

Sea cucumber *Cucumaria frondosa* fucoidan inhibits osteosarcoma adhesion and migration by regulating cytoskeleton remodeling

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Received November 20, 2019; Accepted April 7, 2020

DOI: 10.3892/or.2020.7614

Abstract. Osteosarcoma (OS) has been demonstrated to be difficult to cure due to its potently malignant metastasis. Therefore, new therapeutic approaches blocking the metastatic potential of OS are urgently required to improve the outcomes for OS patients. In the present study, the anti-metastatic capacity of sea cucumber (*Cucumaria frondosa*) fucoidan (Cf-Fuc) was evaluated on osteosarcoma cells by cell adhesion assay, Transwell assay and U2OS cell migration assay. The underlying mechanism on the dynamic remodeling of the cytoskeleton was also explored. The present data indicated that Cf-Fuc could block the U2OS osteosarcoma cell adhesion to fibronectin and significantly inhibit U2OS cell migration. Cf-Fuc greatly impaired the migration capacity of U2OS cells, and the migrated distance and velocity of Cf-Fuc-treated cells were markedly reduced. Also, Cf-Fuc could impair the dynamic remodeling of the cytoskeleton possibly by suppressing the phosphorylation of focal adhesion kinase and paxillin, as well as the activation of the Rac1/PAK1/LIMK1/cofilin signaling axis. Collectively, the present findings provide a novel therapeutic potential of *C. frondosa* fucoidan for osteosarcoma metastasis.

Introduction

Fucoidan, a type of marine polysaccharide containing substantial percentages of L-fucose and sulfate ester groups, is an essential constituent of brown seaweed (1) and certain marine invertebrates (such as sea urchins and sea cucumbers) (2-4). For the past decade, fucoidan has been widely studied owing to its various biological activities, including anticoagulant,

antitumor, antiviral, anti-inflammatory, immunomodulatory, and antioxidant properties (5-7). Therefore, fucoidan has been considered as a promising potential marine resource for the development of medications or functional foods beneficial to human health.

Osteosarcoma (OS) is the most primary malignant bone cancer commonly found in children and adolescents worldwide (8). The frequent sites of OS are the femur, the tibia and the humerus, and osteosarcoma represents more than half of all bone cancers (8). The 5 year cumulative survival rate of primary OS has been greatly improved by combining surgery with multi-agent chemotherapy in the past decades (9,10). However, almost 80% of OS patients eventually develop pulmonary metastasis, which is the major cause of fatal outcomes (11,12). Hence, new therapeutic approaches blocking the metastatic potential are urgently required to improve the outcomes for OS patients.

Sea cucumber is a widely used traditional Chinese medicine (TCM) with numerous healthy benefits (13). It is rich in a variety of bioactive components, such as polysaccharide, polypeptide and saponins (13-16). Sea cucumber fucoidan has been reported to have anticoagulant, anti-hyperglycemic, anti-inflammatory, and immunomodulatory activity (17-19). Growing evidence demonstrates that fucoidan possesses marked anticancer and anti-metastatic effects (20,21). Unfortunately, the underlying mechanism that accounts for the anti-metastatic effect of sea cucumber *Cucumaria frondosa* fucoidan (Cf-Fuc) remains largely unknown and requires further investigation. Therefore, in the present study the inhibitory effect and mechanism of Cf-Fuc on the *in vitro* migration of human bone osteosarcoma epithelial cells (U2OS) were examined. The present findings may reveal the potential targets affected by *C. frondosa* fucoidan and promising therapeutic implications in treating osteosarcoma metastasis.

Materials and methods

Materials and chemicals. Dry sea cucumber *C. frondosa* was purchased from a local market (Changchun, Jilin Province, China). Antibodies against focal adhesion kinase (FAK) (product code ab131435), phospho-FAK (product code ab81298),

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Key words: *Cucumaria frondosa*, fucoidan, osteosarcoma metastasis

paxillin (product code ab32115) and phospho-paxillin (product code ab4833) were purchased from Abcam. Antibodies against p21-activated kinase 1 (PAK1) (cat. no. sc-166887) and phospho-PAK1 (sc-135755) were purchased from Santa Cruz Biotechnology, Inc. LIM domain kinase 1 (LIMK1) (product no. 3842), phospho-LIMK1 (product no. 3841), cofilin (product no. 5175) and phospho-cofilin (product no. 3311) were obtained from Cell Signaling Technology, Inc. ECL chemiluminescent detection reagent and Glutathione Sepharose 4B affinity chromatography resin were obtained from GE Healthcare Life Sciences. Fibronectin, DMSO, EDTA, cysteine and TIRTC-conjugated phalloidin were purchased from Sigma-Aldrich; Merck KGaA. Fetal bovine serum, penicillin, streptomycin and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Gibco Life Technologies; Thermo Fisher Scientific, Inc. All other chemical reagents used in this study were analytical grade.

Preparation of Cf-Fuc. The preparation of sea cucumber (*C. frondosa*) fucoidan was performed according to a previous method (22) with some modifications. Briefly, the dry body wall of the sea cucumber (200 g) was ground and degreased by reflux extraction with anhydrous acetone at 60°C for 4 h. Then, the sample was digested with 0.5% papain solution containing 5 mM EDTA and 5 mM cysteine at 60°C for 10 h. Subsequently, the digested sample was centrifuged at 8,000 x g for 15 min at 4°C. Polysaccharides were precipitated from the supernatant with 200 ml of 10% cetylpyridinium chloride solution at 4°C overnight. The precipitate obtained by centrifugation was re-dissolved with 1.5 l of 3 M NaCl:ethanol (100:15, v/v), and then 1 l of 95% ethanol was added into the mixture. After centrifugation at 8,000 x g for 15 min at 4°C and removal of the precipitate chondroitin sulfate, another 1.5 l of ethanol was added to the supernatant. The precipitate collected by centrifugation was re-dissolved and dialyzed (MWCO 1000 Da) against deionized water for 48 h. The sample was lyophilized to obtain sea cucumber *C. frondosa* fucoidan named Cf-Fuc. Cf-Fuc was composed of L-fucose and a sulfate ester group. The purity of Cf-Fuc was determined as 98.6% (total carbohydrate content 72.5% quantified by phenol-sulfuric acid method (23); the sulfate content 26.1% determined by BaCl₂-gelatin method (24).

Cell culture. U2OS cells were purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology. U2OS cells were routinely cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, incubated in a 5% CO₂ incubator at 37°C.

Cytotoxicity of Cf-Fuc. Cells (1x10⁴) were seeded into each well of 96-well plates. After incubation at 37°C for 24 h, Cf-Fuc was administered to the cells at 50 and 100 µg/ml. After 24 h of incubation at 37°C, the medium was replaced with 100 µl fresh medium containing 0.5 mg/ml of MTT. Subsequently, the cells were incubated at 37°C for another 4 h, and 150 µl of DMSO was added after removal of the supernatant. The absorbance at 570 nm was determined using a microplate reader.

Cell adhesion assay. U2OS cells were detached by trypsin digestion and suspended in serum-free medium with or without Cf-Fuc (50 and 100 µg/ml) for 30 min. Then, the cells were

seeded into a 96-well plate coated with fibronectin (10 µg/ml), and incubated for 0.5, 1, 2 or 4 h. After washing with PBS, the adherent cells were fixed with 4% paraformaldehyde at room temperature (RT) for 30 min, and stained with 0.5% crystal violet at RT for 15 min. The dye, extracted with 33% acetic acid after washing with PBS, was quantified using a microplate reader.

Transwell migration assay. U2OS cells incubated with or without Cf-Fuc (50 and 100 µg/ml) at 37°C for 4 h were then placed in 24-well Transwell™ (Corning Costar, Corning, NY) filter inserts (8 µm pore diameter). DMEM containing 10% FBS as the chemoattractant was added in the lower well. After incubation for 12 h, the non-migrated U2OS cells inside the inserts were gently removed using cotton swab. The migrated cells were then fixed and stained with 0.1% crystal violet at RT for 15 min. Migrated cells were counted in five random fields under a light microscope at x400 magnification.

U2OS cell migration assay. U2OS cells were seeded into a CELLview cell culture dish (Greiner Bio-One) pre-coated with fibronectin (10 µg/ml), then incubated with or without Cf-Fuc (50 and 100 µg/ml) at 37°C for 4 h. U2OS cells were then placed in a 37°C heating chamber with CO₂ supply. Images were captured at 5 min intervals with a CCD camera for 4 h. Image stacks were quantitatively analyzed and wind-rose plots of tracked migration paths of U2OS cells were plotted by NIH ImageJ software (version 1.30; National Institutes of Health).

Determination of F-actin content. Six-well plates were pre-coated with fibronectin (10 µg/ml) at 37°C overnight, and then U2OS cells were seeded into each well and incubated at 37°C for 2 h. Cells were further treated with or without Cf-Fuc (50 and 100 µg/ml) for 0.5, 1, 2 or 4 h. After being washed with PBS the cells were fixed and stained with rhodamine phalloidin (0.1% Triton X-100, 3.7% formaldehyde, and 2 µM rhodamine phalloidin in PBS) for 1 h. Cells were incubated with methanol to extract the dye and rhodamine fluorescence (excitation wavelength: 530 nm, emission wavelength: 590 nm) was further assessed with a fluorescence microplate reader (TECAN GENios; Tecan Austria GmbH). F-actin content at time 0 was considered as 1; F-actin content at 0.5, 1, 2 and 4 h are expressed as a relative fold change of mean fluorescence intensity at time 0.

Ras-related C3 botulinum toxin substrate 1 (Rac1) activation assay. Rac1 activation was determined according to a previous method (25). Briefly, U2OS cells were lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology) and the lysate supernatant was then incubated with GST-PAK1 PBD fusion proteins immobilized on Glutathione Sepharose 4B affinity chromatography resin. After washing with lysis buffer, the resin was boiled in Laemmli sample buffer. Then, Rac1 protein bound to GST-PAK1 PBD as well as the endogenous total Rac1 were analyzed by SDS-PAGE and immunoblotting.

Immunoblotting. Cells were harvested and lysed with RIPA lysis buffer. Cell lysates were collected and the concentration

of proteins was quantified by BCA protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (30 μg of total protein per lane) were subjected to 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 3% BSA for 2 h at room temperature and then incubated with indicated primary antibodies (1:1,000 dilution) at 4°C overnight. HRP-conjugated secondary antibody (Beyotime Institute of Biotechnology) was incubated at RT for another 1 h after washing with TBST three times. Protein bands were subsequently visualized using ECL Western blotting chemiluminescent detection reagent. The band intensities for quantitative analysis were measured by densitometric analysis using NIH ImageJ software (version 1.30; National Institutes of Health).

Statistical analysis. Data were expressed as the mean \pm SD. Significant differences were determined by one-way ANOVA, followed by Bonferroni post hoc test using GraphPad Prism 6 (GraphPad Software, Inc.). P-values <0.05 were considered to indicate a statistically significant difference.

Results

Cf-Fuc inhibits U2OS cell adhesion. As revealed in Fig. 1, U2OS cell viability was not impacted by Cf-Fuc treatment at concentrations of 50, 100, and 200 $\mu\text{g}/\text{ml}$ for 24 h. When the concentration of Cf-Fuc increased to ≥ 400 $\mu\text{g}/\text{ml}$, the U2OS cell viability was significantly decreased compared to the control group. Therefore, the non-cytotoxic concentrations of 50 and 100 $\mu\text{g}/\text{ml}$ were selected for the following experiment to evaluate the inhibitory effect of Cf-Fuc on U2OS cell adhesion and migration, in case the phenotype presented in the present study was caused by cytotoxicity.

As revealed in Fig. 2, the adherent percentage of U2OS cells without treatment gradually increased, and eventually reached 83.2% at 4 h. While, the adherent percentage of Cf-Fuc-treated cells increased slowly, and only reached 59.0 and 43.8% at 4 h, respectively, with the Cf-Fuc concentration of 50 and 100 $\mu\text{g}/\text{ml}$, indicating that Cf-Fuc treatment could inhibit U2OS cell adhesion.

Cf-Fuc impairs the migration capacity of U2OS cells. First, the inhibitory effect of Cf-Fuc on U2OS cell migration was evaluated by Transwell assay. Cf-Fuc caused strong inhibition on U2OS cell migration, leading to a significant decrease in the number of migrated cells compared with the control group (Fig. 3). Then, the migratory capacity of Cf-Fuc-treated U2OS cells was quantitatively analyzed by NIH ImageJ. As revealed in Figs. 4 and 5, the control cells exhibited a strong migration ability, and the velocity reached 64.8 $\mu\text{m}/\text{h}$. Notably, in comparison to the cells in the control group, Cf-Fuc impaired the migratory capacity of U2OS cells. The migratory distance of Cf-Fuc-treated cells significantly decreased and the velocity was reduced to 28.5 and 18.1 $\mu\text{m}/\text{h}$, respectively, at concentrations of 50 and 100 $\mu\text{g}/\text{ml}$.

Cf-Fuc inhibits the formation of F-actin. Cytoskeleton remodeling is a critical event involved in cancer metastasis (26). During the adhesion and migration of cancer cells, G-actin polymerizes into F-actin filaments, which acts as stress fiber of

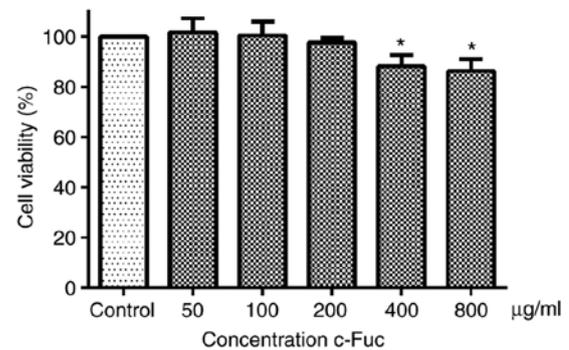


Figure 1. Cytotoxic effect of Cf-Fuc on U2OS cells. Data are expressed as the mean \pm SD. *P<0.05 vs. the control group. Cf-Fuc, *Cucumaria frondosa* fucoidan.

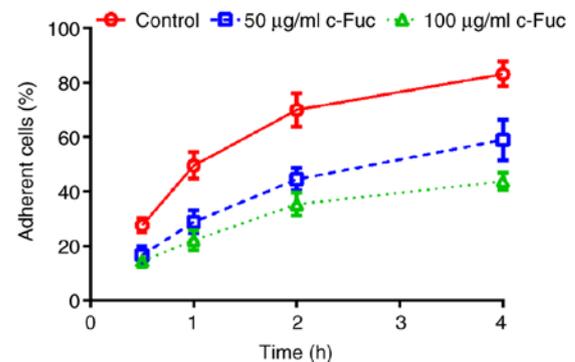


Figure 2. Cf-Fuc inhibits U2OS cell adhesion. U2OS cells were seeded into 96-well plates pre-coated with fibronectin to assess adhesion at indicated time-points (0.5, 1, 2 and 4 h). The adherent cells were fixed and then stained with crystal violet. The dye was quantified using a microplate reader. Cf-Fuc, *Cucumaria frondosa* fucoidan.

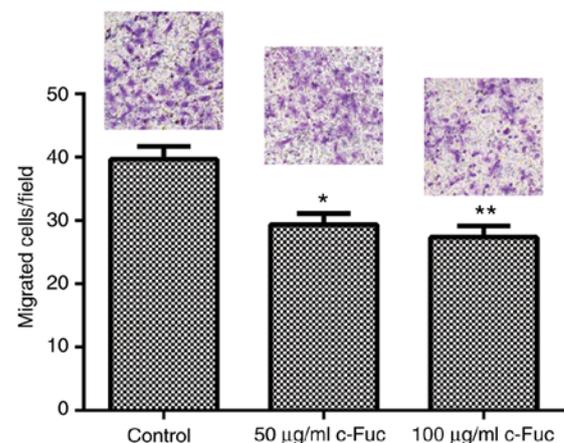


Figure 3. Transwell assay. U2OS cells incubated with or without Cf-Fuc were placed in Transwell inserts. The migrated cells were then fixed and stained with crystal violet. Migrated cells were counted in five random fields. Representative images are presented and data are expressed as the mean \pm SD. *P<0.05, **P<0.01 vs. the control group. Cf-Fuc, *Cucumaria frondosa* fucoidan.

the cytoskeleton and participates in the formation of lamellar pseudopods required for osteosarcoma metastasis (27,28). Therefore, the effect of Cf-Fuc on actin polymerization was

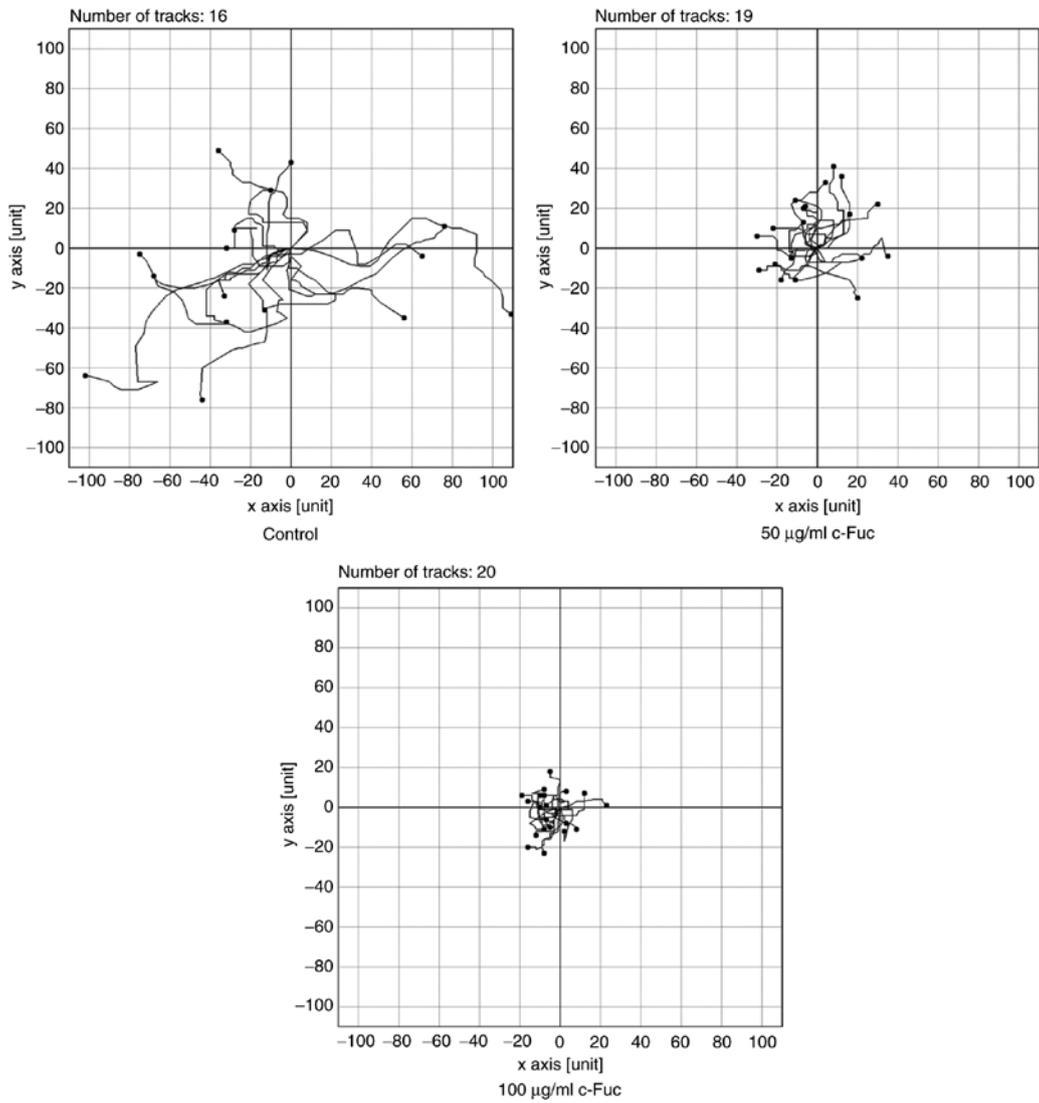


Figure 4. Effect of Cf-Fuc on U2OS cell migration tracks demonstrated by wind-rose plots. Cf-Fuc, *Cucumaria frondosa* fucoidan.

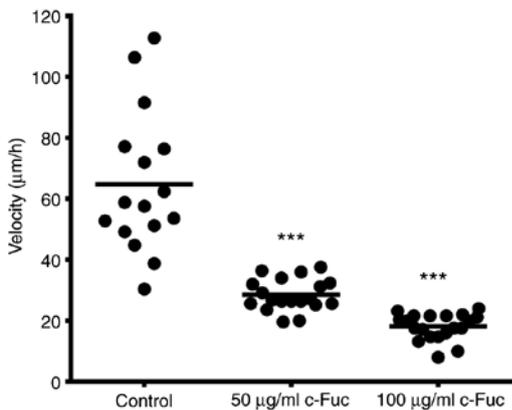


Figure 5. Effect of Cf-Fuc on the velocity of U2OS cells. ***P<0.001 vs. the control group. Cf-Fuc, *Cucumaria frondosa* fucoidan.

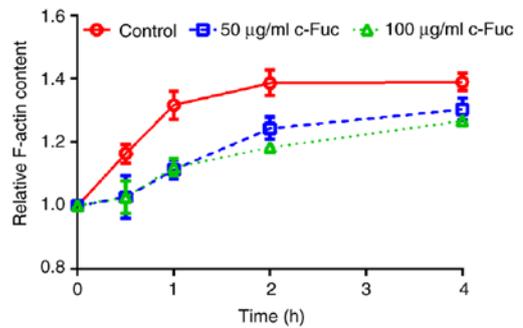


Figure 6. Effect of Cf-Fuc on actin polymerization in U2OS cells. U2OS cells were seeded into 24-well plates pre-coated with fibronectin, and then treated with or without Cf-Fuc. F-actin was calculated by measuring the fluorescence of extracted rhodamine phalloidin with a fluorescence microplate reader. Bar graphs indicate the changes in F-actin content expressed as a relative fold change in the mean fluorescence intensity of the cells at time 0. Data are presented as the mean \pm SD. Cf-Fuc, *Cucumaria frondosa* fucoidan.

further investigated. As revealed in Fig. 6, the actin polymerization of U2OS cells increased in a time-dependent manner. However, F-actin content of Cf-Fuc-treated cells was reduced compared to the control cells.

Cf-Fuc blocks the signaling transduction of cell adhesion. The effect of Cf-Fuc on cell adhesion signaling of U2OS cells was further examined. As revealed in Fig. 7A, Cf-Fuc treatment

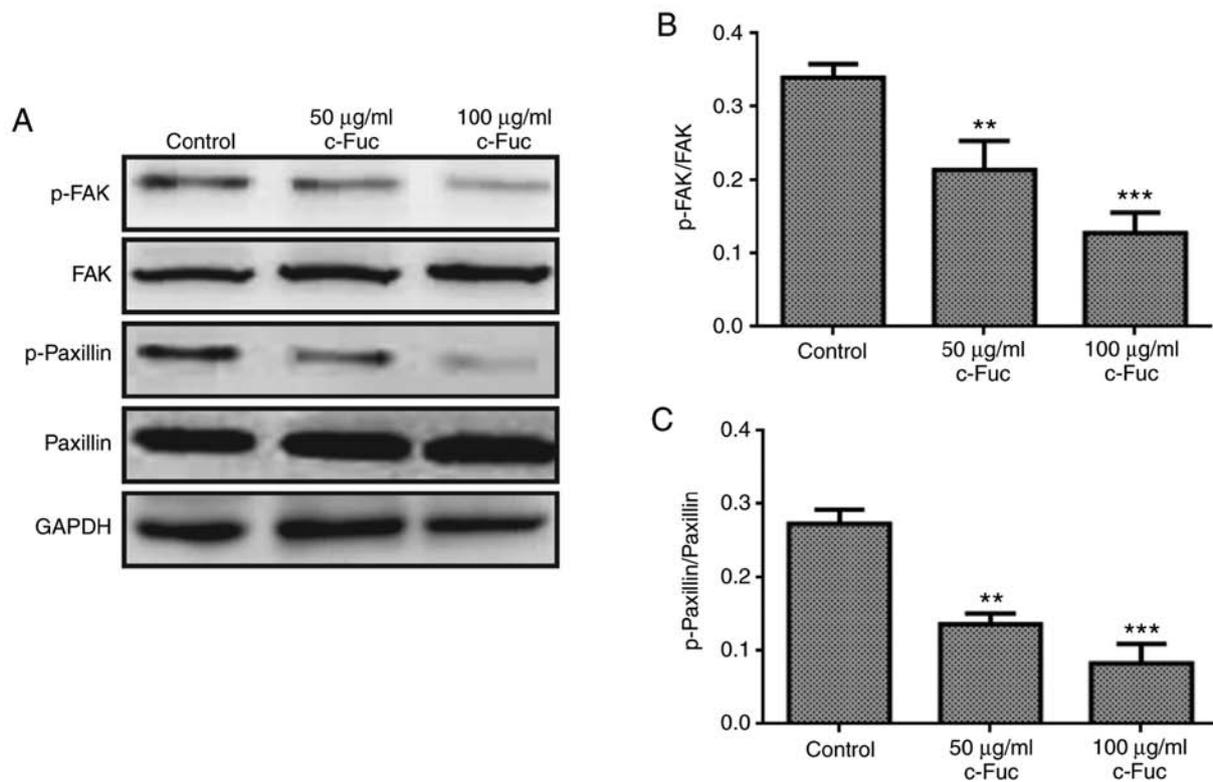


Figure 7. Cf-Fuc inhibits the phosphorylation of FAK and paxillin. (A) U2OS cells were seeded into 6-well plates pre-coated with fibronectin and allowed to adhere for 4 h. The total and phosphorylated proteins were detected by western blotting. The blots for GAPDH demonstrated the equal loading. The quantitative analysis of (B) p-FAK/total FAK and (C) p-paxillin/total paxillin were determined by ImageJ software. Data are presented as the mean \pm SD. **P<0.01, ***P<0.001 vs. the control. Cf-Fuc, *Cucumaria frondosa* fucoidan.

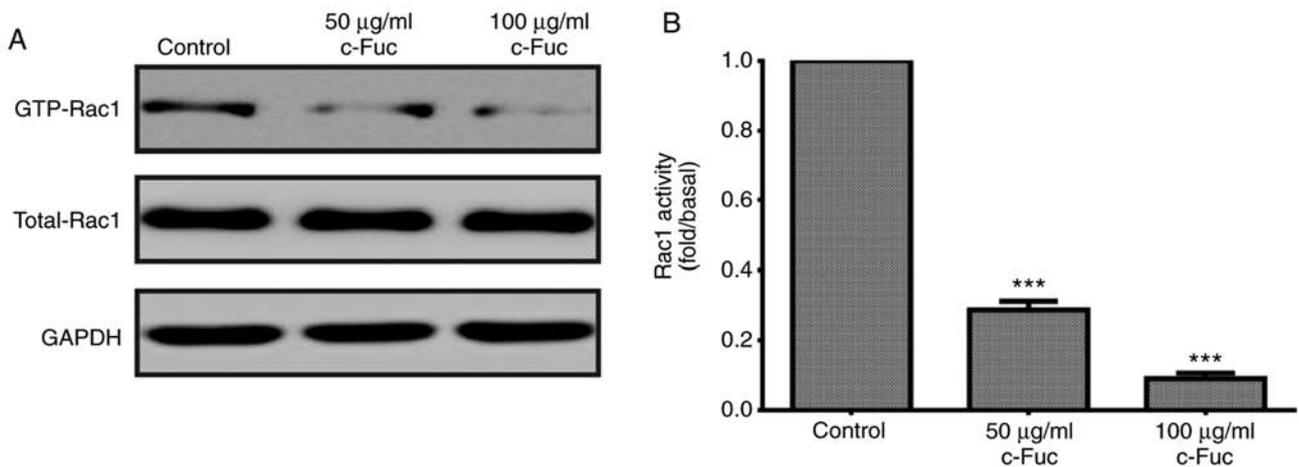


Figure 8. Effect of Cf-Fuc on Rac1 activation in U2OS cells. (A) Rac1 activation assay. (B) Quantitative analysis of Rac1 activation. Data are presented as the mean \pm SD. ***P<0.001 vs. the control group. Cf-Fuc, *Cucumaria frondosa* fucoidan; Rac1, Ras-related C3 botulinum toxin substrate 1.

significantly suppressed the phosphorylation of FAK (Fig. 7B) and paxillin (Fig. 7C) compared to the control group, indicating that Cf-Fuc inhibited the U2OS cell migration possibly by modulating the FAK/paxillin cell adhesion signaling axis.

Cf-Fuc impacts the cytoskeleton remodeling signaling axis. Small GTPase Rac1 plays a crucial role in the remodeling of the actin cytoskeleton. Whether Cf-Fuc could inhibit Rac1 activation in U2OS cells was subsequently examined. As revealed in Fig. 8A, compared to the control group, once treated with

Cf-Fuc, the level of GTP-Rac1 (activated form of Rac1) was reduced by 72 and 91%, respectively, at concentrations of 50 and 100 µg/ml (Fig. 8B).

The Rac1/PAK1/LIMK1/cofilin signaling axis could regulate the assembly of the actin cytoskeleton and has been revealed to be involved in cancer metastasis (29,30). Therefore, to determine whether Cf-Fuc inhibited U2OS cell adhesion and migration via the PAK1/LIMK1/cofilin signaling axis, the phosphorylation levels of PAK1, LIMK1 and cofilin were further investigated. As revealed in Fig. 9, the phosphorylated

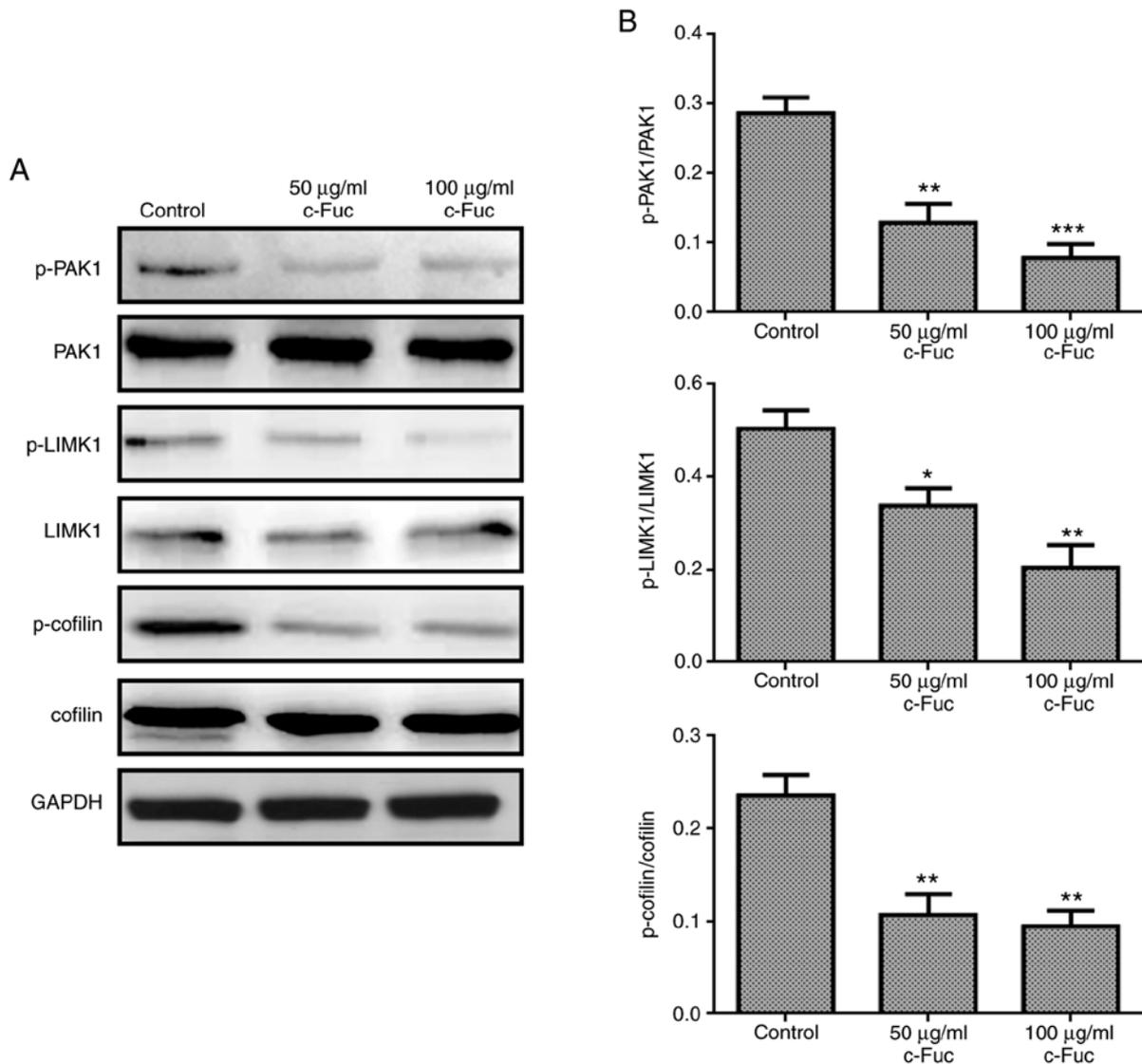


Figure 9. Cf-Fuc impacts the PAK1/LIMK1/cofilin signaling axis in U2OS cells. (A) The total and phosphorylated PAK1, LIMK1, and cofilin were determined by immunoblotting. The blots for GAPDH demonstrated the equal loading. (B) Quantitative analysis was determined by ImageJ. Data are presented as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the control group. Cf-Fuc, *Cucumaria frondosa* fucoidan; PAK1, p21-activated kinase 1; LIMK1, LIM domain kinase 1.

forms of PAK1, LIMK1 and cofilin were significantly inhibited by Cf-Fuc treatment at concentrations of 50 and 100 $\mu\text{g/ml}$ compared to the control group. The results indicated the potential molecular mechanism of Cf-Fuc in inhibiting U2OS cell adhesion and migration by blocking the activation of the Rac1/PAK1/LIMK1/cofilin signaling axis.

Discussion

Osteosarcoma (OS), a primary malignancy of bone, is prone to early metastasis (8). Currently, resection surgery and chemotherapy are standard treatments for OS (9). However, OS patients receive treatment that remains fundamentally unchanged since the 1970s, and accumulative results from clinical trials for OS treatment have proven largely disappointing (31). Outcomes of OS remain largely unimproved; notably, metastasis remains the most fatal complication of OS, and thus, the long-term survival rate of patients with OS is low due to the high risk of metastasis (32,33). Hence, novel approaches are urgently required

to improve the treatment of OS and prevent its metastasis. Compared with current chemotherapy, marine natural active molecules exhibit less adverse effects and improved anti-metastatic effects, and have been considered as a promising strategy to treat cancer metastasis (34,35).

Growing evidence reveals that polysaccharides from marine resources possess extensive health benefits, such as anti-metastatic ability (36,37). Research on the underlying anti-metastatic mechanisms of polysaccharides has revealed that multiple signaling pathways are involved. Wang *et al* (38) reported that a fucoidan derived from *Undaria pinnatifida* sporophylls (Ups-fucoidan) exerted a concentration- and time-dependent inhibitory effect on tumor metastasis *in vivo* and inhibited mouse hepatocarcinoma Hca-F cell growth, migration, invasion, and adhesion capabilities *in vitro*. Their study revealed that Ups-fucoidan inhibited growth by down-regulating VEGFC/VEGFR3, c-MET, cyclin D1, CDK4, PI3K/Akt and ERK, and suppressed adhesion and invasion by down-regulating L-selectin and upregulating TIMPs. Similarly,

other sulfated polysaccharides isolated from marine invertebrates (*Strongylocentrotus droebachiensis* and *Echinometra lucunter*) were revealed to attenuate tumor metastasis by a P-selectin-mediated mechanism (39). Another study (40) also demonstrated that *Undaria pinnatifida* fucoidan significantly inhibited the hypoxia-induced expression, nuclear translocation and activation of HIF-1 α , the synthesis and secretion of VEGF-C and HGF, as well as the invasion and lymphatic metastasis in a mouse hepatocarcinoma Hca-F cell line. In the present study, our findings also provide evidence that sulfated polysaccharide Cf-Fuc, isolated from sea cucumber *C. frondosa*, could inhibit the migratory capacity of U2OS cells *in vitro*, indicating that Cf-Fuc possesses potential anti-metastatic capacity.

As an essential cell adhesion molecule in OS cells, integrin plays important roles in mediating OS metastasis (41,42). Therefore, integrin and its downstream signaling have been considered as promising therapeutic targets for OS metastasis. It is widely accepted that FAK is a crucial kinase in integrin signaling that can phosphorylate multiple substrates, as well as a scaffold protein for protein-protein interactions, regulating adhesion and migration of cancer cells (43). Furthermore, paxillin is a multifunctional adaptor protein phosphorylated by FAK during cell migration, and performs a critical function in coordinating integrin signaling involved in cancer metastasis (44). The present findings revealed that Cf-Fuc could significantly reduce the number of adherent U2OS cells and cause a significant decrease in the migration capacity of U2OS cells. In addition, Cf-Fuc treatment inhibited the phosphorylation of FAK and paxillin, and this may be the major reason for the decrease in the adhesion and migration of U2OS cells.

Cancer metastasis is associated with increased motility characteristics, such as an extensively cross-linked actin network to form pseudopods, which requires appropriate cytoskeleton remodeling (45). Rac1 is an important member of the Rho small GTPase family which classically regulates actin cytoskeleton rearrangement (45). Rac1 activation is correlated with metastatic progression in many types of cancers (45). Growing evidence clearly reveals that Rac1-dependent signaling activation can promote cancer cell adhesion, invasion and metastasis in a variety of cancers, including osteosarcoma (46). Rac1 initially activates PAK1 which further phosphorylates and activates LIMK1. Activated LIMK1 further phosphorylates and inactivates cofilin, leading to the growth of actin filaments and formation of pseudopodia. Therefore, Rac-1 exhibits crucial roles in regulating cancer invasion and metastasis. The present study determined that Cf-Fuc could inhibit actin polymerization as revealed by the decreased content of F-actin, and this may be due to the blockage of the Rac1/PAK1/LIMK1/cofilin signaling axis, which impaired the dynamic reorganization of the actin cytoskeleton. Further research will be carried out to confirm the roles of Cf-Fuc on this pathway during the cytoskeleton remodeling of OS metastasis by RNAi, overexpression or inhibitor treatment, not only on U2OS cells, but also on other OS cell lines such as MG-63 and Saos-2 cells to support the present findings. In addition, considering fucoidans are huge molecules with a complex structure, the bioactivity of Cf-Fuc in the inhibition of adhesion and migration of U2OS cells may not depend on the integral molecule, Cf-Fuc may contain some essential motifs. Therefore, the degradation of Cf-Fuc, the purification and characterization of the functional motif(s),

as well as its pharmacokinetics and the distribution traced by radioactive isotope labeling *in vivo*, will become future research directions. In addition, a blood compatibility test of Cf-Fuc will be performed in our future research to confirm its clinical potential.

In summary, Cf-Fuc significantly inhibited OS cell adhesion and migration, reduced F-actin formation, and downregulated adhesion signaling via suppression of the phosphorylation of FAK and paxillin. Cf-Fuc also impaired Rac1 activation and the PAK1/LIMK1/cofilin signaling axis for reorganization of the actin cytoskeleton, providing the potential anti-metastatic mechanism of Cf-Fuc.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

MZ, LC and YL designed and performed the experiments, analyzed the data and wrote the manuscript. MC and SZ also performed the experiments. DK designed, interpreted and funded the study, and also wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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