Original Article
MACC1 overexpression induces cisplatin resistance in lung adenocarcinoma A549 cells by activating c-Met/Akt pathway

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Abstract: The overexpression of metastasis-associated in colon cancer-1 (MACC1) was correlated with poor prognosis in non-small cell lung cancer (NSCLC), and the development of cisplatin (CDDP) resistance was a frequent event in lung adenocarcinoma therapy. This study aimed to investigate the role of MACC1 in CDDP resistance in human lung adenocarcinoma A549 cells. We first established a CDDP-resistant A549/CDDP cell line from its parental cell line A549. The chemoresistance was confirmed by CCK-8 assay, western blotting of apoptotic proteins and colony formation assay. The CDDP sensitivity of A549 cell after ectopic overexpression of MACC1 by introducing MACC1-expressing cDNA was also determined. Then MACC1 shRNA plasmids and negative control plasmids were transfected into A549/CDDP cells for further researches. We found that A549/CDDP cells developed CDDP resistance following continuous exposure to CDDP. MACC1 was up-regulated in A549/CDDP cells, while the overexpression of MACC1 also induced CDDP resistance in A549 cells. The inhibition of MACC1 in A549/CDDP cells reversed CDDP resistance by inducing apoptosis after CDDP treatment. The mechanism underlying MACC1 induced CDDP resistance was also investigated. We found that c-Met and P-Akt increased in A549/CDDP cells, while the inhibition of c-Met and P-Akt reversed CDDP resistance. In conclusion, we found that MACC1 overexpression induced CDDP resistance in A549 cells by activating c-Met/Akt pathway.

Keywords: Lung adenocarcinoma, CDDP resistance, MACC1, c-Met/Akt pathway

Introduction

Lung cancer is the most frequently diagnosed cancer worldwide. It is also the first leading cause of cancer related death in males and the second in females, causing more than 1.5 million deaths in 2012 [1]. Among all the lung cancer, the NSCLC accounts for over 85% and lung adenocarcinoma is the most diagnosed histological subtype of NSCLC. The overall 5-year survival rate of NSCLC is less than 20% [2]. In China, the incidence of NSCLC is quite high because of indoor and outdoor pollutions [3, 4]. Despite major advances in comprehensive treatment, the prognosis of NSCLC patients is far from satisfying [5]. Targeted therapies has improved the outcomes in a subset of NSCLC patients; however, most patients with metastatic NSCLC receive chemotherapy [6]. CDDP-based doublets are the foundation of chemo-

therapy for patients with advanced lung adenocarcinoma [7]. The anti-cancer effect of CDDP lies in generating DNA lesion and causing apoptotic cell death [8], but the emerging of chemoresistance has limited its clinical utility and led to therapeutic failure and tumor relapse [9]. Thus the study on CDDP resistance will help to overcome the obstacles towards better prognosis of NSCLC patients.

MACC1, firstly described by Stein et al., was a novel tumor biomarker in colon cancer [10]. The overexpression of MACC1 was associated with poor survival rates, regional invasion and lymph-node metastasis in colorectal cancer [11]. MACC1 was also found to be overexpressed in some other human malignancies and correlated with poor prognosis. Its overexpression could promote the development and progression of certain cancer types, such as hepatocel-

lular carcinoma (HCC), epithelial ovarian cancer, breast cancer, nasopharyngeal carcinoma, gastric cancer and pancreatic cancer [12-17]. The high MACC1 expression was found to have an unfavorable impact on relapse-free survival (RFS) and disease-free survival (DFS) in patients [18]. In NSCLC, the overexpression of MACC1 also predicted poor survival. Chundong et al. found that positive MACC1 immunohistochemical staining in resected lung adenocarcinoma specimens was associated with poorer DFS [19]. Wang et al. found that MACC1 protein in NSCLC tissues was higher than that in adjacent normal tissues. MACC1 expression was positively associated with tumor differentiation, postoperative pathological TNM stage and lymph node metastasis. MACC1 was an independent prognostic indicator for DFS and overall survival (OS) in NSCLC patients [5]. The research by Zhou et al. got similar findings that MACC1 overexpression in NSCLC correlated with tumor grade, lymph node metastasis, and tumor node metastasis [20]. High circulating MACC1 mRNA levels were also correlated with tumor stage and lymph node metastasis, and were predictive of poorer DFS and OS in NSCLC patients [21].

Besides its role as a predictor of poor prognosis, MACC1 also functioned in the development of chemoresistance. The research by Li et al. demonstrated that inhibition of MACC1 could enhance CDDP sensitivity and decrease drug resistance in tongue squamous cells [22]. Similar results were revealed in the researches in epithelial ovarian cancer cells [23, 24]. However, the possible function of MACC1 in CDDP resistance in lung adenocarcinoma has not been revealed yet.

In this research, we aimed to discover whether MACC1 played a role in the development of CDDP resistance in lung adenocarcinoma and to reveal the underlying mechanism beneath the drug resistance.

Materials and methods

Cell culture and reagents

Human lung adenocarcinoma cancer A549 cell line was obtained from American Tissue Culture Collection (ATCC, USA) and was cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS, Gibco, Gaithersburg, MD) at a

37°C CO_2 incubator. The CDDP-resistant A549/CDDP cell line was developed by continuous exposure to CDDP as previously described [25]. The primary antibody against MACC1 as obtained from Abcam (Cambridge, UK). Primary antibodies against c-Met, caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9, poly ADP-ribose polymerase (PARP), cleaved PARP, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and B-cell lymphoma-2 (Bcl-2) were obtained from Cell Signaling Technology (Beverly, MA). The c-Met inhibitor crizotinib (PF-02341066) and the p-Akt inhibitor MK-2206 2HCl were purchased from Selleck (Selleck Chemicals, China).

Cell viability assay

The cell viability was detected by commercial Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Cells were seeded on 96-well plates at a density of $5×10^3$ cells per well and treated with CDDP of different concentrations for 48 h. Thereafter, the cells were incubated with CCK-8 reagent at 37°C for 2 h. The absorbance values at 490 nm were evaluated by using a microplate reader (GloMax® Discover System, Promega, WI, USA). The experiments were performed in triplicate.

Colony formation assay

The proliferative capacity was determined by colony formation assay. Briefly, Cells were seeded on a 6-well plate at the density of $2×10^3$ cells and incubated with 1 μM CDDP for 30 days. After being washed with phosphate buffered saline (PBS) and fixed with cold methanol, the cells were stained with crystal violet and photographed.

Quantitative reverse transcription-polymerase chain reaction (qPCR)

Total RNA was extracted by using TRIzol® reagent (Takara Bio, Inc., Otsu, Japan) and the reverse transcription was performed using Primescript RT master mix (Takara Bio, Inc.). The qPCRwas conducted by using the SYBR Green PCR Kit (Takara Bio, Inc.) on an ABI PRISM 7300 Sequence Detector. GAPDH was used as the internal control. The relative expression level was determined by $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows:
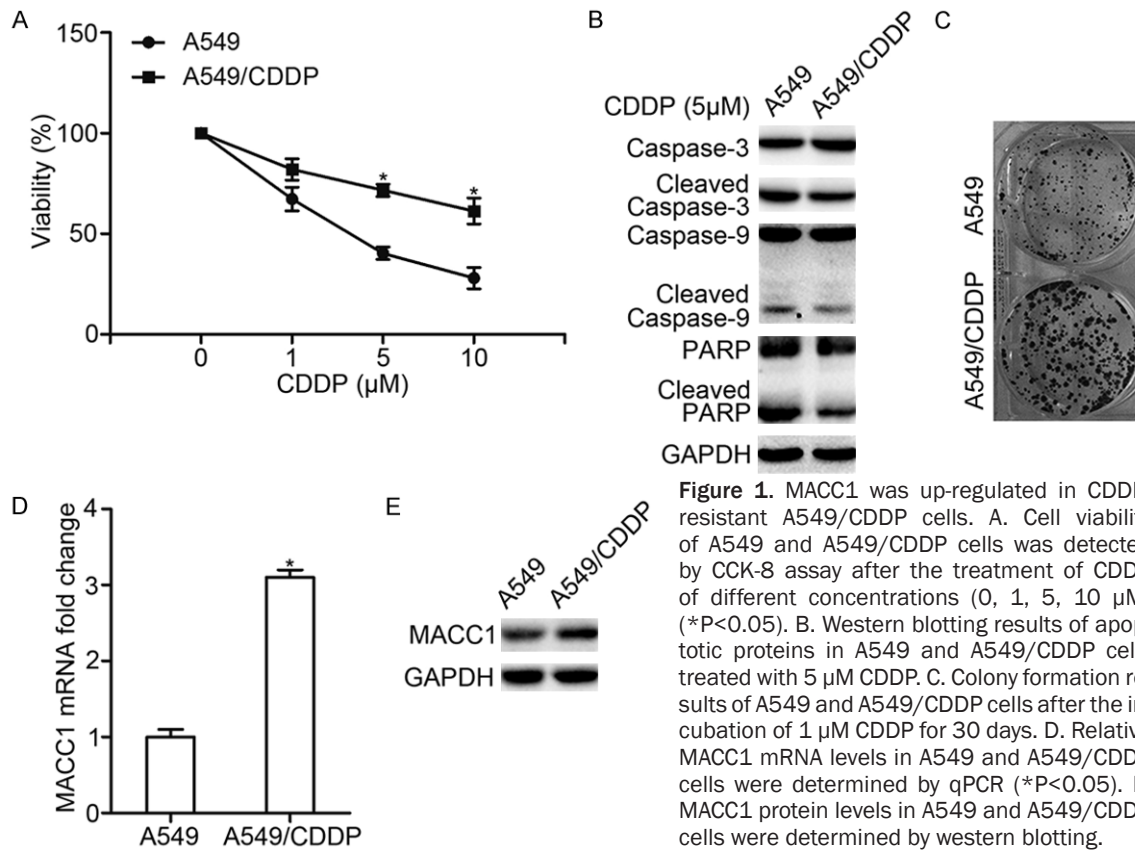
Figure 1. MACC1 was up-regulated in CDDP-resistant A549/CDDP cells. A. Cell viability of A549 and A549/CDDP cells was detected by CCK-8 assay after the treatment of CDDP of different concentrations (0, 1, 5, 10 μM) (*$P<0.05$). B. Western blotting results of apoptotic proteins in A549 and A549/CDDP cells treated with 5 μM CDDP. C. Colony formation results of A549 and A549/CDDP cells after the incubation of 1 μM CDDP for 30 days. D. Relative MACC1 mRNA levels in A549 and A549/CDDP cells were determined by qPCR (*$P<0.05$). E. MACC1 protein levels in A549 and A549/CDDP cells were determined by western blotting.

MACC1 forward (5'-3'): AGGTACAGTAAGAGCCATAGGAC; MACC1 reverse (5'-3'): CTTGGTTGTCAAAATGCCATCAG; GAPDH forward (5'-3'): TGGATTTGGACGCATTGGTC; GAPDH reverse (5'-3'): TTTGCACTGGTACGTGTTGAT.

Western blotting

Total proteins were extracted using NP40 solution. The cell lysates were separated in Tris-glycine gels by SDS/PAGE and transferred to a polyvinylidine fluoride (PVDF) membrane. After being incubated with indicated first antibody overnight at 4°C, the membrane was washed with TBST and incubated with horseradish peroxidase (HRP)-conjugated second antibody for 2 h at room temperature. The signal was developed by enhanced chemiluminescence (ECL) (Millipore, Switzerland) and visualized by a Tanon 5200 Chemiluminescent Imaging System (Tanon Science and Technology Co., Ltd., Shanghai, China).

Ectopic expression of MACC1

The pLenti-MACC1 cDNA lentiviral vector and negative control (NC) were purchased from

GenePharma (Shanghai, China) and transfected into A549 cells following the manufacturer's instructions, respectively.

Transfection of shRNA

Human MACC1 shRNA and a scrambled shRNA control were purchased from GenePharma (Shanghai, China) and transfected into A549/CDDP cells using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Statistical analysis

The data were presented as mean ± standard deviation (SD) and were statistically analyzed with Student's t test and one-way ANOVA with Bonferroni correction. $P<0.05$ was considered significantly different.

Results

MACC1 was up-regulated in CDDP-resistant A549/CDDP cells

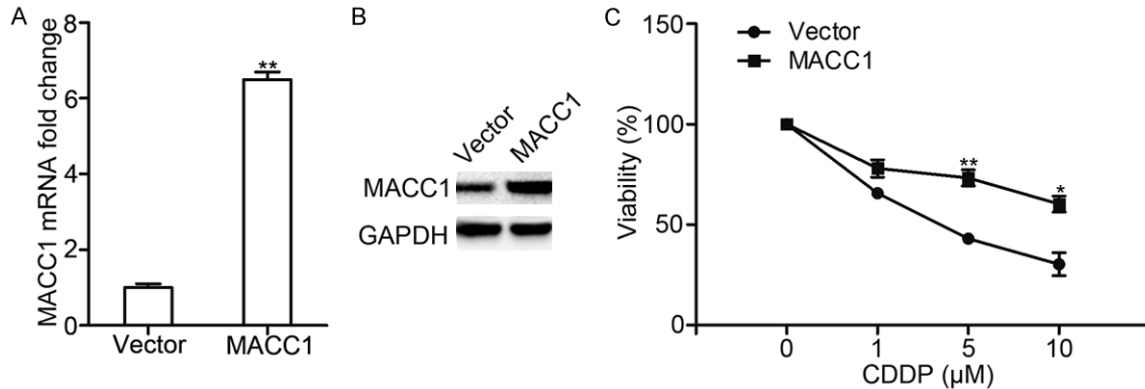We first established a CDDP-resistant lung adenocarcinoma cell line A549/CDDP by continu-

Figure 2. Overexpression of MACC1 induced CDDP resistance in A549 cells. A. A549 cells were transfected with lentivirus carrying MACC1-expressing cDNA sequences. The overexpression efficiency was determined by qPCR. B. The overexpression efficiency of MACC1 was determined by western blotting. C. A549 cells with MACC1 overexpression and control cells were incubated with CDDP of different concentrations (0, 1, 5, 10 μM) for 48 h. The cell viability was detected by CCK-8 assay ($*P<0.05$, $**P<0.01$).

ous exposure to CDDP treatment starting at 0.1 μM and increasing to 5 μM in a stepwise manner. The drug resistance to CDDP of A549/CDDP cells was confirmed by CCK-8 assay. We found that the parental A549 cells were sensitive to CDDP, while the A549/CDDP cells showed increased viability after CDDP treatment (**Figure 1A**). The resistance to CDDP was further confirmed by western blotting. After the administration of CDDP (5 μM), the cleaved form of caspase-3, caspase-9 and PARP were decreased in A549/CDDP cells which showed less apoptosis in A549/CDDP cells (**Figure 1B**). We also evaluated the proliferative capacity of A549 and A549/CDDP cells after the incubation of CDDP. We found that the colony numbers of A549/CDDP cells were higher than that of A549 cells (**Figure 1C**). To explore the effect of MACC1 on CDDP resistance in A549/CDDP cells, we detected the expression level of MACC1 in A549 and A549/CDDP cells. The results demonstrated that the mRNA and protein levels of MACC1 in A549/CDDP cells were higher than that in A549 cells (**Figure 1D** and **1E**). The above results suggested a potential role of MACC1 in CDDP resistance in A549/CDDP cells.

Overexpression of MACC1 induced CDDP resistance in A549 cells

To confirm the role of MACC1 in CDDP resistance in A549 cells, A549 cells were transfected with lentivirus carrying plasmids encoding MACC1. The overexpression efficiency was confirmed by qPCR and western blotting. We found MACC1 mRNA level increased more than 6 times after the transfection (**Figure 2A**). The western blotting results also showed increased MACC1 protein levels after MACC1 overexpression (**Figure 2B**). Then we compared the cell viability of A549 cells and A549 cells with MACC1 overexpression after the incubation with CDDP. The A549 cells with MACC1 overexpression demonstrated increased viability, suggesting the development of CDDP resistance (**Figure 2C**).

Inhibition of MACC1 in A549/CDDP cells reversed CDDP resistance

To further investigate the function of MACC1 in the development of CDDP resistance, the specific stable MACC1 shRNA was adopted to inhibit the MACC1 expression in A549/CDDP cells. The qPCR showed that MACC1 shRNA substantially reduced MACC1 mRNA level in A549/CDDP cells approximately by 65% compared to control shRNA (**Figure 3A**). The western blotting results also confirmed decreased MACC1 protein levels in A549/CDDP cells transfected with MACC1 shRNA (**Figure 3B**). Then A549/CDDP cells transfected with control shRNA or MACC1 shRNA were incubated with CDDP of different concentrations for 24 h, and the cell viability was evaluated. The down-regulation of MACC1 in A549/CDDP cells inhibited cell viability compared to control shRNA (**Figure**
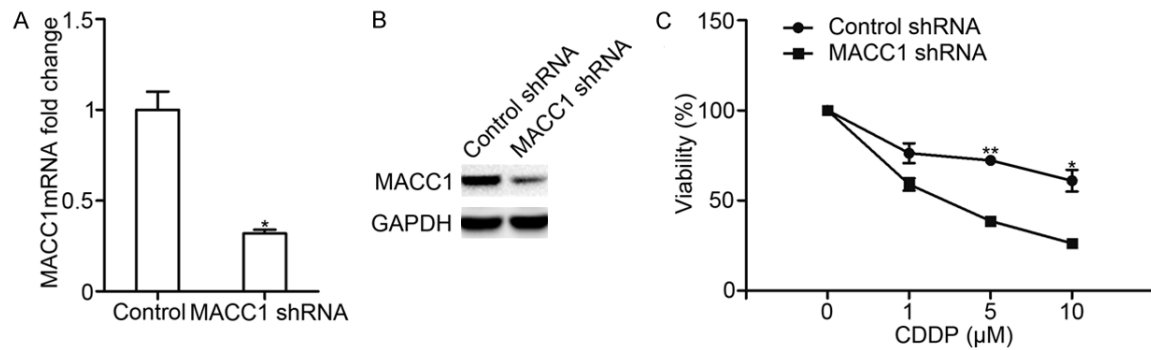
Figure 3. Inhibition of MACC1 in A549/CDDP cells reversed CDDP resistance. A. qPCR analysis of MACC1 mRNA levels in A549/CDDP cells transfected with MACC1 shRNA and control shRNA. B. Western blotting results of MACC1 protein levels in A549/CDDP cells transfected with MACC1 shRNA and control shRNA. C. A549/CDDP cells with stable MACC1 knock-down and control cells were incubated with CDDP of different concentrations (0, 1, 5, 10 μM) for 48 h. The cell viability was detected by CCK-8 assay (*P<0.05, **P<0.01).
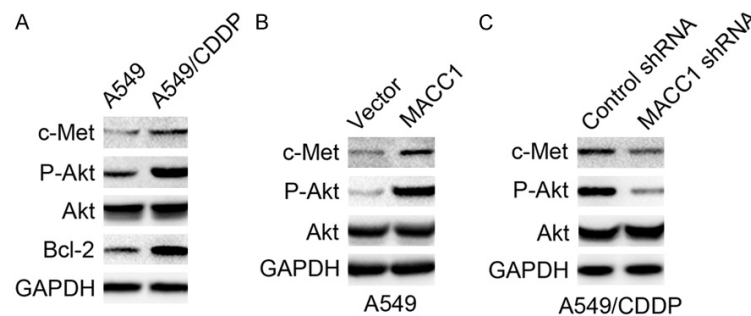

Figure 4. c-Met and P-Akt were increased in A549/CDDP cells. A. c-Met, P-Akt and Bcl-2 protein levels in A549 and A549/CDDP cells were determined by western blotting. B. c-Met and P-Akt protein levels in A549 cells with MACC1 overexpression were determined by western blotting. C. c-Met and P-Akt protein levels in A549/CDDP cells transfected with control shRNA and MACC1 shRNA were determined by western blotting.
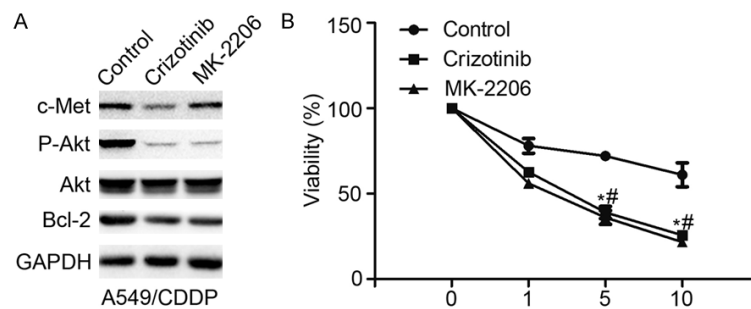

Figure 5. Inhibition of c-Met and P-Akt reversed CDDP resistance in A549/CDDP cells. A. A549/CDDP cells were incubated with the c-Met inhibitor crizotinib (500 nM) or P-Akt inhibitor MK-2206 (5 μM) for 48 h. c-Met, P-Akt and Bcl-2 protein levels were determined by western blotting. B. A549/CDDP cells incubated with crizotinib or MK-2206 were treated with CDDP of different concentrations (0, 1, 5, 10 μM) for 48 h. The cell viability was determined by CCK-8 assay (*P<0.05 crizotinib vs. Control, #P<0.05 MK-2206 vs. Control).

3C), suggesting that inhibition of MACC1 could reverse CDDP resistance in A549/CDDP cells.

c-Met and P-Akt were increased in A549/CDDP cells

The mechanism underlying MACC1-mediated CDDP resistance in A549 cells was also investigated. According to the research by Stein et al., the hepatocyte growth factor (HGF) receptor, c-Met was a transcriptional target of MACC1 [26]. In addition, previous studies showed that MACC1 could activate Akt via the HGF/c-Met pathway [27, 28]. So we detected the level of c-Met in A549/CDDP cells. We found that c-Met was increased in A549/CDDP cells. The downstream Akt was also activated (**Figure 4A**). At the same time, the anti-apoptotic protein Bcl-2 was increased. In A549 cells with MACC1 overexpression, the increased protein levels of c-Met and P-Akt were also detected (**Figure 4B**). However, when inhibiting MACC1 in A549/CDDP cells, the c-Met increase and Akt activation were abrogated (**Figure 3C**).

Inhibition of c-Met and P-Akt reversed CDDP resistance in A549/CDDP cells

To further confirm that c-Met and Akt activation were responsible for MACC1-mediated CDDP resistance, the c-Met inhibitor crizotinib and P-Akt inhibitor MK-2206 were adopted. After incubating A549/CDDP cells with crizotininb (500 nM) for 48 h, c-Met and Akt activation were reversed. The administration of MK-2206 (5 μM) inhibited Akt activation, but had no effect on c-Met levels. The inhibition of c-Met and Akt both reversed the increase of Bcl-2 (**Figure 5A**). Then to find out whether the inhibition of c-Met or Akt could reverse CDDP resistance in A549/CDDP cells, the cell viability after the treatment of crizotinib, MK-2206 and CDDP were evaluated. We found crizotinib and MK-2206 both reversed CDDP resistance in A549/CDDP cells (**Figure 5B**).

Discussion

The present study demonstrated that MACC1 was up-regulated in CDDP-resistant A549/CDDP cells. Ectopic expression of MACC1 in A549 cells also promoted the development of CDDP resistance. By contrast, inhibition of MACC1 in A549/CDDP cells reversed the induced chemoresistance. Furthermore, the mechanism underlying the MACC1-mediated CDDP resistance in A549 cells was also investigated. The up-regulated MACC1 in A549/CDDP cells increased c-Met and activated the subsequent Akt pathway. The same results were also found in A549 cells with MACC1 overexpression. These effects could be attenuated by inhibiting MACC1 in A549/CDDP cells. The incubation of A549/CDDP cells with crizotinib and MK-2206 further confirmed that c-Met/Akt pathway was involved in MACC1-induced CDDP resistance. Thus, MACC1 overexpression in lung adenocarcinoma A549 cells induced CDDP resistance by activating the c-Met/Akt pathway.

MACC1, first identified and characterized by Stein et al. in 2009, was an oncogene promoting the tumor growth and metastasis of colon cancer [10]. Since then, a multiple researches on MACC1 gene functions have been performed in several other cancer types. It was found that MACC1 was involved in many other biological processes more than metastasis, such as epithelial-mesenchymal transition

(EMT), metabolic reprogramming, cell proliferation and angiogenesis [29]. Increasing amount of evidences have made MACC1 a novel remarkable biomarker for disease prognosis for a variety of solid cancers [30]. The overexpression of MACC1 was associated with poor survival of cancer patients by multiple studies [31]. Up-regulated MACC1 was predictive of shorter DFS, OS and RFS in solid cancers [18]. A few researches focused on its role in drug resistance. Liu et al. found that MACC1 mediated trastuzumab resistance by promoting the Warburg effect [32]. The down-regulation of MACC1 was reported to attenuate CDDP resistance in tongue squamous cell carcinoma and epithelial ovarian cancer cells [22, 23]. The role of MACC1 in CDDP resistance in lung adenocarcinoma has not been reported yet. In our research, we found that CDDP-resistant A549/CDDP cells had enhanced expression of MACC1. A549 cells with MACC1 overexpression developed CDDP resistance. Knockdown of MACC1 expression could reverse CDDP resistance.

The mechanism by which MACC1 mediated CDDP resistance was also investigated in our research. We found increased c-Met in A549/CDDP cells. The inhibition of c-Met by crizotinib reversed CDDP resistance. The HGF/c-Met pathway was the firstly-studied pathway regulated by MACC1. Stein et al. found that MACC1 overexpression increased the mRNA and protein levels of c-Met. MACC1 could trigger the expression of c-Met by direct transcriptional regulationthrough its SH3 domain [10]. Their further research demonstrated that MACC1 acted via an Sp1 binding site of the c-Met promoter [33]. Galimi et al. also found that MACC1 controlled c-Met levels as an upstream regulator of c-Met transcription [34].

Our research also showed that Akt was the downstream regulator of c-Met in the regulation of MACC1-mediated CDDP resistance. We found increased Akt activation in A549/CDDP cells and A549 cells with MACC1 overexpression. The inhibition of Akt activation by MK-2206 abrogated MACC1-induced CDDP resistance. Akt activation was one important mechanism that inhibited the DNA damage signal to apoptotic machinery [35]. The Akt signal transduction pathway was implicated in the protection of cells from apoptosis and the promotion of cell proliferation [36]. Yao et al. also found that

MACC1 could suppress cell apoptosis in HCC by targeting the HGF/c-Met/Akt pathway [28] which was consistent with our results. Our research also found increased anti-apoptotic Bcl-2 protein level in A549/CDDP cells. Because overexpression of Bcl-2 was associated with CDDP resistance [37], this might also be a mechanism for MACC1-mediated CDDP resistance.

The reason why MACC1 was up-regulated in A549/CDDP cells was not investigated in our research. The possible mechanism might be the deregulation of some miRNAs. It was reported that miR-1, miR-143, miR-433 and miR-200a and miR-338-3p could directly bind to the 3' untranslated region (UTR) of MACC1 mRNA and inhibit protein expression [27, 38-41]. The down-regulation of these miRNAs might up-regulate MACC1, leading to CDDP resistance. Feng et al. found that miR-338-3p could inhibit EMT in gastric cancer cells by targeting MACC1/c-Met/Akt signaling. MiR-338-3p was also reported to inhibit the growth and invasion of human NSCLC cells [42]. Whether miR-338-3p directly targeted MACC1 and whether its down-regulation was the reason resulting in MACC1 up-regulation in A549/CDDP cells need further investigations.

In conclusion, our study demonstrated that MACC1 overexpression induced CDDP resistance in A549 cells by activating c-Met/Akt pathway. This study provided evidence that MACC1 might be a therapeutic target to overcome CDDP resistance in lung adenocarcinoma.

Disclosure of conflict of interest

None.

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