Abstract. Non-small-cell lung cancer (NSCLC) accounts for ~80% of human lung cancers that result in mortalities worldwide. Metastasis-associated in colon cancer-1 (MACC-1) has been demonstrated to be significantly expressed in cases of NSCLC and promotes tumor cell migration and metastasis through transactivation of the metastasis-inducing hepatocyte growth factor/MET proto-gene, receptor tyrosine kinase (HGF/MET) signaling pathway. The present study constructed a chimeric antibody (Chanti-MACC-1) targeting MACC-1 and investigated its potential as a molecular therapeutic target in the treatment of NSCLC therapy. The expression of MACC-1 was detected by reverse transcription-quantitative polymerase chain reaction and western blotting in lung cancer cell lines and tissues. MTT assay was used to detect proliferation of A549 cells treated by Chanti-MACC-1, whereas the functional and regulatory effects of Chanti-MACC-1 in the migration and metastasis of NSCLC cells was investigated by a cell invasion assay. The therapeutic effect and survival time was observed in animal models. The results demonstrated that MACC-1 expression was increased and overexpression of MACC-1 promoted the progression of the cell cycle, significantly promoted NSCLC cell growth and enhanced tumor migration and invasion through the HGF/MET signaling pathway. It was further demonstrated that Chanti-MACC-1 efficiently suppressed MACC-1 expression and significantly inhibited NSCLC cell proliferation, migration and invasion by blocking the HGF/MET signaling pathway. The data revealed that Chanti-MACC-1 was not only beneficial for tumor remission, however additionally contributed to the long-term survival of NSCLC-bearing mice. The findings of the present study indicated that MACC-1 was significantly upregulated and promoted tumor cell growth and migration in NSCLC cells and tissues via transactivation of the metastasis-inducing HGF/MET signaling pathway. However, Chanti-MACC-1 significantly inhibited tumor growth and metastasis, which suggested that MACC-1 may be essential for tumor initiation and progression by negatively regulating tumor suppressors.

Introduction

Target immunotherapies have been suggested to be important in the termination of tumor initiation, development, progression and deterioration, via immunologic cytotoxicity and personalized adoptive cellular immunotherapy (1,2). It has previously been demonstrated that various target molecules specific to tumor antigens may be used to suppress tumor growth, migration, invasion or metastasis via targeting metastasis-associated pivotal proteins, and may act as potential therapeutic strategies with tumor-inhibition and anti-metastatic properties in human cancer treatments (3,4). Cafarotti et al (5) reported that the personalized target therapy era ideally involves therapeutically treating each individual human disease case, including cancers, infections and hereditary diseases, in different ways that are most efficient and in accordance with the patient’s unique genome.

Lung cancer is a primary public health concern and the leading cause of cancer-associated mortalities worldwide (6). Non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) are two particular variations of lung cancer, which account for ~85 and 15% of the incidence rate, respectively, in human cancer clinical statistical analysis (7). NSCLC includes large cell carcinoma, squamous cell carcinoma and adenocarcinoma that additionally present an increasing trend and incidence rate (8-10). Various investigations regarding therapeutic treatments for NSCLC have previously been conducted, however the overall 5-year survival rate is <15% in patients with NSCLC, and this is of primary clinical concern (9,11,12).

NSCLC is one of the most frequently occurring cancers resulting from poor air quality and high levels of air contamination (13). Migration and invasion are the predominant features of tumor metastasis and development. The migratory, invasive and metastatic capabilities of NSCLC result in the
poor survival rate during treatment and subsequent recurrence of the disease in patients (14,15). Therefore, the development and identification of effective agents for inhibition of migration and invasion, and individualized medication for NSCLC, is of primary concern regarding treatment of cancer patients (16,17). Lung cancer spectral histopathology statistical analysis indicates that standard bio-therapy protocols, including cell therapy and target therapy, results in beneficial outcomes in 95% of patients, compared with traditional treatments (7). A previous study suggested that bio-therapy stiffness modulates lung cancer cell migration via focal adhesion signaling as opposed to epithelial mesenchymal transition (EMT) signaling (18).

Significant advances have been made, particularly with the discovery of targeted agents. Metastasis-associated in colon cancer-1 (MACC-1) is a protein that promotes human lung cancer cell metastasis and is associated with poor patient prognosis in NSCLC (19). Expression levels of MACC-1 have been observed to be increased in human colorectal cancer, and promote tumor migration and metastasis through transactivating the metastasis-inducing hepatocyte growth factor/MET proto-gene, receptor tyrosine kinase (HGF/MET) signaling pathway (20).

The present study investigated if targeting of MACC-1 is a reliable strategy for the inhibition of NSCLC migration and metastasis in vitro and in vivo. Furthermore, the regulatory pathway of MACC-1 and its functional role in NSCLC was investigated. Overall, the results of the present study suggest that targeting of MACC-1 may act as a promising potential therapeutic strategy for intervention in metastasis formation and treatment of NSCLC.

Materials and methods

Ethics statement. The present study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the First Affiliated Hospital of Soochow University (Suzhou, China). The study was approved by the ethics committee of the Third People's Hospital of Yancheng (Yancheng, China). All surgery and euthanasia were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The animals were anesthetized with 35 mg/kg sodium pentobarbital by tail vein injection.

Cell culture. CHO-K1SV, NCI-H520, A549, H358 and MRC-5 cells were purchased from American Type Culture Collection (Manassas, VA, USA). NCI-H520, A549 and H358 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). MRC-5 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). All cells were cultured at 37°C in an environment containing 5% CO₂.

Lung cancer tissues. Lung cancer tissue samples of large cell carcinoma, squamous cell carcinoma and adenocarcinoma were obtained from patients that underwent tumorectomies in the First Affiliated Hospital of Soochow University. The ethical committee members reviewed the experimental designs and protocols and gave ethical approval. A total of 9 male lung cancer patients were enrolled in the study during May 2015 to July 2015, with large cell carcinoma (n=3), squamous cell carcinoma (n=3) and adenocarcinoma (n=3). The mean age of the patients was 48.3 years old (range, 35.6-68.4). All patients agreed to participate in the present study and gave written informed consent.

Construction of full length Anti-MACC-1 antibody. The mouse anti-human MACC-1 monoclonal antibody was constructed using a conventional approach. The chimeric antibody (Chanti)-MACC-1 was constructed as previously described (21) The single chain variable fragments of the MACC-1 monoclonal antibody were cloned and inserted into the Pklight vector (Hengfei Bioscience, Inc., Shanghai China). The constant domain heavy chain (CH)-Fc and light chain (CL) fragments were subcloned into the Pklight-anti-MACC-1 vector, which were subcloned into the Peedual 12.4 vector (Hengfei Bioscience, Inc.). Subsequently, this was transfected into CHO-K1SV cells using Lipofectamine® 2000 (Sigma-Aldrich; Merck KGaA) at room temperature for 20 min. The production of Chanti-MACC-1 extracted from the CHO-K1SV cells was confirmed by using SDS-PAGE western blotting.

Enzyme-linked immunosorbent assay (ELISA). The affinity of Chanti-MACC-1 for its target antigens mouse-MACC-1 (m MACC-1) and human-MACC-1 (hMACC-1) was determined using ELISA kit (cat. no. bs-4293R; BIOSS, Beijing China). A total of 0.2-1.2 mg/ml mMACC-1 and hMACC-1 protein was added into a microplate and incubated with Chanti-MACC-1 at 4°C for 12 h. Chanti-MACC-1 (3 µg/ml) was subsequently added to the mMACC-1 and hMACC-1 microplate and incubated for 120 min at 37°C and PBS was used as a control. The protocol was conducted as previously described (22) and the results were analyzed at a wavelength of 450 nm by an ELISA plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

MTT assay. A549 cells were cultured in 6-well plates and grown to ~90% monolayer cells. Subsequently, Chanti-MACC-1 was added into the 6-well plates for 12 h. A total of 10 µl MTT was used, and formazan crystals were subsequently dissolved in 100 µl dimethyl sulfoxide, then the procedure was conducted as previously described (23). The results were determined by a spectrophotometer (Bio-Rad Laboratories, Inc.) at a wavelength of 570 nm.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was obtained from NCI-H520, A549, H358 and MRC-5 cells prior or the tumor tissues with Chanti-MACC-1 monoclonal antibody were cloned and inserted into the Pklight vector (Hengfei Bioscience, Inc., Shanghai China). A total of 0.2-1.2 mg/ml mMACC-1 and hMACC-1 protein was added into a microplate and incubated with Chanti-MACC-1 at 4°C for 12 h. Chanti-MACC-1 (3 µg/ml) was subsequently added to the mMACC-1 and hMACC-1 microplate and incubated for 120 min at 37°C and PBS was used as a control. The protocol was conducted as previously described (22) and the results were analyzed at a wavelength of 450 nm by an ELISA plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

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Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was obtained from NCI-H520, A549, H358 and MRC-5 cells prior or the tumor tissues with Chanti-MACC-1 by using RNAeasy Mini kit (24) (Qiagen Sciences, Inc., Gaithersburg, MD, USA). A total of 1 µg total RNA was then transcribed into cDNA using the PrimeScript™ RT Master mix (Perfect Real Time; Takara Biotechnology Co., Ltd.) in an ABI PRISM 7900 real time system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The quality of the synthetic cDNA was verified by electrophoresis. Subsequently, the synthetic cDNA (10 ng) was subjected to RT-qPCR using SYBR-Green Master Mix system (Bio-Rad Laboratories, Inc.). The protocol of thermos cycling was as follows: Denaturation,
95°C for 2 min; annealing, 40 repetitions of 95°C for 30 sec and 60°C for 60 sec; and final extension, 72°C for 10 min. The primers used in the present study were synthesized by Shanghai Shenggong Biology Engineering Technology Service, Ltd., Shanghai, China. MACC-1 forward, 5'-AGTGGGATTGTG GAGACGGTG-3' and reverse, 5'-AGTTAAAGAAGTTG GCAAAGC-3'; GAPDH forward, 5'-GTGGACATCCGCAA A GC-3' and reverse, 5'-AAGGTTGTAACGCAA CTACT-3'. GAPDH was included as an internal control. Differences in mRNA expression alterations were calculated by $2^{-ΔΔCt}$ (25).

The results are expressed as the n-fold way compared with control.

Cell invasion assay. A549 cells were treated with Chanti-MACC-1 and non-treated cells served as control. Cultured cells were suspended at a density of 5x10⁴ in 1,000 µl serum-free DMEM medium for 12 h in the upper chamber of a BD BioCoat Matrigel chamber (BD Biosciences, Franklin Lakes, NJ, USA) whereas the lower chamber was filled with 600 µl culture medium supplemented with 10% FBS. According to the manufacturer's protocol. Following incubation for 12 h at 37°C, cells in each group were fixed with ice-cold methanol for 10 min and stained with crystal violet for 5 min at room temperature. Images were captured under a light microscope (magnification, x200; Nikon Corp., Tokyo, Japan). A549 cell invasion was determined in at least three randomly stained-fields using a microscope for each sample.

SDS-PAGE and western blot analysis. The supernatant of serum-free CHO-K1SV cells was harvested. Proteins were resolved by 12% SDS-PAGE under reducing conditions with β-mercaptoethanol (Yuanmu Bioscience Inc., Shanghai, China). A549 cells were treated with Chanti-MACC-1 at a concentration of 100 ng/ml for 12 h. Protein samples from colorectal tumors and A549 cells and were homogenized using radioimmunoprecipitation assay lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.) and samples were centrifuged at 12,000 x g for 10 min. The supernatant of cell extracts were then measured using Bradford protein dye reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

A total of 30 µg/lane protein was loaded and separated by 12% SDS-PAGE under reducing conditions with β-mercaptoethanol (Yuanmu Bioscience Inc., Shanghai, China) for 10 min and stained with crystal violet for 1 min at room temperature. Images were captured under a light microscope (magnification, x200; Nikon Corp., Tokyo, Japan). A549 cell invasion was determined in at least three randomly stained-fields using a microscope for each sample.

Statistical analysis. All data were presented as the mean ± standard error of the mean of three independent replicates. Analysis was performed using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA). Unpaired data was determined by Student's t-test and comparisons of data between multiple groups were analyzed by analysis of variance followed by a Student-Newman-Keuls test. A Kaplan-Meier test was used to estimate the survival rate during a 150-day observation. *P<0.05 and **P<0.01 were considered to indicate a statistically significant difference.

Results

MACC-1 expression increases in NSCLC cells and tissues. In order to investigate the role of MACC-1 in NSCLC and normal lung cells, RT-qPCR was used to analyze expression levels. The results in Fig. 1A and B demonstrated that mRNA and protein expression of MACC-1 was increased in NCI-H520, A549 and H358 cells compared with normal lung MRC-5 cells. In addition, relative mRNA and protein expression levels were additionally studied from patients with large cell carcinoma, squamous cell carcinoma and adenocarcinoma. It was observed that MACC-1 expression levels were upregulated at the mRNA and protein level in NSCLC tumor tissues (Fig. 1C and D). MACC-1 expression in A549 cells was the

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Figure 1. Expression of MACC-1 in non-small-cell lung cancer cells and tissues. (A) RT-qPCR and (B) western blot analysis of MACC-1 mRNA and protein expression levels in NCI-H520, A549 and H358 lung cancer cells, compared with MRC-5 normal lung cells. (C) RT-qPCR and (D) western blot analysis of the mRNA and protein expression level of MACC-1 in large cell carcinoma, squamous cell carcinoma, adenocarcinoma and normal lung tissues. Data are presented as the mean ± standard error of mean, from triplicate samples. *P<0.01 vs. control; †P<0.05 vs. other groups. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MACC-1, metastasis-associated in colon cancer-1.

Figure 2. Inhibitory effects of Chanti-MACC-1 on MACC-1 expression and non-small-cell lung cancer cell growth in vitro. (A) MACC-1 mRNA expression levels were decreased in A549 cells following treatment with Chanti-MACC-1. (B) MACC-1 expression alterations in A549 cells transfected with Chanti-MACC-1, determined via immunofluorescence. (C) MTT assays analyzed the inhibitory effects of Chanti-MACC-1 on A549 cells. (D) Migration analysis was performed to detect the efficacy of Chanti-MACC-1 on A549 cells. Student t-tests revealed a significant difference. *P<0.01, vs. control. MACC-1, metastasis-associated in colon cancer-1; Chanti-MACC-1, chimeric antibody-MACC-1.
greatest in all cell lines. These results suggested that MACC-1 expression was upregulated in NSCLC, and expressed at a low level in MRC-5 cells.

**ChAnti-MAC-1 directly targets MACC-1 in NSCLC cells.** In consideration of the increased expression of MACC-1 in NSCLC cells, the authors hypothesized that targeting of MACC-1 was beneficial for NSCLS tumor cell inhibition. As presented in Fig. 1, MACC-1 expression in A549 cells was greatest among NSCLS cells. Therefore, A549 cells were used to further study the efficacy of Chanti-MACC-1 in NSCLC cells. The results in Fig. 2A demonstrated that Chanti-MACC-1 efficiently decreased MACC-1 mRNA expression levels. The immunofluorescence assay revealed that MACC-1 expression was suppressed in A549 cells following treatment with Chanti-MACC-1 (Fig. 2B). In addition, the present study further investigated the efficient effects of Chanti-MACC-1 on NSCLS tumor cell growth and migration. The results in Fig. 2C demonstrated that A549 cell growth was significantly inhibited in the Chanti-MACC-1-treated group. However, it was observed that MACC-1 greatly promoted A549 cell growth compared with non-treated control. Chanti-MACC-1 treatment efficiently suppressed A549 cell migration, whereas MACC-1 significantly promoted A549 cell migration (Fig. 2D). These data indicated that Chanti-MACC-1 directly targets MACC-1 in NSCLC cells.

**ChAnti-MAC-1 inhibits the EMT process via the HGF/MET signaling pathway.** A previous study indicated that MACC-1 is significantly associated with the EMT and tumor progression (27). The MACC-1-induced EMT signaling pathway was first analyzed, and observed to be blocked by Chanti-MACC-1. Therefore, the underlying mechanism of the effects of Chanti-MACC-1 on HGF/MET and EMT marker expression levels were analyzed, including Vimentin, E-cadherin and Slug. The results in Fig. 3A demonstrated that HGF/MET expression was downregulated in A549 cells following Chanti-MACC-1 treatment. In addition, EMT marker expression was analyzed...
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Figure 4. Therapeutic and metastasis-inhibitory effects of Chanti-MACC-1 in A549-bearing mice. (A) Tumor volume was analyzed following Chanti-MACC-1 treatment in a 24-day short term observation. (B) Long-term survival probability was performed in a 150-day observation between Chanti-MACC-1 and PBS treatment groups. (C) NSCLC metastasis was analyzed between Chanti-MACC-1 and PBS treatment animals. (D) MACC-1 expression was analyzed via histological staining in tumors following treatment with Chanti-MACC-1. Student’s t-test revealed a significant effect. *P<0.05 and **P<0.01 vs. control. MACC-1, metastasis-associated in colon cancer-1; Chanti-MACC-1, chimeric antibody-MACC-1; NSCLC, non-small-cell lung cancer cell.

and Fig. 3B demonstrated that Chanti-MACC-1 treatment decreased Vimentin, E-cadherin and Slug expression in A549 cells. Furthermore, migration-associated matrix metalloproteinase (MMP)-1 proteins, collagen type I (CT-I) and fibronectin were studied in A549 cells. The data presented in Fig. 3C revealed that MMP-1 proteins, CT-I and fibronectin protein expression levels appeared to be markedly decreased following treatment with Chanti-MACC-1 in A549 cells. These results suggested that Chanti-MACC-1 suppressed the migration-promoting proteins in the MACC-1-induced EMT process, which may be beneficial to treatment of cancer cell migration in the HGF/MET pathway.

Chanti-MACC-1 exhibits a therapeutic effect in tumor-bearing mice. Following confirmation of the in vitro effects and mechanism, the present study proceeded to analyze the in vivo effects of Chanti-MACC-1 in xenogeneic NSCLC in C57BL/6 mice. As presented in Fig. 4A, tumor growth was significantly decreased in Chanti-MACC-1-treated xenograft mice, compared with those treated with PBS. In addition, the results in Fig. 4B demonstrated that Chanti-MACC-1 treatment prolonged the survival of NSCLC-bearing mice in a 150-day observation, compared with control mice (n=30 in each group). Tumor metastasis was additionally observed and Fig. 4C demonstrated that Chanti-MACC-1 treatment inhibited 80% metastasis tumor-bearing mice compared to control group. Furthermore, the data in Fig. 4D indicated that MACC-1, HGF/MET expression levels were significantly downregulated in tumors following treatment with Chanti-MACC-1. These data suggested that inhibition of tumor metastasis in NSCLC-bearing mice was enhanced due to Chanti-MACC-1 therapy.

Discussion

The occurrence of lung cancer has previously been demonstrated to be associated with industrial pollution and destruction of the ecological environment in developing countries (28). NSCLC is the primary manifestation of human lung cancer (accountable for >80% of lung cancer cases) that are frequently diagnosed at an advanced stage. Therefore, numerous patients with NSCLC are informed of the limited survival time associated with the diagnosis (29). In addition, the relative increased morbidity and mortality rates of NSCLC among human cancers is currently of primary concern (28). Previous studies have demonstrated that NSCLC is exhibiting a significantly increasing trend in recent years, and is gradually becoming the focus of public opinion and a significant hazard to human health maintenance (30).

Currently, curative treatment of NSCLC is limited to surgical resection or orthotopic lung transplantation. However, not all patients with NSCLC benefit from a surgical approach and suffer from increased recurrence rates. Furthermore, conventional radiation and chemotherapy demonstrate little efficacy for NSCLC and more frequent recurrence and metastasis occurs. Patients generally exhibit a poor response to chemotherapy and surgery, and as of yet, no targeted therapy has been established. In addition, the poor survival rate of NSCLC patients is <15% following the overall 5-year
census (31). Therefore, identification of novel therapeutic protocols to cure patients with NSCLC is of primary concern.

A previous study suggested that migration and metastasis are associated with a poor prognosis of patients with NSCLC (32). Numerous reports have indicated that migration and metastasis contribute to the short-term survival period and are important contributing factors to the relapse and retreatment of patients with NSCLC (33,34). Therefore, anti-tumor agents targeting metastasis-promoting factors are currently of great research interest for the treatment of patients with NSCLC.

MACC-1 is human colon cancer cell metastasis gene located on human chromosome 7 (35). Increased expression of MACC-1 is positively correlated with patient disease progression and prognosis in colorectal cancer (36). Previous biostatistics have revealed that the majority of NSCLC patients observed exhibit an increased expression of MACC-1 in tumors, that may contribute to a short-term survival period (37). Clinical studies have additionally demonstrated that MACC-1 is overexpressed in colorectal cancer (38). In addition, a previous study indicated that targeted therapy for MACC-1 in patients with NSCLC has been investigated in various human tumor cells (39-44). Furthermore, MACC-1 may be useful in the identification of poor prognosis subjects with NSCLC in clinical diagnosis, and may act as a potential molecular target for intervention in metastasis formation. However, the mechanism of the signaling pathway of MACC-1 in the growth, migration and metastasis of NSCLC remains to be elucidated.

The results of the present study verified those of a previous study, identifying MACC-1 as a tumorigenesis and developmental metastasis-associated gene, which enhances NSCLC cell proliferation, migration and invasion (45). The data from the present study demonstrated that MACC-1 regulated proliferation, migration and invasion through HGF-induced scattering and the metastasis-inducing HGF/Met signaling pathway in tumor cells and xenograft models. MACC-1 was reported as a regulator of HGF/Met/mitogen-activated protein kinase pathway and induces proliferation, migration and metastasis. In the present study, migration and invasion-associated MMP-3, CT-1 and fibronectin were studied and were demonstrated to be significantly decreased both in vitro and in vivo, following treatment with Chanti-MACC-1.

In conclusion, the results of the present study suggested that MACC-1 was overexpressed in NSCLC cells and induced the EMT signaling pathway to enhance growth, migration and metastasis. The data additionally indicated that MACC-1 was associated with a poor prognosis of mice with NSCLC. In addition, Chanti-MACC-1 targeting MACC-1 revealed beneficial outcomes for NSCLC-bearing mice, through inhibition of the HGF/Met/mitogen-activated protein kinase pathway. Furthermore, the results have identified the potential of Chanti-MACC-1 to suppress growth, migration and metastasis via regulating metastasis-associated genes, and to act as a novel therapeutic in anti-metastasis treatment strategies.

References


