

Effect of MST1 on Invasion and Migration of Colon Cancer through Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase Signal Pathway

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Wenmin *et al.*: To Explore the Effect of MST1 on Colon Cancer

To explore the effect of mammalian STE20 like protein kinase 1 on colon cancer invasion and metastasis through mitogen-activated protein kinase/extracellular signal-regulated kinase signaling pathway. Three groups of cells were set up; blank control group, colon cancer group and mammalian STE20 like protein kinase 1 overexpression group. The proliferation ability of the three groups of cells was assessed using cell counting kit 8, protein expression was detected using Western blot, the expression level of relevant messenger ribonucleic acid was determined using quantitative polymerase chain reaction, and the migration and invasion ability of the cells was evaluated using Transwell. The expression level of B-cell lymphoma 2 was markedly significantly lower reduced than that of colon cancer group. The relative expression of mitogen-activated protein kinase, extracellular regulated kinase messenger ribonucleic acid and protein in the colon cancer group was markedly significantly higher than that in the blank control group; the relative expression of mitogen-activated protein kinase, extracellular regulated kinase messenger ribonucleic acid and protein in the mammalian STE20 like protein kinase 1 overexpression group was markedly significantly lower reduced than that in the colon cancer group. Overexpression of mammalian STE20 like protein kinase 1 can block the mitogen-activated protein kinase-extracellular regulated kinase signal transduction pathway, reduce the viability of colon cancer cells, restrain the proliferation growth, migration and invasion of colon cancer cells, and induce apoptosis of colon cancer cells, thus ultimately contributing to the establishment of a theoretical foundation for the development of targeted therapies for colon cancer.

Key words: Mammalian STE20 like protein kinase 1, mitogen-activated protein kinase/extracellular signal-regulated kinase, colon cancer, migration, invasion, colon cancer

Colon Cancer (CC) is a prevalent malignancy affecting the digestive system, specifically the colon, within the realm of clinical practice, with high morbidity and mortality^[1]. As the aging population continues to grow and societal living habits undergo transformation, the incidence of CC is gradually increasing, which seriously threatens people's life and health^[1]. There exists a deficiency in curative modalities for the clinical management of patients diagnosed with CC, and radiotherapy and surgical resection are mainly used. In recent years, with the progress of targeted therapy, more and more malignant tumors have been gradually treated with targeted therapy. Therefore, conducting comprehensive investigations into the pathogenesis and etiology of CC, alongside the exploration of novel therapeutic targets,

holds immense clinical importance in enhancing patient's clinical manifestations, slowing down the progression of cancer, and improving patient's quality of life^[2]. The Hippo pathway, which is considered to be related to the growth of tissues and organs, was firstly found in *Drosophila*, and the later study confirmed that the abnormal activity of the Hippo signaling pathway is closely associated to the occurrence and development of colorectal cancer^[3]. Mammalian STE20-like Kinase 1 (MST1) is one of the core members of the Hippo pathway,

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which was found to play an important role in cell growth, proliferation, apoptosis, maintenance of organ size and tumorigenesis^[4]. In addition, the Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase (MAPK-ERK) signaling pathway is closely related to tumorigenesis and progression, which is an important proliferative and anti-apoptotic pathway in cells^[5]. This study aimed to examine the impact MST1 on CC invasion and metastasis through the MAPK-ERK signaling pathway, with a view to providing potential possible therapeutic targets for clinical CC treatment.

MATERIALS AND METHODS

Materials and reagents:

Both the human normal intestinal mucosal cell line (FHC) and the human rectal cancer cell line SW837 were purchased from the American Type Culture Collection (ATCC) cell bank in the United States. MST1, MAPK, ERK and Beta (β)-actin primers (Sigma, Inc., USA); Cell Counting Kit 8 (CCK-8) detection kit (Shanghai Biyuntian Company); Transwell chambers (Corning, USA); artificial reconstructed basement film glue (Matrigel) purchased from BD Company in the United States; MST1, MAPK, ERK, Fas, Bcl-2-Associated X protein (BAX), Fas ligand (FasL), B-Cell Lymphoma 2 (Bcl-2) primary antibody (Abcam Biotechnology Ltd.); the cell MST1 gene required for this study overexpresses lentivirus (Guangzhou Saiye Biotechnology Co., Ltd.).

Methods:

Cell culture and treatment: The cells were cultured in T25 culture flasks using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum, and incubated at a constant temperature of 37° with 5 % Carbon dioxide (CO₂) for cultivation, and then trypsin digested, passaged, and frozen in accordance with the growth of the cells. The blank control group were human normal intestinal mucosa cell line (FHC); and the cells in the CC group were human rectal cancer cell line SW837 and were cultured routinely without any treatment; and the cells in the MST1 overexpression group were cultured in the CC group as the progeny of the human rectal cancer cell line SW837 infected with lentiviral overexpression of the MST1 gene. Each experiment

was repeated 6 times.

Western blot: Adjust the cell density to 1×10^6 cells/well inoculated in 6-well plates, incubated in a constant temperature incubator for culture, MST1 overexpression group of cells by MST1 gene overexpression lentivirus infection after the stable passaging, add the cell protein lysate at 4° homogenate made of 10 % homogenate, centrifugation of the supernatant to be examined, the Bicinchoninic Acid (BCA) assay method to determine the concentration of protein, gel, electrophoresis for 90 min, cut the gel, transfer membrane 90 min, milk closed, washed and sequentially with MST1, MAPK, ERK, Fas, BAX, and FasL. The gel was cut, electrophoresed for 90 min, milk was sealed, washed, and then sequentially incubated with primary and secondary antibodies, developed, and Bio-Rad Image Lab software was used to analyze the results.

Quantitative Polymerase Chain Reaction (qPCR) detection of relevant messenger Ribonucleic Acid (mRNA) expression level: Adjust the cell density to 1×10^6 cells/well inoculated in 6-well plates and cultured in a constant temperature incubator, cells were cultured in the incubator after adding p-nitrophenylethyl caffeic acid 10 μ g/ml for 48 h, following the instructions of the TRIzol reagent, Then RNA was reversely transcribed into complementary Deoxyribonucleic Acid (cDNA). We performed the amplification reactions as per the instructions provided with the Tras Start Top Green qPCR super mix. After the reaction was completed, a software program was used to calculate relative mRNA expression.

CCK-8 method: After the cells in the MST1 overexpression group were stably passaged by MST1 gene overexpression lentivirus infection, 10 μ l of CCK8 solution was added to each well of the blank control group, CC group, and MST1 overexpression group, and then cultured in incubator for 4 h, and then detected the proliferative capacity of the cells in each group in an enzyme labeler (Optical Density (OD) 450 nm).

Transwell assay: After the cells were added with resveratrol 15 μ g/ml and cultured for 48 h, each group was adjusted to have a cell density of 5×10^5 cells/well inoculated in the upper chamber of the Transwell, in the lower chamber, medium containing 10 % fetal bovine serum was added.

The control group was given the same amount of DMEM culture medium, fixed, stained, and then microscopically count the purple stained perforated cells and calculate the cell migration ability. For the detection of cell invasion ability, the upper chamber of the Transwell was first covered with Matrigel in an ultra-clean bench, and the subsequent steps were the same as that of cell migration.

Statistical methods:

Statistical methods analysis and processing of data were carried out using the Statistical Package for the Social Sciences (SPSS) 22.0 statistical software, and the measurement information was expressed by ($x \pm s$), and the comparison was made by t-test. In comparison to the blank control group, ^a $p < 0.05$ and in comparison to the TNBC group, ^b $p < 0.05$.

RESULTS AND DISCUSSION

The levels of MST1 protein and mRNA were markedly elevated in the CC group in comparison to the blank control group. Furthermore, MST1 protein and mRNA were significantly higher in the MST1 overexpression group compared to the CC group as shown in Table 1.

The CC group exhibited significantly higher levels

of cell, growth and cell metastasis compared to the blank control group ($p < 0.05$). Conversely, the MST1 overexpression group demonstrated significantly markedly lower levels of cell proliferation, cell migration, and cell invasion compared to the CC group as shown in Table 2.

The levels of apoptotic proteins Fas, Bax and FasL in the group with MST1 overexpression exhibited a statistically significant increase compared to the CC group. Conversely, Bcl-2 was markedly decreased in the MST1 overexpression group compared to the CC group ($p < 0.05$), as indicated in Table 3.

The mRNA levels of MAPK and ERK in cells of the CC group were found to be markedly higher compared to the blank control group. Conversely, the MAPK and ERK in cells of the MST1 overexpression group were significantly markedly lower reduced than those in the CC group, as shown in Table 4.

The levels of MAPK and ERK proteins of the CC group demonstrated a statistically significant elevation in comparison to the blank control group. Conversely, the expression levels of MAPK and ERK proteins in the cells of the MST1 overexpression group demonstrated a statistically significant elevation in comparison to the CC group ($p < 0.05$), as depicted in Table 5.

TABLE 1: ESTABLISHMENT OF OVEREXPRESSING MST1 CELL LINES

Group	n	MST1 protein	MST1 mRNA
Control	6	0.97±0.23	0.99±0.25
CC	6	0.51±0.13 ^a	0.59±0.17 ^a
MST1 overexpression	6	1.34±0.25 ^b	1.36±0.33 ^b
F		23.524	13.327
p		0.000	0.001

Note: Compared to the blank control group, ^a $p < 0.05$ and compared to the TNBC group, ^b $p < 0.05$

TABLE 2: EFFECT OF OVEREXPRESSION OF MST1 ON GROWTH AND METASTASIS OF CC CELLS

Group	n	Cell proliferative activity	Number of cell migration	Number of cell invasions
Control	6	0.10±0.01	73.25±15.70	51.72±10.31
CC	6	0.22±0.04 ^a	142.74±24.34 ^a	121.05±20.26 ^a
MST1 overexpression	6	0.18±0.03 ^b	106.48±17.44 ^b	93.12±16.35 ^b
F		25.846	19.022	26.157
p		0.000	0.000	0.000

Note: Compared to the blank control group, ^a $p < 0.05$ and compared to the TNBC group, ^b $p < 0.05$

TABLE 3: EFFECT OF OVEREXPRESSION OF MST1 ON APOPTOTIC PROTEIN EXPRESSION IN CC CELLS

Group	n	Fas	BAX	FasL	Bcl-2
Control	6	0.35±0.14	0.37±0.09	0.41±0.14	0.54±0.03
CC	6	0.33±0.17 ^a	0.43±0.14 ^a	0.44±0.14 ^a	0.49±0.04 ^a
MST1 overexpression	6	0.84±0.18 ^b	0.86±0.16 ^b	0.78±0.12 ^b	0.31±0.03 ^b
F		13.446	20.294	18.641	72.177
p		0.001	0.000	0.000	0.000

Note: Compared to the blank control group, ^ap<0.05 and compared to the TNBC group, ^bp<0.05

TABLE 4: EFFECT OF OVEREXPRESSION OF MST1 ON THE RELATIVE EXPRESSION OF MAPK AND ERK mRNA IN CC CELLS

Group	n	MAPK mRNA	ERK mRNA
Control	6	1.24±0.32	0.83±0.35
CC	6	2.47±0.54 ^a	2.59±0.67 ^a
MST1 overexpression	6	1.65±0.40 ^b	1.76±0.43 ^b
F		12.744	18.451
p		0.000	0.000

Note: Compared to the blank control group, ^ap<0.05 and compared to the TNBC group, ^bp<0.05

TABLE 5: EFFECT OF OVEREXPRESSION OF MST1 ON THE EXPRESSION LEVELS OF MAPK AND ERK PROTEINS IN CC CELLS

Group	n	MAPK	ERK
Control	6	0.18±0.04	0.23±0.03
CC	6	0.85±0.27 ^a	0.83±0.25 ^a
MST1 overexpression	6	0.44±0.11 ^b	0.49±0.17 ^b
F		23.716	17.656
p		0.000	0.000

Note: Compared to the blank control group, ^ap<0.05 and compared to the TNBC group, ^bp<0.05

In recent years, there has been a noticeable shift in individual's lifestyle choices, eating habits, and occupational settings, the incidence of CC has been on the rise year by year, and it has become a more common digestive malignant tumor in clinical practice^[6]. The pathogenesis and etiology of CC have not yet been fully elucidated, but according to a recent study, age, genetic susceptibility, inflammatory response, immune disorders, physical and chemical radiation, and other factors are all related to the development of CC^[7]. Currently, clinical treatment is mainly based on radiotherapy as well as surgical resection, which can help some patients to cure their cancer, but some patients are diagnosed late, lose the opportunity of surgical resection of tumor treatment in late stage, and are insensitive to radiotherapy treatment, which leads to limited clinical treatment effect^[8]. CC is characterized by high morbidity, invasiveness, metastatic potential and recurrence rate, and it is of great clinical significance to carry out radical treatment targeting cancer cells for CC patients^[9].

Therefore, in-depth study of the pathogenesis and etiology of CC, determining the molecular mechanisms of growth and metastasis of CC cells, and the exploration of novel therapeutic targets holds significant clinical importance in enhancing the clinical manifestations experienced by patients, slow down the pathological progression of CC, and improve the quality of life of patients. This study aimed to examine the impact of MST1 on CC invasion and metastasis through the MAPK-ERK signaling pathway, with the aim of providing potential possible therapeutic targets for clinical CC treatment.

The Hippo pathway is a highly conserved signal transduction pathway, named after the *Drosophila* Hpo kinase because it was initially discovered in *Drosophila*, and tumor suppression, stem cell function, immune response, and tissue regeneration, etc.^[10]. MST1 is a serine/threonine kinase consisting of 487 amino acids, threonine kinase; MST1, being a constituent of the Hippo

pathway, assumes a significant role as a protein kinase within the Hippo pathway, which has been confirmed to be an important signaling molecule affecting cell proliferation, cell polarity, cell migration, cell proliferation and organ size^[11]. Recently, more and more research evidence suggested that regulating MST1 activity to dysregulate the Hippo pathway has the ability to contribute to cancer development. Silencing the expression of the MST1/2 gene in mice, which results in a lack of MST1/2 protein, can lead to the development of hepatocellular carcinoma in mice^[12]. In addition, patients with reduced MST1 expression in early-stage rectal cancer have a poor prognosis. All of these studies suggest that the dysregulation of MST1 has been found to be linked to the onset and progression of CC^[13]. The results of this research found that the relative expression of MST1 protein as well as mRNA in the CC group was markedly higher than the blank control group; the MST1 protein as well as mRNA in the MST1 overexpression group was markedly higher than the CC group. Cell proliferation activity, metastasis were markedly significantly higher in CC group than in blank control group; cell proliferation activity, cell migration and cell invasion metastasis were markedly significantly lower reduced in MST1 overexpression group than in CC group. It is suggested that overexpression of MST1 may lead to a decrease in the viability of CC cells, as well as hinder the proliferation and metastasis of these cells.

The aberrant expression of the Fas pathway, which is a pathway involved in programmed cell death, plays a significant role in inhibiting apoptosis. Fas and its ligand FasL are members of the tumor necrosis factor family of receptors located on the cell surface, and their interaction triggers cell death mediated by the ligand. Decreased levels of Fas and FasL, or weakened signaling, are associated with the advancement of tumors^[14]. BAX is considered a highly representative pro-apoptotic molecule, while Bcl-2 functions as an inhibitor of apoptosis molecule. Both molecules are intricately associated with the process of tumor cell apoptosis. In clinical practice, the Bcl-2/BAX ratio is frequently employed as a means to assess the extent of apoptosis in tumor cells^[15]. The findings of this study indicate that the MST1 overexpression group exhibited markedly elevated expression levels of apoptotic proteins Fas, BAX

and FasL compared to the CC group. Additionally, the bcl-2 was markedly diminished in the MST1 overexpression group when compared to the CC group. It is suggested that overexpression of MST1 can induce apoptosis in CC cells. It was found that when MST1 was stimulated by apoptotic signals, it was easily cleaved by caspase into an N-terminal active region of about 34KD-36KD, which significantly enhanced the kinase activity of MST1 and caused the cleaved N-terminal active region to be transferred to the nucleus of cells, and ultimately led to apoptosis^[16].

The MAPK-ERK signaling pathway, as a signal transduction channel with a wide range of roles in the human body, is an important signal transduction system mediating extracellular stimuli to intracellular responses, regulating cell proliferation, differentiation, migration, apoptosis, etc., and playing a significant role in the manifestation playing an important role in the occurrence and infiltration of malignant tumor cells, and participating in the regulation of cell growth, development and other processes^[17]. Numerous studies have demonstrated the significant involvement of the MAPK-ERK signaling pathway in both tumor progression and anti-tumor therapeutic interventions^[18]. MAPK is a direct target gene of MAPK and a downstream effector of MAPK, and ERK phosphorylation can be used as an indicator of MAPK activity, and ERK phosphorylation can modulate downstream proteins, thus regulating the basal functions of cells^[19]. It has been found that ERK can promote tumor angiogenesis and destroy extracellular matrix through phosphorylation reaction, and can promote the transcription and expression of a variety of oncogene-related genes, which in turn facilitates the movement of tumor cells and metastasis^[20]. Studies have confirmed that the excessive activation of the MAPK-ERK signaling pathway assumes a crucial function in governing the proliferation, viability and invasive potential of endometrial, breast, and gastric cancer cells^[21]. The findings of this study indicate that the MAPK and ERK mRNA in cells belonging to the CC group exhibited a statistically significant increase compared to the blank control group. Conversely, MAPK and ERK mRNA in cells of the MST1 overexpression group demonstrated a statistically significant decrease when compared to the CC group. MAPK and ERK proteins in cells

of CC group were markedly higher than the blank control group; MAPK and ERK proteins in cells of MST1 overexpression group were markedly reduced than the CC group. The present study proposes that overexpression of MST1 can block the MAPK-ERK signal transduction pathway, thus establishing a theoretical foundation for the therapeutic intervention of tumor metastasis and invasion in clinical settings.

In summary, overexpression of MST1 can block the MAPK-ERK signal transduction pathway, reduce the viability of CC cells, restrain the proliferation and the growth and metastasis ability of CC cells, and induce apoptosis of CC cells, thus providing a theoretical basis for the targeted therapy of clinical CC.

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Conflict of interests:

The authors declared no conflict of interests.

REFERENCES

- Idoudi S, Bedhafi T, Sahir F, Hijji Y, Uddin S, Merhi M, *et al.* Studies on anti-colon cancer potential of nanoformulations of curcumin and succinylated curcumin in mannosylated chitosan. *Int J Biol Macromol* 2023;235:123827.
- Garcia JA, Chen R, Xu M, Comerford SA, Hammer RE, Melton SD, *et al.* Acss2/HIF-2 signaling facilitates colon cancer growth and metastasis. *PLoS One* 2023;18(3):e0282223.
- Trang NT, Lai CY, Tsai HC, Huang YL, Liu SC, Tsai CH, *et al.* Apelin promotes osteosarcoma metastasis by upregulating PLOD2 expression *via* the Hippo signaling pathway and hsa_circ_0000004/miR-1303 axis. *Int J Biol Sci* 2023;19(2):412-25.
- Huang Z, Xiao PY, Chen JY, Zeng Q, Huang BX, Yu J, *et al.* Mammalian sterile 20-like kinase 1 mediates neuropathic pain associated with Its effects on regulating mitophagy in schwann cells. *Oxid Med Cell Longev* 2022;2022:3458283.
- Zhang H, Liu J, Dang Q, Wang X, Chen J, Lin X, *et al.* Ribosomal protein RPL5 regulates colon cancer cell proliferation and migration through MAPK/ERK signaling pathway. *BMC Mol Cell Biol* 2022;23(1):48.
- Rademaker G, Costanza B, Bellier J, Herfs M, Peiffer R, Agirman F, *et al.* Correction: Human colon cancer cells highly express myoferlin to maintain a fit mitochondrial network and escape p53-driven apoptosis. *Oncogenesis* 2023;12(1):11.
- Zhu L, Zhang X, Chen X, Yang D, Nie Y, Pan R, *et al.* Anti-TNFR2 enhanced the antitumor activity of a new HMGN1/3M-052 stimulated dendritic cell vaccine in a mouse model of colon cancer. *Biochem Biophys Res Commun* 2023;653:106-14.
- Wu H, Zhong W, Zhang R, Ding Y, Qu C, Lai K, *et al.* G-quadruplex-enhanced circular single-stranded DNA (G4-CSSD) adsorption of miRNA to inhibit colon cancer progression. *Cancer Med* 2023;12(8):9774-87.
- Matsuno T, Mikami T, Hayashi H, Funahashi K, Okazumi S, Hiruta N, *et al.* Estrogen receptor beta expression in colitis-associated carcinoma in comparison with sporadic colonic tumor: An immunohistochemical study. *JGH Open* 2023;7(2):110-7.
- Imam Aliagan A, Madungwe NB, Tombo N, Feng Y, Bopassa JC. Chronic GPER1 activation protects against oxidative stress-induced cardiomyoblast death *via* preservation of mitochondrial integrity and deactivation of mammalian sterile-20-like kinase/yes-associated protein pathway. *Front Endocrinol (Lausanne)* 2020;11:579161.
- Feng Y, Ci H, Wu Q. Expression of mammalian sterile 20-like kinase 1 and 2 and yes-associated protein 1 proteins in triple-negative breast cancer and the clinicopathological significance. *Medicine (Baltimore)* 2021;100(34):e27032.
- Wang P, Zhang Y, Xia Y, Xu D, Wang H, Liu D, *et al.* microRNA-139-5p promotes functional recovery and reduces pain hypersensitivity in mice with spinal cord injury by targeting mammalian sterile 20-like kinase 1. *Neurochem Res* 2021;46(2):349-57.
- Zuhra K, Tomé CS, Masi L, Giardina G, Paulini G, Malagrino F, *et al.* N-Acetylcysteine serves as substrate of 3-Mercaptopyruvate sulfurtransferase and stimulates sulfide metabolism in colon cancer cells. *Cells* 2019;8(8):828.
- Qi Y, Sun D, Yang W, Xu B, Lv D, Han Y, *et al.* Mammalian sterile 20-like kinase (MST) 1/2: Crucial players in nervous and immune system and neurological disorders. *J Mol Biol* 2020;432(10):3177-90.
- Lu K, Liu X, Guo W. Melatonin attenuates inflammation-related venous endothelial cells apoptosis through modulating the MST1-MIEF1 pathway. *J Cell Physiol* 2019;234(12):23675-84.
- Hu J, Wang S, Xiong Z, Cheng Z, Yang Z, Lin J, *et al.* Exosomal Mst1 transfer from cardiac microvascular endothelial cells to cardiomyocytes deteriorates diabetic cardiomyopathy. *Biochim Biophys Acta Mol Basis Dis* 2018;1864(11):3639-49.
- Cheng XS, Huo YN, Fan YY, Xiao CX, Ouyang XM, Liang LY, *et al.* Mindin serves as a tumour suppressor gene during colon cancer progression through MAPK/ERK signalling pathway in mice. *J Cell Mol Med* 2020;24(15):8391-404.
- Li W, Ke C, Yang C, Li J, Chen Q, Xia Z, *et al.* LncRNA DICER1-AS1 promotes colorectal cancer progression by activating the MAPK/ERK signaling pathway through sponging miR-650. *Cancer Med* 2023;12(7):8351-66.
- Pan Y, Cheung ST, Tong JH, Tin KY, Kang W, Lung RW, *et al.* Granulin epithelin precursor promotes colorectal carcinogenesis by activating MARK/ERK pathway. *J Transl Med* 2018;16(1):150.
- Meng C, Tian G, Xu C, Li X, Zhang Y, Wang Y, *et al.* Hippo kinases MST1 and MST2 control the differentiation of the epididymal initial segment *via* the MEK-ERK pathway. *Cell Death Differ* 2020;27(10):2797-809.
- Zhang SL, Zhu HY, Zhou BY, Chu Y, Huo JR, Tan YY, *et al.* Histone deacetylase 6 is overexpressed and promotes tumor growth of colon cancer through regulation of the MAPK/ERK signal pathway. *Onco Targets Ther* 2019;12:2409-19.