Antitumor effect and molecular mechanism of fucoidan in NSCLC

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Abstract

Background: Fucoidan, a water-soluble polysaccharide, exerts anticoagulant and antiviral functions. It was recently reported that fucoidan also exerts an antitumor function. Lung cancer is one of the most common cancers in the world. The aim of this study was to investigate anti-tumor, apoptosis and anti-metastasis effects of fucoidan in both cell-based assays and mouse xenograft model, as well as to clarify possible role of m-TOR pathway in the protection.

Methods: In vitro: Different concentrations of fucoidan were given to act on non-small cell lung cancer (NSCLC) cell lines A549 and H1650. The effects of fucoidan on cell proliferation were observed by detecting cyclin expression levels, CCK8 and EDU experiments and cloning experiments. The apoptotic level was detected by flow cytometry and the apoptotic protein level was detected by Westernblot. By detecting the expression of adhesion molecules, the expression of matrix metalloproteinase (MMP) family, and Transwell cell invasion and migration experiment, the effect of fucoidan on cell adhesion, invasion and migration was observed. Meanwhile the effect of fucoidan on angiogenesis was observed by detecting the expression of vascular endothelial growth factor (VEGF).

In vivo experiment: An animal model of NSCLC cell mouse subcutaneous xenograft tumor was established to analyze the correlation between the consumption of fucoidan and the size and volume of xenograft tumor through gross observation. Through immunohistochemical staining and immunofluorescence double staining, ki67 and cell adhesion molecules (E-cadherin, N-cadherin and CD31) and VEGF-A in the tumor were detected, and the correlation between the amount of fucoidan and the above indexes was analyzed.

Results: Fucoidan inhibited the proliferation and angiogenesis of NSCLC cells via the mTOR pathway and promoted their apoptosis by increasing the Bax/Bcl-2 ratio. Not only that, fucoidan inhibited NSCLC cell invasion via epithelial–mesenchymal transformation (EMT). The mice fed fucoidan exhibited significant reductions in tumor volumes and weights. These indicators (Ki67, VEGF-A, N-cadherin) were decreased and E-cadherin expression was up-regulated in A549 mice that treated with fucoidan. The results showed that fucoidan inhibited tumor proliferation in vivo by affecting the expression of related proteins.

Conclusion: Fucoidan conveys antitumor effects and our results represent an ideal therapeutic agent for NSCLC.

Keywords: Fucoidan, Non-small cell lung cancer (NSCLC)
Background
The morbidity and mortality rates of lung cancer rank first in the world and are still on the rise in China. Non-small cell lung cancer (NSCLC) accounts for 80–85% of all lung cancers, with a 5-year survival rate of only 18% [1]. The application of traditional chemotherapy drugs, as well as the combination of chemotherapy and molecular targeted drugs, has enabled some patients to achieve significant survival benefits. However, drug side effects are severe, and drug resistance eventually occurs. Based on these results, marine organisms provide new ideas and methods for antitumor treatment.

Fucoidan is a natural derived compound consisting mainly of fucose and sulfuric acid groups and contains small amounts of xylose, mannose, galactose, arabinose and glucuronic [2]. It was found in different species of brown algae and in some animals, that has gained importance and practical application prospects for further research on the prevention and treatment of NSCLC. This project has important theoretical significance and practical application prospects for further research on the prevention and treatment of NSCLC.

Methods

Ethical permission
All animal protocols were approved by the Ethics Committee of Affiliated Hospital of Qingdao University (Qing Dao, China) and conducted according to national regulations in China.

Cell lines and culture
The human lung carcinoma cell lines A549 and H1650 were purchased from the China Center for Type Culture Collection (Beijing, China) in 2019. Cells were validated by short-sequence tandem repeat region analysis. Cells were cultured in DMEM (GIBCO; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 100 U/ml penicillin, 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 100 μg/ml streptomycin. All cells were raised in a 37 °C moist incubator containing 5% CO2.

CCK8 assay
Based on the instructions of the CCK8 kit (Best Bio, Shanghai, China), we digested cells in logarithmic growth phase into a cell suspension and counted them. Then, 2000 cells were inoculated per well of a 96-well plate. Each 96-well plate contained 100 μl of different concentrations of fucoidan. Later, the proliferation ability of A549 and H1650 cells was analyzed at 24, 48, 72 and 96 h. Then, 100 μl of fresh medium containing 10 μl of CCK8 solution was added to each well and cultivated for 2 h. We measured the absorbance at 450 nm with a Thermo Scientific Varioskan Flash spectrophotometer (Thermo Scientific, Inc., Vantaa, Finland).

Colony formation assay
We digested cells into a single-cell suspension and inoculated them at a density of 1000 cells/well in 6-well
plates. Cells were incubated for 7 days, after which they were fixed with methanol and dyed with crystal violet. We calculated the number of cells and defined a colony with more than 50 cells as a clone (ImageJ v1.47 software; NIH; National Institutes of Health, Bethesda, MD, USA).

EdU proliferation assay
A549 and H1650 cells (2.5×10^4 cells/well) were inoculated into 24-well plates. Cells were cultivated at 37 °C overnight and then plated into DMEM containing 10% serum and different concentrations of fucoidan. After cultivation for 48 h, cells were dyed with EdU based on the manufacturer's instructions for the EdU incorporation assay kit (Ribobio; Guangzhou, China). We counted EdU-positive cells from five random areas by fluorescence microscopy (Leica DMi8; Wetzlar, Germany).

Apoptosis assay
A549 and H1650 cells were treated with different concentrations of fucoidan (0, 10, 16 mg/mL) for 48 h at 37 °C. After treatment, the cells were stained with an annexin V-PE/7-AAD apoptosis detection kit (BD Biosciences; San Jose, CA, USA) based on the manufacturer's instructions. In total, 40,000 cells per sample were analyzed by using a flow cytometer.

Western blot analyses
A549 and H1650 cells were inoculated into 6-well plates. After overnight incubation, the cells were harvested following treatment with different concentrations of fucoidan for 48 h. Cells were lysed into protein lysates (20 μg) with RIPA buffer. Then, proteins were subjected to polyacrylamide gel electrophoresis, and those in the agarose gel were then transferred to PVDF membranes. Five percent dried skimmed milk was used to block the membranes at room temperature for 2 h. Then, we used TBST (pH 7.4) to wash the membranes three times, after which we added primary antibodies to the membranes. After cultivation overnight, we added secondary antibodies to the membranes and cultivated them for 1 h. Based on the instructions provided by the manufacturer, we exposed proteins to a chemical reaction. Finally, chemical signals and protein bands were analyzed on a CHEMIDOC XRS+ (Bio-Rad, Hercules, CA, USA) and with Imagej v1.47 software (NIH; National Institutes of Health, Bethesda, MD, USA), respectively. The following primary antibodies were used for Western blot analyses: cyclin D1, Bax, Bcl-2, p-mTOR, p-P70S6K, p-S6K, p-4EBP1, E-cadherin, N-cadherin, vimentin (Cell Signaling Technology; Danvers, MA, USA), VEGF (Santa Cruz; Dallas, TX, USA), and MMP-9 (Abcam; Cambridge, MA, USA). The secondary antibody was goat anti-rabbit IgG H&L (DyLight® 488) (Abcam).

Invasion assays
Cells were inoculated into 6-well plates and then treated with different concentrations of fucoidan for 48 h, after which the cells were digested with trypsin. Then, we counted and seeded the cells into invasion chambers (size: 8 μm, 24-well; BD Biosciences) in medium without serum. We then added the chemical inducer, medium containing 20% FBS, into the bottom wells. After 24 h of incubation, cells were fixed and then dyed with crystal violet. Finally, we counted invaded cells.

In vivo experiments
Four-week-old male mice were obtained from Weitong Lihua Limited Company (Beijing, China). Weight, the general situation of mice is similar. Each mouse was inoculated with A549 cells (5 x10^6 cells per mouse) into the right underarm (forearm). The cells were suspended in serum-free RPMI-1640 medium. Mice with tumor volume of 100±30 mm^3 were enrolled to ensure that the average volume of transplanted tumor in mice before treatment was 100mm^3. After the tumors reached a size of 100 mm^3, the mice were divided into two groups (n = 7) according to volume of mice. One group received a daily oral gavage of fucoidan (25 mg/kg., treatment group), and the other group received a vehicle (ddH2O; control group) every day for 14 days. Weight and tumor size were measured twice a week after administration, and the health of the mice was observed daily. Tumor volume was measured using calipers and calculated as L x W^2 x 1/2, where L is the length and W is the width.

Pentobarbital sodium and cervical dislocation euthanasia were performed to decrease animal suffering in the course of the experiment. The experiment was terminated 1 week after the final dose or the tumor volume of the control group exceeded 2000 mm^3. Mice were anesthetized deeply with intraperitoneal pentobarbital sodium (1%,50 mg/kg) and the anesthetized mice were photographed, then they were euthanized by cervical dislocation. Tumor volumes and body weights were recorded and part of the tumor tissues were stored in a −80 degree refrigerator.

Immunohistochemistry (IHC) and immunofluorescence (IF)
Sections were cut into 4 μm slices from paraffin-embedded tissues. Sections were dewaxed with xylene and rehydrated with graded ethanol, and then antigen retrieval was conducted using the microwave heating technique. Sections were then incubated with the indicated primary antibody overnight at 4 °C. Subsequently, after washing with PBS three times, we added the secondary antibody. The immunological reaction was visualized with diaminobenzidine (DAB) as the chromogenic agent, and the sections were restained with hematoxylin and counterstained with
Fucoidan inhibits angiogenesis.

Cells in a time-dependent manner (Fig. 2a) and that decreased the production of VEGF in A549 and H1650 pathway in A549 and H1650 cells.

indicating that fucoidan suppressed the mTOR signaling stream proteins p-S6K, p-P70S6K and p-4EBP1 (Fig. 2b), protein expression levels of p-mTOR and its down-
fucoidan for 48 h caused a significant reduction in the and 4EBP1 by western blot analysis. Treatment with detected the phosphorylation of mTOR, P70S6K, S6K, examined the activation of cancer-related pathways. We
to investigate the molecular mechanism by which fucoidan inhibits the cancer characteristics of NSCLC, we

Results

Fucoidan inhibits cell proliferation and VEGF expression in A549 and H1650 cells

To clarify whether fucoidan influences the proliferation ability of NSCLC cells, we treated A549 and H1650 cells with 10 and 16 mg/ml fucoidan for 4 days. The OD values of the CCK8 assay showed a significant decrease in the proliferation of A549 and H1650 cells compared with control cells (cells treated without fucoidan) (Fig. 1a). The effect of fucoidan in NSCLC cells was also examined using a colony formation assay. We found that the number of colonies formed in A549 and H1650 NSCLC cells was significantly decreased after fucoidan treatment (10 mg/ml, 16 mg/ml) for 7 days compared with control cells (Fig. 1b). Consistent with this finding, proliferation under fucoidan treatment was examined using EdU incorporation assays. The number of EdU-positive cells decreased in a dose-dependent manner in response to fucoidan (Fig. 1c). In addition, cyclin D1 expression was decreased in the fucoidan treatment group (Fig. 1d). The results also indicated that fucoidan decreased the production of VEGF in A549 and H1650 cells in a time-dependent manner (Fig. 2a) and that fucoidan inhibits angiogenesis.

Fucoidan inhibits NSCLC cell proliferation via the mTOR signaling pathway

To investigate the molecular mechanism by which fucoidan inhibits the cancer characteristics of NSCLC, we examined the activation of cancer-related pathways. We detected the phosphorylation of mTOR, P70S6K, S6K, and 4EBP1 by western blot analysis. Treatment with fucoidan for 48 h caused a significant reduction in the protein expression levels of p-mTOR and its downstream proteins p-S6K, p-P70S6K and p-4EBP1 (Fig. 2b), indicating that fucoidan suppressed the mTOR signaling pathway in A549 and H1650 cells.

Fucoidan inhibits invasion and epithelial–mesenchymal transformation (EMT) in NSCLC cells

We found that fucoidan inhibited the invasion ability of NSCLC cells and EMT markers in NSCLC cells. A549 and H1650 cells were treated with different concentrations of fucoidan (0, 10, and 16 mg/ml) for 48 h. Transwell assays (Fig. 3a) showed that the number of NSCLC cells in the fucoidan-treated groups decreased in a dose-dependent manner. A significant upregulation of E-cadherin and downregulation of N-cadherin, MMP-9 and vimentin were observed by western blot analyses (Fig. 3b). These data suggest that fucoidan effectively inhibits invasion in a dose-dependent manner in A549 and H1650 cells.

Fucoidan induces apoptosis in NSCLC cells

Moreover, to investigate the apoptotic effects of fucoidan in NSCLC cells, A549 and H1650 cells were exposed to various concentrations of fucoidan for up to 48 h, and fucoidan-induced A549 and H1650 cell death was confirmed by annexin V-PE/7-AAD staining by flow cytometry (Fig. 4a). Annexin V-PE/7-AAD double-staining and flow cytometry revealed that fucoidan effectively induced apoptosis in A549 and H1650 NSCLC cells. The proportion of apoptotic cells (lower right quadrant) significantly increased, from 20.02% in untreated A549 cells to 25.35% (10 mg/ml) and 49.82% (16 mg/ml) in fucoidan-treated A549 cells. Similarly, the proportion of apoptotic cells (lower right quadrant) significantly increased, from 21.36% in untreated H1650 cells to 27.30% (10 mg/ml) and 40.08% (16 mg/ml) in fucoidan-treated H1650 cells. In addition, the proportion of apoptotic cells increased significantly when A549 and H1650 cells were treated with fucoidan (16 mg/ml). Furthermore, to explore the underlying molecular mechanisms of fucoidan-induced apoptosis, western blot analysis was performed and revealed that fucoidan downregulated the expression of the antiapoptotic protein Bcl-2 and moderately increased the expression of the proapoptotic protein Bax. Fucoidan also induced a concentration-dependent increase in the Bax/Bcl-2 ratio in both A549 and H1650 cells (Fig. 4b).

Fucoidan suppresses tumor growth in A549 cell xenograft mice in vivo

To verify the inhibitory effect of fucoidan in NSCLC, we examined xenograft mice to verify the anticancer effect of fucoidan by observing tumor development, for which the body weight, tumor weight, and tumor volume were monitored. To investigate the effect of fucoidan feeding on A549 cell-bearing mice in vivo, we hypodermically inoculated A549 cells into the right underarm (forearm). The treatment group received fucoidan (25 mg/kg) or vehicle (ddH2O, control) via oral gavage every day for 14 days. The tumor weight, volume and growth rates

Statistical analysis

The data were presented as the mean ± SE and the differences between the means were analyzed using SPSS software 24.0(IBM Crop,Armonk,NY,USA). Unpaired t-tests and one-way analysis of variance (ANOVA) were conducted to identify significant differences of data. * P < 0.05 were considered statistically significant.
were assessed over 14 days. Tumors were dissected from every group so that we could observe the tumor size visually (Fig. 5a). The mice fed fucoidan exhibited significant reductions in tumor volumes and weights (Fig. 5b). As shown in Fig. 5c, the Ki-67 index was lower in fucoidan-fed A549-bearing mice than in control mice, indicating that fucoidan induced proliferation and the expression of metastasis-related proteins in tumors in vivo (see the following section). For further verification, we examined the expression of the vascular endothelial markers CD31 and VEGF-A in mouse in which tumor tissues were transplanted to further clarify our hypothesis that fucoidan inhibits angiogenesis (Fig. 5d). Consistent with the results that fucoidan inhibited EMT in vitro, we also found that E-cadherin staining was enhanced whereas N-cadherin staining was weakened based on the IF analysis of transplanted tumor tissues (Fig. 5e).
Discussion

Recently, great interest has been raised in developing natural molecules as potential anticancer agents [22]. Many recent studies have been investigated to discover the potential anti-tumor natural products using in vitro and in vivo systems. Calotropis gigantea (CG), a kind of tall waxen flowers, has been shown to induce apoptosis of A549 and NCI-H1299 lung cancer cells [23]. Alnus nitida also showed anti-tumor activity in A549 and H1650 lung cancer cells [24]. Moreover, preclinical and clinical studies revealed that phenolic compounds can prevent multidrug resistance in lung cancer [25]. They are in general easily tolerated by the human body, resulting in few side-effects. Like them, although fucoidan has proven to play an antitumor role in a variety of tumor cells and animal models, the effect of fucoidan on the growth of NSCLC and its mechanism are unclear. In our study, we investigated the inhibitory effect of fucoidan on NSCLC growth in both A549 and H1650 cells and in a mouse xenograft model. We proved that fucoidan inhibits proliferation and invasion and promotes apoptosis in NSCLC cells in vitro and inhibits tumor growth in vivo. Our results are in line with data obtained from previous studies that reported the antiproliferative effects of fucoidan on hepatoma and melanoma [26, 27].

Proliferation and metastasis are two main features of NSCLC progression. Previous studies have shown that the PI3K/Akt/mTOR pathway regulates cell proliferation and inhibits angiogenesis in A549 cells [28–30]. Once activated, Akt phosphorylates its downstream target mTOR, leading to the activation of ribosomal P70S6 kinase (P70S6K1) and translation initiation factor 4E binding protein 1 (4EBP1). The activation of P70S6K1 and 4EBP1 then leads to the upregulation of many growth factors that promote cell growth and cell cycle progression [31]. In our study, we confirmed the involvement of mTOR activation by analyzing the phosphorylated protein levels of mTOR and its downstream targets, including P70S6K1, S6K and 4EBP1, in both A549 and H1650 cells. Although we found that fucoidan inhibits p-mTOR and its downstream effectors, we did not fully illuminate the precise function of fucoidan in mTOR-related molecular networks.

Invasion and EMT are the initial stages of tumor metastasis. During EMT, cancer cells lose their polarity and adhesion to epithelial cells. Subsequently, cancer
cells acquired a mesenchymal cell-like morphology and migration capacity. Once cancer cells stop expressing epithelial markers, including E-cadherin, they start expressing mesenchyme markers, including N-cadherin and vimentin [32]. Degradation of the extracellular matrix (ECM), in which MMP-9 is involved, also plays an important role in invasion [33]. In our study, the upregulation of E-cadherin and the downregulation of N-cadherin, vimentin and MMP-9 revealed that fucoidan inhibits EMT. Moreover, we also confirmed that fucoidan inhibits invasion by transwell assays. In addition, angiogenesis is necessary for metastasis. VEGF is a classic angiogenic factor whose effects on endothelial cells are mediated in part by the mTOR pathway [34]. Although our results showed that fucoidan inhibited the expression of VEGF in vivo and in vitro, they proved only that fucoidan inhibits angiogenesis in NSCLC, and further experimental verification is required.

Apoptosis involves a vastly complicated network of biochemical and cellular processes that are linked to different and intricate groups of regulatory molecules [35]. Remarkably, the downregulation of apoptosis may contribute to different human diseases, such as cancer [36]. In mammals, there are two central apoptotic pathways: the extrinsic pathway (death receptor-mediated pathway) and the intrinsic pathway (mitochondrial-mediated

**Fig. 3** Fucoidan inhibits invasion and EMT in NSCLC cells. **a.** Transwell assays were used to determine the invasive ability of NSCLC cells. Representative fields showing invasive cells following fucoidan treatment on the membrane are presented. We counted the cells separately. **b.** Western blot analyses were used to detect the expression of MMP-9, E-cadherin, N-cadherin and vimentin. **p < 0.05**
The intrinsic pathway is also called the Bcl-2-regulated pathway and is tightly regulated by the Bcl-2 family of proteins. The Bcl-2 family proteins are divided into three groups, antiapoptotic proteins, proapoptotic proteins and BH3-only proteins, which exert both anti- and proapoptotic activities. A disruption in the balance of pro- and antiapoptotic proteins contributes to carcinogenesis by reducing apoptosis in malignant cells [36]. Bax and Bcl-2 are the major members of the Bcl-2 family and play a key role in tumor progression or inhibition of the intrinsic apoptotic pathway triggered by mitochondrial dysfunction [37]. We showed that fucoidan downregulated Bcl-2 protein expression, upregulated Bax protein expression, and increased the Bax/Bcl-2 ratio in a concentration-dependent manner in A549 and H1650 cells. These results are consistent with previous findings. However, more research is required to verify the complete molecular network of apoptosis.
To further explore the effects of fucoidan on A549 cells, we performed in vivo experiments. Based on the most effective inhibitory concentration obtained in vitro, A549 cell xenograft mice were fed a similar concentration of fucoidan. We observed antitumor effects of fucoidan in the mouse xenografts. These results confirmed the anticancer effect of fucoidan once again. However, we verified only the inhibitory effect of fucoidan on transplanted tumors of the A549 cell line, and additional in vitro experiments need to be performed. Our animal experiments lacked metastasis models, so more animal models of antitumor metastasis need to be studied.

**Conclusion**

Our results showed that fucoidan inhibits proliferation, angiogenesis, invasion and promotes apoptosis of NSCLC cells in vitro and in vivo. The underlying mechanism was dependent on the inhibition of the m-Tor pathway. Study provided strong evidence for clinical trials and the favorable effects of fucoidan suggested its potential as a new anticancer drug for the treatment of NSCLC.
Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12906-020-03191-0.

Additional file 1.

Abbreviations
NSCLC: Non-small cell lung cancer; MMP: Matrix metalloproteinase; VEGF: Vascular endothelial growth factor; HNSCC: Head and neck squamous cell carcinoma; HCC: Hepatocellular carcinoma; EMT: Epithelial–mesenchymal transformation; ECM: Extracellular matrix; IHC: Immunohistochemistry; IF: Immunofluorescence

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Not applicable.

Authors’ contributions
LHJ designed the study. CXH, SL, WXJ, TY, SZY, JJU performed the experiments. CXXH prepared the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials
The datasets supporting the conclusions of this article are included within the article.

Ethics approval and consent to participate
All animal protocols were approved by the Ethics Committee of Affiliated Municipal Hospital, Heze 274000, Shandong Province, China. 2Department of Oncology, Heze Municipal Hospital, Heze 274000, Shandong Province, China. 3State Key Laboratory of Bioactive Seaweed Substances, Qingdao Brightmoon Seaweed Group Co Ltd, Qingdao, China.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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