.

It has been shown that a proinflammatory cytokine associated with EMT requires NF-κB to upregulate TWIST1 expression [28]. In this study, NF-κBp65 silencing or overexpression accordingly altered TWIST1 protein level, and increased or attenuated the effects of ChR. TWIST1 silencing or overexpression did not affect NF-κBp65 expression, and it promoted or reduced the effects of ChR. These results provided reliable evidence that NF-KB is located upstream of TWIST1.

In conclusion, the present study shows for the first time that ChR can inhibit proinflammatory cytokine-induced EMT and CSLC characteristics in OVCAR-3 cells, and its mechanism may regulate the NF-κB/Twist axis. This study supports the use of ChR for chemoprevention and treatment of ovarian cancer.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (Grant No. 81172375), Guangdong Provincial Science and Technology Department Program (Grant No. 2017ZC0259), and Guangdong Provincial Science and Technology Plan for Strengthening Traditional Chinese Medicine (Grant No. 20152161).

Disclosure of conflict of interest

None.

Address correspondence to: Jianguo Cao, Department of Pharmaceutical Science, Medical College, Hunan Normal University, Key Laboratory of Study and Discover of Small Targeted Molecules of Hunan Province, Changsha 410013, China. Tel: +86-15084740258; E-mail: caojianguo2005@126.com

References

ChR inhibits OCSLC properties by co-treatment with TNF-α and TGF-β


[23] Das L, Vinayak M. Curcumin attenuates carcinogenesis by down regulating proinflammatory cytokine interleukin-1 (IL-1α and IL-1β) via modulation of AP-1 and NF-IL6 in lymphoma bearing mice. Int Immunopharmacol 2014; 20: 141-147.


