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ORIGINAL ARTICLE

Cortactin and Exo70 mediated invasion of hepatoma carcinoma cells by MMP-9 secretion

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Abstract This study was aimed to evaluate the regulation mechanism of cortactin (CTTN) on matrix metalloproteinases 9 (MMP-9) and its relations with Exo70 in invasion of hepatoma carcinoma (HCC) cells. The expression levels of CTTN, Exo70 and MMP-9 were detected in normal hepatocytes and various HCC cells by real-time PCR. Then the migration and invasion ability of these cells was revealed by scratch and invasion assay. The effects of CTTN on MMP-9 and the ability of migration and invasion were evaluated by silence and overexpress CTTN. During this process, the expression of CTTN was detected by Western blot, the activity and concentration of MMP-9 in supernatant of culture medium was detected by zymography and ELISA assay. Besides, Exo70 was also inhibited to reveal its effects on MMP-9 and the migration and invasion ability of LM3. Increased expression of CTTN, MMP-9, Exo70, reduced scratch area and increased puncture cell numbers were found in HCC cells ($p < 0.05$). The expression of CTTN was significantly correlated with Exo70 and the migration and invasion ability of HCC ($p < 0.05$). In addition, the activity and concentration of MMP-9 were significantly affected by the expression level of CTTN, while the expression of MMP-9 was not influenced. Besides, Exo70-si also exhibited significantly inhibition effects on the activity and concentration of MMP-9 and puncture cell numbers ($p < 0.05$). A synergistic reaction may exhibited on CTTN and Exo70, which could mediate the secretion of MMPs thereby regulate tumor invasion.

Keywords Cortactin · Exo70 · Matrix metalloproteinase 9 · Hepatocellular carcinoma · Invasion

Introduction

Hepatocellular carcinoma (HCC) is one of the main pathological types of liver cancer, which accounts for 70–85 % of primary liver cancer in the world and 90 % in China [1]. As a common human cancer, HCC has ranked the fifth most prevalent tumor type and the third leading cause of deaths in the world [2]. Although remarkable advances have been made in the treatment of HCC, the prognosis of HCC remains poor due to the metastasis [2]. According to statistics, metastasis of HCC occurs in about 30–50 % of patients [3], and the 5-year overall survival rate in advanced-stage patients is <5 % due to the high rates of recurrence and metastasis [4]. Therefore, it is of great significance to uncover the metastasis mechanisms of HCC, and identify potential therapeutic targets for tumor metastasis.

During the metastasis process of tumor cells, the most important physiological barrier is the extracellular matrix (ECM) and basement membrane (BM), and degradation of ECM and BM has been described as one of the most important steps in the tumor invasion [5, 6]. Matrix metalloproteinases (MMPs) are kinds of zinc-dependent proteinases, which exhibit a main function in ECM degradation [7]. In this family, MMP-9 has been considered to be a key

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factor for cell invasion. As reported, MMP-9 could not only degrade ECM and BM proteins by destroying type IV collagen [7], but also contribute to the establishment of metastasis-prone sites at tumor-distant organs [8]. Meanwhile, high expression and secretion of MMP-9 were reported to be always related to poor prognosis of HCC [9]. Thus, MMP-9 has become a therapeutic target in inhibition of tumor invasion in clinical. On the other hand, the intracellular transportation and exocytosis of MMPs need the cytoskeleton remodeling. As a cytoskeletal protein and src kinase substrate that is frequently overexpressed in cancer, cortactin (CTTN) is also considered to be important in cell motility and invasion [9]. It has been reported that CTTN was rich in subcellular protrusions (invadopodia), which could assist the process of ECM degradation [10]. Overexpression of CTTN was always related to increased lymph-node metastasis rate, local recurrence rate, and reduced disease-free survival time and 5-year overall survival rate. Therefore, CTTN is considered to be closely related to MMP-9 and involves in tumor metastasis.

Exocyst is an evolutionarily conserved multisubunit protein complex and could mediate the tethering of secretory vesicles at the plasma membrane for exocytosis and cell-surface expansion. As an important subunit of Exocyst, Exo70 is considered to be particularly important in cell migration. It has been reported that inhibition of Exo70 could reduce the secretion of MMPs [11]. In addition, Exo70 could influence the actin polymerization by interacting with the Arp2/3 during invadopodia formation, while combination of Arp2/3 and CTTN was necessary in cytoskeleton remodeling. Therefore, a close relation may exist among CTTN, MMP-9 and Exo70 in the invasion of HCC.

In this study, the expression of CTTN, Exo70 and MMP-9 in HCC cells was firstly detected and their relations with the ability of migration and invasion were preliminary evaluated. In addition, CTTN and Exo70 were silenced to reveal their effects on MMP-9 and the migration and invasion ability of HCC. Our findings may deeply uncover the synergistic reactions between intracellular transport system and cytoskeleton system during tumor invasion, and provide a new strategy for the prevention and treatment of HCC in future.

Materials and methods

Cell culture

Normal hepatocytes (QSG-7701, kindly given by Dr. Liping Lv, Institute of Field Blood Transfusion, Beijing, China) and HCC cell lines, including HepG2 (given by Dr. Liping Lv, Institute of Field Blood Transfusion, Beijing, China), LM3 (given by Dr. Ang Huang, Central Laboratory of tumor in PLA General Hospital, Beijing, China), MHCG97-L and SK-Hep-1 (given by Dr. Rui Zhang, Institute of Field Blood Transfusion, Beijing, China) were cultured in high glucose DEME containing 10 % fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C with 5 % CO_2.

Construction of vectors and transfection

To reveal the effects of CTTN and Exo70 on MMP-9 and the ability of migration and invasion, two shRNA vectors of CTTN (CTTN-ps-KD1, CTTN-ps-KD2) and a shRNA vector of Exo70 (Exo70-si) were constructed. Simply, special complementation fragments were amplified by PCR using specific primers (Table 1) and inserted into the vector of pSuper (kindly given by Dr. Xinlei Yu, Institute of Field Blood Transfusion, Beijing, China). Meanwhile, a vector of scrambled shRNA oligo was constructed as control. Besides, the CDS of CTTN was also amplified by PCR using specific primers (Table 1), and then an overexpression vector of CTTN (Lenti-CTTN-EGFP) was constructed by recombination of CDS and vector of pCDH-EGFP (given by Dr. Liping Lv, Institute of Field Blood Transfusion, Beijing, China).

For transfection, LM3 cells were digested and inoculated in 24-well plate at a density of 2×10^5 cells/well, and cultured until 90–95 % cells confluence. Simply, liposome 2000 (Invitrogen) and diverse constructed plasmids were added to Opti-MEM medium and incubated for 20 min. Then the plasmid mixture was added into LM3 cells and cultured at 37 °C with 5 % CO_2 for 6 h. Finally, the cultured medium was refreshed with serum medium and cultured for additional 48 h.

Real-time PCR

The expression of CTTN, Exo70, MMP-9 in different groups was detected by real-time PCR. Total RNA of HCC cells in different groups were isolated by Trizol (Invitrogen) and reversely transcribed by RNA PCR Kit Ver.3.0 (TaKaRa). The expression of CTTN, Exo70, MMP-9 was detected by SYBR Premix Ex *Taq*II (TaKaRa) on StepOnePlus (Applied Biosystems, USA) using specific primers (Table 1). The PCR program included 95 °C for 20 s, 40 cycles at 95 °C for 5 s, 55 °C for 20 s and 72 °C for 12 s. Relative expression of these genes was calculated by ΔΔCT value. During this process, GAPDH was used as the internal control.

Protein detection

Total cellular protein of LM3 cells in different groups was directly extracted. Simply, the cells were washed with PBS,

Table 1 Specific primers used in vector construction and real-time PCR

Primers	Sequences
CTTN-pS-KD-1F	GATCCCCccaggagcatatcaacataTTCAAGAGAtatgttgatatgctcctggTTTTTA
CTTN-pS-KD-1R	GATCCCCgaatgtctttcaagagcatTTCAAGAGAatgctcttgaaagacattcTTTTTA
CTTN-pS-KD-2F	GATCCCCgaatgtctttcaagagcatTTCAAGAGAatgctcttgaaagacattcTTTTTA
CTTN-pS-KD-2R	AGCTTAAAAAgaatgtctttcaagagcatTCTCTTGAAatgctcttgaaagacattcGGG
Scrambled oligo-F	GATCCCCtgactagatgtaacccatgTTCAAGAGAcatgggttacatctagtca
Scrambled oligo-R	AGCTTAAAAAtgactagatgtaacccatgTCTCTTGAAcatgggttacatctagtca
Exo70-si–F	GATCCCCggttaaaggtgactgattaTTCAAGAGAtaatcagtcacctttaaccTTTTTA
Exo70-si-R	AGCTTAAAAAggttaaaggtgactgattaTCTCTTGAAtaatcagtcacctttaaccGGG
CTTN-CDS-F	CCGATGTGGGAAAGCTTCAGCAG
CTTN-CDS-R	ATCTGCCGCAGCTCCACATAGT
RT-CTTN-F	AAGCACGAGTCACAGAGAGAT
RT-CTTN-R	CTCCAAACCCTTTCACATAGTC
RT-Exo70-F	CAAGTCCCTGGAGAAGTCTG
RT-Exo70-R	TGCGATGTAATCAGTCACCT
RT-MMP-9-F	TGTACCGCTATGGTTACACTCG
RT-MMP-9-R	GGCAGGGACAGTTGCTTCT
RT-GAPDH-F	CATGAGAAGTATGACAACAGCCT
RT-GAPDH-R	AGTCCTTCCACGATACCAAAGT

and digested by Trypsin. Then RIPA (APPLYGEN) containing 5 % PMSF was added and the cells were lysed after incubation for 30 min on ice. After centrifugation at 12,000 rpm for 15 min at 4 °C, the supernatant (total protein) was isolated.

Western blot was performed to detect the expression of CTTN in cells of each group. The isolated proteins were denatured by boiling in a 100 °C water bath for 10 min with 5× SDS loading buffer (1:4). Then the proteins were separated by SDS–polyacrylamide gel electrophoresis on 8 % polyacrylamide gels and transferred to a polyvinylidene fluoride membrane (Millipore). The samples were blocked with 5 % skim milk in TBST for 2 h, and special diluted primary antibody anti-CTTN (1:1000, Cell Signaling) was added. After incubation overnight at 4 °C, the samples were washed by TBST and horseradish peroxidase-labeled goat anti-rabbit antibodies (1:1000, CWbiotech) were added. After incubation for 1 h at 25 °C, the samples were washed with TBST, and semi-quantitative gel image was analyzed by Imaging Systems (Bio-Rad ChemiDoc XRS). During this process, β-actin was used as control (1:1000, CWbiotech).

The activity and the concentration of MMP-9 in supernatant of the culture medium in each groups were detected by MMP Zymography assay kit (APPLYGEN) and Human MMP-9 ELISA Kit (4A Biotech) according to the instructions, respectively.

Cell migration assay

Scratch assay (wound healing assay) was performed to detect the migration ability of cells in each group [12]. Simply, cells were cultured in 24-well plates and grew to 90 % confluence. A wound track was scored in each dish with transferpettor, and then the debris was removed by washing with PBS. After 48 h culturing at 37 °C, the scratch area was photographed and measured by WCIF ImageJ. This experiment was performed in triplicate.

Cell invasion assays

Invasion assay (transwell assay) was carried out using transwell cell culture chambers to detect the invasion ability of cells in each group [13]. Simply, cell suspension (5×10^5/mL) was added to the upper compartment of the chamber, and high glucose DEME containing 10 % fetal bovine serum was placed in the lower compartment of the chemotaxis chamber as a chemoattractant source. After incubation at 37 °C for 24 h, cells on the upper surface of the filter were removed with cotton swabs, and those on the lower side were fixed in methanol and then stained with hematoxylin. The stained cells were observed through an inverted microscope (OPTEC BDS300, China) and quantified by the software of Cell Counter (puncture cell numbers). More than five randomly selected fields of views at ×100 magnifications were counted, and each group was performed in triplicate.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS 15.0 software package (SPSS Inc., Chicago, IL). Comparisons

between normal hepatocytes and various HCCs were respectively analyzed by unpaired *t* test. A *p* value less than 0.05 was considered to be significantly different. Besides, the relations between the expression levels of CTTN, MMP-9, Exo70 and the ability of migration and invasion in HCC cells were analyzed through radar chart by office Excel 2007 (Microsoft).

Results

The expression levels of CTTN, MMP-9, Exo70 in HCC, and their relations with migration and invasion

The expression levels of CTTN, MMP-9, Exo70 in HCC were firstly detected by real-time PCR. As a result, CTTN, MMP-9, Exo70 were all significantly up-regulated in cell lines of HepG2, LM3 and MHCG97-L when compared with normal hepatocytes QSG-7701 ($p < 0.05$). Differently, significantly decreased expression of CTTN and Exo70, and increased MMP-9 were found in SK-Hep-1 ($p < 0.05$) (Fig. 1a–c). Then scratch and invasion assay was performed to reveal the migration and invasion ability of these cells. As shown in Fig. 1d, e, the scratch areas were found to be significantly reduced in HepG2, LM3 and SK-Hep-1 when compared with QSG-7701 ($p < 0.05$), while the scratch area was not significantly changed in MHCC97-L. Meanwhile, the puncture cell numbers were also found to be significantly increased in HepG2, LM3 and SK-Hep-1 cells ($p < 0.01$), and no significantly different was found in MHCC97-L. Then we further analyzed the relations of the expression levels of CTTN, MMP-9 and Exo70 expression, and the relations of their expression with migration and invasion ability of HCC cells. As shown in Fig. 1f, the expression of CTTN was significantly correlated with Exo70 expression and the migration and invasion ability of HCC cells ($p < 0.05$). However, the elevated expression of MMP-9 was not interrelated with the expression CTTN and Exo70, and the ability of migration and invasion (Fig. 1f).

The effects of changed expression of CTTN on MMP-9 and the ability of migration and invasion

As to evaluate the relations between CTTN and MMP-9 in HCC cells, a deletion and overexpression mode of CTTN was established in LM3 (Fig. 2). As shown in Fig. 3a, the expression of CTTN was significantly reduced in CTTN-pSuper (CTTN-ps-KD1, CTTN-ps-KD2, ps-KD2/Lenti-EGFP) when compared with that in the control groups (LM3, pSuper, Scrambled oligo) ($p < 0.01$). Meanwhile, lenti-CTTN significantly increased the expression of CTTN in LM3 cells (Lenti-CTTN-EGFP, ps-KD2/Lenti-CTTN-EGFP) ($p < 0.01$). Besides, the influence of deletion and overexpression of CTTN on the expression of MMP-9 did not be observed (Fig. 1b). However, the activity and concentration of MMP-9 were significantly influenced by the expression of CTTN. As shown in Fig. 1c, d, the activity of MMP-9 was significantly reduced by CTTN-si-pSuper and increased by lenti-CTTN when compared with the control groups ($p < 0.05$). Meanwhile, the changed trend of MMP-9 concentration in supernatant of the culture medium was similar with the activity of MMP-9, while no differences were revealed on cells transfected with ps-KD2/Lenti-CTTN-EGFP.

Scratch and invasion assay was performed to evaluate the effects of changed expression of CTTN on migration and invasion ability of HCC. As a result, inhibition of CTTN significantly elevated the scratch area of LM3 in CTTN-ps-KD1, CTTN-ps-KD2, ps-KD2/Lenti-EGFP group ($p < 0.01$). Meanwhile, overexpression of CTTN could significantly reduce the scratch area of LM3 (Lenti-CTTN-EGFP, ps-KD2/Lenti-CTTN-EGFP) ($p < 0.01$) (Fig. 3a). Besides, the changed trends of puncture cell numbers were similar with scratch area (Fig. 3b).

The effects of Exo70 inhibition on MMP-9 and migration and invasion

As Exo70 was correlated with the expression of CTTN, its effects on MMP-9 and the ability of migration and invasion was further evaluated by Exo70-si. As a result, the activity and concentration of MMP-9 was significantly reduced by Exo70-si in either CTTN inhibition or overexpression group ($p < 0.01$) (Fig. 4a, b). In addition, puncture cell numbers were also revealed to be significantly reduced by Exo70-si ($p < 0.05$) (Fig. 4d). However, no significantly differences were exhibited on scratch area of cells transfected with Exo70-si (Fig. 4c).

Discussion

As the poor and lethal outcome of HCC is always caused by spreading of primary tumor cells and the outgrowth of secondary tumors at distant sites, inhibition of tumor metastasis has become a hot topic in prevention and treatment of HCC [8]. Recently, CTTN, MMP-9 and Exo70 are emerged and considered to play important roles in tumor metastasis [14]. In this study, the expression levels of CTTN and Exo70 were revealed to be closely related with the MMP-9 activity and/or concentration as well as the migration and invasion ability of HCC.

As cytoskeleton remodeling and directed transportation of secretory vesicles were necessary in intracellular

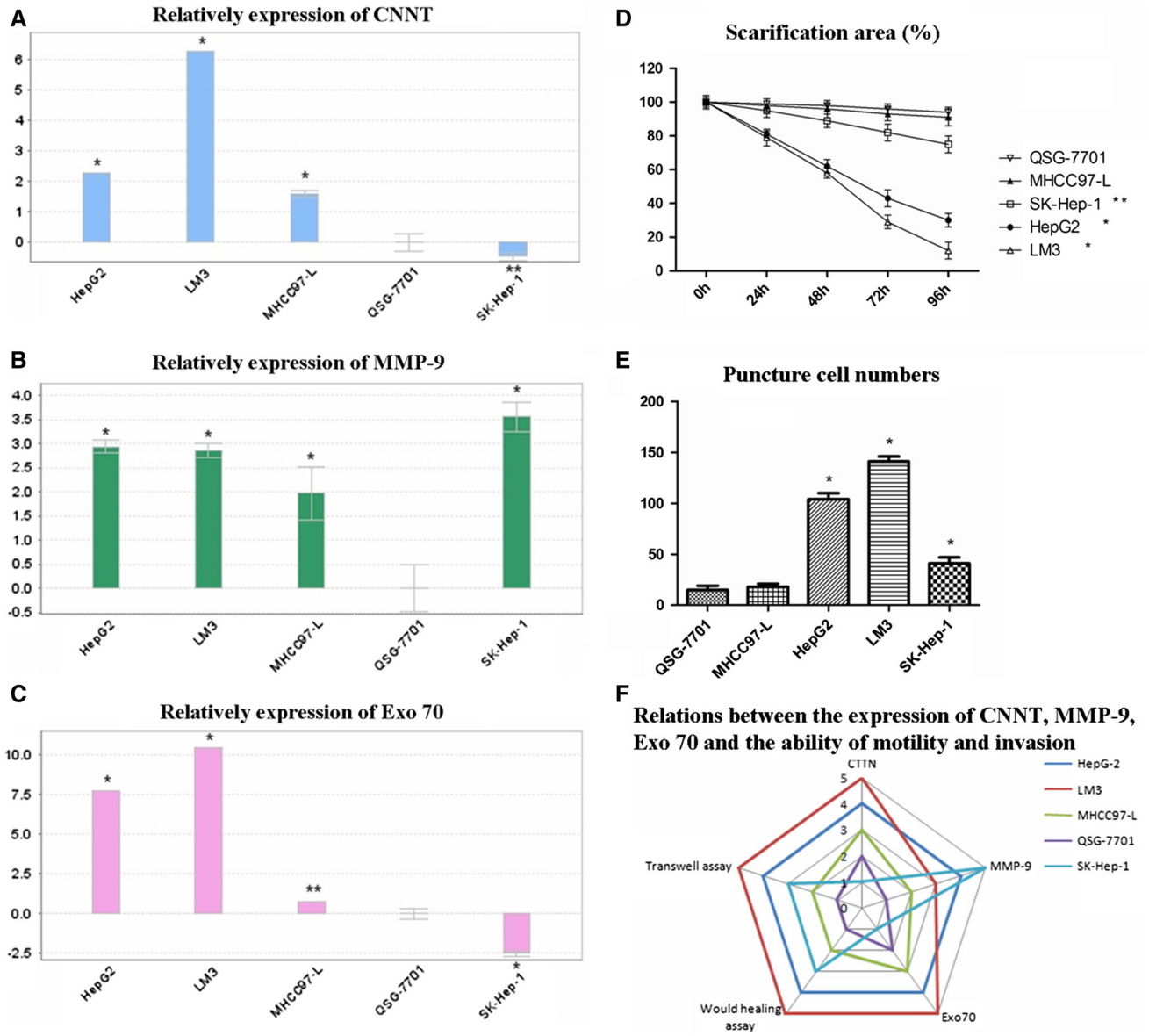

Fig. 1 The mRNA expression level of CTTN **(a)**, MMP-9 **(b)**, Exo70 **(c)** in different hepatoma carcinoma cells (HepG2, LM3, MHCG97-L, SK-Hep-1), and normal hepatocytes (QSG-7701); Scratch assay of migration ability **(d)** and invasion assay of invasion ability **(e)** in different hepatoma carcinoma cell lines and normal hepatocytes; Relations between the expression of CTTN, MMP-9, Exo70 and the migration and invasion in different hepatoma carcinoma cell lines **(f)**. *asterisk*, *double asterisk* represent significantly different at $p < 0.01$, and $p < 0.05$, respectively

transportation and exocytosis of secretory proteins, the elevated expression of CTTN and Exo70 just met the needs of the above biological process. Therefore, CTTN and Exo70 were illustrated to be consistent with the formation of invasive phenotype of HCC cells. On the other hand, MMP-9 was known to be closely related to the invasiveness of HCC [15]. However, the elevated expression of MMP-9 was not revealed to be related to the migration and invasion ability of HCC cells in this study. This phenomenon may be explained by the increased invasive capacity and secretion of MMP-9 but not increased expression. Besides, as this part of experiment was performed on various HCC cells, different sources and genetic background of these cells may influence part of our results. For example, the migration and invasion ability of SK-Hep-1 was medium, while the expression of CTTN and Exo70 was relatively low.

In this study, a deletion and overexpression mode of CTTN was established to reveal the relations between CTTN and MMP-9. To pay attention, CTTN significantly influenced the activity and concentration of MMP-9, as well as the ability of migration and invasion of HCC. These

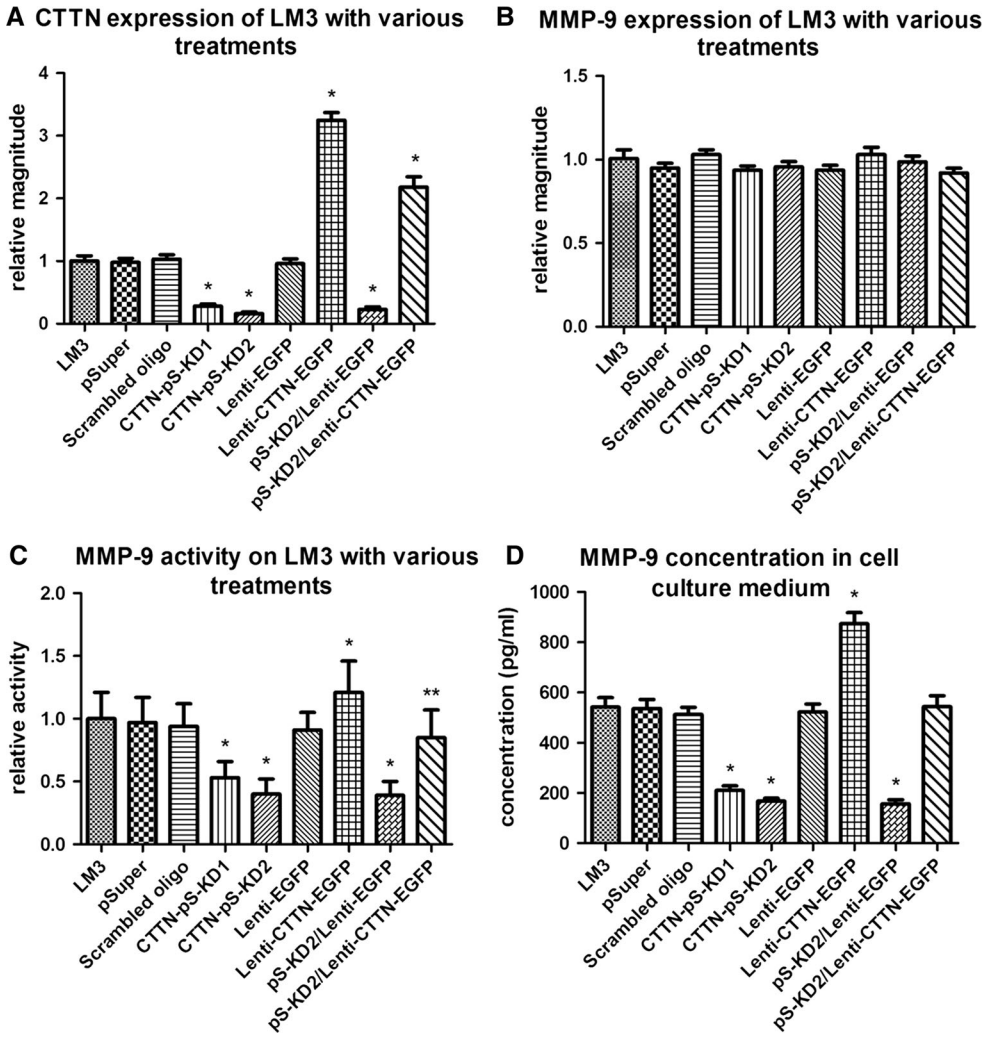

Fig. 2 The expression of CTTN **(a)**, MMP-9 **(b)**, and the activity **(c)** and concentration **(d)** of MMP-9 in LM3 of different groups. Untreated LM3, empty vector pSuper, scrambled oligo was used as the control of CTTN-ps-KD1 and CTTN-ps-KD2; Lenti-EFGP was used as the control of Lenti-CTTN-EFGP. *asterisk*, *double asterisk* represent significantly different at $p < 0.01$, and $p < 0.05$, respectively

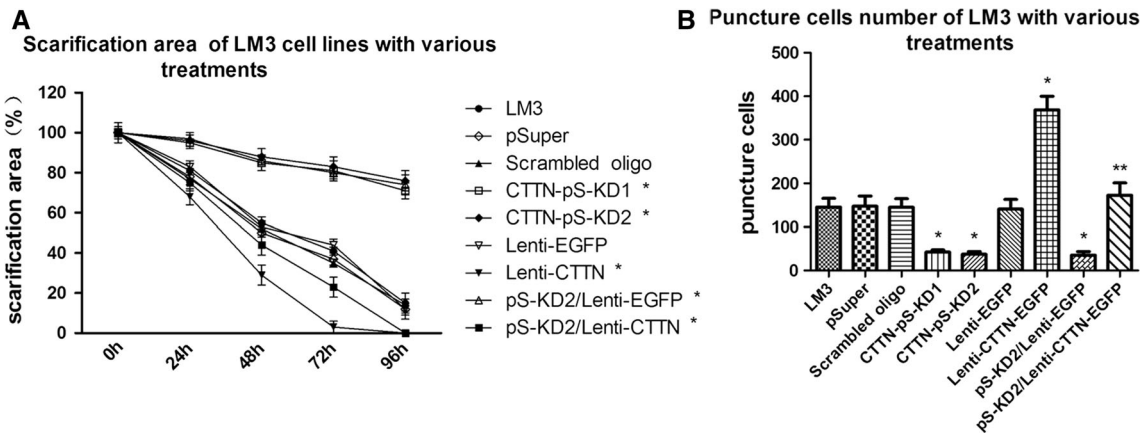

Fig. 3 Scratch assay of migration ability **(a)** and invasion assay of invasion ability **(b)** in LM3 of different groups. Untreated LM3, empty vector pSuper, scrambled oligo was used as the control of CTTN-ps-KD1 and CTTN-ps-KD2; Lenti-EFGP was used as the control of Lenti-CTTN-EFGP. *asterisk*, *double asterisk* represent significantly different at $p < 0.01$, and $p < 0.05$, respectively

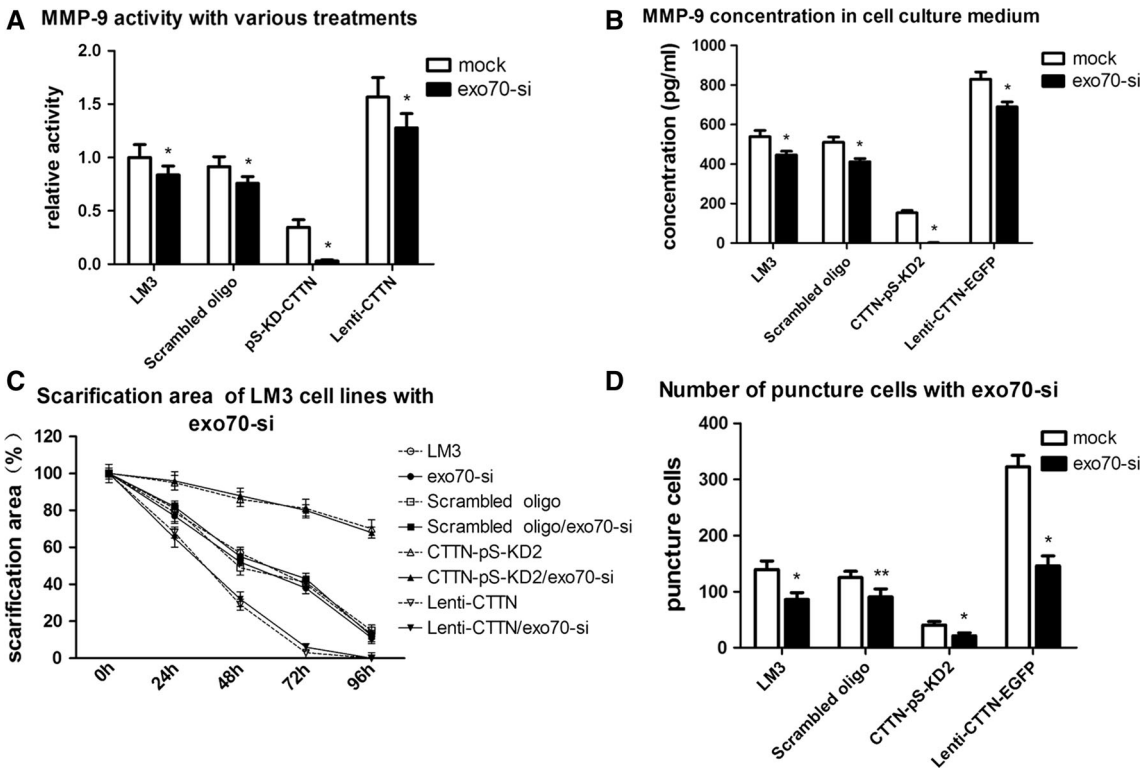

Fig. 4 The activity **(a)** and concentration **(b)** of MMP-9 in LM3 treated by Exo70-si. Scratch assay of migration ability **(c)** and invasion assay of invasion ability **(d)** in LM3 treated by Exo70-si. Untreated LM3, scrambled oligo, CTTN-ps-KD2 and Lenti -CTTN was used as the control. *asterisk*, *double asterisk* represent significantly different at $p < 0.01$, and $p < 0.05$, respectively

results indicated CTTN could regulate the secretion of MMPs, and thereby involved in invasion of HCC. During the invasion process of tumor cells, CTTN was reported to be able to assist the formation and separation of secretory vesicle containing MMPs, guide the directional movement of secretory vesicle, and promote the secretion of MMPs in invadopodia by regulate cytoskeleton remodeling. Therefore, CTTN was illustrated to be a key regulator of MMP-9 in tumor invasion. However, the expression of MMP-9 was not influenced by deletion and overexpression of CTTN in this study. This phenomenon indicated CTTN could regulate the secretion of MMPs in the absence of changed expression level. CTTN mediated cytoskeleton remodeling was the main cause of MMP-9 transportion and secretion.

As the expression of Exo70 was closely correlated with CTTN, Exo70 was also considered to be involved in the process of tumor invasion [16]. In this study, the activity and concentration of MMP-9 were significantly reduced by Exo70-si, which was consisted with previously studies. In addition, the invasion ability of HCC cells was also significantly inhibited by Exo70-si. As known, the effects of exocyst were always related to the process of intracellular transportation. For example, exocyst was found to be important in polarized delivery of membrane proteins [17]. Exocyst could mediate the tether and endocytosis of post-Golgi, which enable cyclic utilization of internal particles in the plasma membrane [18]. Therefore, the influence of Exo70 on tumor invasion may also relate to the formation and separation of secretory vesicle containing MMP-9. As CTTN was known to be closely related to Arp2/3, interactions between exocyst and Arp2/3 were reported to be able to influence actin polymerization during the formation of invadopodia. Therefore, Exo70 may involve in tumor invasion by regulation of MMP-9 secretion through synergistic reaction with CTTN. Only simultaneously silencing of CTTN and Exo70 could block the secretion of MMPs effectively, thereby inhibited the tumor invasion. Besides, Exo70 was also reported to be able to promote cell migration by interacting with Arp2/3 [19]. However, Exo70-si exhibited no effect on migration ability of cells in each group. This indicated the migration ability may also regulate by some other factors with complex mechanisms, and further researches were still needed.

We have to acknowledge the limitations of our study. For instance, the levels of CTTN and Exo70 in high metastatic HCC tissues have not been determined due to the limited funding and unavailable materials. This section will contribute to the clinical relevance of CNNT and Exo70 with HCC invasion and migration, and therefore will be included in our future studies.

In conclusion, the activity and concentration of MMP-9 could be significantly affected by the expression of CTTN, and the secretion of MMP-9 induced by high expression of CTTN could directly influence the ability of migration and invasion in HCC cells. Besides, a synergistic reaction was exhibited on CTTN and Exo70, which could simultaneously stimulate the secretion of MMPs thereby regulate tumor invasion.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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