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# S100A4 promotes squamous cell laryngeal cancer Hep-2 cell invasion via NF-kB/MMP-9 signal

W. ZHANG[1], Y. LIU[2], C.-W. WANG[2]

[1]Department of Head and Neck Tumor Surgery, [2]Department of Radiotherapy, Linyi Cancer Institute Hospital, Linyi, China

**Abstract. – OBJECTIVE: S100A4 is a member of the S100 family of calcium-binding proteins, which possesses a wide range of biological functions, such as regulation of angiogenesis, cell survival, motility, and invasion. Here, we demonstrate for the first time a major role of S100A4 in the cell invasion properties of the human laryngeal squamous carcinoma cells (LSCC) and evaluated the mechanism.**

**MATERIALS AND METHODS: Cultured human LSCC cell line Hep-2 was overexpressed by transfection of pcDNA3.1-S100A4 plasmid. For this, cellular Hep-2 expression was quantified by Western blot analysis. Moreover, cell invasion and migration assays were performed. Furthermore, the impact of the S100A4 on NF-kB activity and MMP-9 expression was detected.**

**RESULTS: We found S100A4 potently promoted Hep-2 invasion, by increasing cell motility and matrix metalloproteinase-9 (MMP-9) production. The increase in MMP-9 production was mediated by activation of nuclear factor-kB transcriptional activity by S100A4. After MMP-9 and NF-kBp65 was inhibited by BB94 treatment and NF-kBp65 siRNA transfected, pcDNA3.1-S100A4 induced cell invasion and migration was decreased.**

**CONCLUSIONS: Our findings thus establish S100A4 as a major factor in the invasive abilities of Hep-2 cells.**

*Key Words:*
Laryngeal squamous carcinoma, Invasion, S10044, Nuclear factor-kB, MMP-9.

## Introduction

Laryngeal squamous cell carcinoma (LSCC), one of the most common tumors of the head and neck, occurs mainly in adult males who abuse tobacco and alcohol, and is characterized by squamous differentiation. Although early-stage glottic cancer has a favorable prognosis, with 5-year survival rates of over 70%[1], many supraglottic and subglottic cancers are not diagnosed until severe signs develop, by which time the 5-year survival rate has dropped to less than 50%. Locoregional recurrence, cervical lymph node metastases and distant metastases are the factors significantly affecting prognosis in LSCC patients[2]. The recognition and identification of tumor markers associated with metastasis are key elements in predicting the biological behavior of the tumor and deciding on the most appropriate therapeutic strategy.

The metastasis-promoting protein S100A4 belongs to the S100 family of calcium-binding proteins. Many studies have verified S100A4 as an important player in the metastatic process, and increased expression of the protein has been associated with poor prognosis in various human cancer types[3-7]. The protein seems to have multiple intracellular and extracellular functions that may contribute to its prometastatic effects (reviewed in several papers)[8,9].

Since the cloning of S100A4 from highly metastatic murine mammary carcinoma cells[10], its importance in the process of cancer metastasis has been corroborated by several experimental approaches. 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[11], and, correspondingly, the nonmetastatic human breast cancer cell line MCF-7 acquired a metastatic phenotype upon S100A4 transfection[12]. Furthermore, decreased expression of S100A4 in highly metastatic human osteosarcoma cells produced a significant suppression of experimental metastasis formation after intracardial injection in rats[13], and S100A4 antisense-transfected Lewis lung carcinoma cells displayed reduced metastatic capacity upon tail vein injection in syngeneic mice[14]. In an orthotopic model of bladder cancer, S100A4 overexpression induced lymph node and lung metastasis[15], and increased metastatic

*Corresponding Author:* Cuiwei Wang, MD; e-mail: cancerhly@126.com

growth was also observed after intrasplenal and intracardial injection of colon cancer cells[16].

Cao et al[17] has found S100A4 dependent matrix metallopeptidases (MMPs) regulation was related with the in-vitro invasive potential of human breast cancer cells. Jia et al[18] has found S100A4 could inhibit angiogenesis, proliferation and invasion by regulating the expression of MMP-9 and VEGF in thyroid cancer cells. Yang et al[19] has foundS100A4 plays a crucial role in migratory/invasive processes in human renal cancer cells by a mechanism involving activation of NF-kB-MMP-2 pathway. Zhang et al[20] has found S100A4 contributes to hepatocarcinoma metastasis by activation of NF-kB dependent MMP-9 expression.

It has previously found S100A4 was overexpressed in laryngeal cancer tissues and cancer cells[21,22], and S100A4 overexpression increases the invasiveness of laryngeal squamous cell carcinoma cells[23]. However, the mechanism of the metastasis-promoting function of S100A4 is, however, not well defined. In the current project, we present evidence to demonstrate that S100A4 is a key regulator for Hep-2 cell invasion and metastasis mediated by controlling NF-kB/MMP-9 signal.

## Materials and Methods

### Cell culture and Stable pcDNA3.1-S100A4 Transfection

Laryngeal carcinoma Hep-2 cells was purchased from ATCC, Shanghai, China, and were cultured in RPMI-1640 medium supplemented with 10% new-born calf serum with 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a homeothermic incubator with a 5% $CO_2$ atmosphere. Cells were transfected with pcDNA3.1-S100A4 and control pcDNA3.1 plasmid [gifted by Dr Jia[18]] by using the LipofectAMINE 2000 (Invitrogen) according to the methods before.

### BB94 treatment

To study the effect of re-inhibition of MMP-9 on the invasion in Hep-2 cells in vitro, pCDNA3.1-S100A4 transfected Hep-2 cells were plated at a density of $1\times10^6$ cells per well in six-well plates and incubated for 24 hs in complete medium, then treated with 0.1 mmol/mL BB94 (a specific MMPs inhibitor) for 24 hs.

### NF-kB p65 siRNA Transfection

SignalSilence NF-κB p65 siRNA was purchased from Cell Signaling Technology (Danvers, MA, USA). nontargeting siRNA purchased from Dharmacon (Chicago, IL, USA) was used as a control siRNA. To study the effect of re-inhibition of NF-kBp65 on the invasion in Hep-2 cells in vitro, stably transfected Hep-2(pcDNA3.1-S100A4/Hep-2) cells were transfected with NF-κB p65 siRNA using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA) for 48 h according to the manufacturer's instructions. To confirm the efficacy of NF-κB p65 siRNA, NF-κB p65 protein were analyzed Western Blotting.

### Western Blot Analysis

After the different treatments, cells were collected and washed with PBS and lysed on ice in 500 uL of lysis buffer per dish (10 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 0.5 mmol/L DTT, 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 25% glycerol, and 0.2 mmol/L EDTA). Protein concentrations were determined using BCA Protein Assay kit (Pierce, Rockford, IL, USA). Equal aliquots of protein (40 ug/lane) were electrophoresed on 10% SDS-polyacrylamide gel and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The blot was first incubated with appropriate primary antibody at a dilution of 1:100 to 1:1,000 and then with horseradish peroxidase-conjugated secondary antibody at a dilution of 1:5,000. The enhanced chemiluminescence system (Amersham Life Science, Arlington Heights, IL, USA) was used to visualize the specific protein. The specific S100A4 and MMP-9 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). NF-kBp65 antibody was purchased from Upstate (Lake Placid, NY, USA).

### Electrophoretic Mobility Shift Assay (EMSA)

To detect the DNA binding activity of NF-kB, electrophoretic mobility shift assay was done according to the manufacturer's instructions (Panomics, Inc., Redwood City, CA). Briefly, nuclear proteins were prepared using a nuclear extraction kit and their concentrations determined by protein estimation procedure. A biotin-labeled NF-kB probe with a 5'-AGTTGAGGGGACTTTCCCAGGC-3' sequence or an unlabeled cold probe was used to bind nuclear proteins at 15°C to 20°C for 30 min. Products were run on a 6%

nondenaturing polyacrylamide gel in 0.5 × Tris-borate EDTA at 120 V for 60 min at 4°C; the shifted bands corresponding to the protein/DNA complexes were separated relative to the unbound dsDNA. The gel was then transferred onto a presoaked membrane at 300 mA for 30 min at 4°C. Following the immobilization of bound oligonucleotides in the membrane by a UV-cross-linking oven for 5 min, the shifted bands were visualized after exposure to film.

### Cell Migration Assays

The invasiveness of Hep-2 cells was tested after transfection as previously described. The cells ($1\times10^6$/mL) were added to the upper wells coated with Matrigel (1 mg/mL; Collaborative Research, Inc., Boston, MA, USA) with serum-free medium containing 25 ug/mL fibronectin as a chemoattractive agent in the lower wells. After a 24-h incubation period, cells that migrated through the filters into the lower chamber were counted by the number of cells on the lower side of the membrane in five random fields after staining with Hema-3 kit.

### Cell Invasion Assays

Cell invasion assays were performed as described for the cell migration assays, except that the Transwell filters were additionally coated on the upper side with 1 mg/mL of Matrigel (Becton Dickinson, Bedford, MA, USA).

### Statistical Analysis

Statistical analysis was performed using the Graphpad Prism (version 5). The two-tailed Student's *t* test was used for the statistical analysis of the results. The differences were considered to be significant when $p < 0.05$.

## Results

### Effect of pCDNA3.1 S100A4 cDNA Transfection on S100A4 Expression in Hep-2 Cells

As shown in Figure 1*A*, high (80%) transfection efficiency of pCDNA3.1 was observed in Hep-2 cells. As determined by Western blot analysis, cells transfected with S100A4 cDNA displayed a significant increase in the expression levels of S100A4 protein (Figure 1*B*). pCDNA3.1 did not exhibit any effect on protein levels of S100A4 (Figure 1*B*). These data confirmed that pCDNA3.1 was successful transfected into the Hep-2 cells.

### S100A4 Promotes Invasion and Migration in Hep-2 Cells

We analyzed the effect of S100A4 overexpression on the migration. As shown in Figure 2A overexpression of S100A4 nearly three times the migration rate of Hep-2 cells, whereas pCDNA3.1 has no significant role on the migration rate of Hep-2 cells. An increase in the migratory ability of a cell usually leads to an increase in invasive ability. Because S100A4 was found to modulate the migration of Hep-2 cells, we examined whether S100A4 also affects their invasiveness, by analyzing their ability to invade a reconstituted ECM (Matrigel). As shown in Figure 2B, the invasiveness of Hep-2 cells was increased nearly fourfold by S100A4. Thus, the effects of S100A4 on invasion were similar to its effects on migration but were more dramatic. These data further support our hypothesis that S100A4 confers the invasive characteristics to cells during human Hep-2 development.

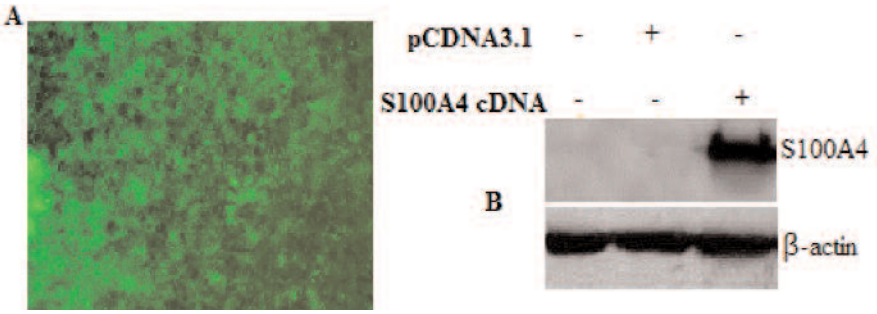


**Figure 1.** S100A4 expression by pCDNA3.1 S100A4 cDNA transfection in Hep-2 cells. ***A,*** Photomicrographs showing transfection of pCDNA3.1 in Hep-2 cells (Magnification: ×100). ***B,*** Representative images showing expression of S100A4 protein in pCDNA3.1 transfected cells as analyzed by Western blotting. Equal loading of protein was confirmed by stripping the blots and reprobing with β-actin antibody.

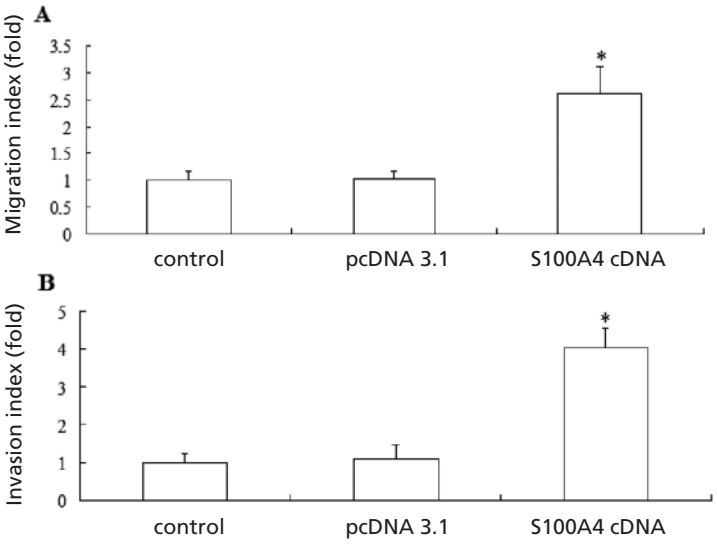


**Figure 2.** Role of S100A4 in Hep-2 cells migration and invasion. ***A,*** Cells were transfected with pCDNA3.1 S100A4 cDNA and pCDNA3.1, and were assayed for migration through Transwell filters. ***B,*** Cells were transfected with pCDNA3.1 S100A4 cDNA and pCDNA3.1, and were assayed for invasion through Matrigel on Transwell filters as described before. All experiments were repeated three times with similar results. Each bar represents mean±SE ($n$= 3); vs control and pCDNA3.1; *, $p < 0.05$.

### *S100A4 are Involved in the Regulation of MMP-9 production*

For the many known proteases, the expression of MMP-9 is regulated by NF-Kb transcriptional activity[18,24-25]. NF-kB transcriptional activity, in turn, has been shown in recent studies to be activated by S100A4[19,20]. Therefore, we suspected that MMP-9 plays a role in the promotion by S100A4 of cancer cell invasion. The effect of S100A4 on the production of MMP-9 in Hep-2 cells was determined by using western blot assay. As shown in Figure 3*A*, MMP-9 was significantly increased by pcDNA3.1-S100A4 transfection.

### *S100A4 Induces MMP-9 via Activation of NF-kB Transcription Activity*

Because S100A4 has been shown to up-regulate NF-kB transcriptional activity in a variety of

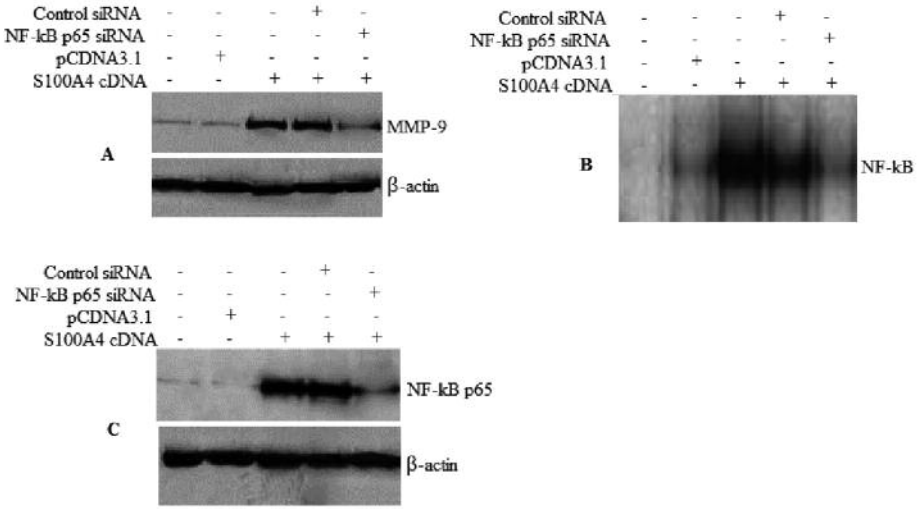


**Figure 3.** Effect of S100A4 overexpression by transfection of S100A4-pcDNA3.1 plasmid on MMP-9, NF-kBp65 expression and NF-kB activity. ***A,*** Representative image showing MMP-9 expression in transfected cells as determined by western blot analysis. ***B,*** Representative images showing expression of NF-kBp65 in vector (pcDNA3.1) and pcDNA3.1-S100A4 transfected cells as analyzed by Western blot analysis. ***C,*** Representative images showing expression of NF-kB activity in vector (pcDNA3.1) and pcDNA3.1-S100A4 transfected cells as analyzed by EMSA analysis. All experiments were repeated three times with similar results.

cells[19,20], we suspected that the up-regulation of MMP-9 production in Hep-2 cells by S100A4 might occur via increased NF-kB transcriptional activity. Therefore, we examined whether S100A4 increases NF-kB transcriptional activity in Hep-2 cells, by using western blot and EMSA assay. As shown in Figure 3B and 3C, S100A4 increased NF-kBp65 expression and NF-kB activity compared with the control.

To determine whether the modulation of NF-kB transcriptional activity by S100A4 is responsible for its regulation of MMP-9 production, we detected MMP-9 expression by western blot assay either with or without the NF-kBp65 siRNA (to inhibit NF-kBp65). Transfection with pcDNA3.1-S100A4 plasmid significantly increased expression levels of MMP-9 (Figure 3A). After NF-kB p65 was blocked by NF-kB p65 siRNA transfection, pcDNA3.1-S100A4 induced NF-kB p65 and MMP-9 expression was significantly reduced (Figure 3). However, pcDNA3.1 transfection has no effect on NF-kB p65 and MMP-9 expression and NF-kB activity (Figure 3). We also found after pcDNA3.1-S100A4/Hep-2 cells was treated with BB94, pcDNA3.1-S100A4 induced MMP-9 expression was significantly reduced, however, BB94 has no effect on pcDNA3.1-S100A4 induced NF-kB p65 expression and NF-kB activity (data not shown). These data demonstrated that S100A4 induces MMP-9 via activation of NF-kB transcription activity.

### *S100A4 Promotes Migration and Invasion Via Activation of NF-kB Dependent MMP-9 in Hep-2 cells*

Because S100A4 has been shown to promotes migration and invasion in Hep-2 cells, and S100A4 is related with NF-kB dependent MMP-9 upregulation, we suspected that the S100A4 promotes migration and invasion via activation of NF-kB dependent MMP-9 in Hep-2 cells. To determine the suspection, pcDNA3.1-S100A4 transfected Hep-2 cells was treated with BB94 or transfected with NF-kBp65 siRNA to inhibit MMP-9 or NF-kBp65 expression and NF-kB activity. Transfection with NF-kBp65 siRNA inhibited pcDNA3.1-S100A4 induced NF-kBp65 expression (Figure 3 B) and NF-kB activity (Figure 3 C). Treatment with BB94 inhibited pcDNA3.1-S100A4 induced MMP-9 expression (data not shown). As shown in Figure 4A and 4 B, treated with BB94 or transfected with NF-kBp65 siRNA significantly decreased the migration and invasive ability of the pcDNA3.1-S100A4 /Hep-2 cells. These data further support our hypothesis that S100A4 confers the invasive characteristics to Hep-2 cells via activation of NF-kB dependent MMP-9 signal.

## Discussion

Our present results demonstrated that S100A4 stimulated invasion and metastasis; up-regulated

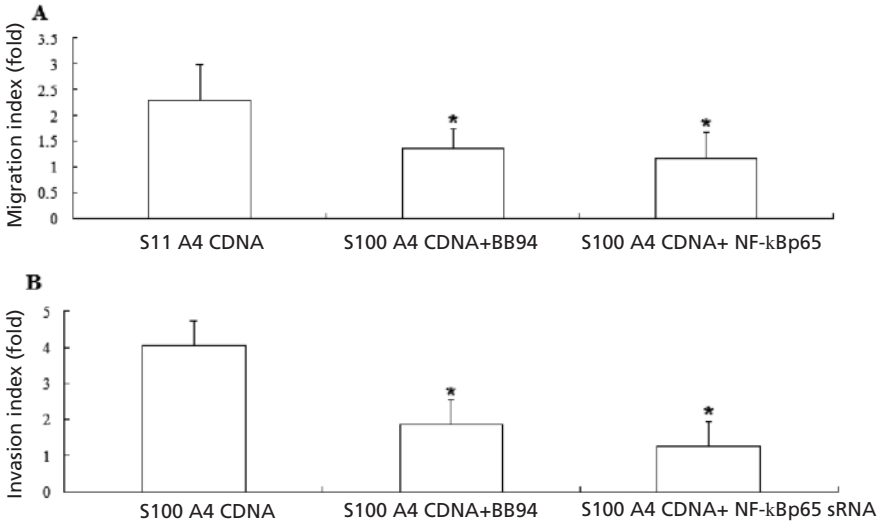


**Figure 4.** Role of MMP-9 and NF-kBp65 in S100A4 cDNA/Hep-2 cells migration and invasion. Cells were transfected with pCDNA3.1 S100A4 cDNA, and then treated with BB94 or transfected with NF-kBp65 siRNA. A, Transwell filters assay; B, Matrigel assay. Each bar represents mean±SE ($n$= 3); vs pCDNA3.1-S100A4 cDNA $^{*}$, $p < 0.05$.

MMP-9 expression; and promoted the nuclear translocation of NF-kBp65 in Hep-2 cells. MMP-9 was abrogated in the presence of the NF-kBp65 siRNA, targeted NF-kBp65. The invasive and metastatic effects of S100A4 were abrogated in the presence of the NF-kBp65 siRNA, the MMP-9 inhibitor BB94. These results demonstrated that the invasion and metastasis of Hep-2 cells is mediated through the S100A4/NF-kB/MMP-9 signalling pathway.

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[11-12]. In a previous study, it has also identified that S100A4 has a critical role in regulating the invasion of Hep-2[21,23]. Here, we showed that S100A4 was present at a fairly low level in quiescent Hep-2, but was rapidly up-regulated upon pCDNA3.1S100A4 cDNA transfection. The invasive and metastatic of Hep-2 was extensively improved after transfection with S100A4. However, the mechanism responsible for S100A4 signalling pathway activation in Hep-2 is largely unknown.

Recent studies have indicated that S100A4 is linked to the invasion of many types of human cancers via its regulation of the NF-kB/MMP-2/9 signalling pathways following the initial effects on cellular invasion[19,20].

In this study, we determined the effects of S100A4/ NF-kB/MMP-9 signalling on Hep-2 invasion and metastasis using Matrigel assays. The present results identified that S100A4 induced the activation of the NF-kB/MMP-9 signalling pathway during Hep-2 cells invasion. The *in vitro* transfection of Hep-2 with S100A4 induced the MMP-9 expression by NF-kB activity. Therefore, the evidences suggest that the pro-invasive property of S100A4 is attributable to the NF-kB activity, the nuclear translocation of NF-kBp65, as well as the MMP-9 upregulation. In addition, blockage of the NF-kB/MMP-9 signalling pathway abrogated S100A4-induced Hep-2 cells invasion.

However, (1) our observations were based on an *in vitro* experiment and not an animal model, and thus, the conclusions drawn may be opened to cell experiments; (2) thus far, we do not know whether the down-regulation of S100A4 can inhibit NF-kB/MMP-9 signaling and invasion *in vitro* and *vivo*.

## Conclusions

We demonstrated that S100A4 is a critical promotor of Hep-2 invasion, and the S100A4/NF-kB/MMP-9 signaling pathway may play an important role during Hep-2 invasion.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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