

# S100A4 regulates migration and invasion in hepatocellular carcinoma HepG2 cells via NF- $\kappa$ B-dependent MMP-9 signal

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**Abstract.** –**BACKGROUND AND OBJECTIVES:** We previously showed that the calcium-binding protein S100A4 is overexpressed and related to metastasis in hepatocellular carcinoma (HCC). However, whether S100A4 participates in the regulation of metastasis and its mechanisms in HCC is mostly unknown. Given the associations of S100A4, nuclear factor- $\kappa$ B (NF- $\kappa$ B/RelA) and MMP-9 with metastasis in a variety of malignancies, we explored a potential role of S100A4 in HCC metastasis and its mechanism.

**METHODS:** 20 patients with HCC invasion (Lymph node metastasis, microvascular invasion, major portal vein invasion and intrahepatic metastasis) and 20 patients without HCC invasion were included. These tissues were detected for the expression of S100A4, NF- $\kappa$ B/RelA and MMP-9 by immunohistochemistry and quantitative real time polymerase chain reaction (Q-PCR). Correlation between the expressions of S100A4, NF- $\kappa$ B/RelA and MMP-9 with the invasion was analysed. The expressions of S100A4, nuclear factor- $\kappa$ B and MMP-9 was evaluated in HepG2 cells by western blot and immunohistochemistry. HepG2 cells were stably transfected with S100A4-specific small interfering RNA (S100A4 siRNA) to knockdown of S100A4, then transiently transfected with S100A4 cDNA to rescue the S100A4 level and evaluated for effects on invasion and expression analysis for molecules involved in invasion. After the HepG2 cells recurred the S100A4 levels, the HepG2 cells was treated with 5  $\mu$ M Pyrrolidine Dithiocarbamate (PDTC) (a selective NF- $\kappa$ B inhibitor) to inhibit the NF- $\kappa$ B activity, or treated with Batimast (BB94: a MMPs inhibitor) to inhibit the MMP-9 activity. The expression analysis for molecules involved in invasion was analyzed.

**RESULTS:** A significant increase of S100A4, NF- $\kappa$ B/RelA and MMP-9 expression in HCC tissues with invasion than that of without invasion. A positive correlation was observed between S100A4, NF- $\kappa$ B/RelA, MMP-9 and invasion, respectively. In addition, S100A4 was positively correlated with NF- $\kappa$ B and MMP-9. S100A4 siRNA mediated knockdown of S100A4 in HepG2 cells resulted in significant reduction in the NF- $\kappa$ B activity and MMP-9 expression, and dramatically decreased its invasion. Moreover, the HepG2 cell metastatic potential was rescued by overexpression of S100A4 completely,

at the same time, the NF- $\kappa$ B activity and MMP-9 expression was also increased. Pretreatment with PDTC or BB94 was observed to significantly reduce NF- $\kappa$ B activity and MMP-9 expression and dramatically decreased S100A4-induced invasion.

**CONCLUSIONS:** Our findings indicate that S100A4 contributes to HCC metastasis by activation of NF- $\kappa$ B dependent MMP-9 expression, suggesting S100A4 as a novel diagnostic biomarker and therapeutic target in HCC.

*Key words:*

Hepatocellular carcinoma; Metastasis; S100A4, Nuclear factor - $\kappa$ B; MMP-9

## Introduction

Hepatocellular carcinoma (HCC) is a significant cause of cancer-related morbidity and mortality worldwide. Despite improvements in local therapies, including surgical resection, liver transplantation, and transarterial embolization, the prognosis remains poor for the majority of patients who develop recurrence or particularly for patients with distant metastasis<sup>1</sup>. Mechanisms involved in regulation of metastasis in HCC are likely complex and poorly understood.

S100A4, also known as metastasis-associated protein Mts1, belongs to the family of small calcium-binding S100 proteins containing two EF-hand calcium-binding motifs<sup>2</sup>. It is a symmetric homodimer, and undergoes a relatively large conformational change upon the typical EF-hand binding calcium, which is necessary for S100A4 to interact with its protein targets and generate biological effects<sup>3</sup>. It can bind the already known targets p53, F-actin, liprin  $\beta$ , myosin heavy chain II, and prevent their phosphorylation and multimerization. It has been demonstrated that S100A4 is directly involved in tumor metastasis including cell motility, invasion, apoptosis, angiogenesis and differentiation, and appears to be a metastasis factor and a molecular marker for clinical prognosis<sup>4-5</sup>.

Overexpression of S100A4 in a benign rat mammary epithelial cell line was shown to promote subcutaneous tumor growth and metastasis to the lungs and lymph nodes<sup>6</sup> and, correspondingly, the nonmetastatic human breast cancer cell line MCF-7 acquired a metastatic phenotype upon S100A4 transfection<sup>7</sup>. Furthermore, decreased expression of S100A4 in highly metastatic human osteosarcoma cells produced a significant suppression of experimental metastasis formation after intracardial injection in rats<sup>8</sup> and S100A4 antisense-transfected Lewis lung carcinoma cells displayed reduced metastatic capacity upon tail vein injection in syngeneic mice<sup>9</sup>. Altogether, the above-mentioned studies provide compelling evidence that S100A4 is directly involved in the formation of metastasis from several different tumor types.

Numerous studies have investigated the potential use of S100A4 as a prognostic marker. In a panel of 349 patients with breast cancer, Rudland et al<sup>10</sup> identified S100A4 protein expression as the most significant predictor of patient survival, even when compared with well-established markers of disease progression. Of patients with S100A4-negative tumors, 80% were alive after 19 years of follow-up, whereas only 11% of those with S100A4-positive tumors were still alive. Furthermore, an association between S100A4 protein expression and patient survival has been shown in several other tumor types, such as ovarian carcinoma, pancreatic cancer, malignant melanoma, bladder cancer, non-small cell lung cancer, gallbladder cancer, esophageal squamous cell carcinoma, and gastric cancer<sup>5,11</sup>. We have recently found S100A4 levels were significantly increased in HCC samples compared with controls. Moderately or poorly differentiated tumours had significantly higher levels of S100A4 protein than well-differentiated tumours, and those with tumour vascular invasion showed significantly higher S100A4 levels than those without invasion. Risk of recurrence increased in patients positive for S100A4, and S100A4 positivity correlated with a shorter overall survival time<sup>12</sup>. Though S100A4 is a well established marker of metastatic HCC, but the exact mechanisms responsible for the metastasis promoting effects are less well defined.

Matrix metalloproteinases (MMPs) are known to play an important role in cell migration during cancer invasion by degrading extracellular matrix proteins. Several experimental strategies have established an intimate connection between S100A4 and certain members of the MMP family. Down-

regulation of S100A4 expression in osteosarcoma cells led to reduced expression of MMP-2 and MT1-MMP, with a subsequent reduction in MMP-2 activity and a reduced ability to migrate through Matrigel-coated filters<sup>13</sup>. The invasive capability of human prostate cancer cells is also stimulated by S100A4, at least partly through transcriptional activation of MMP-9<sup>4</sup>. Our and others recently studies found S100A4 regulates motility and invasiveness in diffecient cancer cells by various signal pathways including MMP-9<sup>14-18</sup>.

The mechanisms by which S100A4 participates in the regulation of MMPs are mostly unknown. Nuclear factor-kappaB (NF- $\kappa$ B) has been shown to play an important role in the development and progression of cancer. Inhibition of NF- $\kappa$ B activity significantly reduced proliferation and invasion of HCC cells as well as down-regulated the expression of invasion-related molecules including matrix metalloproteinase MMP-9<sup>18</sup>, NF- $\kappa$ B could play an important role in MMP-9 regulation<sup>19-20</sup>.

S100A4 has been shown to activate the transcription factor nuclear factor-kB<sup>21-22</sup>, indicating that nuclear factor-KB activation and subsequent regulation of target genes might be involved in S100A4-mediated metastatic progression<sup>23</sup>. We, therefore, suggested that S100A4 might regulate invasion by NF- $\kappa$ B-dependent MMP-9 signal.

In this study, we first investigated the expression of S100A4, NF- $\kappa$ B and MMP-9 in HCC patients and the potential functional relationship in tumor metastasis and prognosis. Then, we investigated the mechanism of how S100A4 to regulate MMP-9.

## Patients and Methods

### Cell Culture

Human hepatocellular carcinoma (HCC) cell lines HepG2 was obtained from American Type Culture Collection and cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution. Cells were grown in a humidified incubator containing 5% CO<sub>2</sub> at 37°C.

### Selection of Cases

All HCC specimens along with complete clinical and pathological data were obtained from 40 HCC patients who underwent surgical resection at Department of Surgery, the Affiliated Hospital of medical college, Qingdao University, Qingdao,

China, between Mar. 2008 and Dec 2012. Of the 40 HCC patients, 20 patients with HCC invasion<sup>24</sup> (Lymph node metastasis, microvascular invasion, major portal vein invasion and intrahepatic metastasis) and 20 patients without HCC invasion were included. Part of the 40 HCC tissues immersed in RNAlater (Ambion, Inc., Austin, TX, USA) immediately after surgical resection and stored at  $-80^{\circ}\text{C}$  were subjected to quantitative real-time RT-PCR. Part of the 40 HCC tissues were paraformaldehyde fixed for immunohistochemistry analysis. None of the patients had received adjuvant therapies before surgery.

### ***Immunohistochemical Staining and Evaluation***

Immunohistochemical staining was carried out using the primary antibody to human S100A4 (Ab-8, LabVision-Neomarker, Inc., Fremont, CA, USA), NF- $\kappa$ B/RelA (Santa Cruz, CA, USA) and MMP-9, respectively. The sections were dewaxed through xylene and ethanol. Following the blocking of endogenous peroxidase and antigen retrieval (microwave heating in a citrate buffer for 40 min), the sections were exposed to the primary antibody at  $48^{\circ}\text{C}$  overnight and stained using a streptavidin-biotin-peroxidase kit (Nichirei, Tokyo, Japan). The labeled antigen was then visualized by 3,3'-diaminobenzidine tetrahydrochloride, followed by counterstaining with hematoxylin. S100A4, NF- $\kappa$ B/RelA and MMP-9 was evaluated within the tumor by three clinical pathologists who were unaware of patient outcome. The various results were unified by consensus. The immunoreactivity was evaluated according to the percentage of positively stained cells. Immunohistochemical staining was performed for S100A4<sup>12</sup>, RelA<sup>25</sup> and MMP-9<sup>26</sup> as described previously. Immunohistochemistry for specific detection of activated RelA was performed using monoclonal anti-human RelA antibody that recognizes an epitope overlapping the nuclear localization signal and I $\kappa$ B- $\alpha$  binding site of RelA. Secondary antibody for the detection of primary antibody: anti-rabbit IgG. The negative controls were processed in a similar manner with PBS instead of primary antibody.

### ***Quantitative Real-Time PCR***

Total RNA was extracted by RNeasy extraction kit (Qiagen, Valencia, CA, USA) and reverse transcribed according to standard protocols. Real-time PCR was carried out using an ABI PRISM 7000 Sequence Detection System (Ap-

plied Biosystems, Forst City, CA, USA). Two micrograms of total RNA was subjected to reverse transcription to synthesize cDNA using the Proto-Script M-MuLV Taq RT-PCR Kit (New England Biolabs), according to the manufacture's instruction, followed by real-time PCR using the TransStart Green qPCR SuperMix (TransGen Biotech, Beijing, China). The transcription of GAPDH was used as an internal control for normalization. Reactions were run in triplicate in two independent experiments. The primer sequences were as followed: S100A4-forward, 5'-CAGATCCTGACTGCTGCCATGGCG-3'; S100A4-reverse, 5'-ACGTGTCTGAAGGAGC-CATGGTGG-3'. MMP-9- forward, 5'-CCCACT-TACTTTGGAAACG- 3', reverse ,5'-GAAGAT-GAATGGAAATACGC- 3'.

### ***S100A4 siRNA Transfection***

Validated S100A4-specific and scrambled small interfering RNAs (siRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HepG2 cells in 6-well plates or 96-well plates were grown to 50% confluence, and using electroporation (Amaxa, Inc., Gaithersburg, MD, USA), HepG2 cells were transiently transfected with S100A4 siRNA in concentrations ranging from 30 to 120 nmol/L and scrambled siRNA (120 nmol/L). Cells were incubated under these conditions for 48 h and silencing was then confirmed by reporter assays as well as Western blotting. HepG2/S100A4 siRNA cells were selected with 600  $\mu\text{g}/\text{mL}$  G418 for 14 d to acquire the stably transfected HepG2/S100A4 siRNA cells.

### ***pcDNA3.1-S100A4 cDNA Plasmid and Transfection***

The pcDNA3.1-S100A4 cDNA plasmid was kindly gifted by Doc. W Jia, Department of Hepatobiliary and Pancreatic Surgery, Huaxi Hospital Sichuan University, Chengdu, Sichuan, China<sup>18</sup>. The stably transfected HepG2/S100A4 siRNA cells were transiently transfected with pcDNA3.1-S100A4 cDNA and pcDNA3.1 for 48 hours, using Lipofectamine 2000, then for further analysis.

### ***PDTC or BB94 Treatment***

The stably transfected HepG2/S100A4 siRNA cells were pretreated with pyrrolidine dithiocarbamate (PDTC) at a concentration of 5  $\mu\text{M}$  or BB94 (a MMPs inhibitor, British Biotech Ltd., Oxford, UK) at a concentration of 0.5 $\mu\text{g}/\text{mL}$  for

24 hours, then transiently transfected with pcDNA3.1-S100A4 cDNA and pcDNA3.1 for another 48 hours.

### **Electromobility Shift Assay (EMSA)**

Nuclear extracts were prepared by lysing adipocytes in a buffer containing 20 mmol/L HEPES, pH 7.9, 10 mmol/L NaCl, 0.2 mol/L EDTA, 2 mmol/L DTT, and Complete (Roche Diagnostics GmbH, Mannheim, Germany). The cells were centrifuged and the pellet was resuspended in a buffer containing 2 mmol/L HEPES, pH 7.9, 0.75 mmol/L spermidine, 0.15 mmol/L spermin, 420 mmol/L NaCl, 0.2 mol/L EDTA, 2 mmol/L DTT, 25% glycerol, and Complete (Roche Diagnostics GmbH). Nuclear fragments were incubated for 30 minutes on ice and centrifuged; after that, the supernatant was used for the EMSA or the supershift assay. The NF- $\kappa$ B probe had the following consensus sequence: 5'-AGT TGA GGG GAC TTT CCCAGG C (top strand only). The labeling of the probe and the binding reaction were performed using the DIG Gel shift kit (Roche Diagnostics GmbH). Samples were separated in a nondenaturing 6% PAGE gel, and bands were detected using a Lumi-imager device (Roche Diagnostics GmbH).

### **Western Blotting**

Cells were collected with sample buffer. Cell lysates were centrifuged at 10000 g for 10 min at 4°C and the supernatant was stored at -70°C. Protein concentrations were determined with a Bio-Rad (Hercules, CA, USA) kit. 40  $\mu$ g aliquots of protein were subjected to 12% and 6% SDS-PAGE gels. Then the protein was blotted onto a polyvinylidene fluoride (PVDF) membrane. Primary antibodies against MMP-9 (1:1000, Becton Dickinson, San Diego, CA, USA), RelA (1:1000, Cell Signaling, Beverly, MA, USA), S100A4 (1:1000, Cell Signaling) and  $\beta$ -actin (1:2000, Becton Dickinson) were used according to the manufacturer's recommendations. After washing the membrane, a second antibody (HRP-conjugated anti-mouse IgG) (1:2000, Becton Dickinson) was used to detect S100A4, MMP-9 and RelA and  $\beta$ -actin. The bands were visualized using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA) with 5 to 30 min exposure after washing the membrane.  $\beta$ -actin was used as the protein loading control.

### **Cell Migration and Cell Invasion Assay**

For the Boyden chamber assay,  $2.5 \times 10^5$  cells were seeded into transwell filter membrane chambers (pore size, 12.0  $\mu$ m; Millipore, Billerica, MA) and allowed to accommodate for 24 h. The number of cells that migrated to the lower chamber was counted in a Neubauer chamber. For the invasion assays, transwell membranes were coated with 1:3 diluted Matrigel (BD Biosciences, Heidelberg, Germany).

### **Statistical Analysis**

The values reported are means  $\pm$  standard error of the mean (SEM). Statistical analysis between two samples was performed using Student's *t*-test. Statistical comparisons of more than two groups were performed using one-way analysis of variance (ANOVA) with Bonferroni's post-hoc test. In all cases,  $p < 0.05$  was considered significant.

## **Results**

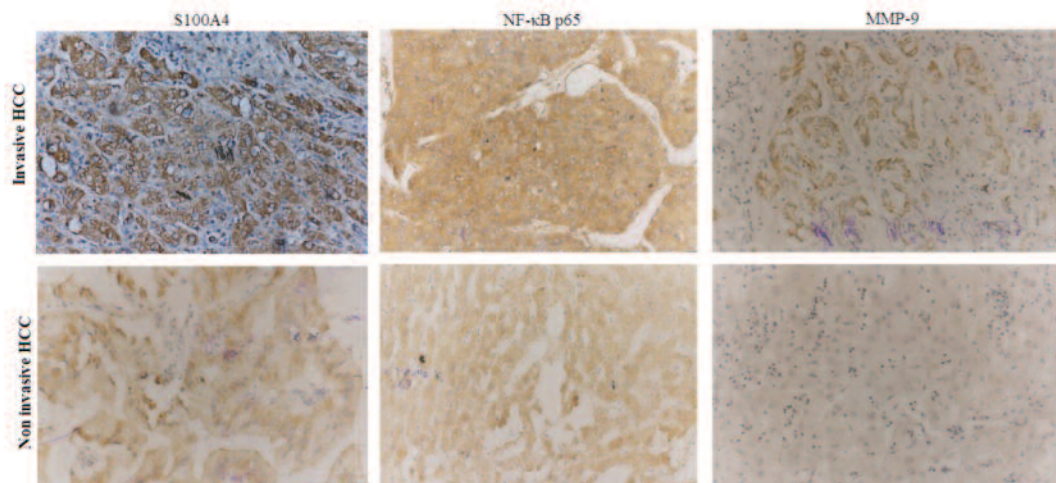
### **Increased Expression of S100A4, NF- $\kappa$ B p65 and MMP-6 is associated with invasion in HCC**

Results of immunohistochemistry (IHC) staining showed that S100A4, NF- $\kappa$ B p65 and MMP-9 was strongly expressed in 20 cases of invasive (lymph node metastasis, microvascular invasion, major portal vein invasion and intrahepatic metastasis) HCC tissues, and weakly expressed in 20 cases of non invasive HCC tissues (Figure 1A, Table I). S100A4, NF- $\kappa$ B p65 and MMP-9 had a significantly higher prevalence of overexpression in HCC with invasion than those without invasion (Table I). Significantly, S100A4 expression was positively with NF- $\kappa$ B p65 and MMP-9 expression (Table II).

### **Effect of S100A4 Silencing on NF- $\kappa$ B p65 Activation and Nuclear Translocation in Human HepG2**

To address the question whether the S100A4 is associated with stimulation of the NF- $\kappa$ B pathway, we measured mRNA and protein levels of NF- $\kappa$ B p65 in the S100A4 siRNA and scrambled siRNA transfected HepG2 cells in concentrations ranging from 30 to 120 nmol/L and scrambled siRNA (120 nmol/L) for 48 h using real-time PCR, western blotting and EMSA assay. S100A4 mRNA and protein was significantly inhibited in a concentration-dependent as compared to scrambled siRNA by real-time PCR (Figure 2A) and western blot assay (Figure 2B). As shown in





**Figure 1.** Immunohistochemistry (IHC) staining of S100A4, NF-κB p65 and MMP-9 in invasive and non-invasive HCC tissues. Strongly expressed was found in invasive HCC tissues, and weakly expressed was found in non-invasive HCC tissues.

Figure 2A, NF-κB p65 mRNA expression decreased significantly following S100A4 siRNA transfection in a concentration-dependent manner as compared to scrambled siRNA. Additionally, NF-κB p65 activation in S100A4 siRNA transfected HepG2 cells was confirmed at the protein level (western blot) as shown in Figure 1B. Also, NF-κB p65 nuclear translocation following S100A4 siRNA transfection was inhibited in a concentration-dependent manner as compared to scrambled siRNA by EMSA (Figure 2C) assay. When HepG2 cells were transiently transfected with S100A4 siRNA in concentration over 90 nmol/L, NF-κB p65 activation and nuclear translocation was almost completely inhibited.

**Effect of S100A4 Silencing on mRNA and Protein Levels of MMP-9 in Human HepG2**

To address the question whether the effect of S100A4 is associated with changes at the transcriptional and protein level, we measured mRNA

**Table I.** Correlation of S100A4 expression with NF-κB p65 and MMP-9 in invasive and non-invasive HCC tissues.

	S100A4		NF-κB p65		MMP-9	
	+	-	+	-	+	-
Invasive (n=20)	17	3	16	4	18	2
Non-invasive (n=20)	5	15	6	14	5	15
	$p < 0.05$		$p < 0.05$		$p < 0.05$	

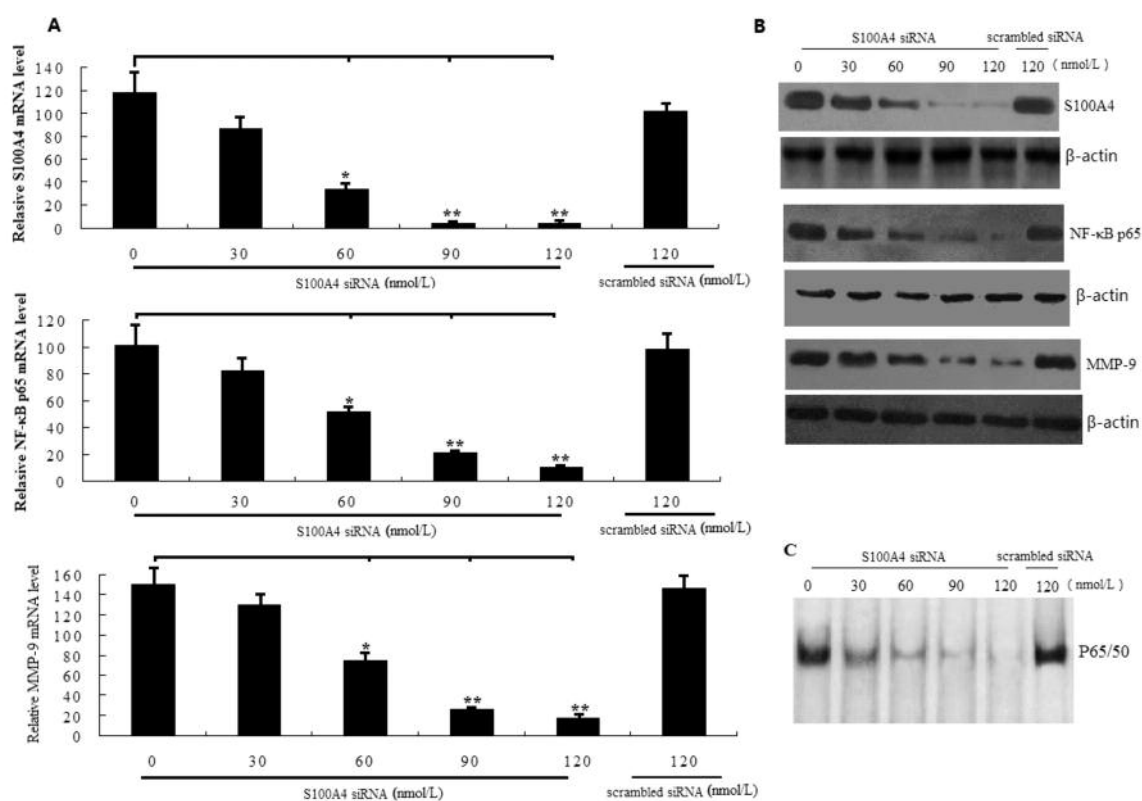
and protein levels of MMP-9 in the absence and presence of S100A4 siRNA. HepG2 cells were transiently transfected with S100A4 siRNA in concentrations ranging from 30 to 120 nmol/L and scrambled siRNA (120 nmol/L) for 48 h. The S100A4 siRNA inhibited a concentration-dependent decrease in MMP-9 mRNA (Figure 2A) and protein (Figure 2B) levels compared with scrambled siRNA. When HepG2 cells were transiently transfected with S100A4 siRNA in concentration of 90-120 nmol/L, S100A4 and MMP-9 mRNA and protein was almost completely inhibited.

**Effect of S100A4 Overexpression on NF-κB p65 Activation and Nuclear Translocation in Human HepG2**

In the stably transfected HepG2/S100A4 siRNA cells, NF-κB p65 activation and nuclear translocation was almost completely inhibited. However, when the stably transfected HepG2/S100A4 siRNA cells were transiently transfected with S100A4 cDNA for 48 hours to rescue S100A4 levels, NF-κB p65 mRNA ex-

**Table II.** Relevance of S100A4 with NF-κB p65 and MMP-9 in 40 cases of HCC tissues.

	NF-κB p65		MMP-9	
	+(n=22)	-(n=18)	+(n=23)	-(n=17)
S100A4 +(n=22)	16	6	17	5
S100A4 -(n=18)	6	12	6	12
	$p < 0.01$		$p < 0.01$	



**Figure 2.** Effect of S100A4 silencing on MMP-9, NF-κB p65 activation and nuclear translocation in human HepG2. HepG2 cells were transiently transfected with S100A4 siRNA and scrambled siRNA in concentrations ranging from 30 to 120 nmol/L and scrambled siRNA (120 nmol/L) for 48 h. **A**, S100A4 mRNA, NF-κB p65 mRNA and MMP-9 mRNA levels were detected using quantitative real-time PCR. **B**, S100A4 and MMP-9 protein and NF-κB p65 nuclear translocation levels were detected using western blot assay. **C**, NF-κB p65 activation in human HepG2 was detected using EMSA. \* $p < 0.05$ , \*\* $p < 0.01$ .

pression increased significantly following S100A4 cDNA transfection as compared to scrambled siRNA (Figure 3A). Additionally, NF-κB p65 nuclear translocation following S100A4 cDNA transfection was increased as compared to scrambled siRNA by western blot (Figure 3B) and EMSA (Figure 3C) assay.

#### **Effect of S100A4 Overexpression on mRNA and Protein Levels of MMP-9 in Human HepG2**

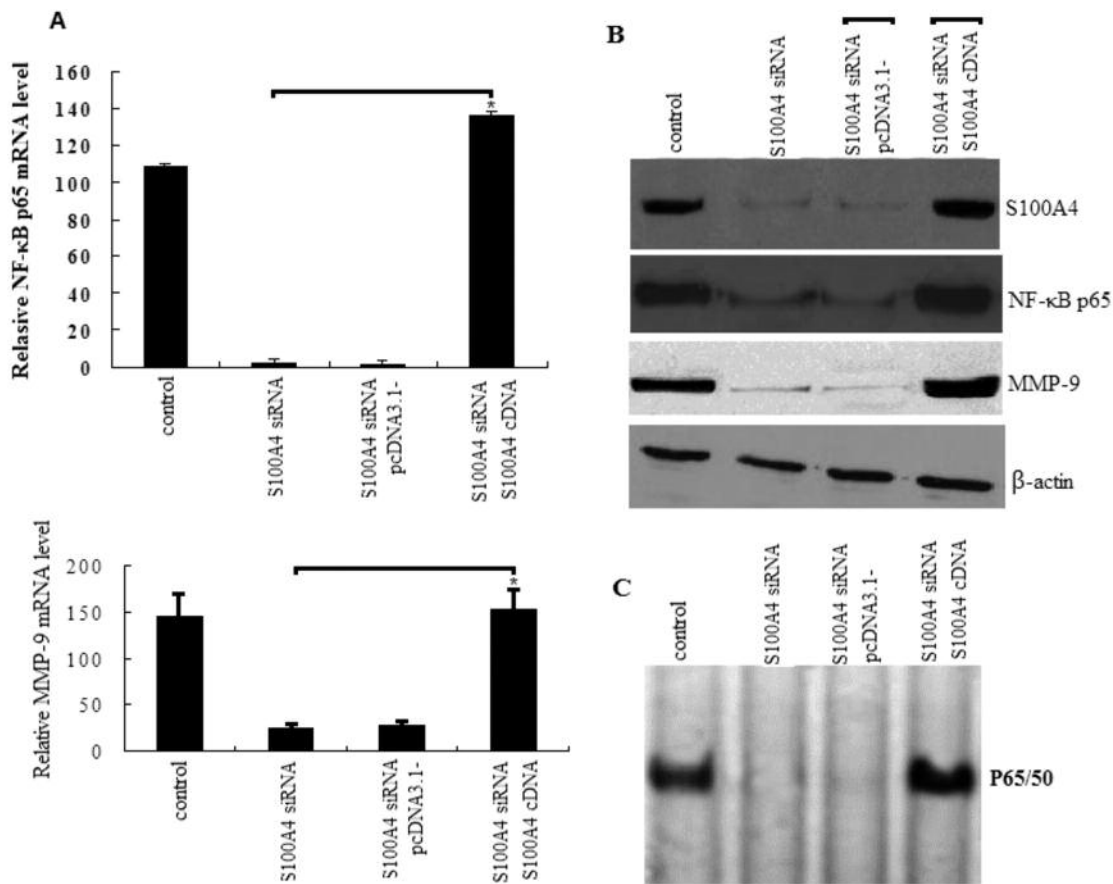
S100A4 and MMP-9 mRNA and protein was inhibited in the stably transfected HepG2/S100A4 siRNA cells. However, when stably transfected HepG2/S100A4 siRNA cells were transiently transfected with S100A4 cDNA for 48 hours to rescue S100A4 levels, S100A4 and MMP-9 protein (Figure 3B), MMP-9 and NF-κB p65 mRNA (Figure 3A) and NF-κB p65 activation (Figure 3C) was significantly increased compared with scrambled siRNA.

#### **S100A4 Upregulates MMP-9 via NF-κB p65 Dependent Signal**

The stably transfected HepG2/S100A4 siRNA cells were pretreated with pyrrolidine dithiocarbamate (PDTC) at a concentration of 5 μM for 24 hours, then following by S100A4 cDNA transfection for 48 hours. NF-κB p65 mRNA expression (Figure 4A), NF-κB p65 activation (Figure 4C) and NF-κB p65 nuclear translocation (Figure 4D) was decreased as compared to scrambled siRNA. Also, MMP-9 mRNA (Figure 4B) and protein (Figure 4E) was almost completely inhibited as compared to scrambled siRNA.

#### **Knockdown of S100A4 Inhibits Migration and Invasion of HepG2 Cells**

To examine the effects of S100A4 silencing on cell migration and invasion, we used a Boyden chamber assay to detect cell motility. Figure 5A shows that S100A4 siRNA significantly in-



**Figure 3.** Effect of S100A4 overexpression on NF-κB p65 activation, nuclear translocation and MMP-9 expression in human HepG2. The stably transfected HepG2/S100A4 siRNA cells were transiently transfected with S100A4 cDNA for 48 hours. **A**, NF-κB p65 mRNA and MMP-9 mRNA levels were detected using quantitative real-time PCR. **B**, S100A4 and MMP-9 protein and NF-κB p65 nuclear translocation levels were detected using western blot assay. **C**, NF-κB p65 activation in human HepG2 was detected using EMSA. \*  $p < 0.01$ .

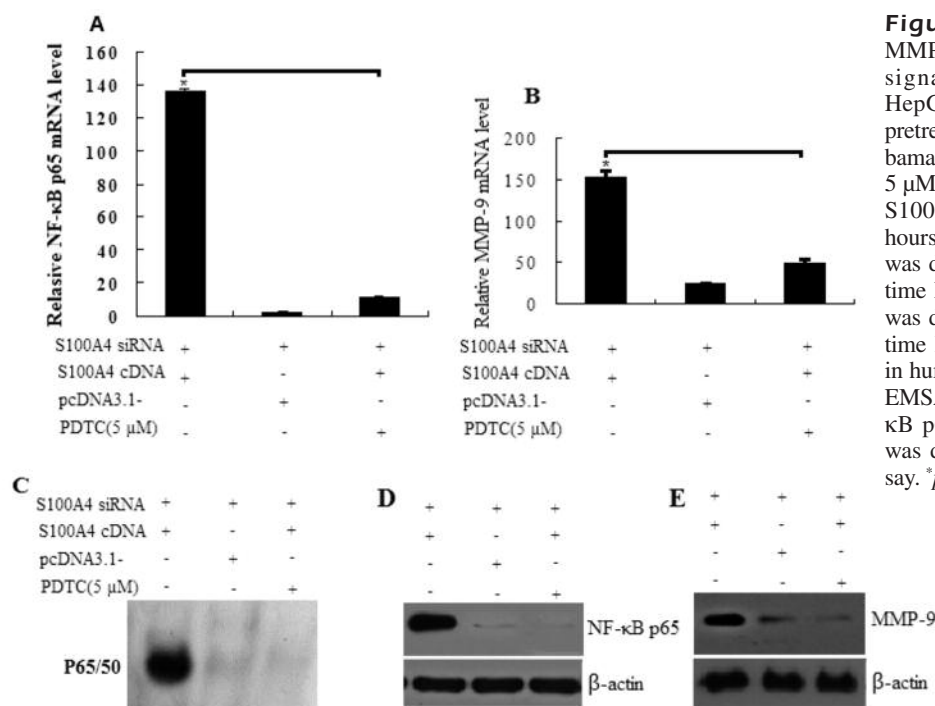
hibited migration in a concentration-dependent manner for 24 hours. Similarly, Figure 5B indicates that the invasiveness of HepG2 cells was also reduced after transfection of S100A4 siRNA for 24 hours.

### **S100A4 Overexpression Promotes Migration and Invasion of HepG2 Cells**

As shown in Figure 5 A-B, migration and invasion was significantly inhibited in the stably transfected HepG2/S100A4 siRNA cells. However, when the stably transfected HepG2/S100A4 siRNA cells were transiently transfected with S100A4 cDNA for 48 hours to rescue S100A4 levels, the migration and invasion was significantly increased (Figure 5 A-B).

### **S100A4 Regulates Migration and Invasion of HepG2 cells via NF-κB p65 Dependent MMP-9 Signal**

Thought the stably transfected HepG2/S100A4 siRNA cells that transiently transfected with S100A4 cDNA for 48 hours significantly increased the migration and invasion ability (Figure 5 A-B). However, when the stably transfected HepG2/S100A4 siRNA cells were pretreated with PDTC (a selective NF-κB inhibitor) at a concentration of 5 μM for 24 hours, or BB94 (MMPs inhibitor) at a concentration of 0.5 μg /mL for 24 hours, then following by S100A4 cDNA transfection for 48 hours, the migration and invasion was significantly decreased (Figure 5 A-B).



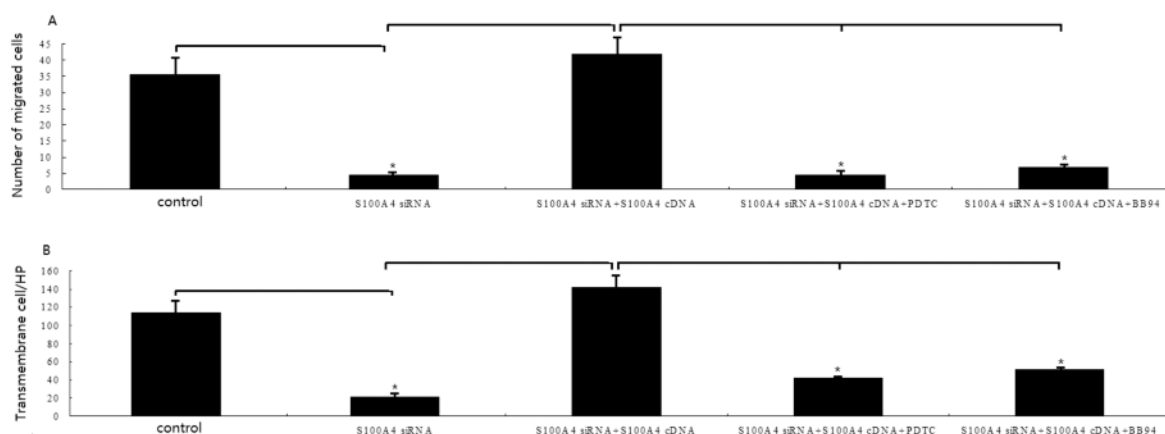
**Figure 4.** S100A4 upregulates MMP-9 via NF-κB p65 dependent signal. The stably transfected HepG2/S100A4 siRNA cells were pretreated with pyrrolidine dithiocarbamate (PDTC) at a concentration of 5 μM for 24 hours, then following by S100A4 cDNA transfection for 48 hours. **A**, NF-κB p65 mRNA levels was detected using quantitative real-time PCR. **B**, MMP-9 mRNA levels was detected using quantitative real-time PCR. **C**, NF-κB p65 activation in human HepG2 was detected using EMSA. **D**, MMP-9 protein and NF-κB p65 nuclear translocation levels was detected using western blot assay. \**p* < 0.01.

### Discussion

S100A4 expression has shown significant increase in breast, pancreatic, esophageal and thyroid carcinomas<sup>27-29</sup>. In these cancers S100A4 expression is found at elevated levels compared with normal tissue, suggesting that enhanced S100A4 expression contributes to manifestation of a metastatic phenotype. Studies in rodents

have provided evidence supporting the direct involvement of S100A4 in tumor progression and metastasis. The role of S100A4 in cancer has been examined most widely in breast cancer models, which have demonstrated that overexpression of S100A4 in nonmetastatic mammary tumor cells confers a metastatic phenotype<sup>6-7</sup>.

The association between S100A4 expression and metastasis observed in animal studies has led to a number of studies examining the utility of S100A4



**Figure 5.** Effect of S100A4 on cell migration and invasion in HepG2 cells. **A**, The cell migration and **B** cell invasion were measured using a Boyden chamber for 24 h with polycarbonate filters respectively. The migration and invasion abilities of HepG2 cells were quantified by counting the number of cells that invaded to the underside of the porous polycarbonate as described in the Materials and Methods section. The values represented the means ± SD of at least three independent experiments. \**p* < 0.01.



expression as a prognostic marker in human cancers. In a retrospective study of 349 invasive human breast cancer specimens, S100A4 expression and other variables were evaluated for their prognostic significance over a period of 14-20 years<sup>10</sup>. Analysis of patients with carcinomas that stain positively for S100A4 expression demonstrated that S100A4 expression is highly correlated with patient death. In addition to breast cancer, S100A4 has been shown to be a prognostic marker in a number of human cancers, including pancreatic, esophageal and thyroid carcinomas<sup>28-30</sup>. The universality of S100A4 expression in a variety of cancers illustrates the potential use of S100A4 as a marker for tumor metastasis and disease progression.

In the present study, we analyzed the S100A4 expression by immunohistochemistry in 20 cases of invasive HCC tissues, and 20 cases of non invasive HCC tissues, and found S100A4 was strongly expressed in invasive HCC tissues, and weakly expressed in the non invasive HCC tissues. Furthermore, S100A4, NF- $\kappa$ B p65 and MMP-9 had a significantly higher incidence of overexpression in HCC with invasion than those without invasion. Significantly, S100A4 expression was positively with NF- $\kappa$ B p65 and MMP-9 expression. We, therefore, suggested S100A4 may be the potential marker for HCC tumor metastasis. However, whether S100A4 participates in the regulation of metastasis and its mechanisms in HCC is mostly unknown.

In this work we found S100A4 silencing inhibited invasion, and promoted invasion when overexpressed in the HepG2 cells. This study also demonstrates a role for NF- $\kappa$ B in mediating the S100A4 silencing and overexpression on the HepG2 invasion *in vitro*, and strongly implicates NF- $\kappa$ B as a key mediator of the effects of S100A4 on the invasive phenotype. Transcription factors of the Rel/NF- $\kappa$ B family regulate diverse aspects of pancreatic adenocarcinoma tumor biology, including cellular invasiveness<sup>31-34</sup>. Grotterød et al<sup>35</sup> have shown that overexpression of S100A4 in the human osteosarcoma cell line II-11b exhibits increased NF- $\kappa$ B binding activity and invasion when compared to controls. Hua et al<sup>36</sup> has found S100A4 inhibition decreased the expression of both NF- $\kappa$ B p65 and phosphor (Ser32)-I- $\kappa$ B- $\alpha$ . Our study found S100A4 silencing inhibited NF- $\kappa$ B p65 activation and nuclear translocation followed by decreased invasion in the HepG2 cells. Furthermore, S100A4 overexpression increased NF- $\kappa$ B p65 activation and nuclear translocation followed by increased invasion in the HepG2

cells. However, when NF- $\kappa$ B p65 activation was inhibited by PDTC treatment, S100A4 overexpression could not increase invasion in the HepG2 cells. We, therefore, suggested that S100A4 regulate invasion by NF- $\kappa$ B dependent pathway.

The NF- $\kappa$ B transcription factor modulates expression of metalloproteinases in a range of human cancers<sup>37-40</sup>. We focused on MMP-9 as its promoter contains motifs homologous to the binding sites for the NF- $\kappa$ B p65 protein and levels of NF- $\kappa$ B p65 activity have been shown to modulate MMP-9 transcription<sup>41-42</sup>. We have also found MMP-9 activity to be decreased by S100A4 silencing<sup>16,18,43</sup>. Our study found S100A4 silencing inhibited NF- $\kappa$ B p65 activation and MMP-9 expression, and overexpression increased NF- $\kappa$ B p65 activation and MMP-9 expression. When NF- $\kappa$ B p65 was inhibited by PDTC, MMP-9 was also inhibited, and S100A4 overexpression could not increase invasion in the HepG2 cells. Furthermore, S100A4 overexpression could not increase invasion in the HepG2 cells when the cells was treated with BB94 to inhibit the MMP-9 expression. These observations together with those reported here are consistent with a model in which NF- $\kappa$ B p65 activation, resulting from S100A4 overexpression, enhances MMP-9 activity and promotes cellular invasiveness, and vice versa.

However, we interpret these observations cautiously. While MMP-9 is an important mediator of HCC cellular invasiveness, other proteases and modulators of cellular motility not studied here play additional roles in determining tumor cell invasiveness. The nature of the mechanisms through which S100A4 influences nuclear transcription factor activity requires further investigation.

## Conclusions

Increased invasiveness induced by S100A4 overexpression is associated with, and dependent upon, NF- $\kappa$ B p65 activation. MMP-9 appears to be an important effector of the enhanced invasiveness that S100A4 overexpression induces. Knockdown of S100A4 lead to significant decrease in cellular invasiveness. Targeting S100A4 and its downstream signaling intermediaries represents a rational approach for developing novel anticancer therapeutics.

## Conflict of interest

The Authors declare that they have no conflict of interests.

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