Trans-Cinnamaldehyde inhibition of cytoskeleton-induced invasion and migration by regulating the Rho/ROCK pathway in adenomyosis

Die Yu[1], Fengxin Cui[1], Yiran Zhang[1], Keke Zhang[1], Xin Wang[1], Yinuo Zhang[1], Zilu Wang[1], Yang Liu[1], and Wei Shi[1]

[1]Affiliation not available

June 14, 2022

Abstract

Some of the key steps in adenomyosis (AM) are the migration and invasion of adenomyosis derived cells (AMDC); these processes require rearrangement of the cytoskeleton which Rho/ROCK signaling pathway is involved. The aim of the current study was to verify AMDC characterization and the effect of Trans-Cinnamaldehyde (TC) on the expression of the cell viability and cytotoxicity, cytoskeleton, invasion, migration, RhoA, RhoB, RhoC, ROCK1, ROCK2 in AMDC, which by immunofluorescence, CCK-8, immunofluorescence, cell wound scratch assay, transwell invasion assay and western blot analysis. Human endometrium tissues were obtained from AM patients who underwent total hysterectomy for AM in the Linyi Central Hospital from September 2019 to May 2021 with the first patient 41 years old and second one 45 years old. AMDC was characterized with strongly positive staining for Vimentin and almost negative for CK7, VWF, E-cadherin, SMA, and the patient samples were in secretory phases. IC50 = 28.93±0.44 μg/mL at 48 h with first patient, and IC50 = 27.67±0.72 μg/mL with second one. The final concentrations of TC (0, 6, 12μg/mL) were selected of the cell cytotoxicity. The T-text test group >0.05, so there was no difference between the two patients. These experiments were significantly downregulated and in dose-dependent fashion after treatment with TC. The data suggest that TC can inhibition the viability of cell, regulate cytoskeleton-induced invasion and migration may related to Rho/ROCK signaling pathway are involved in the pathophysiology of adenomyosis. Keyword: Cytoskeleton, Invasion, Migration, Rho/ROCK pathway, Trans-Cinnamaldehyde, Adenomyosis

ORIGINAL RESEARCH

Mengdie Yu

The Research of Trans-Cinnamaldehyde inhibits invasion and migration in adenomyosis derived cells

Mengdie Yu[1], Fengxin Cui[1], Yiran Zhang[1], Keke Zhang[1], Xin Wang[1], Yinuo Zhang[1], Zilu Wang[1], Yang Liu[1], Wei Shi[2]

Shandong University of Traditional Chinese Medicine, Jinan City, Shandong, China.

Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan City, Shandong, China.

Correspondence: Wei Shi, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, No.16369 Jingshi Road, Jinan, Shandong 250014, China

Tel/ Fax: 0531-68616800 Email: sw19781214@163.com

Objective: To investigate the efficacy of Trans-Cinnamaldehyde (TC) in inhibiting the invasion and migration of adenomyosis (AM) and to explore its mechanism of action.

Design: Original research.

Population or Sample: Human endometrium tissues were obtained from AM patients who underwent total hysterectomy for AM in the Linyi Central Hospital from September 2019 to May 2021 with patients 41 years old and 45 years old.

Method: The effect of TC in AMDC by CCK-8, immunofluorescence, transwell invasion assay, cell wound scratch assay and western blot analysis the protein for Rho/ROCK signaling pathway.

Results: AMDC was characterized with strongly positive staining for Vimentin and almost negative for CK7, VWF, E-cadherin, SMA. CCK-8 for IC50 = 28.93±0.44 μg/mL and IC50 = 27.67±0.72 μg/mL at 48h. The final concentrations of TC (0, 6, 12 μg/mL) were selected of the cell cytotoxicity, T-text test group >0.05. With increasing TC concentration, cytoskeleton fluorescence intensity, cell invasion and migration ability diminished, and Rho/ROCK-related protein expression was significantly downregulated.

Conclusions: TC inhibits the invasive migratory ability of AMDC by regulating the Rho/ROCK signaling pathway to affect cytoskeletal rearrangement with a dose-dependent, which are involved in the pathophysiology of adenomyosis.

Introduction

Adenomyosis (AM) means to a type of estrogen-dependent disease in which active endometrial tissue (glandular and interstitial) appears in the myometrium[1], accompanied by hypertrophy and hyperplasia of the surrounding myometrial tissues[2], severe endometriosis is associated with adenomyosis with a deeper myometrial invasion[3]. The condition can present with debilitating symptoms (heavy menstrual flow, prolonged menstrual periods, progressive aggravation dysmenorrhea, large uterine) that have considerable adverse impacts on quality of life[4], including an increased risk of secondary infertility[5]. Studies have shown that the incidence of AM is 10 to 15% and equating to 190 million women worldwide[6, 7]. At present, the pathogenesis of adenomyosis remains unclear. Adenomyosis is a benign disease, but it has some pathophysiological characteristics similar to the malignant tumor, such as regulating cytoskeleton inducing migration and invasion[8]. Therefore, the regulation of cytoskeleton, migration and invasion ability of the endometrium may be the critical factor in the occurrence of adenomyosis, but the exact mechanisms need further exploration.

Cell migration requires cytoskeletal reorganization, which plays a critical role in cancer metastasis[9, 10]. In particular, some classical signaling pathways are involved during cytoskeletal reorganization, such as Rho/ROCK signaling pathway[11, 12]. Guizhi can regulate the cytoskeleton through the Rho/ROCK signaling pathway. Therefore, the interactions among Guizhi, Rho/ROCK signaling, and the cytoskeleton underlie the ability of a cell to become motile, eventually leading to tumor migration[13-16]. Trans-cinnamaldehyde (TC) is the main active constituent of Guizhi which is a traditional Chinese herbal medicine[17].however, their role and mechanism in AMDC remain unclear.

Our research group support the theory of AM derived from the invasion and migration of the endometrium and identified cell subcluster with tumour-associated characteristics[18]. What's more, Traditional Chinese medicine prescription Guizhi Fuling Pills in the treatment of endometriosis is supported[19]. Even though the underlying mechanism is not known yet, based on this research basis, the current evidence does encourage us to dig out the role of TC to AMDC may relate to Rho/ROCK signaling pathway to regulate cytoskeleton-induced invasion and migration.

Material and methods

Patients

The study protocol was approved by the Ethics Committee of Affiliated Hospital of Shandong University of Traditional Chinese Medicine (2018) Clinical Research Application No. (021) and written informed consent was obtained from every individual participant. All the experimental procedures were performed in accordance with the Declaration of Helsinki. Human endometrium tissues were obtained from AM patients who underwent total hysterectomy for AM in the Linyi Central Hospital from September 2019 to May 2021 with the first patient 41 years old and second one 45 years old.

Primary Cell Isolation and Culture

In brief, AMDC was obtained using enzymatic digestion and by mechanical means[20], harvested from excised uterus with AM completely which have not used hormone therapy in the past three months. Ectopic adenomyotic samples were collected from hysterectomy specimens by cutting the obvious adenomyotic tissue at least 2.5 mm below the endometrial-myometrial interface. The presence of AM was confirmed by frozen section and histopathologic examination. Each specimen is divided into two parts, one put in 10% formaldehyde (Biosharp, BL539A, Chongqing, China) for pathological examination, as shown in*Figure 1* , the other was placed in put in with sterile D-Hanks (without calcium, magnesium, with phenol red) (Solarbio, H1045, Beijing, China) several times to wash away impurities and blood stains, put it into fresh tissue protection solution with 10% penicillin-streptomycin (Solarbio, P1400, Beijing, China) antibiotics and then transferred to the laboratory. Removal postoperative medical examination did not meet the pathological changes of AM timely. The tissues were gently cut into small pieces (1 mm^3) and added 4 times the volume of 2mg/mL collagenase IV (diluted with D-Hanks) ((Solarbio, C8160, Beijing, China), digested in a 37°C water bath for 4 hours, shaking the tissue every 10 minutes during the digestion process to make the tissue and enzyme fully mix and contact. Adding 3-5 times the volume of 10% Fetal Bovine Serum (UY; Lonsera S711-001S, South America) after digesting to chyle for completing medium to neutralize the digestive activity of collagenase. The dispersed cells were filtered through 70-μm and 40-μm (Becton Dickinson, Franklin Lakes, NJ, NY, USA) filter screens respectively to remove the undigested tissue pieces. The AMDC was collected by centrifugation (Baiyang, Beijing, China) at 1000 rpm for 5 min and washed 2–3 times with Phosphate-Buffered Saline solution (PBS) sterile.

The isolated AMDC was cultured in medium solution completely, using 35.6mL DMEM/F-12 (1:1) basic (1×) (C11330500BT, Gibco, Shanghai, China) with 10% UY 4 mL and 1% penicillin-streptomycin antibiotics 400μl. These cells in culture dishes were incubated at 37 °C in a humidified atmosphere of 5% CO_2-95% air (Germany,Memmert). Cells from the sixth to the tenth passages were used for further experiments.

Drug Dilution

Trans-cinnamaldehyde(TC, Ltd B-50765, Shanghai yuanye Bio-Technology Co., China)dissolved in dimethyl sulfoxide (DMSO; D8371, Solarbio, China), the ratio of DMSO¡1

Immunofluorescence for Cell Characterization

Identification of the isolated AMDC was performed by immunofluorescence cell staining with AM patients. In brief, cells were seeded on climbing piece and fixed with 4% paraformaldehyde universal tissue fixative for 10 mins at Room temperature, followed by 0.5% Triton X-100 (Solarbio, T8200, Beijing, China) for 10 min, Normal Goat Serum, 10% (Bioss, C01-03001, Beijing, China) for 30 min. Thereafter, with primary antibody with the whole night at 4, Rabbit Anti-Vimentin antibody (VIM, 1:100, bioss, bs-0756R, Beijing, China), Rabbit Anti-CK7 antibody (1:100, bioss, bs-1610R, Beijing, China), Rabbit Anti-Von Willebrand Factor antibody (VWF, 1:100, bioss, bs-10048R, Beijing, China), Rabbit Anti-Ecadherin antibody(1:100, bioss, bs-10009R, Beijing, China), Rabbit Anti-alpha smooth muscle Actin antibody(SMA, 1:100, bioss, bs-10196R, Beijing, China), PBS (Servicebio, G4202, Wuhan, China), respectively. Followed by incubated with the secondary antibody of goat anti-rabbit IgG (H +L) (1:400; YEASEN, Shanghai, China) at 1h on second day, DAPI (Solarbio, C0065, Beijing, China)5 min. Before each next step, washing with PBS 3 times for 5 min.

Determination of Cell viability

The cell viability effect of TC on AMDC was tested by Cell Counting Kit-8 assay (CCK-8 Sparkjade, CT001, Shandong, China) assay. Briefly, AMDC was dispensed into 96-well plates (3.5 x 10^3cells/well, 50% cells/well for 24h) in 100 μL of complete DMEM and various concentrations of TC were added (0, 8, 16, 32, 64μg/mL). After incubated at 37 ° C for 48h, 10 μL of CCK-8 solution in 90 μL complete DMEM was added to each well and incubation continued for 4 h. The absorbance of each well was determined at 450 nm by Microplate Reader (Thermo, Shanghai, China). The cell viability was calculated using the following equation:

Cellviability (%)

= (Absorbance individual test group – Absorbance blank group)/ (Absorbance control group – Absorbance blank group)

The IC_{50} values (concentration that inhibits cell growth by 50%) were determined using regression analysis. We reset new concentration to analyze the maximum drug concentration that does not affect cell viability (Compared with 0 drug concentration, cell viability P>0.05), 0, 6, 12, 18, 24 μg/mL, according to the maximum drug concentration measured and decline turning concentration in this study, using immunofluorescence for cytoskeleton, scratching, transwell and Western Blot were used on AMDC in subsequent experiments respectively.

Immunofluorescence for Cell Cytoskeletal

Immunofluorescence for cell cytoskeletal rearrangement was performed using Glass Bottom Cell Culture Dish (801001; Nest, Wuxi, China) with Laser scanning confocal microscope (leica, TCS SP8, Shanghai, China). Before the experiment, TC was pretreated with different concentrations (0, 6, 12 μg/mL) for 48 h. Then 2.5×10^4 of AMDC was resuspended in 2 mL complete DMEM containing 10% UY at Glass Bottom Cell Culture Dish. Cells were then incubated at 37 °C in a 5% CO_2 for 24 h with 50% AMDC. In brief, cells were fixed with 4% paraformaldehyde for 10 min at Room temperature, followed by 0.5% Triton X-100 for 5 min, 150 nM of TRITC-Phalloidin (Solarbio, CA1610, Beijing, China) with 1% BSA (Albumin Bovine V) (Solarbio, A8020, Beijing, China) in PBS for 30 min avoid light, DAPI 5 min. Before each next step, washing with PBS 3 times for 5 min. Then under the Laser scanning confocal microscope.

Cell Wound Scratch Assay

Cells were plated at a density of 1.2×10^5 cells/well onto 6-well plates and cultured at 37 °C in 5% CO_2until a monolayer was formed. After 24 h, artificial wound tracks were created in the confluent monolayers with a 1000 mL blue. Cells were then gently washed with 1% UY DMEM f12 to remove debris and further incubated with different concentrations of TC (0, 6, 12 μg/mL) at 1% UY DMEM f12, took photos with Axiovert 40 (Germany, Carl zeiss) for 0, 24 and 48 h. The migration distance was measured and analyzed using Image pro plus 6.0 software.

Transwell Invasion Assay

Cell invasion assay was performed using transwell chambers with polycarbonate filters (8.0 μm pore size; Costar, USA) in 24-well plates. In brief, the upper transwell chambers were precoated with 35 μL Matrigel (BD Biosciences, USA) and serum-free DMEM medium mixtures at a ratio of 1:9. Before the experiment, TC was pretreated with different concentrations (0, 6, 12 μg/mL) for 48 h. Then 2.5×10^5 of AMDC was resuspended in 300 μL serum-free DMEM and plated on the upper side of the filter, while 600 μL complete DMEM f12 containing 10% UY was placed in the lower plate. Cells were incubated at 37 °C in a 5% CO_2 for 48 h. The invaded cells in the lower chamber were fixed with 4% paraformaldehyde for 25 min and stained with 0.1% crystal violet (Beyotime, Beijing, China) for 30 min at room temperature, after which non-invasive cells on the upper surface of the membrane were gently removed with a cotton swab. Dry at room temperature for 24h. Next the microscope (Nikon, Japan) was employed to capture the images of the cells invaded to the lower chamber. Three fields per filter were randomly selected under the microscope to count the invaded cell numbers.

Western Blot Analysis

The protocol of Western blot analysis was described previously. In brief, AMDC seeded into 6-well plates at a density of 1×10^5 cells/well and treated by TC with varying concentrations described above (0, 6, 12 μg/mL). After 48 h incubation, total proteins in cells were extracted using radioimmunoprecipitation assay (RIPA buffer high) lysis buffer (60 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS) (Solarbio, R0010, Beijing, China), PMSF 100 Mm (Solarbio, P0100, Beijing, China), Aprotinin from bovine lung (Solarbio, 9087-70-1, Beijing, China), Protein Phosphatase Inhibitor (All-in-one,100x) (Solarbio, P1260, Beijing, China) with the proportion of 100:1:1:1 on ice and quantified using BCA protein concentration determination kit (Beyotime, P0012, Beijing, China) according to the manufacturer's instruction. Subsequently, proteins were in polyacrylamide gels, transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, NY, USA), and incubated with primary antibodies at 4 °C overnight. After washing with Tris Buffered Saline Tween (TBST; Solarbio, T1086, Beijing, China) for 3 times, the PVDF membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The GAPDH antibody (1:10000; Proteintech, Cat No.:10494-1-AP, Wuhan, China) signal was used as a loading control. Finally, the bound antibodies were detected with Immobilon Western Chemiluminescent HRP Substrate (Millipore, NY, USA). Images were obtained with Bio-Rad ChemiDocTM XRS system (Bio-Rad, Shanghai, China). The intensity of the target proteins blots was quantified by Image J software version 1.8.0.

Statistical Analysis

All statistical analyses were performed using SPSS 25.0 soft-ware (Chicago, USA). Before comparing, all data sets were first analyzed with Shapiro–Wilk normality test and considered normally distributed with $P > 0.05$. All normally distributed values were expressed as mean ± standard deviation (SD) and were evaluated by Student's t-test (two groups) or ANOVA (three or more groups) for the difference. If variances were homogeneous, then the least significance method was employed to compare between two groups. Dunnett's T3 method was used to compare between two groups if variances were nonhomogeneous. Non-normally distributed data were presented as median (range) and were analyzed comparably by Mann–Whitney testing. A two-tailed P-value less than 0.05 was defined as statistically significant.

Results

Phenotypic Characterization of AMDC Isolated from Human Uterine Tissues

Immunofluorescence cell staining for vimentin was performed to identify whether the isolated cells were AMDC. As shown in*Figure 2* , AMDC was strongly positive for Vimentin, and nearly negative for E-cadherin, CK7, Von Willebrand Factor and alpha smooth muscle Actin, which confirmed that these cells were actually AMDC.

Cell viability Effects and cytotoxicity of TC on AMDC

In order to confirm the Cell viability activity of these extracts on AMDC, the different concentrations of TC at 48h that inhibit cell growth by 50% were determined and maximum drug concentration that does not affect cell viability. The Cell viability effect of TC of AMDC. TC treatment at concentrations between 0 and 128 μg/mL significantly increased the inhibition rate in AMDC in a dose -dependent manner. With the maximum drug concentration that does not affect cell viability, 12 μg/mL. The Cell viability results were listed in*Figure 3* and*Figure 4* . Thus, the final concentration of TC in AMDC (12 μg/mL) was selected in further experiments.

TC Destroy the cytoskeleton of AMDC

To determine whether TC destroy the cytoskeleton. AMDC was pretreated with varying concentrations of TC (0, 6, 12 μg/mL) for 48 h before the confocal microscope experiment. *Figure 5* demonstrates that TC decreased the destroy the cytoskeleton of AMDC in a dose-dependent manner.

TC Suppresses the Migration of AMDC

To determine whether TC affected the migration of AMDC, wound healing assay was performed, respectively. AMDC was formed firstly, with varying concentrations of TC (0, 6, 12 μg/mL) for 0, 24 and 48 h after the cell scratch experiment. *Figure 6*demonstrates that TC decreased the wound healing of AMDC in a dose-dependent manner.

TC Suppresses the Invasion of AMDC

Transwell chambers covered with matrigel were employed to detect the effects of TC on AMDC invasion. AMDC was pretreated with varying concentrations of TC (0, 6, 12 μg/mL) for 48 h.*Figure 7* revealed that the number of invaded cells was significantly decreased following TC treatment in a dose-dependent manner in AMDC.

TC Downregulates the Expression related of Rho/ROCK signal pathway Proteins in AMDC

To further explore the effect of TC may on the Rho/ROCK signal pathway of AMDC, the expression of several proteins which were crucial for Rho (RhoA, RhoB, RhoC) and ROCK (ROCK1, ROCK2) was detected after treatment with different concentrations of TC (0, 6, 12 μg/mL) for 48 h. As shown in *Figure 8* , Western blot analysis showed that TC dose-dependently decreased the expression of RhoA, RhoB, RhoC, ROCK1, ROCK2 in AMDC. All of these proteins decreased and in dose-dependent manner.

Discussion

Adenomyosis is a kind of benign disease, but it exhibits many characteristics that are similar to malignant tumors. Many tumors signaling pathways also participate in the development of adenomyosis. Current studies show that Rho/ROCK signaling pathways consisting of RhoA, RhoB, RhoC, ROCK1 ROCK2 induces a specific type of cytoskeleton and migration of tumor cells[21]. However, the possible role(s) of Rho/ROCK signaling pathways in adenomyosis remain unclear. In the present study, the effects of regulating cytoskeleton-induced invasion and migration may relate to Rho/ROCK signaling pathway on human endometrial cells and underlying mechanisms were investigated.

At present, there are certain results about the regulation of cytoskeleton-induced invasion and migration in malignant tumors. For example, nasopharyngeal carcinoma migration and invasion via cytoskeletal remodeling[22]. Reorganization of the cytoskeleton, cell migration and invasion contribute to colorectal cancer[23]. Cytoskeleton reorganization acquires the migration and invasion potential typical of mesenchymal cells with the Role in Cancer and Fibrotic Diseases[24]. Tumor transcription factors could suppress the expression of epithelial cell markers (E-cadherin, CK7, Von Willebrand Factor and alpha smooth muscle Actin) and maintain the phenotype of mesenchymal cells that express Vimentin [25-27]. TC is the main active constituent of Guizhi serves as a tumor suppressor such as cutaneous[28], cervical[29], colorectal ovarian[30-32], bladder[33], and breast cancers[34, 35]. What's more, Rho/ROCK signaling pathway serves as an oncogene in cancer, has inhibitory effects on the cytoskeleton, migration and invasion of tumor cells, such as colorectal cancer[36]. In this study, we found that the AMDC purified by AM with positive Vimentin and negative E-cadherin, CK7, Von Willebrand Factor and alpha smooth muscle Actin and our results suggest that downregulation of cytoskeleton, migration, invasion and may relate to Rho/ROCK (RhoA, RhoB, RhoC, ROCK1, ROCK2) signaling pathway treating with TC may also be involved in the pathogenesis of adenomyosis.

Conclusion

In conclusion, we propose that regulating cytoskeleton may play an important inhibitory role in the migration and invasion of AMDC, which may relate to Rho/ROCK signaling pathway. TC may be a potential element target for the treatment of adenomyosis. In additional there was still a lot of room for development in basic research.

Acknowledgments

Author Contributions

Wei Shi conceived and designed the study. Fengxin Cui assisted in wound scratch, transwell experiments. Zilu Wang assisted in Western blot experiments. Keke Zhang and Yinuo Zhang assisted in Immunofluorescence experiments. Yiran Zhang assisted in cell experiments. Mengdie Yu did the whole experiments. Mengdie Yu and Yang Liu analyzed and interpreted the data. Mengdie Yu, Xin Wang and Wei Shi revised the manuscript. All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Funding

This work was financed by National Natural Science Foundation of China (81873330). Taishan Scholars Young Expert Project(NO.tsqn201909185)Jinan Science and Technology Development Plan(201907009).

Disclosure

The authors report no conflicts of interest in this work.

Notes:

Abbreviations:

CT: Guizhi; TC: Trans-Cinnamaldehyde; AMDCs: denomyosis Derived Cells; AM: adenomyosis; VIM: Vimentin; VWF: Von Willebrand Factor; E-cad: E-cadherin; SMA: alpha smooth muscle Actin; CCK-8: Cell Counting Kit-8 assay; UY: Fetal Bovine Serum; PBS: Phosphate-Buffered Saline solution; DMSO: dimethyl sulfoxide.

Reference:

1. Bird CC, McElin TW, Manalo-Estrella P. The elusive adenomyosis of the uterus–revisited. Am J Obstet Gynecol 1972;112:583-593.

2. Benagiano G, Brosens I. History of adenomyosis. Best Pract Res Clin Obstet Gynaecol 2006;20:449-463.

3. Larsen SB, Lundorf E, Forman A, Dueholm M. Adenomyosis and junctional zone changes in patients with endometriosis. Eur J Obstet Gynecol Reprod Biol 2011;157:206-211.

4. Nnoaham KE, Hummelshoj L, Webster P, et al. Impact of endometriosis on quality of life and work productivity: a multicenter study across ten countries. Fertil Steril 2011;96:366-373.e368.

5. Prescott J, Farland LV, Tobias DK, et al. A prospective cohort study of endometriosis and subsequent risk of infertility. Hum Reprod 2016;31:1475-1482.

6. Kennedy S, Bergqvist A, Chapron C, et al. ESHRE guideline for the diagnosis and treatment of endometriosis. Hum Reprod 2005;20:2698-2704.

7. Kvaskoff M, Mahamat-Saleh Y, Farland LV, et al. Endometriosis and cancer: a systematic review and meta-analysis. Hum Reprod Update 2021;27:393-420.

8. Liu L, Luo N, Guo J, Xie Y, Chen L, Cheng Z. Berberine inhibits growth and inflammatory invasive phenotypes of ectopic stromal cells: Imply the possible treatment of adenomyosis. J Pharmacol Sci 2018;137:5-11.

9. Xiang T, Fan Y, Li C, et al. DACT2 silencing by promoter CpG methylation disrupts its regulation of epithelial-to-mesenchymal transition and cytoskeleton reorganization in breast cancer cells. Oncotarget 2016;7:70924-70935.

10. Leduc C, Etienne-Manneville S. Regulation of microtubule-associated motors drives intermediate filament network polarization. J Cell Biol 2017;216:1689-1703.

11. Yu FX, Guan KL. The Hippo pathway: regulators and regulations. Genes Dev 2013;27:355-371.

12. Halon A, Donizy P, Surowiak P, Matkowski R. ERM/Rho protein expression in ductal breast cancer: a 15 year follow-up. Cell Oncol (Dordr) 2013;36:181-190.

13. Bo H, Gong Z, Zhang W, et al. Upregulated long non-coding RNA AFAP1-AS1 expression is associated with progression and poor prognosis of nasopharyngeal carcinoma. Oncotarget 2015;6:20404-20418.

14. Zeng Z, Bo H, Gong Z, et al. AFAP1-AS1, a long noncoding RNA upregulated in lung cancer and promotes invasion and metastasis. Tumour Biol 2016;37:729-737.

15. Tang Y, He Y, Zhang P, et al. LncRNAs regulate the cytoskeleton and related Rho/ROCK signaling in cancer metastasis. Mol Cancer 2018;17:77.

16. Nan L, Yang L, Zheng Y, et al. Effects of Gualou Guizhi Decoction Aqueous Extract on Axonal Regeneration in Organotypic Cortical Slice Culture after Oxygen-Glucose Deprivation. Evid Based Complement Alternat Med 2017;2017:5170538.

17. Lin J, Song Z, Chen X, et al. Trans-cinnamaldehyde shows anti-depression effect in the forced swimming test and possible involvement of the endocannabinoid system. Biochem Biophys Res Commun 2019;518:351-356.

18. Liu Z, Sun Z, Liu H, et al. Single-cell transcriptomic analysis of eutopic endometrium and ectopic lesions of adenomyosis. Cell Biosci 2021;11:51.

19. Wang X, Shi Y, Xu L, et al. Traditional Chinese medicine prescription Guizhi Fuling Pills in the treatment of endometriosis. Int J Med Sci 2021;18:2401-2408.

20. Chan RW, Schwab KE, Gargett CE. Clonogenicity of human endometrial epithelial and stromal cells. Biol Reprod 2004;70:1738-1750.

21. Narumiya S, Tanji M, Ishizaki T. Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion. Cancer Metastasis Rev 2009;28:65-76.

22. Fan C, Qu H, Xiong F, et al. CircARHGAP12 promotes nasopharyngeal carcinoma migration and invasion via ezrin-mediated cytoskeletal remodeling. Cancer Lett 2021;496:41-56.

23. Henstra C, van Praagh J, Olinga P, Nagelkerke A. The gastrointestinal microbiota in colorectal cancer cell migration and invasion. Clin Exp Metastasis 2021;38:495-510.

24. Ciszewski WM, Wawro ME, Sacewicz-Hofman I, Sobierajska K. Cytoskeleton Reorganization in EndMT-The Role in Cancer and Fibrotic Diseases. Int J Mol Sci 2021;22.

25. Liu Y, Wang X, Wan L, et al. TIPE2 inhibits the migration and invasion of endometrial cells by targeting β-catenin to reverse epithelial-mesenchymal transition. Hum Reprod 2020;35:1377-1390.

26. Sica GL, Choi IH, Zhu G, et al. B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. Immunity 2003;18:849-861.

27. Darenfed H, Dayanandan B, Zhang T, Hsieh SH, Fournier AE, Mandato CA. Molecular characterization of the effects of Y-27632. Cell Motil Cytoskeleton 2007;64:97-109.

28. Zhang B. Guizhi Fuling pills inhibit the proliferation, migration and invasion of human cutaneous malignant melanoma cells by regulating the molecular axis of LncRNA TPT1-AS1 / miR-671-5p. Cell Mol Biol (Noisy-le-grand) 2020;66:148-154.

29. Yao Z, Shulan Z. Inhibition effect of Guizhi-Fuling-decoction on the invasion of human cervical cancer. J Ethnopharmacol 2008;120:25-35.

30. Chen D, Zhao J, Cong W. Chinese Herbal Medicines Facilitate the Control of Chemotherapy-Induced Side Effects in Colorectal Cancer: Progress and Perspective. Front Pharmacol 2018;9:1442.

31. Han L, Cao X, Chen Z, et al. Overcoming cisplatin resistance by targeting the MTDH-PTEN interaction in ovarian cancer with sera derived from rats exposed to Guizhi Fuling wan extract. BMC Complement Med Ther 2020;20:57.

32. Han L, Guo X, Bian H, et al. Guizhi Fuling Wan, a Traditional Chinese Herbal Formula, Sensitizes Cisplatin-Resistant Human Ovarian Cancer Cells through Inactivation of the PI3K/AKT/mTOR Pathway. Evid Based Complement Alternat Med 2016;2016:4651949.

33. Lu CC, Shen CH, Chang CB, et al. Guizhi Fuling Wan as a Novel Agent for Intravesical Treatment for Bladder Cancer in a Mouse Model. Mol Med 2016;22:64-73.

34. Dai Y, Qiang W, Yu X, et al. Guizhi Fuling Decoction inhibiting the PI3K and MAPK pathways in breast cancer cells revealed by HTS(2) technology and systems pharmacology. Comput Struct Biotechnol J 2020;18:1121-1136.

35. Jiang Y, Fan L. Evaluation of anticancer activities of Poria cocos ethanol extract in breast cancer: In vivo and in vitro, identification and mechanism. J Ethnopharmacol 2020;257:112851.

36. Bin J, Nie S, Tang Z, et al. Long noncoding RNA EPB41L4A-AS1 functions as an oncogene by regulating the Rho/ROCK pathway in colorectal cancer. J Cell Physiol 2021;236:523-535.

Hosted file

`Figure.docx` available at https://authorea.com/users/489049/articles/572935-trans-cinnamaldehyde-inhibition-of-cytoskeleton-induced-invasion-and-migration-by-regulating-the-rho-rock-pathway-in-adenomyosis