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Knockdown of MMP-7 inhibits cell proliferation and enhances sensitivity to 5-Fluorouracil and X-ray irradiation in colon cancer cells

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Running head: The possible roles of MMP-7 in colon cancer cells

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Abstract

The role of Matrix metalloproteinase-7 (MMP-7) in the pathogenesis of colon cancer is not understood thoroughly. Previous studies from our group have shown that the expression levels of MMP-7 were highly elevated in colorectal cancer patient specimens and were correlated with Dukes Staging, histological differentiation grade and CEA level. The goal of this study was to investigate the cellular impact of MMP-7 in colon cancer. In this study, we used the SW480 colon cancer cell lines of MMP-7 knockdown by lentivirus-mediated RNA interference as a model system to investigate the impact of MMP-7 on cell proliferation and sensitivity to 5-Fluorouracil (5-FU) and X-ray irradiation (IR). Cell proliferation and sensitivity to 5-FU and IR was measured by MTT assay and colony formation assay. Cell cycle was evaluated by flow cytometry. We showed that the down regulation of MMP-7 inhibits colon cancer cell proliferation and sensitizes tumor cells to 5-FU and IR ($P<0.05$). Decreased MMP-7 expression in SW480 cells by RNA interference triggered cell cycle arrest at G1 phase ($P<0.05$). Down regulation of MMP-7 may inhibit the cell proliferation of colon cancer cells and increase tumor cells sensitivity to radiotherapy and chemotherapy. RNAi-mediated silencing of MMP-7 may represent a powerful therapeutic approach for controlling human colorectal cancer growth.

Key words

MMP-7; colon neoplasm; proliferation; 5-Fluorouracil; X-ray irradiation

Introduction

The matrix metalloproteinases (MMPs) comprise a family of zinc-dependent endopeptidases which are typically described as enzymes capable of degrading many, if not all, components of the extracellular matrix (ECM). In view of their matrix-degrading capabilities, this group of enzymes has been extensively studied in in tumour invasion and metastasis [1–4]. Massive MMPs have been identified that show up-regulation in tumours and most tumours show enhanced MMP activity [1, 3, 5-7]. This provided the rationale for clinical trials of MMP inhibitors, unfortunately, the results of these trials have been disappointing[8].However, it is also becoming increasingly recognized that the matrix metalloproteinases are a multifunctional group of biologically important molecules with diverse roles in normal cell growth, differentiation, and cell regulation, and with involvement in the early stages of tumourigenesis. These new MMP functions are distinct from their well-defined role in degrading individual components of the extracellular matrix, and in some cases they have substrates other than components of the extracellular matrix [9-10].

MMP-7 (matrix metalloproteinase-7, matrilysin), a member of MMPs, in contrast to other MMP family members, is usually overproduced by carcinoma cells instead of stromal cells. Previous studies suggested that MMP-7 plays an important role in the invasion and progression of

tumor because of its tumor-associated expression [5-7]. Studies have shown that MMP-7 may be involved in the early stage of tumor development. For example, overexpression of MMP-7 significantly promotes tumor formation in mouse mammary glands [11]. Concordantly, ablation in MMP-7 expression results in a dramatic reduction in intestinal cancer growth in animals [12]. In addition, MMP-7 may confer apoptosis resistance to chemotherapeutic agents in colon cancer cells [13-14]. However, it remains to be determined what actual roles MMP7 plays in colon cancer development.

To address this issue, we used the lentivirus-mediated RNA interference to specifically suppress the MMP-7 expression in SW480 colon cancer cells, and then analysed the alterations of these cells in growth, colony formation ability, as well as the sensitivity to 5-Fluorouracil (5-FU) and X-ray irradiation(IR).

Materials and methods

Cell culture

Human colon cancer cell line SW480, and 293T cells were purchased from ATCC (American Type Culture Collection, Manassas, VA) and maintained in DMEM medium (Genom BioMed Technology, Inc. Hangzhou, China) supplemented with 2% penicillin-streptomycin, 1.5mM L-glutamine, 2% NaHCO₃ and 10% fetal bovine serum in a humidified atmosphere of 5% CO₂ at 37 °C.

Lentivirus-mediated RNA interference

We designed and cloned a shRNA template into a third generation self-inactivating lentivirus vector containing a CMV-driven GFP reporter and a U₆ promoter upstream of the cloning restriction sites (HpaI and XhoI). We constructed three shRNA-MMP-7 lentivirus vectors, namely LV-MMP-7shRNA-1, LV-MMP-7shRNA-2, LV-MMP-7shRNA-3, respectively (Table1), and we purchased the package plasmid, the envelop plasmid and the plasmid containing shRNA without RNA interference effect designated as LV-con (Addgene,USA). Briefly, oligonucleotides were annealed, digested and inserted between the Hpa I and Xho I restriction sites of the plasmid vector. Some mutations were introduced in the sense sequence of the hairpin structure to facilitate sequence and avoid destruction by bacteria during amplification in the bacterial host. Correct insertions of shRNA cassettes were confirmed by direct DNA sequencing. The lentivirus carrying shRNA, targeting MMP-7 or without RNA interference effect, was produced by 293T cells with lentiviral plasmids designated as LV –MMP-7 and LV-con, respectively. The SW480 cells were seeded at 2.0×10^5 cells per well into six-well plates in triplicate. After 24 h of incubation, the medium of each well was replaced with 2ml viral suspension supplemented with 8 mg/ml polybrene (Sigma-Aldrich). Then, the plates were centrifuged for 1h (25°C, 1200g), followed by a 10h incubation in standard condition, and

media were replaced with fresh DMEM. After 48 h of additional incubation, cells were passaged into 25 cm² flasks and maintained under standard conditions. The enhanced green fluorescent protein expression profile of transduced cells was observed using fluorescent microscope and was determined at 48 h after transduction using FACS Aria Cell Sorter (BD, USA).

Quantitative Realtime RT-PCR

For screening, the vectors of LV-MMP-7siRNA-1, LV-MMP-7siRNA-2, LV-MMP-7siRNA-3 or LV-con were transfected into SW480 cells as described above, compared with the untreated cells (WT), MMP7 mRNA expression was detected with quantitative real-time RT-PCR as described elsewhere[7]. Briefly, total cellular RNA was isolated with Trizol reagent (Jinmei Biotech Co., Ltd, Shenzhen, China), followed by reverse transcription with RT Reagents Kit (TaKaRa). Subsequently quantitative real-time polymerase chain reaction (qRT-PCR) was carried out with relative quantification protocol on an iCycler iQ System (BioRad Laboratories, Ltd., Hercules, CA), using TaqMan probe (TaKaRa) for amplification detection. The PCR master mix containing 1× PCR buffer, 2.5 mM MgCl₂, 0.3 mM dNTP, 0.33 mM of Taqman probe and each primer, 1.25 U Taq DNA polymerase (TaKaRa) and 1 µl cDNA in 25 µl volume were processed as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 20 sec, 53.1°C for 30 sec and 72°C for 30 sec. The expression

ratios(R) of MMP-7 mRNA were calculated by using the comparative threshold cycle (Ct) method ($\Delta\Delta C_t$) as described before[7].

Western Blotting

Total proteins were extracted using RIPA lysis buffer and the concentrations were assayed by the Bradford method using BCA Protein Assay Reagent. Protein samples (20ug/well) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), electrophoretically transferred to a nitrocellulose membrane. The membranes were blocked with 5% skim milk in TBST (20 mmol/L Tris-Hcl at PH8.0, 150 mmol/L NaCl, and 0.05% Tween 20) for 1 h at room temperature , and then incubated with 1:800 mouse anti-human MMP-7 monoclonal antibody (Santa Cruz Biotech, Inc., Santa Cruz, CA) overnight at 4°C, followed by incubation in 1:2500 dilution of secondary antibodies against rabbit or mouse IgG conjugated to horseradish peroxidase (Zhongshan Golden Bridge Biotech. Co., Ltd., Beijing, China) for 2 h at room temperature. Protein bands were detected using ECL detection system (Zhongshan Golden Bridge Bio technology, China), and β -actin staining served as loading control.

Cell proliferation assay

Cell proliferation in vitro was assayed with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) C. Briefly, The log-phase cells were seeded in

96-well plates (5×10^3 /well) in 200 μ l of medium. After 24, 48, 72 and 96 h of incubation respectively, then added 20 μ l MTT to cells and incubated for a further 4 hours at 37°C. Subsequently, the medium was removed, formazan crystals were solubilized with 150 μ l of dimethyl sulphoxide (DMSO, Sigma). Finally, cells were shaken well for 10 minutes, and the absorbance (A) value was measured at 490 nm wavelength on an automatic Microplate Reader (model 490, BioRad) with DMSO as the blank.

Colony formation assay

Cells were seeded in triplicate in six-well plates at a density of 600 cells /well and incubated at 37°C in a humidified incubator. At 12 days after plating, positive colony formation (>50 cells /colony) was counted and colony formation rate was calculated.

Cell cycle analysis

The cell cycle was analyzed by flow cytometry. Cells were grown in 24-well plates at a density of 2.7×10^4 and were either treated with 5-FU or exposed to IR. After 24 h incubation, cells were harvested by trypsin, washed by PBS, fixed with 70% ethanol overnight at 4°C. Next, the cells were washed twice with PBS and resuspended in PBS containing RNase A, finally stained by propidium iodide (PI) for 30 minutes at room temperature. Finally, analysis of cell cycle distribution was performed by flow cytometry (FCM) by using a FACS Aria Cell Sorter (BD, USA).

Drug sensitivity assay

To investigate the potential impact of MMP-7 on chemosensitivity to anti-tumor drug 5-FU, the cells were seeded in triplicate on 96-well plates at 1×10^4 cells/well and incubated for 24 h. The medium was then removed and replaced with fresh medium containing 5-FU with serial dilution at a final concentration of 2, 4, 6, 8 and 10 μ M. After another 48 h incubation, cells were treated with MTT as described earlier.

Radiosensitivity assay

To investigate the potential impact of MMP-7 on radiosensitivity to X-ray irradiation, MTT assay was employed. SW480 cells were plated onto 96-well plates and incubated for 24h. The cells were then X-ray irradiated with 5, 10, 15, and 20 Gy, respectively, with X-rays (X-RAD320, PRECISLON X-RAY, ZNCN. BRANFORD, CT USA). All doses were delivered without interruption and with the same focus–target distance. After 48h, cell proliferation was analyzed by MTT assay as described earlier.

Statistical analysis

The relative expression analysis of MMP-7mRNA was performed by using the software REST-XL on the basis of the $\Delta\Delta C_t$ method(available at <http://www.wzw.tum.de/gene-quantification/>). Continuous variables were compared using the ANOVA and post hoc multiple comparison (LSD) and non-continuous variables were compared using the chi-square

test by using SPSS 17.0 software (SPSS, Inc., Chicago,IL), $P < 0.05$ was taken as the significance level. Data shown represent at least three replicates of each experiment performed in triplicate.

Results

LV-MMP-7siRNA-3 most efficiently mediated MMP-7 knockdown

To determine the effect of LV-MMP-7shRNA1, 2, 3 on the expression of MMP-7, GFP expression was observed under a fluorescent microscope in SW480 cells at 72 h after infection (Fig.1a). Next, real-time RT-PCR and western blotting were performed to determine the mRNA and protein levels of MMP-7 in LV-MMP-7shRNA1, 2, 3, LV-con and WT (untreated cells) cell groups. We showed that LV-MMP-7shRNA1,2,3 reduced MMP-7mRNA expression by approximately by 71.2%, 52.3% and 72.3% in SW480 cells, respectively, relative to that of WT cells($P < 0.05$), whereas LV-con had no significant effect on the MMP-7 mRNA level ($P > 0.05$, Fig.1b) Western blot revealed an obvious reduction in MMP-7 protein expression in the LV-MMP-7shRNA3 group of the cell lines and showed no obvious difference between the LV-con group and untreated group(Fig.1c) . These analyses demonstrated that LV-MMP-7shRNA3 was the most effective lentivirus vector and, thus, was used in the following research.

Down regulation of MMP-7 expression inhibited the cancer cells' proliferation and colony formation

By MTT assay, the SW480 cell group with stably infected with LV-MMP-7shRNA3 disclosed a significant decrease in proliferation at 48h,72h,96h after incubation compared with LV-con and WT SW480 cell groups ($P<0.05$; Fig.2a). Meanwhile, the colony formation assay further revealed effects of MMP-7 knockdown on growth properties of SW480 cells. LV-MMP-7shRNA3 group had lower positive colony formation than LV-con and WT groups ($P<0.05$; Fig.2b). There was no significant difference between LV-con and WT groups ($P>0.05$).

Down regulation of MMP-7 expression enhanced the cancer cells' susceptibility to 5-Fluorouracil (5-FU) and X-ray irradiation (IR)

To determine whether decreasing MMP-7 expression in SW480 cells would enhance the anticancer effects of radiotherapy and chemotherapy, LV-MMP-7shRNA3, LV-con and WT cells were either exposed to IR or treated with 5-FU. Cell viability/proliferation was measured by MTT assay, and the cell colony formation efficiency was measured by cell colony formation assay. We showed the number of surviving cells in LV-MMP-7shRNA3 cell group of either exposure to IR or treatment with 5-FU was significantly lower than that of LV-con and WT cell groups ($P<0.05$, Fig.3a, Fig.3b). Meanwhile, the colony formation assay also revealed effects of MMP-7 knockdown on the cancer cells' susceptibility to 5-FU and radiation. LV-MMP-7shRNA3 group of either exposure to IR(10Gy) or treatment with 5-FU (6uM) had lower positive colony

formation than LV-con and WT groups ($P < 0.05$; Fig.3c, Fig.3d). There was no significant difference between LV-con and WT cell groups ($P > 0.05$).

Cell cycle assay

Finally, we used flow cytometric analysis to examine changes in cell cycle distribution in each cell group either exposed to IR or treated with 5-FU. We observed that the decreased MMP-7 expression SW480 cells were more able to remain largely in the G1 phase without cell cycle re-entry compared with LV-con and WT cells ($P < 0.05$, Fig.4). This result was consistent with that of the MTT assay, and suggested that the cell cycle control after DNA damage by 5-FU or IR is depending on the presence of wild type MMP-7.

Discussion

It is now becoming recognized that MMP-7 plays important biological roles in tumour development and progression including in apoptosis, cell proliferation, and cell differentiation, which are distinct from their well-defined role in matrix degradation. However, the definitive involvement of MMP-7 in colorectal carcinogenesis has not yet been identified. In this study, we found that the knockdown of MMP-7 inhibited SW480 colon cancer cell proliferation and colony formation and enhanced tumor cells sensitivity to 5-FU and IR. These findings indicate that MMP-7 may facilitate proliferation and decrease sensitivity to

chemotherapeutic agents and irradiation, likely having a promotive role in the carcinogenesis of colon cancer.

Studies suggested that MMP7 may be implicated in the early stages of colorectal cancer tumourigenesis. Both MMP7 mRNA and MMP7 activity have been shown to be present in colorectal adenomas [15] and immunoreactive MMP-7 has been localized to the cytoplasm of colorectal adenoma cells [16]. Heslin MJ et al. reported that MMP-7 gene expression was significantly increased in adenomas but did not increase further in carcinomas[17].Studies from Takanori Kitamura et al. showed that lack of MMP7 reduces the number and size of the adenomas in *Apc^{Min}* mice[18]. In this study, we found that down regulation of MMP-7 expression inhibited cell proliferation by MTT assay. In line with this finding, the colony formation assay showed that knockdown of MMP-7 inhibited cell colony formation ability. These findings indicated that MMP-7 may promote cancer cell growth and suggested that MMP-7 may play an important role in the early stage of colon tumor development. The mechanism(s) by which MMP-7 influences early-stage colorectal tumourigenesis is now being elucidated. MMP-7 is one of the target genes transcriptionally activated by the beta-catenin–tcf-4 complex, one of the key transcription factor complexes involved in the early stages of colorectal tumourigenesis[19].The uncontrolled cell proliferation, resulting from the dysregulation of cell cycle, is a hallmark of cancer

cells. Our finding that MMP-7 knockdown increase the percentage of G1-phase cells suggested MMP-7 may accelerate the progress of cell cycle from G1 to S phase.

Previous studies suggested that a significant role for MMP-7 in tumor invasion and metastasis at late stages of colon cancer progression. However, the clinical data showed no significant correlation between MMP-7 expression and depth of invasion in the early colorectal carcinomas, recently [20]. Disappointing results of several clinical trials of MMPs inhibitors in various tumors may provide an important framework of properly testing these drugs in the right group of patients [8].

It has been shown that anti-cancer drugs or irradiation often kill tumor cells, yet putative cancer stem/progenitor cells are resistant to these agents[21-23]. MMP-7 was identified as a potential downstream target of the Wnt/ β -catenin signaling pathway. Recent studies showed that the Wnt/ β -catenin pathway plays a role in radiation and/or chemotherapy resistance of various malignancies such as leukaemia, head and neck tumours, prostate cancer and hepatocellular carcinoma [24-27]. In this study, we showed that MMP-7 knockdown plus 5-FU or IR significantly inhibited the cancer cells' growth and colony formation efficiency. In addition, we found that MMP-7 knockdown plus 5-FU or IR markedly increased the percentage of G1-phase cells, mechanisms related to their

anti-proliferative effects, this suggested that MMP-7 may weaken the inhibitory effects of 5-FU or IR on DNA synthesis, by accelerating the progress of cell cycle from G1 to S phase. This is consistent with the recent report that activation of Wnt/ β -catenin signalling resulted in reduction of the inhibitory effects of IFN- α /5-FU on DNA synthesis, by decreasing the accumulation of cells in S-phase [27]. Further studies are needed to examine the molecular mechanisms of MMP-7-related enhancement of resistance to 5-FU or IR.

Conclusions

In conclusion, we showed, in this study, that down regulation of MMP-7 not only inhibits the cell proliferation of colon cancer cells but also increases tumor cells sensitivity to radiotherapy and chemotherapy. Therefore, we believe that RNAi-mediated silencing of MMP-7 may represent a powerful therapeutic approach for controlling human colorectal cancer growth.

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Figures Captions

LV-MMP7siRNA-1	Oligo1	5'CCGGGCCTACCTATAACTGGAATGTCTCGAGacattccagttataggtaggcTTTTTG3'
	Oligo2	5' AATTCAAAAAGCCTACCTATAACTGGAATGTCTCGAGacattccagttataggtaggc3'
LV-MMP7siRNA-2	Oligo1	5'CCGGGATGGTAGCAGTCTAGGGATTCTCGAGaatccctagactgctaccatcTTTTTG3'
	Oligo2	5' AATTCAAAAAGATGGTAGCAGTCTAGGGATTCTCGAGaatccctagactgctaccatc3'
LV-MMP7siRNA-3	Oligo1	5'CCGGGTGATGTATCCAACCTATGGACTCGAGtccataggtggatacatcacTTTTTG3'
	Oligo2	5' AATTCAAAAAGTGTATGTATCCAACCTATGGACTCGAGtccataggtggatacatcac3'

Table1 *Interfering sequence specified for MMP-7 gene*

Fig.1 The efficacy of LV-MMP-7shRNA3-mediated RNA interference (RNAi). (a) The efficient transductions of LV-con and LV-MMP-7shRNA3 in SW48 cells were detected using light microscopy (x100), fluorescent microscopy (x100). (b) LV-MMP-7shRNA1,2,3 reduced MMP-7mRNA expression by approximately 71.2, 52.3 and 72.3%

in SW480 cells, respectively, relative to that of WT cells(*P<0.05), whereas LV-con had no significant effect on the MMP-7 mRNA level (*P>0.05, using the software REST-XL).(c) Western blot. The expressions of MMP-7 protein were obviously suppressed by LV-MMP-7 and were not affected by LV-con. Data shown represent at least three independent experiments. WT: untreated SW480 cells; LV-con: transfection with control; LV-MMP-7:transfection with LV-MMP-7shRNA3

Fig.2 Downregulation of MMP-7 expression inhibited the cancer cells' growth and colony formation.

(a). By MTT assay, LV-MMP-7shRNA3 cells disclosed a significant decrease in proliferation compared with LV-con and WT SW480 cell cells (* $P < 0.05$, ANOVA and LSD test). (b). Colony formation assay further revealed LV-MMP-7shRNA3 cells had lower positive colony formation than LV-con and WT cells (* $P < 0.05$, ANOVA and LSD test).

There was no significant difference between LV-con and untreated cell groups (* $P > 0.05$, ANOVA and LSD test). Data shown represent at least three independent experiments.

Fig.3 Down regulation of MMP-7 expression enhanced the cancer cells' susceptibility to 5-Fluorouracil (5-FU) and X-ray irradiation (IR)

. LV-MMP-7shRNA3, LV-con and WT SW480 cells were treated with

5-FU (2, 4, 6, 8 and 10 μ M) or exposed to IR(5, 10, 15, and 20 Gy). After 48 h, surviving cells were identified by MTT assay. For each cell group, the number of cells in the absence of either treatment with 5-FU or exposure to IR was taken as 100%. Fig.3a and Fig.3b showed growth of SW480 cells with reduced MMP-7 expression was significantly lower than that of LV-Con or WT $*(P<0.05, \text{chi-square test})$. Meanwhile, LV-MMP-7shRNA3, LV-con and WT SW480 cells were treated with 5-FU (6 μ M) or exposed to IR(10Gy), then, the cell colony formation assay was employed. Fig.3c and Fig.3d showed positive colony formation of SW480 cells with reduced MMP-7 expression was significantly lower than that of LV-Con or WT $*(P<0.05, \text{ANOVA and LSD test})$. Data shown represent at least three independent experiments.

Fig.4 Effect of reduced MMP-7 expression in G1/S cell cycle checkpoint control in SW480 cells by flow cytometry analysis. G1/S

rate in SW480 cells with reduced MMP-7 expression was significantly higher than that of LV-Con or WT *($P < 0.05$, chi-square test). Data shown represent at least three independent experiments.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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