Recombinant water stress protein 1 (Re-WSP1) suppresses colon cancer cell growth through the miR-539/β-catenin signaling pathway

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Abstract
Background Nostoc commune Vauch. is a nitrogen-fixing blue-green algae that expresses a large number of active molecules with medicinal properties. Our previous study found that a water stress protein (WSP1) from N. commune and its recombinant counterpart (Re-WSP1) exhibited significant anti-colon cancer activity both in vitro and in vivo. This study is to investigate the effects of Re-WSP1 on proliferation of colon cancer cells and to elucidate the relevant mechanisms.

Methods Real-time quantitative PCR was used to detect the expression of miR-539 in colon cancer HT-29 and DLD1 cells. Colon cancer cells were transfected with miR-539 mimics and negative controls, and cell proliferation were detected by CCK8 and clonogenic assays. The target gene of miR-539 was predicted, and the dual luciferase reporter gene experiment was used to verify the target gene. After colon cancer cells were transfected with miR-539 mimics or inhibitors, the expression of target gene β-catenin was detected by Western blot. miR-539 inhibitor confirmed cell proliferation.

Results Re-WSP1 inhibited colon cancer cell growth in a dose-dependent manner. Re-WSP1 inhibited the expression of β-catenin, which was partly reversed by LiCl treatment. Quantitative PCR analysis showed that the expression of miR-539 was significantly upregulated after Re-WSP1 treatment. Moreover, miR-539 negatively regulated the expression of β-catenin by directly binding to the 3′UTR of β-catenin mRNA. The cell growth inhibition and the decrease in β-catenin expression induced by Re-WSP1 were significantly reversed by miR-539 inhibitor.

Conclusion Re-WSP1 suppresses colon cancer cell growth via the miR-539/β-catenin axis.

Keywords Re-WSP1 · Nostoc commune Vauch. · microRNA-539 · β-catenin · Colon cancer

Introduction
N. commune also known as landraces, is a nitrogen-fixing blue-green algae that is rich in nutrients, including amino acids, proteins, lipids, vitamins, minerals and pigments. Potts et al. [1] described a set of acidic proteins expressed by N. commune. These proteins are synthesized under natural conditions of constant drying and rehydration because these kinds of proteins are involved in the water stress response of N. commune. Potts named these proteins water stress proteins (WSPs). Water stress proteins are the main component of the extracellular matrix [2]. Our preliminary study first reported that natural soil water stress protein (WSP1) exerts a significant anti-CRC effect in vitro and in vivo. Then, the recombinant protein was expressed by genetic engineering technology to obtain recombinant water stress protein (Re-WSP1). The in vitro experimental data showed that Re-WSP1 exerts a significant anti-colon cancer effect and has no obvious inhibitory effect on human normal colonic epithelial
cells [3]. However, the mechanism underlying this anti-colon cancer effect still requires further research.

Colorectal cancer (CRC) is one of the most common malignancies in the world, and the CRC incidence in males and females ranks third and second, respectively, among the incidence of all cancers [4]. Activation of the Wnt/β-catenin signaling pathway plays an important role in the development and progression of CRC. Studies have shown that more than 90% of human CRC tumors have aberrant activation of the Wnt/β-catenin signaling pathway, which is accompanied by the accumulation of intracellular β-catenin [5]. With the rapid development of molecular oncology and molecular pharmacology, a new strategy for developing modern anti-cancer drugs from the active ingredients in natural products has emerged.

MicroRNAs (miRNAs) are endogenous noncoding RNAs that regulate gene expression by binding to the 3′-untranslated regions (UTRs) of specific messenger RNAs (mRNAs), resulting in either mRNA degradation or translational inhibition; miRNAs are small and are only 18–25 nucleotides in length [6, 7]. Emerging evidence suggests that miRNAs contribute to the development and progression of CRC as either tumor suppressors or oncogenes [8, 9].

In the present study, our results demonstrate that Re-WSP1 suppresses CRC growth via the miR-539/β-catenin axis, which provides new insights into the molecular mechanisms underlying the anti-CRC effect of Re-WSP1.

Materials and methods

Cell lines

The human colon cancer cell lines HT29 and DLD1 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Evergreen, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C in a humidified incubator with 5% CO₂.

Expression and purification of the recombinant Re-WSP1 protein

Primers were designed according to the coding sequence of the WSP1 gene. His tag were added to the N terminal of WSP1. The enzyme digestion sites Bam HI and Xho I were introduced at the ends of the two primers, and the primers were synthesized by Takara Company. cDNA was used as the template for PCR amplification, and then, the PCR products were treated with both the restriction enzymes Xho I and Bam HI. After gel recovery, the target fragment was ligated into pET28a. The constructed vector was transformed into E. coli DH5α cells. Positive recombinants were selected with LB plates containing kanamycin. Single colonies were verified by PCR, and the selected positive recombinants were sequenced by Shanghai Sangon. The recombinant plasmid was named Re-WSP1-pET28a and transformed into the E. coli BL21 (DE3) cell expression system. The transformed cells were grown in LB agar containing kanamycin. The bacteria were cultured in LB medium containing kanamycin. When the bacteria reached the logarithmic stage of growth (A600 = 0.5), the expression of the recombinant vector plasmid was induced by IPTG, and the control group was treated without IPTG. Then, the bacterial cells were collected by centrifugation and subjected to ultrasonication. The supernatant was filtered with a 0.45-μm filter for later use. Separation and purification were performed according to the instructions for affinity chromatography. After obtaining the target protein, it was analyzed by SDS-PAGE electrophoresis.

Cell proliferation and colony formation analyses

Cell proliferation was determined using a Cell Counting Kit-8 assay (Dojindo Laboratories, Kumamoto, Japan). Briefly, cells were seeded in a 96-well plate at a density of 5 × 10³ cells/well and then treated with different concentrations (0, 0.05, 0.1, 0.15, and 0.2 μg/μL) of Re-WSP1 for 24 h. At the indicated time points, 20 μL CCK8 reagent was added to each well and incubated for 4 h at 37 °C. The absorbance was detected at a wavelength of 450 nm using a Benchmark PlusTM microplate spectrometer (Bio-Rad, CA, USA).

For the colony formation assay, cells were seeded in a 6-well plate at a density of 1000 cells/well and cultured for two weeks. The cell colonies were then fixed with 10% formaldehyde (Sigma, USA), stained with 0.1% crystal violet (Sigma), and counted using a microscope (Olympus, Tokyo, Japan).

Western blotting

CRC cells were harvested and lysed with RIPA buffer (Beyotime, Jiangsu, China), and the protein concentration was determined with a BCA Protein Assay Kit (Pierce, Bonn, Germany). Protein expression was determined by western blotting as previously described [10]. The following primary antibodies were used in this study: β-catenin (1:1000, Santa Cruz, CA, USA), c-Myc (1:1000, Santa Cruz), cyclin D (1:1000, Santa Cruz, CA, USA) and GAPDH (1:3000, Santa Cruz). GAPDH was used as the control.
RNA extraction and quantitative PCR (qPCR)

Total RNA was isolated from HT29 and DLD1 cells using the mirVana miRNA Isolation Kit (Ambion, Carlsbad, CA, USA), and qPCR was performed using the microRNA Assay Kit (Ambion) and an Applied Biosystems 7900HT system (Applied Biosystems, CA, USA). U6 as the internal control. The expression level was calculated by the $2^{-\Delta\Delta C_t}$ method.

Dual-luciferase reporter assay

The β-catenin 3′UTR was amplified by PCR and subcloned into the psiCHECK2.0 vector (Ambion, Austin, TX, USA), and the resulting plasmid was named β-catenin-3′UTR. For the luciferase reporter assay, HT29 cells were cotransfected with the miR-539 mimic (GenePharma Co., Shanghai, China), corresponding negative control mimic (miR-NC, GenePharma), miR-539 inhibitor (GenePharma) and negative control inhibitor (NC inhibitor, GenePharma) using Lipofectamine 2000. The relative luciferase activity was determined using a Dual-Luciferase® Reporter Assay Kit (Promega, Madison, WI, USA) according to the manufacturer’s protocol 48 h after transfection. The Renilla luciferase activity was normalized to the firefly luciferase activity.

Statistical analysis

All the statistical analyses were carried out using SPSS statistical software for Windows Version 20 (SPSS, Chicago, IL, USA). The values are expressed as the mean ± standard deviation (SD) from at least three independent experiments. Comparisons between groups were analyzed by Student’s t-test or one-way ANOVA with Dunn’s least significant difference test. In all cases, P < 0.05 was considered to indicate statistical significance.

Results

Re-WSP1 inhibits proliferation and colony formation in CRC cells

To assess the biological effects of Re-WSP1 on CRC cells, CCK8 and colony formation assays were performed. The results demonstrated that cell viability gradually decreased with increasing Re-WSP1 concentration, and the number of cell clones in the Re-WSP1-treated group was substantially lower than that in the control group (Fig. 1A, B).

β-catenin mediates the inhibitory effect of Re-WSP1 on CRC cells

Aberrant activation of the β-catenin signaling pathway plays an important role in the development and progression of colon cancer. Thus, we detected the expression levels of β-catenin and its downstream factors, c-Myc and cyclin D, after treatment with different concentrations of Re-WSP1. The results showed that Re-WSP1 treatment decreased the expression levels of β-catenin, c-Myc and cyclin D in HT-29 and DLD1 cells (Fig. 2A, B). To further validate the involvement of β-catenin in the Re-WSP1-induced inhibition of CRC cell growth, cells were treated with an activator of the Wnt signaling pathway (LiCl). LiCl treatment markedly reversed the changes in the expression levels of β-catenin, c-Myc and cyclin D, indicating that β-catenin mediates the inhibitory effect of Re-WSP1 on CRC cells (Fig. 2C, D).

miR-539 directly inhibits β-catenin expression

It is known that the expression of β-catenin is regulated by miRNAs at the posttranscriptional level. By screening the miRanda and TargetScan databases, it was found that miR-543, miR-212, miR-539, miR-132, miR-129 and...
miR-221 were predicted to target β-catenin, so we assessed the expression levels of these miRNAs after Re-WSP1 treatment using qPCR. The results showed that among the miRNAs mentioned above, miR-539 expression was significantly increased in a dose-dependent manner, while the other miRNAs were not significantly changed in either the HT-29 or DLD1 cells (Fig. 3A, B).

To determine the relationship between miR-539 and β-catenin, the expression levels of miR-539 and β-catenin were detected after cells were transfected with the miR-539 mimic and inhibitor. The results showed that miR-539 mimic treatment increased miR-539 expression and decreased β-catenin expression, while miR-539 inhibitor treatment exerted the opposite effects (Fig. 3C, D), indicating that miR-539 negatively regulates the expression of β-catenin.

Based on the evidence described above, we focused on whether miR-539 repressed the expression of β-catenin by directly binding to the 3'UTR of β-catenin mRNA. Putative miR-539 binding sites in the 3'UTR of β-catenin were predicted through bioinformatics analysis (Fig. 4A). The dual-luciferase reporter system was used to determine whether miR-539 directly regulates β-catenin expression. A dual-luciferase reporter vector containing the β-catenin 3'UTR was constructed, and then, cells were cotransfected with the miR-539 mimic and inhibitor. The cells transfected with the miR-539 mimic showed decreased luciferase activity, and the cells transfected with the miR-539 inhibitor showed increased luciferase activity, indicating that miR-539 can directly target the β-catenin 3'UTR, thereby inhibiting the expression of β-catenin (Fig. 4B).
To verify the role of miR-539 in the growth inhibition induced by Re-WSP1, cells were treated with both Re-WSP1 and the miR-539 inhibitor, and the cell survival rate was markedly increased compared with that of the cells treated with Re-WSP1 alone. This result indicates that the cell growth inhibition induced by Re-WSP1 can be partly neutralized by miR-539 inhibitor (Fig. 4C). Furthermore, after the cells were transfected with the miR-539 inhibitor, Western blot results showed that the decrease in β-catenin expression induced by Re-WSP1 was significantly reversed, indicating that miR-539 mediates the Re-WSP1-induced inhibition of β-catenin expression (Fig. 4D).

**Discussion**

The land vegetable is a combination of fungi and algae, generally grows in dark and humid places, is dark black, and is slightly soft like Black fungus. Scientists from the Weizmann Institute of Israel have found that a component of the genus *Dioscorea* could inhibit the activity of acetylcholinesterase in the human brain, thereby producing a therapeutic effect on Alzheimer’s disease [11]. Our results confirmed that Re-WSP1 could inhibit the proliferation and colonization of CRC cells (Fig. 1) and that the expression of miR-539 in CRC cells notably increased when the cells were treated with increasing concentrations of Re-WSP1 (Fig. 4). These results indicate that the anti-CRC effects of Re-WSP1 are associated with the expression of miR-539.
We know that β-catenin is a critical component of the well-studied Wnt/β-catenin signaling pathway. Studies have shown that the abnormal expression of β-catenin is positively correlated with the conversion of colon adenoma to CRC [12–14].

miRNAs participate in multiple biological processes, including metabolic homeostasis, cell proliferation, invasion, cell cycle progression and apoptosis [15, 16]. In papillary thyroid carcinoma, the upregulation of miR-155 expression promotes tumor cell invasion by activating β-catenin [17]. In neuroblastoma, miR-155 is able to promote disease progression by activating β-catenin [18]. In addition, hepatitis virus can also regulate the Wnt/β-catenin signaling pathway by upregulating the expression of miR-155, thereby initiating liver cancer [19]. However, in CRC, whether miR-539 is involved in the pathogenesis of CRC and its role in the proliferation of CRC cells have not yet been reported. In the present study, we found that miR-539 negatively regulated the expression of β-catenin by directly binding to its 3′UTR, suggesting a tumor suppressor role for miR-539 in CRC.

In summary, we preliminarily established that miR-539 played an important role in the anti-CRC effect induced by Re-WSP1. Re-WSP1 negatively regulated the expression of β-catenin by upregulating the expression of miR-539 and then participated in the regulation of the β-catenin signaling pathway, resulting in the inhibition of CRC cell growth (Fig. 5). This study revealed a novel mechanism underlying the Re-WSP1-induced toxicity in CRC cells.

Fig. 4 Re-WSP1 suppresses colon cancer cell proliferation through the miR-539/β-catenin signaling pathway. A Schematic of the 3′-UTR of β-catenin containing the miR-539 binding site. B Dual-luciferase reporter assay of cells cotransfected with miR-539 or NC miRNAs and the 3′-UTR of β-catenin. *p < 0.05 vs. NC miRNAs. C Cell proliferation after the cells were treated with Re-WSP1 and transfected with the miR-539 inhibitor. *p < 0.05 vs. control. D β-catenin expression detected by Western blotting after the cells were treated with Re-WSP1 and transfected with the miR-539 inhibitor.
that was mediated by miR-539 and shed light on the function of miR-539 in the regulation of CRC cell proliferation. This study might open up some new possibilities for Re-WSP1 as a future therapeutic strategy.

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**Data availability** The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

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