RESEARCH ARTICLE

Influence of the MACC1 Gene on Sensitivity to Chemotherapy in Human U251 Glioblastoma Cells

Chao Shang[1], Yang Hong[2], Yan Guo[3], Yun-Hui Liu[2], Yi-Xue Xue[1]*

Abstract

<u>Background</u>: This study was conducted to determine the influence of MACC1 expression on chemotherapy sensitivity in human U251 glioblastoma cells. <u>Materials and Methods</u>: Expression of the MACC1 gene in 49 cases of human brain glioma was determined by quantitative real-time PCR. Silencing effects of RNA interference on MACC1 was detected by Western-blotting. Flow cytometry methods and methyl thiazolyl tetrazolium assay (MTT) were used to determine the apoptosis and growth inhibitory rates of the U251 cells with MACC1 silencing. before and after treatment with cisplatin (DDP). <u>Results</u>: MACC1 mRNA in gliomas was up-regulated remarkably, to 158.8% of that in peri-cancerous tissues ($P<0.05$). The siRNA-MACC1 could inhibit the expression of MACC1 protein significantly ($p<0.05$), associated with an increase in apoptosis rate from 2.57% to 5.39% in U251 cells and elevation of the growth inhibitory rate from 1.5% to 17.8% ($p<0.05$ for both). After treatment with DDP at various concentrations (1, 3, 5μg/ml), compared with control U251 cells, the apoptosis rate of MACC1-silenced U251 cells rose from 8.41%, 13.2% and 19.5% to 12.8%, 17.8% and 25.8%; the growth inhibitory rate increased from 16.2%, 19.3% and 24.5% to 23.7%, 28.4% and 36.3%. <u>Conclusions</u>: There is a notable relationship between over-expression of MACC1 and the characteristics of glioma cells. Silencing of MACC1 was found to enhance the apoptosis and growth inhibitory rates of U251 glioma cells, and thereby increase their sensitivity to DDP chemotherapy.

Keywords: Brain glioma - chemotherapy sensitivity - MACC1 gene - U251 cell

Introduction

Glioma is the most general primary tumors in nervous system, and show the highest mortality and mortality among endocranial tumors, because of the marked characteristics of malignant proliferation and invasion (Kuhnt et al., 2011). Gliomas are rarely curable. Treatment for a gioblastoma is customized to the individual patient and may include surgery, radiation therapy, chemotherapy, or observation. So far, surgery is the most important initial approach. Chemotherapy and radiotherapy after initial surgical resection is regarded as an effective treatment plan to prevent recurrence and metastasis (Mrugala et al., 2013). However, chemotherapy does not work for everyone with a glioma. It helps about half the people treated. For this reason, the prognosis for patients especially with high-grade glioma is still gloom, despite being given prompt and comprehensive treatment. The median survival time for adults with an anaplastic astrocytoma is about two to three years, and for those with more aggressive glioblastomas, median survival drops off to about 12~14.6 months with a two-year median survival rate of 30% (Nieder et al., 2008).

In this new era, investigating the molecular mechanisms in carcinogenesis might shed light on this deadly disease (Vlachostergios et al., 2013; Burgio et al., 2014; Ugur et al., 2014). In our previous researches, the gene chips were used to detecte the up-regulation of metastasis-associated in colon cancer-1 (MACC1) gene in human glioma. MACC1 gene was firstly identified by Stein et al in 2009 (Stein et al., 2009). MACC1 could activate the HGF/MET signal pathway and mediate the metastasis and recurrence of colorectal cancer (Wang et al., 2014). The current reports suggested that MACC1 participated in regulation of cell proliferation, apoptosis, migration and invasion (Meng et al., 2013; Zhang et al., 2014; Zhen et al., 2014). Proximally, MACC1 has been identified to act as a key biomarker for the prognosis of kinds of cancer, including colorectal cancer, gastric carcinoma and non-small cell lung cancer (Ma et al., 2013; Wang et al., 2014; Yamamoto et al., 2014). Therefore, the object of this study was to first determine the correlation between the expression abnormality of MACC1 and the carcinogenesis and development of human glioma, and then search for its possible influence on chemotherapy sensitivity of human glioblastoma U251 cells.

*[1]Department of Neurobiology, College of Basic Medicine, [2]Department of Neurosurgery, Shengjing Hospital, [3]Department of Central Laboratory, School of Stomatology, China Medical University, Shenyang, China *For correspondence: xueyixue2012@163.com*

Materials and Methods

Materials

The study was approved by the Ethics Committees, and we obtained patient's permission before surgery. Total 49 glioma and corresponding paracancerous tissues provided by the Department of Neurosurgery, Shengjing Hospital of China Medical University from May 2011 to April 2014. The tumors with at least 1cm margin from the corresponding peri-cancerous tissues were obtained from all patients through surgical resection and further histologically proven to be gliomas.

All patients had not experienced radiation or chemotherapy before surgery. The patients include 32 men and 17 women (mean age: 54.1±3.8 years, age range: 47-69 years); and included 23 cases of astrogliomas (Grade I-II), 14 of anaplastic gliomas (Grade III), 12 of glioblastomas (GBM, Grade IV). Human glioblastoma cell line U251 was obtained from Biological Sciences Cell Resource Center (China). Real-time PCR reagents were from Takara Bio (Japan), TransMessenger from Qiagen (Germen), siRNA-MACC1 and siRNA-control from Invitrogen (Carlsbad, CA, USA), DDP from Sigma (USA). The MTT Cell Proliferation Assay Kit was from Beyotime Company (Shanghai, China), Annexin V Cell Apoptosis Assay Kit from Biosea Company (Beijing, China). The PCR primers of MACC1 gene were synthesized by Takara Bio (Japan).

Quantitative real time -PCR (qRT-PCR)

After total RNA was extracted from tissue and cell samples, cDNA was synthesized and used to detect the mRNA expression (Shang et al., 2014). The MACC1 primer was designed by Primer5 as follows: forward primer, 5'- AGGAGGTCAGCATTGGTTTCA -3', reverse primer, 5'- GAGCCCAGCAGTCTGTTTCA -3', and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as reference. The samples were normalized to 18s and the $18 < CT < 30$ were calculated with $2^{-\triangle\triangle CT}$ using the Applied Biosystems 7500.

Cell transfection

24h before transfection, appropriate concentration (about 80%) resuspended U251 cells were seeded on 6-well plates. Then 1 mg of siRNA-MACC1 or siRNA-control was mixed with Enhancer R, followed by mixing with 4 μl TransMessenger, and then 900μl Serum-free medium was further added for incubating the non-transfected U251 cells. The transfected cells were incubated for 4h, and normal media was added. After 48h, the cells were harvested to detect further.

Western blot analysis

Cells were harvested and extracted protein. SDS-PAGE electrophoresis and antibody hybridization were practiced as describe previously. The ECL analysis system (Santa Cruz, USA) was used for detection in accordance with the manufacture's protocol. Western blot quantification was performed using Image Processing and Analysis software. GAPDH was selected to be reference protein.

Flow cytometry detection

Cells (5×10^6) were harvested, and the apoptosis detection kit (Biosea, China) was used to examine the apoptosis rate in accordance with manufacturer's instruction. Then the cells were read by flow cytometry (BD, USA) (Ex 488 nm, Em 635 nm), and the obtained numerical values were analyzed with CELLQuest 3.0 software (BD, USA). Annexin V positive cells were regarded as apoptosis cells. The cells were counted by a dual-color flow cytometric method.

Cell proliferation assay

Cells were seeded in 96-well plate with 2×10^4 cells per well. 20 μl of 0.5mg/ml MTT solution (Sigma, USA) was added to each well, and the 96-well plate was incubated at 37°C. We cleaned up the media after 4 hr, and added 0.2 ml DMSO to each well. The 96-well plate was incubated 30 min, and read on an enzyme-labeled instrument (Bio-Rad, USA) with 570 nm wavelength. The obtained numerical values were used to construct the cell growth curve.

U251 cells treated by DDP

U251 cells were treated with DDP of three different concentrations (1, 3, 5μg/mL) (Jiang et al., 2000; Zhou et al., 2013), and the cell growth inhibitory rate and apoptosis rate was detected after 24h of incubation.

Statistical analysis

All results were obtained from independent experiments in three times. All numerical data were presented as mean±standard deviation (SD) and dealt with SPSS 13.0 software. A Student's t-test was performed to determine the significant differences between two groups. Pearson's correlation was analyzed between the grade of glioma and relative expression levels of MACC1 mRNA. One-way ANOVA and post hoc comparisons (LSD test) were used to determine the significant differences among multiple groups. $p < 0.05$ was considered as significant

Results

MACC1 mRNA up-regulated in human brain glioma specimens

Amplification of the MACC1 gene was shown in human brain gliomas using the primer melting curve analysis. The qRT-PCR analysis showed the ΔCt of glioma and peri-cancerous tissues were 2.947±0.314 and 3.614±0.297 respectively, and the ΔΔCt was -0.667. Compared with corresponding peri-cancerous tissues, the MACC1 expression increase 158.78% in the glioma ($p < 0.05$) (Figure 1). Further, there is an positive relationship between the MACC1 expression and the pathologic grades of gliomas (Table 1).

Influence of MACC1 silencing on apoptosis and growth inhibition of U251 cell

The western blot images showed clear bands of MACC1 protein in all groups (Figure 2). The analytical results confirmed that there was no significant difference between two control groups ($p > 0.05$). However, compared

Table 1. Correlation between MACC1 mRNA Expression in Brain Glioma Tissue and Pathological Differentiation

Differentiation (Grade)	Number of cases	MACC1 mRNA expression relative quantification (gliomas/ peri-cancerous tissues)	*p* value
Medium-well differentiated (Grade I-II)	23	1.467±0.148	<0.05*
Anaplastic glioma (Grade: III)	14	1.635±0.153	
Glioblastoma (GBM, Grade: IV)	12	1.765±0.161	

*A one-way ANOVA showed that FUBP1 mRNA expression in brain glioma tissue was significantly different among the three groups with different pathological differentiation of glioma ($p<0.05$); LSD test showed significant difference between groups of medium-well differentiated glioma (Grade I-II) and anaplastic glioma (Grade: III) ($p=0.03$); as well as groups of medium-well differentiated glioma (Grade I-II) and Glioblastoma (GBM, Grade: IV) ($p=0.01$)
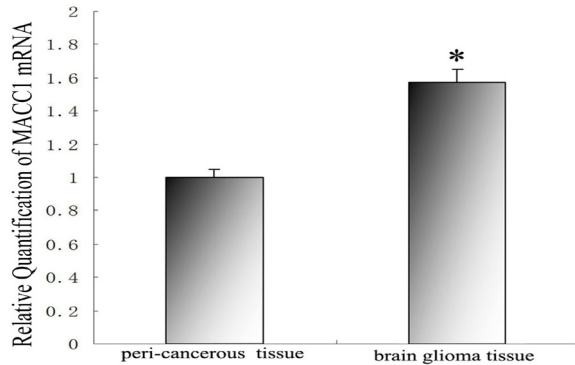

Figure 1. Real Time-PCR Analysis for MACC1 Expression in Brain Glioma Tissue. The expression of MACC1 was higher in brain glioma tissue compared to paracancerous tissue ($p<0.05$). *$p<0.05$ *vs* paracancerous tissue
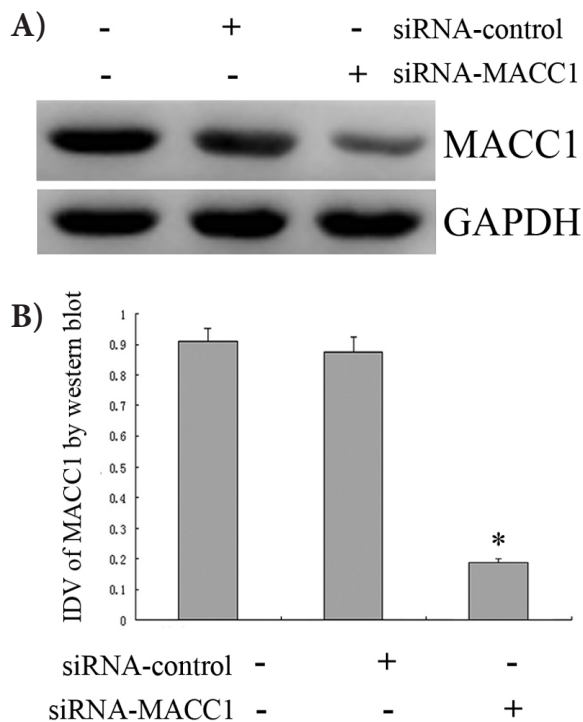

Figure 2. A) Representative Image of the Protein Level of MACC1. GAPDH was used as a reference control; **B)** quantitative analysis of the relative protein levels of MACC1 normalized to those of GAPDH was shown. Data were mean±SD of three independent experiments. *$p<0.05$; #$p<0.05$

to the other two groups, the MACC1 expression down-regulated remarkably in MACC1 silence group ($p<0.05$).

The apoptosis rate of the blank control, negative control and MACC1 silence groups were (2.57±0.19)%, (2.69±0.17 and (5.39±0.14)% respectively (Figure 3A).
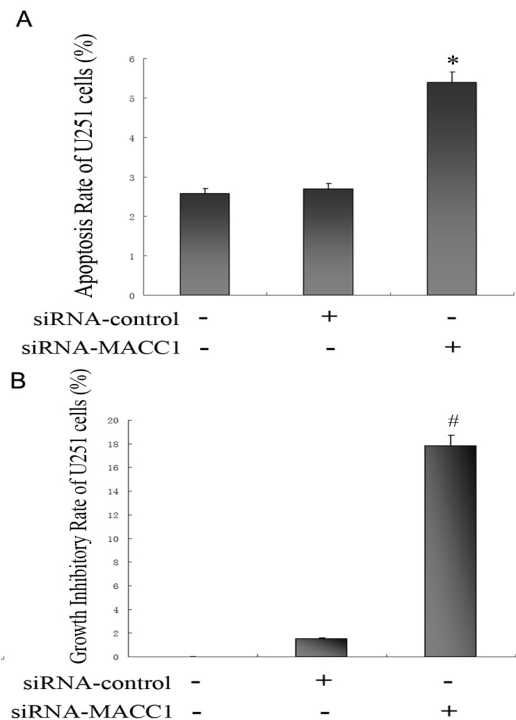

Figure 3. A) Impact of MACC1 Gene Silencing on the Apoptosis Rate of U251 cells. B) Impact of MACC1 gene silencing on the growth inhibitory rate of U251 cells. *,#$p<0.05$ *vs* control U251 cells

No significant difference was found between two control groups ($p>0.05$). The apoptosis rate in MACC1 silence group was significantly elevated relative to two control groups ($p<0.05$).

The growth inhibitory rate increased from 1.5% in negative control group to 17.8% in MACC1 silence group (Fig.3B), presenting significantly higher inhibition of cell proliferation in MACC1 silence group ($p<0.05$).

Influence of MACC1 gene silencing on chemotherapy sensitivity of human glioma U251 cells

Impact of MACC1 gene silencing on apoptosis rate of U251 cells: In the U251 cells treated with DDP, the apoptosis rate with different DDP concentrations DDP (1, 3, 5μg/ml) were (8.41±0.41)%, (13.24±0.47)% and (19.53±0.51)%. And in the MACC1-slienced U251 cells treated with DDP, the apoptosis rates were (12.87±0.52)%, (17.75±0.46)% and (25.82±0.29)% respectively (Figure 4A). Compared with the U251 cells treated with DDP, the apoptosis rate was significantly higher than that in the MACC1-slienced U251 cells at each DDP concentration (all $p<0.05$).
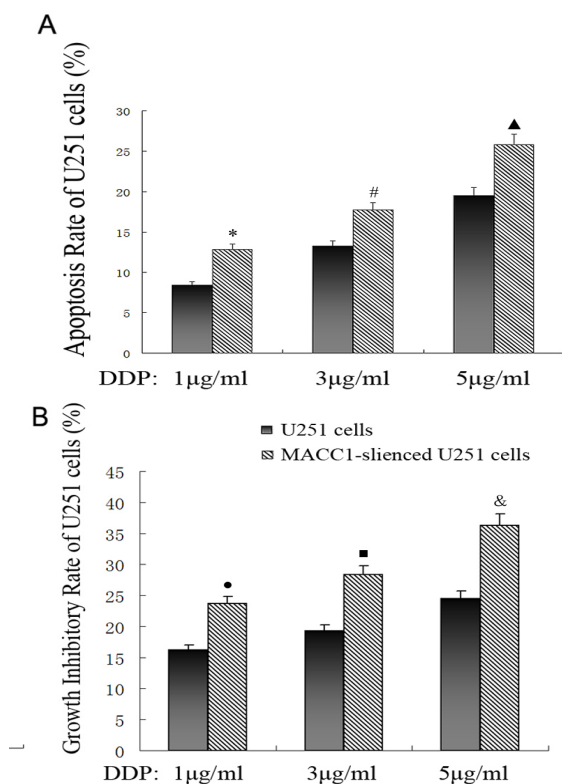
A

B

Figure 4. A) Impact of different Concentration of DDP on the Apoptosis Rate of U251 Cells and MACC1-Slienced U251 Cells. B) Impact of different concentration of DDP on the growth inhibitory rate of U251 cells and MACC1-slienced U251 cells. *,#,▲,●,■,& *p<0.05 vs* corresponding control U251 cells

Influence of MACC1 gene silencing on growth inhibitory rate of U251 cells: Following similar trends, in the U251 cells treated with DDP, the growth inhibition rates with different DDP concentrations DDP (1, 3, 5μg/ml) were (16.17±0.13)%, (19.32±0.11)% and (24.46±0.16)%. And in the MACC1-slienced U251 cells treated with DDP, the growth inhibition rates were (23.69±0.16)%, (28.37±0.12)% and (36.29±0.17)% respectively (Figure 4B). Compared with the U251 cells treated with DDP, the growth inhibition rate was remarkably higher than that in the MACC1-slienced U251 cells at each DDP concentration (all *p<0.05*). The IC_{50} of DDP decreased from (5.26±0.39) μg/mL to (3.74±0.32) μg/mL (*p<0.05*).

Discussion

MACC1 gene was a new carcinogenesis and metastasis related gene which firstly found in colorectal cancer. The recent researches affirmed MACC1 gene act as an oncogene and metastasis-inducing gene, it could activate Met to promote HGF/Met signal pathway which had been identified necessary for carcinogenesis and metastasis. Furthermore, overexpression of MACC1 gene had been reported in many kinds of cancers, including glioma (Hagemann et al., 2013; Yang et al., 2014). However, there was not a conclusive report about chemotherapy sensitivity of MACC1 gene in glioma.

In this study, we performed qRT-PCR and observed the up-regulation of MACC1 mRNA in brain glioma,

indicating the underlying correlation between MACC1 and the carcinogenesis of glioma. Furthermore, the MACC1 expression remarkably correlated with the pathologic grade of glioma. Our data demonstrated that over-expression of MACC1, which promoted tumor growth and migration, correlated with poor differentiation and high grade of glioma patients. This suggests that MACC1 plays an important role in glioma carcinogenesis and development. Consistent with these results, our study further demonstrates that MACC1 genes are positively correlated with pathologic grades, and functions as an oncogene in tumors. Moreover, we silenced MACC1 gene expression in U251 cells and investigated the apoptosis rate and growth inhibitory rate. Our results showed that the gene silencing of MACC1 induced remarkable apoptosis and reduced the proliferation ability of gliomas. Studies on the correlation between MACC1 and apoptosis are few in literature, calling for future efforts to investigate the mechanisms of how MACC1 influences cell apoptosis and proliferation.

Up to now, therapeutic approaches are tailored for individual patients, depending on the nature of the tumor, the growth rate, the location and the patient's state. Upon initial diagnosis of glioma, standard treatment consists of maximal surgical resection, combined with optional chemotherapy and radiotherapy. Chemotherapy and radiotherapy are recommended for reducing the risk of recurrence and metastasis. Chemotherapy is the treatment of cancer with one or more cytotoxic anti-neoplastic drugs ("chemotherapeutic agents") as part of a standardized regimen. At present, dosage of chemotherapy can be difficult due to a compromise between toxicity and efficacy. For this reason, chemotherapy sensitivity is of vital importance to give guidance on customized dose for individuals to maximize the effectiveness and minimize side-effects (Yang et al., 2009).

MACC1 is proven to be involved in regulating cell apoptosis and proliferation, and MACC1 silencing could induce remarkable apoptosis and reduced the proliferation ability of U251 cells. Therefore, we hypothesized that MACC1 might influence the effect of chemotherapy on glioma. And, there is not a conclusive report about the influence of MACC1 gene on chemotherapy sensitivity in glioma. For this reason, the MACC1-silenced U251 cells were treated with different concentrations of DDP (1, 3, 5μg/ml) (Jiang et al., 2000; Zhou et al., 2013) and then characterized. The apoptosis rate and growth inhibitory rate increased dramatically as compared with normal U251 cells, indicating the inhibition of cell proliferation and indicating the possibility of better chemotherapy for patients. On these grounds, we presumed that silencing or low-expression of MACC1 gene could enhance the chemotherapy effectiveness in the same concentration of DDP. In addition, if MACC1 gene was down-regulated or silenced, the chemotherapy with lower concentration DDP could obtain similar effectiveness, in order to reduce the side-effects of chemotherapy.

In conclusion, MACC1 functions as an oncogene in glioma carcinogenesis. The silencing of MACC1 could enhance the chemotherapy sensitivity of DDP in U251 cells. In this context, MACC1 would be a new potential

target gene for glioma treatment, or provide guidance to show chemosensitivity for chemotherapy.

Acknowledgements

References

Burgio E, Migliore L (2014). Towards a systemic paradigm in carcinogenesis: linking epigenetics and genetics. *Mol Biol Rep*, [Epub ahead of print].

Hagemann C, Fuchs S, Monoranu CM, et al (2013). Impact of MACC1 on human malignant glioma progression and patients' unfavorable prognosis. *Neuro Oncol*, 12, 1696-709.

Jiang S, Yuan S, Wang Y, et al (2000). Study on sensitivity of neuroglioma to chemotherapeutic drugs. *Hua Xi Yi Ke Da Xue Xue Bao*, **31**, 191-2.

Kuhnt D, Becker A, Ganslandt O, et al (2011). Correlation of the extent of tumor volume resection and patient survival in surgery of glioblastoma multiforme with high-field intraoperative MRI guidance. *Neuro Oncol*, **12**, 1339-48.

Ma J, Ma J, Meng Q, et al (2013). Prognostic value and clinical pathology of MACC-1 and c-MET expression in gastric carcinoma. *Pathol Oncol Res*, **4**, 821-32.

Meng F, Li H, Shi H, et al (2013). MACC1 down-regulation inhibits proliferation and tumourigenicity of nasopharyngeal carcinoma cells through Akt/β-catenin signaling pathway. *PLoS One*, **4**, 60821.

Mrugala MM (2013). Advances and challenges in the treatment of glioblastoma: a clinician's perspective. *Discov Med*, **83**, 221-30.

Nieder C, Astner ST, Mehta MP, et al (2008). Improvement, clinical course, and quality of life after palliative radiotherapy for recurrent glioblastoma. *Am J Clin Oncol*, **31**, 300-5.

Shang C, Zhang H, Guo Y, et al (2014). MiR-320a down-regulation mediates bladder carcinoma invasion by targeting ITGB3. *Mol Biol Rep*, **4**, 2521-7.

Stein U, Walther W, Arlt F, et al (2009). MACC1, a newly identified key regulator of HGF-MET signaling, predicts colon cancer metastasis. *Nat Med*, **1**, 59-67.

Ugur HC, Taspinar M, Ilgaz S, et al (2014). Chemotherapeutic resistance in anaplastic astrocytoma cell lines treated with a temozolomide-lomeguatrib combination. *Mol Biol Rep*, **2**, 697-703.

Vlachostergios PJ, Voutsadakis IA, Papandreou CN (2013). Mechanisms of proteasome inhibitor-induced cytotoxicity in malignant glioma. *Cell Biol Toxicol*, **4**, 199-211.

Wang Y, Hong Q, Wang J, et al (2014). Downregulated expression of metastasis associated in colon cancer 1 (MACC1) reduces gallbladder cancer cell proliferation and invasion. *Tumour Biol*, **4**, 3771-8.

Wang Z, Li Z, Wu C, et al (2014). MACC1 overexpression predicts a poor prognosis for non-small cell lung cancer. *Med Oncol*, **1**, 790.

Yamamoto H, Miyoshi N, Mimori K, et al (2014). MACC1 expression levels as a novel prognostic marker for colorectal cancer. *Oncol Lett*, **5**, 2305-9.

Yang I, Aghi MK (2009). New advances that enable identification of glioblastoma recurrence. *Nat Rev Clin Oncol*, **6**, 648-57.

Yang T, Kong B, Kuang YQ, et al (2014). Overexpression of MACC1 protein and its clinical implications in patients with glioma. *Tumour Biol*, **1**, 815-9.

Zhang K, Tian F, Zhang Y, et al (2014). MACC1 is involved in the regulation of proliferation, colony formation, invasion ability, cell cycle distribution, apoptosis and tumorigenicity by altering Akt signaling pathway in human osteosarcoma. *Tumour Biol*, **3**, 2537-48.

Zhen T, Dai S, Li H, et al (2014). MACC1 promotes carcinogenesis of colorectal cancer via β-catenin signaling pathway. *Oncotarget*, **11**, 3756-69.

Zhou YT, Li K, Tian H (2013). Effects of vinorelbine on cisplatin resistance reversal in human lung cancer A549/DDP cells. *Asian Pac J Cancer Prev*, **14**, 4635-9.