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Original Research

Vincristine promotes migration and invasion of colorectal cancer HCT116 cells through RhoA/ROCK/ Myosin light chain pathway

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Abstract: Vincristine is an antitumor vinca alkaloid isolated from vinca rosea, and is a medication used to treat a number of types of cancer. In this study, we investigated the impact of vincristine on oncogenic phenotypes of human colorectal cancer HCT116 cells. MTT assay demonstrated that vincristine showed a obviously inhibitory effect on cell growth compared to non-treated cells. However, Transwell assay showed that vincristine promoted migration and invasion of HCT116 cells *in vitro* in a concentration-dependent manner between 0.5 and 15 μM vincristine treatment, whereas cell growth showed no remarkable difference within the same concentration range. Additionally, Western blot analysis showed that vincristine significantly elevated RhoA activity and Myosin light chain (MLC) phosphorylation, suggesting the involvement of RhoA/ROCK pathway in the vincristine-induced enhancement of cellular motility. Furthermore, we found that both the siRNA for RhoA and ROCK inhibitor Y27632 attenuated the phosphorylation of MLC, as well as vincristine-induced migration and invasion. These data indicate that vincristine enhanced migration and invasion of HCT116 cells possibly through stimulating RhoA/ROCK/MLC signaling pathway.

Key words: Vincristine, colorectal cancer, migration, invasion, RhoA/ROCK signal pathway.

Introduction

Conventional therapeutic methods for cancer such as surgical treatment and radiotherapy are being used currently, but both have certain limitations. It has been found that radiotherapy and surgery can trigger a undesirable invasion or metastasis in some cases (1-5). Therefore, chemotherapy is frequently applied in cancer therapy coupled with surgery and/or radiatiotherapy. Most anti-cancer drugs are investigated and used based on their inhibitory effect on growth of tumor cells. However, the effect of anti-cancer drugs on cell invasion and metastasis ability is still unclear.

Vincristine, also known as leurocristine, is a chemotherapeutic drug used to treat a variety of human cancers, including neuroblastoma, acute myeloid leukemia, acute lymphocytic leukemia, small cell lung cancer, and Hodgkin's disease (6), because of lack of significant bone-marrow suppression (at recommended doses) and of unique clinical toxicity. It is given intravenously (6) and works by inducing microtubule perturbation and inhibiting mitosis, the cell then undergoes apoptosis (7). There are also studies showing vincristine to affect the metastasis and motility of tumor cells (8-10). For example, it has been indicated vincristine can decrease invasive ability of MO_4 mouse fibrosarcoma cells by abolishing the cytoplasmic microtubule complex (9).

The efficacy of chemotherapeutic drug is commonly diverse to different types of cancer. For example, some cancers show good sensitivity to specific types of chemotherapy drugs, while others do not. 5-fluorouracil has been reported to be eutherapeutic to breast cancer, while it isn't to cholangiocarcinoma (11,12). Colorectal cancer is known as a widespread malignant tumor, representing the third most common cancer in both men and women (13). It was reported that the incidence of colorectal cancer tended to be younger age (14). While the incidence rate in people 50 years or older has declined, the incidence among people 20 to 49 years has increased. By 2020 and 2030, the incidence rate for colorectal cancer are expected to increase by about 44% and 107%, respectively, for patients 20 to 34 years old (14). If this disease is diagnosed in the early stage, non-muscle invasive colorectal cancer can be cured with surgery or radiation. However, the operation is not enough for metastatic tumors, so chemotherapy becomes necessary for the high grade cancer treatment. However, some anticancer drugs such as doxorubicin stimulate invasion or metastasis of tumor cells in certain conditions as their side effects (3,13). Therefore, investigating the effect of chemotherapeutic drugs on the motility activity of tumor cells is significant.

In this study, we detected the effect of chemotherapeutic drug vincristine on migration and invasive ability of colorectal cancer HCT116 cells and explored the underlying molecular mechanism.

Materials and Methods

Cell cultures

Human colon cancer HCT116 cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Lonza, Levallois-Perret, France) and added with 100 μg/ml penicillin/streptomy-

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cin. Cell lines were cultured at 37°C in 5% CO_2 incubator.

Proliferation assay

Cell proliferation was assessed by MTT dye conversion. Briefly, 10^4 cells were seeded in a 96-well flat bottom plate after transfection. Cells were cultured in a 37°C, 5% CO_2 incubator. Once cell confluence reached 80%, the supernatant was replaced with fresh medium and cells were treated with 5 μM vincristine (Hao-xuan Bio-tech Co., Xi'an, China) dissolved in dimethyl sulfoxide (DMSO) at different concentrations for 24 h, followed by another 4 h after 20 μL MTT (5 mg/mL) was added to each well. 200 μL dimethylsulfoxide (DMSO) was added to the washed well to lyse cells. Absorbance was detected using an enzyme-linked immunosorbent assay (ELISA) spectrophotometer at 570 nm.

Migration and invasion assays

Cell migration was evaluated by Transwell assay using Transwell chambers (BD Bioscience) (15). The lower chamber was filled with 600 μL of cell culture medium with or without 5 μM vincristine / 50 μM Y27632 . Cells (1×10^5) were suspended with 100 μL of culture medium with 1 % FCS and plated into the upper chamber with or without vincristine / Y27632. After 20 h, the cells on the undersurface of the upper chamber were presented by crystal violet (Amresco, USA) staining for 10 minutes at room temperature, and then were observed using a light microscope (Olympus, Japan) and chose six random fields at 100× magnification to score the average cell coverage. Invasion assay was performed as the same procedure as migration assay. Differently, the upper face of the polycarbonate membrane in the upper chamber was covered with 1 mg/mL Matrigel (BD Biosciences) and the invasive cells were detected after incubation for 24 h.

Western blot analysis

Cells were washed twice with PBS and were lysed in lysis buffer (50mM Tris-HCl (pH 7.4), 1mM EDTA, 1% NP40, 150mM NaCl, 10mM NaF, 1mM Na_3VO_4) containing a protease inhibitor cocktail (Roche, NJ, USA). Proteins were separated by 10% SDS-PAGE (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and transferred to Immobilon-P membranes (Millipore, MA, USA). Anti-RhoA and anti-GAPDH antibodies (Santa Cruz, CA, USA), anti-MLC, anti-phosphorylated MLC (pMLC) antibodies, and IgG secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) were used. ECL-detecting reagent (Amersham Biosciences, Buckinghamshire, England) was used to development.

RhoA activity detection

The activity of RhoA was analyzed using Rhotekin RBD (Upstate Biotechnology) bound to glutathione agarose beads to pulldown the active form GTP-RhoA from cell lysates (16). GTP-RhoA and total RhoA were evaluated by Western blot analysis using anti-RhoA antibody.

MLC phosphorylation detection

Cells were firstly starved for 24 h in serum-free medium and were treated with or without 5 μM vincristine for the indicated time in 37°C cell incubator. The cells were lysed in cell lysis buffer (100 mM NaCl, 1 mM Na_3VO_4, 40 mM $Na_4P_2O_7$, 20 mM NaF, 30 mM HEPES NaOH (pH 7.4), 1% Triton X-100, 1 mM EGTA, 1 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin and 10 μg/ml aprotinin), and the cell extracts were used for Western blot analysis using anti-MLC antibody and anti-pMLC antibody.

RNA interference

siRNA targeting RhoA sequence AAGCAGATGAGAATGACGTCGGTG and negative control siRNA were purchased from Ambion (Austin, TX, USA). siRNAs were transfected into HCT116 cells using Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After transfection for 24 h, cells were lysed and analyzed by RT-PCR and Western blot analysis respectively for mRNA and protein expressions of RhoA.

Real-time quantitative PCR (RT-qPCR)

Total RNA in cells was extracted using RNA isolation kit (A&A Biotechnology, Poland). cDNA was obtained by reverse transcription PCR (RT-PCR) using RevertAid™ First Strand cDNA synthesis kit (Fermentas International, Lithuania) and was amplified using TaqMan® Gene Expression Assay (Applied Biosystems), and the specific primers for target proteins were used. mRNA expression of RhoA were calculated using the formula $2^{\Delta\Delta Ct}$ and was normalized to the level of GAPDH. The value of mRNA in siRhoA cells was demonstrated as the relative value of mRNA in control cells.

Statistical analysis

All of the experiments were repeated at least 3 times. SPSS 16.0 was used to assay the experimental data, which are presented as the mean±SEM. $P < 0.05$ was considered statistically significant difference.

Results

Vincristine inhibits growth and promotes motility ability of HCT116 cells

To determine the roles of vincristine in colorectal cancer cells, HCT116 cells were treated with various concentration of vincristine for 24 h. MTT assay was performed to evaluate cell proliferation. The results demonstrated that vincristine treatment significantly reduced the proliferation of HCT116 cells, but there was no significant difference in the cell proliferation between 0.5 and 15 μM vincristine treatment (Fig. 1A). Cell migration and invasion were often used to evaluate the motility ability of tumor cells. Transwell assays indicated that vincristine treatment enhanced migration and invasion of HCT116 cells in a concentration-dependent manner between 0.5 and 15 μM, respectively relative to the DMSO-treated control (Fig. 1B and C). These data suggested that vincristine promoted cellular migration and invasion independently of the effect on cell growth.

Vincristine influences RhoA/ROCK/MLC pathway

It has been reported that Rho GTPases play important roles in cell motility. RhoA, as a member of Rho

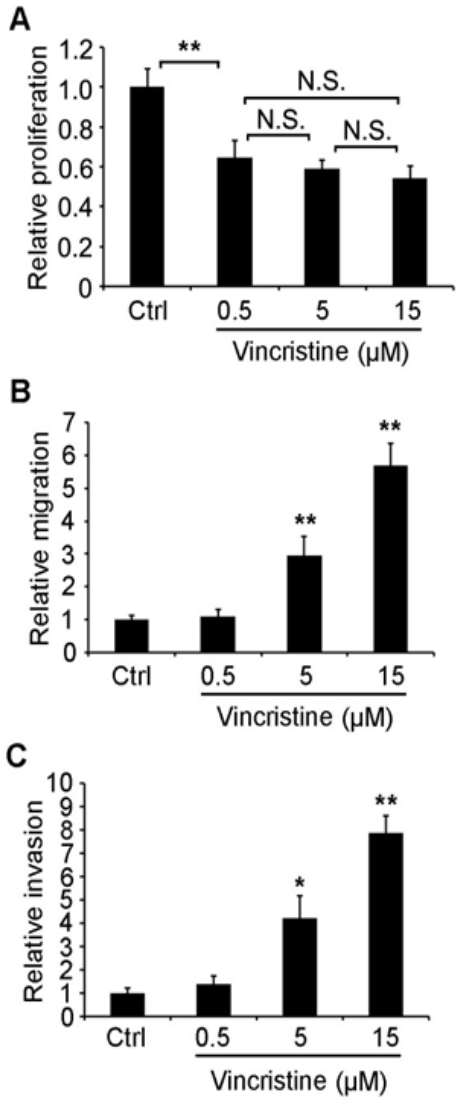

Figure 1. Effects of vincristine on growth and motility of colorectal cancer HCT116 cells. HCT116 cells were grown in medium for 24 h, and then cells were treated with or without 0.5, 5 and 15 μM vincristine for 24 h. (A) Cell proliferation was evaluated by MTT assay. Vincristine-treated cells showed decreased proliferation compared to the DMSO-treated control cells, and there was no significant difference in proliferation between 0.5 and 15 μM vincristine treatment. (B,C) Cell migration and invasion were assessed by Transwell assay. Vincristine-treated cells showed a increased migration and invasion ability in a concentration-dependent manner compared to the control cells. *, $P < 0.05$; **, $P < 0.01$ versus control; N.S., no significance.

GTPase family, is able to stimulate its downstream targets ROCK and Myosin light chain (MLC) to regulate these events of cells (17-22). To determine whether vincristine affects RhoA activity in HCT116 cells, cells were treated with or without 5 μM vincristine for the indicated time. RhoA activity was detected by pull-down assay. The results showed that RhoA activity increased transiently after 5 μM vincristine treatment, reaching the peak at 10 min after the treatment, followed by a gradual decrease up to 60 min (Fig. 2A). It has been known that the phosphorylation of MLC can be stimulated by RhoA through ROCK activation (20), so MLC phosphorylation was analyzed by Western blotting. The results indicated that vincristine treatment increased the phosphorylation of MLC, peaking at 10 min after the treatment, followed by a decrease (Fig. 2B). These results suggested that RhoA/ROCK/MLC signalling might be involved in the modulation of vincristine on biological functions of colorectal cancer HCT116 cells.

Silence of RhoA attenuates vincristine-induced cell migration and invasion

To determine whether vincristine affected HCT116 cell motility through RhoA/ROCK signalling, RhoA was knocked down by RNA interference. RNA expression level of RhoA was assessed using RT-qPCR and protein expression level was assessed using Western blotting to determine the interference efficiency. The results showed that both RNA and protein expression of RhoA in RhoA-silenced cells were efficiently reduced relative to that in non-silenced control cells (Fig. 3A and B). RhoA silenced or non-silenced cells were treated with or without 5 μM vincristine. Western blot analysis showed that RhoA silencing reversed vincristine-induced RhoA activation and MLC phosphorylation (Fig. 3C). Moreover, cell migration and invasion were evaluated by Transwell assay. The results showed that RhoA silencing significantly blocked vincristine-induced migration and invasion (Fig. 3D), suggesting that RhoA plays an important role in the modulation of vincristine on the motility of colorectal cancer HCT116 cells.

Y27632 inhibited vincristine-induced cell migration and invasion

To verify the participation of RhoA/ROCK pathway in vincristine-induced motility, HCT116 cells were treated with 5 μM vincristine, plus 50 μM ROCK inhibitor Y27632 for 24 h. Western blot analysis showed that Y27632 treatment attenuated vincristine-induced MLC phosphorylation in HCT116 cells (Fig. 4A). Transwell assay indicated that vincristine combined with Y27632 remarkably inhibited the induction of vincristine on migration and invasion (Fig. 4B). These data suggest that vincristine promotes the migration and invasion of colorectal cancer HCT116 cells via stimulating RhoA/

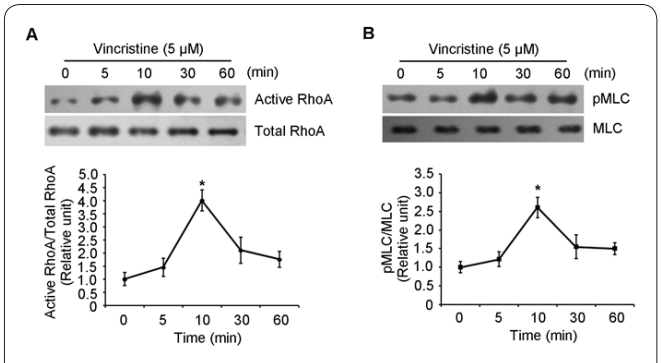

Figure 2. Vincristine influences RhoA activity and MLC phosphorylation. Cells were treated with 5 μM vincristine for 5, 10, 30, 60 min, and then cells were collected. (A) RhoA activity was evaluated by pull-down assay, and active RhoA adsorbed on the beads and total RhoA in the cell lysates were identified using RhoA antibody by Western blotting. (B) MLC phosphorylation and MLC were assessed by Western blotting using pMLC and MLC antibodies. The blots were quantified by densitometry, and the results were expressed as ratio relative to the values obtained in control cells (0 min). The graphs show means ± SEM of three independent experiments. *, $P < 0.05$ versus control.

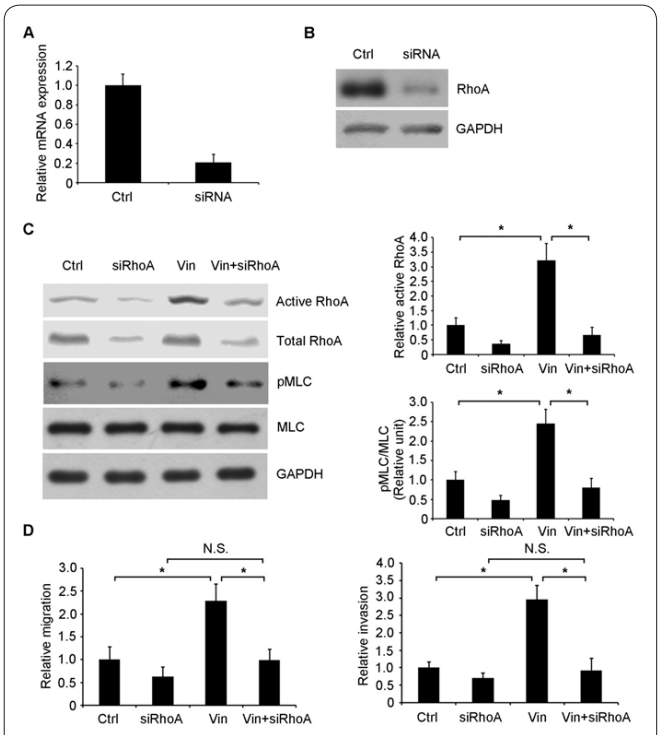

Figure 3. RhoA siRNA blocks vincristine-induced migration and invasion. Cells were transfected with RhoA siRNA or control siRNA for 24 h, and then were collected and lysed to detect (A) mRNA expression of RhoA by RT-qPCR and (B) protein expression by Western blotting. (C) RhoA silenced or non-silenced cells were treated with or without 5 μM vincristine. RhoA activity and MLC phosphorylation were detected by Western blotting. GAPDH was used to internal reference. Equal amounts of intracellular total proteins were put into active RhoA assay. Total RhoA in cell lyates was detected and active RhoA protein existing in total cell lysates were quantified by densitometry relative to GAPDH to exhibit the amounts of active RhoA in different cell group. The blots were quantified and the results were expressed as a ratio relative to the values obtained in control cells. (D) Cell migration and invasion were evaluated by Transwell assay. *, $P < 0.05$; N.S., no significance.

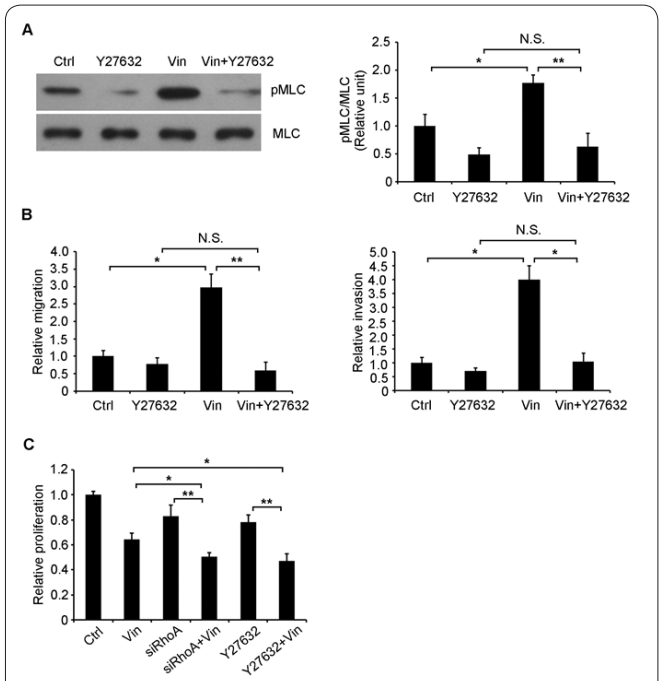

Figure 4. Y27632 abolishes vincristine-induced migration and invasion. vincristine treated and untreated HCT116 cells were treated with or without Y27632 for 24 h. (A) MLC phosphorylation was detected by Western blotting. The blots were quantified by densitometry, and the results were expressed as a ratio relative to the values obtained in control cells. (B) Migration and invasion were analyzed by Transwell assay. (C) Cell proliferation was evaluated by MTT assay. *, $P < 0.05$; **, $P < 0.01$; N.S., no significance.

ROCK/MLC signal pathway. Additionally, cell proliferation was detected by MTT assay. The result demonstrated that vincristine combined with RhoA siRNA or ROCK inhibitor Y27632 significantly enhanced the inhibition of proliferation compared to single treatments (Fig. 4C).

Discussion

Metastasis, the final step in the progression of many solid tumors, is one of the most fatal factors of cancer. Colorectal cancer exhibits the rather high mortality in all of the malignancies due to its high metastasis, commonly invading or spreading to other parts of body (23). Surgical treatment and radiotherapy, as conventional therapeutic methods for cancer, are not enough for metastatic tumors. Therefore, chemotherapy remains to be the alternative treatment strategy. Vincristine, an antitumor vinca alkaloid isolated from vinca rosea, has attracted a lot of attention as a chemotherapeutic drug in vivo and in vitro studies on multiple cancers (6). However, most studies so far focus on the impact of anti-cancer drugs on tumor cell growth and death, while there is little information about the effect on cellular metastasis and motility ability. In this study, we explored the effect of vincristine on the growth and motility ability of colorectal cancer HCT116 cells. On the one hand, we found vincristine significantly inhibited cellular growth. On the other hand, it was shown that vincristine enhanced the cellular motility ability such as migration and invasion in a concentration-dependent manner.

Several studies showed that some anti-cancer drugs inhibited the invasion and metastasis of tumors. For example, Taxol has been shown to block the essential processes of invasion and metastases in prostate tumor PC-3 ML cells (24). Etoposide was reported to inhibit cell invasion in human lung adenocarcinoma A549 cells by stimulating the metastasis suppresser KAI1 (25) Paclitaxel was demonstrated to inhibit metastasis and invasion of human ovarian Ovcar-3 cells by downregulating extracellular matrix degrading factors (26). Vincristine was also found to inhibit the migration of mouse fibrosarcoma MO_4 cells through the destruction of microtubule complex (9). Our finding seems to be contradictory to these results. However, our result is supported by the data reported by Masato Eitaki et al. indicating that vincristine promoted cellular invasive ability and amoeboid-like motility in a concentration-dependent manner between 0.1 and 15 μM in human gastric cancer MKN45 cells (27). Considering each cancer is unique and different types of tumor respond differently to chemotherapeutic drug, we think it may be one possible factor leading to the different effect of vincristine on cellular invasion.

To attenuate this side effect such as the increased cellular motility vincristine induced, the underlying molecular mechanism was explored. Rho family members of

GTPases have been reported to play important roles in the regulation of some biological functions associated with cell movement and actin cytoskeleton rearrangement (28). RhoA, as a member of GTPase family, shows active and inactive states by the exchange between GTP-bound and GDP-bound states. RhoA is closely involved in cancer progression. High expression level of RhoA was reported in several human cancers, including breast (29), gastric (30), ovarian (31), testicular (32) and bladder (33). ROCK affects tumor cells growth, colon formation, migration, invasion and metastasis by modulating cell stress-fiber formation and intercellular connection (33-38). However, RhoA mediated signaling pathway, expecially ROCK/MLC pathway participated in regulating cell motility (18,19,33). In this study, we detected the RhoA/ROCK/MLC signal pathway. The data demonstrated that vincristine treatment elevated RhoA activity and MLC phosphorylation and then activated RhoA/ROCK signal pathway. Furthermore, the pathway was blocked by RhoA silencing or ROCK inhibitor Y27632 treatment, which contributed to the inhibition on migration and invasion induced by vincristine. Our results firstly suggested that vincristine modulated colorectal cancer HCT116 cells motility by affecting RhoA/ROCK signalling. Masato Eitaki et al. have showed that vincristine promoted amoeboid-like motility and invasive ability through activating GEF-H1/RhoA signaling in human gastric cancer MKN45 cells (27), where GEF-H1 was silenced using siRNA and ROCK inhibitor Y27632 was used to block RhoA/ROCK signaling without RhoA silencing to study the underlying mechanism. In our study, RhoA silencing or ROCK inhibitor Y27632 was applied to block RhoA/ROCK signaling, which more directly confirmed the involvement of RhoA/ROCK pathway in vincristine modulated colorectal cancer HCT116 cells motility. However, RhoA activity can be regulated by multiple proteins. RhoA/ROCK signalling can be activated by GEF-H1 to regulate cell contractility (39). p27-Rho activates RhoA and induces invadopodia to regulate tumor cell invasion (40). p27 regulates the activation of RhoA/ROCK signal pathway to affect cell biological functions by binding with RhoA (41). Whether the change of RhoA activity in HCT116 cells is attributed to the modification of these factors is still unclear. What should be responsible for the regulation in colorectal cancer cells needs to be clarified. At last, cell proliferation assay demonstrated that vincristine combined with siRNA for RhoA or ROCK inhibitor Y27632 significantly enhanced the inhibition of proliferation compared to single treatments, indicating that vincristine coupled with gene therapy targeting RhoA/ROCK pathway is able to not only significantly attenuate vincristine induced side effects in HCT116 cells, but also enhance the inhibition of vincristine on tumor cell growth, and then improve the efficacy.

Taken together, vincristine activated RhoA/ROCK signalling to promote the migration and invasion of colorectal cancer HCT116 cells, suggesting valuable targets to control the cellular metastatic ability and laying theoretical foundation for the treatment of colorectal cancer.

Acknowledgments

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