Inhibitory effect and mechanism of exogenous mammalian sterile 20-like kinase 1 on the growth of human colorectal cancer

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Abstract. The present study aimed to observe the inhibitory effect and preliminary mechanism of exogenous mammalian sterile 20-like kinase 1 (MST1) on the growth of colorectal cancer SW480 cells. The SW480 cells were randomly divided into the following groups: Control, empty enhanced green fluorescent protein (EGFP) plasmid (pEGFP-N1), MST1 EGFP plasmid (pEGFP-MST1), 20 µmol/l fluorouracil (5-FU) and pEGFP-MST1 + 5-FU. An MTS colorimetric assay was used to detect cell viability, Hoechst 33342 staining was used to observe cell apoptosis, and western blotting and immunohistochemistry were used to detect the levels of the proteins MST1, yes-associated protein (YAP), phospho-YAP1 (Ser127), p53 and p53 upregulated modulator of apoptosis (PUMA). In addition, nude mice were injected with SW480 cells to assess the tumor inhibition rates. Compared with the control group, the growth inhibition and apoptosis rates, the levels of MST1, p53 and PUMA, and the ratios of phospho-YAP1/YAP in the pEGFP-MST1 and pEGFP-MST1 + 5-FU groups were increased significantly (P<0.01). Additionally, relative to the control group, the tumor inhibition rates in the nude mice transplanted with SW480 cells of the pEGFP-MST1 and pEGFP-MST1 + 5-FU groups were 48.52±1.63 and 87.28±2.58%, respectively, and the positive rates of phospho-YAP1 (Ser127) protein in nuclei increased significantly (P<0.01). Overall, exogenous MST1 effectively inhibited the proliferation and growth of transplanted human colorectal cancer cells and promoted cancer cell apoptosis. The mechanism involved may be associated with the increase of intracellular phospho-YAP1 (Ser127) protein.

Introduction

Colorectal cancer is one of the most common types of malignant tumor in humans, ranking third in the world with respect to incidence (1). The incidence and mortality of colorectal cancer in China is increasing, and the mortality rate has risen to the fourth highest of all types of malignant tumor (2). It has been confirmed that the occurrence and development of colorectal cancer is closely associated with the abnormal activity of the Hippo signaling pathway (3-5). The Hippo signaling pathway is a highly conserved growth control signaling pathway that was identified in Drosophila in 1995 (6), for which mammalian sterile 20-like kinase 1 (MST1) is one of the core members. MST1 and MST2 are members of the class II germinal center kinase family, with 76% of their amino acid sequence in common (7). In 2005, Huang et al (8) first identified the signaling transduction pathway of the Hippo pathway from the cytoplasm to cell nuclei in Drosophila, thus regulating the expression of downstream genes, cell growth, proliferation, and apoptosis. Dong et al (9) subsequently established the yes-associated protein (YAP) conditional transgenic mouse model, and determined the signal transduction orders of the MST1-YAP pathway in mammals. Zhou et al (10) found that mice lacking MST1/2 could not inhibit the proliferative and anti-apoptotic activity of the carcinogenic gene YAP1, resulting in the formation of liver cell carcinoma. However, subsequent to the transfection of MST1 into HepG2 cells, MST1 was re-expressed, resulting in the loss of the proliferative activity of YAP1, the promotion of apoptosis and the elimination of tumorigenicity (11).

The effect of exogenous MST1 on preventing and treating colorectal cancer has not yet been reported. With the development of molecular targeting therapy, numerous tumor-associated signaling molecules have become therapeutic targets, and targeted therapy may become a novel direction in the treatment of colorectal cancer. The present study transfected the MST1 gene into SW480 cells, aiming to investigate the inhibitory effect and mechanism of exogenous MST1 on the growth of human colorectal cancer SW480 cells grafted...
in nude mice, and to provide novel experimental evidence for targeted therapy against colorectal cancer.

Materials and methods

Transfection. PolyJet™ in vitro DNA transfection reagent (cat. no. SL100688; SignaGen Laboratories, Rockville, MD, USA) was used to mediate the transfection of the MST1 recombinant enhanced green fluorescent protein (EGFP) plasmid pEGFP-N1-MST1 and empty pEGFP-N1 plasmids (Clontech Laboratories, Inc., Mountainview, CA, USA) into human colorectal cancer SW480 cells (purchased from Cell Library Of Typical Culture Collection Committee, Chinese Academy Of Sciences). Firstly, SW480 cells were cultured in high-glucose (H)-Dulbecco’s modified Eagle’s medium (DMEM) complete culture medium (Solarbio, Beijing, China) plus 10% fetal calf serum (TransGen Biotech, Beijing, China), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in 6-well plates until 60-70% of the culture area was covered. The original medium was then discarded, 900 µl fresh complete medium was added into each well, and the cells were incubated at 37°C for 30-60 min. Transfection solution (50 µl) was added to each well, which was then gently mixed and cultured at 37°C and 5% CO₂ for 5 h. The transfection solution was subsequently replaced with DMEM containing 10% fetal bovine serum and the cells were re-cultured at 37°C for 24-48 h. The transfection efficiency was observed and images were captured with a fluorescence microscope.

The transfection solution was prepared as follows: Solution A, 1 g plasmid was dissolved in 50 µl serum-free DMEM culture and pipetted 3-4 times; solution B, 3 µl PolyJet reagent solution was added to 50 µl serum-free DMEM and pipetted 3-4 times; and solution C (transfection solution), solution B was quickly added to solution A, rapidly pipetted 3-4 times and left to stand at room temperature for 15 min.

Experimental grouping. The colorectal cancer SW480 cell line were cultured in H-DMEM complete culture medium containing 100 U/ml penicillin and 100 U/ml streptomycin at 37°C and 5% CO₂ for 48-78 h. Cells in the logarithmic growth phase were then randomly divided into 5 groups: i) Control group, subsequent to seeding the cells into the plate, the culture medium was discarded the next day and replaced by serum-free culture medium for a 6-h culture, then the culture medium was discarded and replaced by complete medium for another 48-h culture; ii) the pEGFP-N1 group, transfected with the empty pEGFP-N1 plasmid and cultured for 48 h; iii) the pEGFP-MST1 group, transfected with the pEGFP-N1-MST1 plasmid and cultured for 48 h; iv) the fluorouracil (5-FU) group, 20 µmol/l 5-FU (Ameresco Inc., Framingham, MA, USA) was added 1 day after seeding, then cultured for 48 h; and v) the pEGFP-MST1 + 5-FU group, transfected by the pEGFP-MST1 plasmid on the next day of seeding and cultured for 24 h, prior to adding 20 µmol/l 5-FU for another 24-h culture.

MTS colorimetric assay. The cells were harvested and a single cell suspension was prepared with H-DMEM, at a density of 1x10⁵-1x10⁶ cells/well. The cells were seeded into 96-well plates, with 200 µl in each well and the edge well filled with sterile phosphate-buffered saline (PBS). The cells which were divided into 5 groups and cultured at 37°C and 5% CO₂ for 24, 48 or 72 h. A total of 20 µl MTS (Sigma-Aldrich; EMD Millipore, Billerica, MA, USA) was then added to each well, and the culture was terminated subsequent to 4 h and agitated for 10 min to fully dissolve the crystals. The absorbance of each well was measured at 490 nm and the inhibition rate was calculated. Cell growth inhibition rate (%)=1-cell survival rate OR=(1-absorbance of the experimental group at 490 nm/absorbance of the control group at 490 nm) x100.

Hoechst 33342 staining. The SW480 cells were collected and seeded into 12-well plates at a density of 1x10⁵ cells/well; the cell concentrations were adjusted so that the adherent cells reached 70-90% of the culture area when transfected. The 12-well plates were cultured at 37°C and 5% CO₂ for 48 h, then the culture medium was discarded and the plates were washed with PBS 2-3 times. A total of 500 µl Hoechst 33342 (Beyotime Institute of Biotechnology, Haimen, China) dye/well was added in darkness and cultured at 37°C and 5% CO₂ for 20-30 min. The Hoechst 33342 dye was then discarded and the plates were washed with PBS 2-3 times. The level of apoptosis was observed and images were captured under a fluorescence microscope, with the number of total and apoptotic cells counted in 10 randomly selected high-power fields to calculate the cell apoptotic rate. Apoptotic rate=apoptotic cells/total cells x100.

Western blot analysis. SW480 cells were washed with PBS and lysed with radioimmunoprecipitation assay (RIPA) cell lysis reagent containing proteinase and phosphatase inhibitors (Solarbio) at 4°C for 30 min. Cell extracts were centrifuged at 12,000 x g for 20 min at 4°C, and the supernatants containing total proteins were mixed with an equal volume of 5X sodium dodecyl sulfate (SDS) loading buffer. Samples were heated at 95°C for 5 min, separated by 12% gel electrophoresis, and then electrotransferred onto 0.22-mm polyvinylidene difluoride membrane. The membranes were then incubated with antibodies (dilution, 1:200) against p53-upregulated modulator of apoptosis (PUMA; cat. no. 55120-1-AP; ProteinTech Group, Inc., Chicago, IL, USA), p53 (cat. no. 2524S; Cell Signaling Technology, Inc., Danvers, MA, USA), MST1 (cat. no. ab51134; Abcam, Cambridge, UK), YAP1 (cat. no. 4912; Cell Signaling Technology, Inc.) and phospho-YAP (cat. no. ab76252; Abcam) overnight at 4°C. The membranes were then cultured with horseradish peroxidase-conjugated goat anti-rabbit (cat. no. ZDR-5306) and goat anti-mouse (cat. no. ZDR-5307) secondary antibodies (ZSGB-BIO, Beijing, China; dilution, 1:2,000) for 1.5 h. An anti-β-actin monoclonal antibody (cat. no. SC-47778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; dilution, 1:1,000) was used as an internal control. The protein signals were detected by the enhanced chemiluminescence (ECL) detection system (Pierce; Thermo Fisher Scientific, Inc.). The gray scale of the protein bands was scanned using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). The intensity of the western blot bands was quantified using ImageJ 1.43.67 Launcher Symmetry software (National Institutes of Health, Bethesda, MD, USA). The relative protein level was calculated as follows: Relative protein level=0.25x (gray scale ratio between target gene
product bands and β-actin protein bands in the experimental group (gray scale ratio between target gene product bands and β-actin protein bands in the control group).

Animal experiments. A total of 40 BALB/c mice (specific pathogen-free, 4-week-old, female mice, weighing ~20 g and purchased from Hunan SJA Laboratory Animal Co., Ltd., Changsha, China) were raised in a sterile environment in laminar flow cabinets with disinfectant-treated baskets and bedding, adequate feed and drinking water, aseptic operation, constant temperature (18-22˚C), constant humidity (50-60%). The tumors were cut into 3-μm sections with scissors, fixed with 4% paraformaldehyde overnight and embedded in paraffin. The tissues were then dewaxed, and the expression levels of MST1, phospho-YAP1 (Ser127) and PUMA proteins were detected by immunohistochemistry, according to the protocol of the immunohistochemistry kit (cat. no. PV-9000; ZSGB-BIO, Beijing, China). The appearance of deep yellow to dark-brown particles inside the nuclei or cytoplasm was considered as a positive result.

Statistical analysis. SPSS 19.0 (IBM SPSS, Armonk, NY, USA) statistical software was used to analyze the experimental data. The parameters amongst the groups were analyzed with a one-way analysis of variance or unpaired Student’s t-test and the pairwise comparison among groups was analyzed using Dixon’s q test. P<0.05 was considered to indicate a statistically significant difference.

Results

Transfection efficiency of plasmids. As shown in Fig. 1, cells transfected with pEGFP-N1 and pEGFP-MST1 plasmids exhibited green fluorescence under the fluorescence microscope, while the control group exhibited no fluorescence, suggesting that the plasmids containing pEGFP-N1 or pEGFP-MST1 had entered the SW480 cells.

Effect of overexpression of MST1 on colorectal cancer SW480 cell proliferation. Fig. 2 shows that compared with the control or pEGFP-N1 groups, the rates of inhibition of cell growth of the pEGFP-MST1, 5-FU and pEGFP-MST1 + 5-FU groups were significantly increased (P<0.01). In addition, compared with the pEGFP-MST1 group or the 5-FU group, the inhibition rate of the pEGFP-MST1 + 5-FU group increased significantly (P<0.01), suggesting that the combination of MST1 and 5-FU exhibited the greatest inhibitory effect towards the proliferation of the SW480 cells.

Effect of overexpression of MST1 on colorectal cancer SW480 cell apoptosis. The results of the Hoechst 33342 staining (Fig. 3) showed that the nuclei of the control group were pale blue, and present as full circles, while the
apoptotic nuclei were dark blue, and apoptotic bodies were observed in certain nuclei. Compared with the control and pEGFP-N1 groups, the pEGFP-MST1 group exhibited a significantly greater number of apoptotic cells (P<0.01). The pEGFP-MST1 + 5-FU group exhibited the largest number of apoptotic cells and, compared with the pEGFP-MST1 or the 5-FU group, the differences were statistically significant (P<0.01), indicating that high MST1 gene expression promoted the apoptosis of SW480 cells.

Effect of MST1 overexpression on the expression of YAP, p53, PUMA and phospho-γAPI protein in SW480 cells. The results of the western blot analysis (Fig. 4) showed that subsequent to transfection with the pEGFP-MST1 plasmid, the level of intracellular MST1 protein increased 2.2-times more than that of the control or pEGFP-N1 groups (both P<0.01), the level of phospho-YAP1 (Ser127)/γYAP increased 4.5-times (P<0.01), and the level of p53 (P<0.05) and PUMA proteins (P<0.01) also significantly increased. Compared with the control and pEGFP-N1 groups, the total level of MST of the pEGFP-MST1 + 5-FU group increased by 2.6 times (both P<0.01), the level of phospho-YAP1 (Ser127)/γYAP increased 17 times (both P<0.01), and the levels of p53 and PUMA proteins also significantly increased (all P<0.05). Compared with the pEGFP-MST1 group, the total amount of MST1 in the pEGFP-MST1 + 5-FU group increased by 16.5%,
phospho-YAP1 (Ser127)/YAP by 2.86% and the level of p53 and PUMA proteins also significantly increased (P<0.05). These results suggested that the high expression of MST1 promoted the phosphorylation of YAP protein in the SW480 cells and increased the expression levels of p53 and PUMA proteins, and that the combined application of MST1 and 5-FU exhibited a marked promotion of the phosphorylation of YAP protein in SW480 cells, as well as of the expression levels of p53 and PUMA proteins.

Effect of MST1 on the growth of human colorectal cancer SW480 cell nude mice xenografts. The results showed that the tumor inhibition rates of the pEGFP-MST1, 5-FU and pEGFP-MST1 + 5-FU groups were 48.52±1.6, 74.56±4.15 and 87.28±2.58%, respectively; compared with the control group and the negative control group, the inhibition rates of the aforementioned three groups were significantly different (P<0.01), and the inhibition rate of the combination treatment group was significantly higher than the pEGFP-MST1 and 5-FU groups (P<0.05) (Fig. 5). The results showed that exogenous MST1 exhibited a greater inhibitory effect towards human colorectal cancer nude mice xenografts, and that the combined effects of the MST1 gene + 5-FU were the greatest.

Expression levels of MST1, phospho-YAP1 (Ser127) and PUMA in nude mice xenografts. Immunohistochemistry was performed to analyze the expression of MST1, phospho-YAP1 (Ser127) and PUMA proteins in nude mice xenografts. The results showed that the positive rates of MST1 protein in the nuclei and cytoplasm of the pEGFP-MST1, 5-FU and pEGFP-MST1 + 5-FU groups increased, exhibiting a significant difference compared with the control or the empty vector treatment groups (both P<0.01), although the degree of increase in the nuclei was not as high as that in the cytoplasm (Fig. 6). The positive rate of MST1 protein in the cytoplasm of the pEGFP-MST1 + 5-FU group was the highest (both P<0.01). Compared with the control or the pEGFP-N1 treatment groups, the positive rate of phospho-YAP1 (Ser127) protein in the nuclei of the pEGFP-MST1, 5-FU and pEGFP-MST1 + 5-FU groups significantly increased (both P<0.01) (Fig. 7). The positive rates of PUMA protein in the nuclei and cytoplasm of
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pEGFP-MST1, 5-FU and pEGFP-MST1 + 5-FU groups increased (Fig. 8), and while the degree of increase in the cytoplasm was the most significant, exhibiting significant differences compared with the control or empty vector treatment groups (P<0.01), the positive rate of PUMA protein in the cytoplasm of the combination treatment group was the highest (P<0.01).

**Discussion**

The MST1 protein is distributed in the cytoplasm, is highly conserved, and possesses the function of regulating the size of organs during development, cell proliferation and apoptosis, and inhibiting tumor growth (12). MST1 gene abnormalities are commonly observed in colorectal (13,14),...
hepatocellular (11,15) and gastric (16) cancer. It has also been found that the expression of YAP in the Hippo pathway is elevated in human primary colorectal cancer (17). Previous studies had found that the role of MST1 is mainly associated with the size of the liver and heart (18–20). Since then, the number of studies investigating the associations between
MST1 and tumor cell proliferation and apoptosis has increased, and the low expression of MST1/2 (21), human Salvador 1 and Mob1 have subsequently been found in colorectal cancer (11). Additionally, it was also found that MST1-knockout mice did not inhibit the proliferation and anti-apoptosis activities of Yap1, resulting in embryonic developmental abnormality and the formation of primary hepatocellular carcinoma (10). However, one study found that liver cells lacking MST1 resisted tumor necrosis factor-induced apoptosis (16). Our previous study (11) found that the exogenously induced high expression of the MST1 gene improved MST1 kinase domain expression in HepG2 cells, upregulated the levels of Caspase-3 (CL) and phospho-YAP1 (Ser127) proteins, downregulated the levels of connective tissue growth factor, amphiregulin and survivin, and inhibited the proliferation and promoted the apoptosis of HepG2 cells. Similar results were also found in lung cancer (22). The in vitro experimental results of the present study showed that the proliferation rate of SW480 cells with MST1 overexpression significantly decreased compared with the control group (Figs. 2 and 3; P<0.01), while the apoptosis rate significantly increased (P<0.01). The animal experimental results (Fig. 5) showed that the exogenous MST1 gene exhibited an inhibitory effect with respect to the growth of SW480 cells in nude mice, and the combined antitumor effect of the MST1 gene with 5-FU increased, indicating that MST1 inhibits the proliferation and promotes the apoptosis of human colorectal cancer SW480 cells.

Numerous studies have hypothesized that the occurrence of tumors is the co-result of multiple signaling pathways, including the Hippo signaling and p53 apoptosis pathways, possessing certain functional associations (23-26). A previous study found that in adrenal pheochromocytoma PC12 cells, YAP was phosphorylated on the no. 127 site of serine, therefore entering the nuclei and interacting with p73, a protein of the p53 family, resulting in apoptosis (27). In addition, p73 was revealed to activate the cytoplasmic apoptosis-associated protein PUMA in a transcription-dependent manner, thus promoting the release of cytochrome c inside mitochondria, resulting in apoptosis (28). The present study found that the overexpression of MST1 promoted the phosphorylation of YAP protein in human colorectal cancer SW480 cells, and increased the expression levels of intracellular pro-apoptotic proteins, specifically p53 and PUMA. Furthermore, the combination of MST1 and 5-FU markedly promoted the phosphorylation of YAP protein and the expression of p53 and PUMA proteins in the SW480 cells. The immunohistochemical results in nude mice xenografts confirmed that the overexpression of MST1 promoted the phosphorylation of YAP protein, and increased the expression of p53 and PUMA proteins in human colorectal cancer SW480 cells in nude mice xenografts (Figs. 6-8), thus resulting in an increased level of apoptosis and the inhibition of the proliferation of cancer cells. This may be one of the important mechanisms of the overexpression of MST1. Therefore, the present study hypothesizes that in the occurrence and development of colorectal cancer, there may be mutual intersections with respect to the Hippo, p53 and PUMA-mediated mitochondrial apoptosis signaling pathways. As no study had investigated this previously in colorectal cancer, this theory was worthy of in-depth study.

In conclusion, the present study considered that exogenous MST1 inhibited the growth of colorectal cancer SW480 cells in nude mice xenografts, and that the mechanism of this may be via MST1 promoting the activation of cytoplasmic YAP, resulting in phospho-YAP1 (Ser127) entering the nuclei and upregulating the expression levels of p53 and PUMA, promoting the activation of caspase-3 and inducing apoptosis. An increase in the level of MST1 may also inhibit the entry of cytoplasmic YAP into the nuclei, causing the expression levels of the pro-proliferation-associated genes to decrease, weakening the proliferation ability of the cells. Therefore, MST1 may become a novel target for the prevention and treatment of human colorectal cancer or other types of tumor.

Acknowledgements

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