Hypomethylation-induced expression of *S100A4* increases the invasiveness of laryngeal squamous cell carcinoma

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Abstract. The present study aimed to identify genes related to 5AZA-CdR in laryngeal squamous cell carcinoma (LSCC) and to investigate the role of *S100A4* in the development and aggression of LSCC. Differentially expressed proteins were identified in Hep-2 cells treated with 5AZA-CdR by two-dimensional gel electrophoresis combined with MALDI-TOF-MS. mRNA, protein levels and DNA methylation status of *S100A4* were assessed by RT-PCR, Western blotting and methylation-specific PCR, respectively. The invasiveness of Hep-2 cells transfected by siRNA S100A4 was determined by transwell migration assay. Protein profiles from Hep-2 cells treated with 5AZA-CdR were obtained, and several differentially expressed proteins such as S100 calcium-binding protein A4 (S100A4) were identified. Results of RT-PCR and Western blotting revealed that both mRNA and protein levels of *S100A4* were significantly higher in the metastatic lymph nodes than those in paired adjacent normal laryngeal (PANL) or tumor tissues. The DNA methylation status displayed significant differences between the LSCC and the PANL tissues. The expression level of *S100A4* decreased in Hep-2 cells undergoing RNA interference of *S100A4*. The number of cells which crossed the basement membrane filter was significantly lower in the RNAi *S100A4* group when compared with the number in the control group. The abnormal expression of *S100A4* identified in Hep-2 cells treated with an inhibitor of DNA methyltransferase appeared to result from the aberrant DNA methylation status of *S100A4*. The abnormal expression of *S100A4* altered the invasiveness of LSCC.

Introduction

Head and neck cancer is the eighth leading cause of cancer-related death worldwide (1). As one of the most common cancers in the head and neck (2), more than 95% of LSCC are squamous cell carcinomas. In China, the incidence of LSCC has been rising gradually, particularly in the northeast region. The main treatment for LSCC has been surgery; usually total laryngectomy in advanced cases, followed by radiotherapy. This seriously impairs laryngeal function and the quality of life of patients. Therefore, there is an urgent need to develop chemotherapy and molecular diagnostic biomarkers of LSCC, which may help to improve treatment and survival of LSCC patients (3).

Malignant tumors are endangering human health, and the associated mortality rate has been rising. At present, cancer is recognized as a genetic and epigenetic disease (4). The critical role of epigenetics in gene expression regulation and individual development has shifted the focus of cancer research to a brand new field. DNA methylation, histone modifications and RNA interference are three connected epigenetic cellular memory mechanisms in mammalian cells, and the first two have now been firmly linked to neoplastic transformation (5). Evidence suggests that DNA methylation, which has been extensively studied, is a crucial process in embryonic development, transcription, chromatin structure, X chromosome inactivation, genomic imprinting and chromosome stability. A growing number of human diseases have been found to be associated with aberrant DNA methylation (6-9). Hypermethylation of DNA in tumor cells may result in reduced expression of tumor-suppressing genes, whereas hypomethylation of DNA may be involved in elevating the expression of tumor-promoting genes. Studying the methylation status of genes thought to be involved in the development of carcinoma is therefore important. Identification of methylation-related genes and their functional study in LSCC will provide a base for the further exploration of molecular mechanisms related to LSCC.

S100A4 is a small (11.5 kDa) acidic calcium-binding protein and belongs to the S100 protein family. The human variant of S100A4 consists of 101 amino acids and is characterized by two calcium-binding EF-hands which are the hinge region connected by the intermediate region. S100A4 protein has been associated with many biological functions. For example, S100A4 interacts with cytoskeletal proteins involved in cell motility and provokes degradation of the extracellular matrix (10). The mechanism whereby S100A4 exerts its many reported and partly contradictory biological functions is not well understood. Complex molecular mechanisms might be involved in the regulation of S100A4. In the present study, we investigated the genes related to 5AZA-CdR in LSCC and the role of S100A4 in the development and aggression of LSCC.

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Materials and methods

Cell culture and treatment with 5AZA-CdR. The human laryngeal carcinoma Hep-2 cell line was obtained from the Cell Biology Institute of Shanghai, Chinese Academy of Science. The cells were maintained in RPMI-1640 with 10% FBS and antibiotics at 37°C in a 5% CO_2 humidified atmosphere. After culture in a 6-well plate for 24 h, Hep-2 cells were treated with 2 μM 5AZA-CdR (Sigma Chemical Co.) for 3 days. DMSO (0.1%) served as the vehicle control.

Samples. Seventy-three LSCC tissue samples were obtained from patients treated at the Ear, Nose and Throat Department of The 463 Hospital of PLA of China after obtaining informed consent and the approval of the hospital authorities. None of the patients had received radiotherapy or chemotherapy prior to the genetic analysis. The clinicopathological characteristics of the patients were confirmed according to the International Union Against Cancer. All of the specimens, which included cancerous and paired adjacent normal laryngeal (PANL) tissues, typically 4-15 mm in diameter, were frozen immediately after collection and stored at -80°C.

Protein extraction and two-dimensional (2-D) gel electrophoresis. Proteins were isolated from the Hep-2 cell line treated with 5AZA-CdR. Protein concentrations were then determined using the BCA protein assay system (Pierce, Rockford, IL, USA). First-dimensional separation was carried out using immobiline dry strips (linear). The strips were rehydrated in a rehydration solution containing 8 M urea, 4% CHAPS, 0.2% (w/v) Bio-Lyte, 65 mM DTT and 0.001% bromophenol blue. IEF was carried out on the Protean IEF cell (Bio-Rad) consisting of five phases of stepped voltages from 250 to 10,000 V with total focusing of 60 kVh in a rapid voltage ramping mode with a maximum current of 50 μA per strip. Strips were equilibrated in buffer containing 0.375 M Tris-Cl (pH 8.8), 6 M urea, 20% glycerol, 2% SDS, reduced with 2% (w/v) DTT, and subsequently alkylated with 2.5% (w/v) iodoacetamide. After equilibration, proteins were separated on a 12% gel (20 cm x 20 cm) at a constant voltage of 30 V for 30 min, followed by 120 V until the bromophenol blue reached the bottom of a Protean Plus Dodeca cell (Bio-Rad). After 2-D electrophoresis, the gels were fixed with 50% methanol, 10% ethanol for 2 h and washed once with Milli-Q water for 30 min. The 2-D gels were then stained with Coomassie Blue R-250 (10% acetic acid, 0.25% Coomassie Brilliant Blue and 45% methanol) for at least 10 h with gentle shaking. The gels were then washed with an aqueous solution of 50% methanol, 10% ethanol and stored in 20% ethanol for mass spectrometry identification.

Image analysis, protein identification and database search. The stained gels were imaged using a Umax Power Look III (Hewlett Packard, GE Healthcare). Raw scans were processed by the 2-D gel analysis software, PDQuest 7.0.1 (Bio-Rad). Several differentially expressed spots were excised and identified by mass spectrometry.

The samples were analyzed by MALDI-TOF mass spectrometry on a Voyager DE Pro Instrument (Amersham Biosciences-GE Healthcare). Peak list files were searched against a non-redundant protein database NCBInr, SWISS-PROT and MSDB databases using the MASCOT search engine (http://www.matrixscience.com).

Semi-quantitative RT-PCR analysis. Total RNA was isolated by Trizol reagent according to the instructions, and cDNA was reversibly transcribed from the isolated mRNA using an AMV RNA PCR Kit (Takara) in line with the standard operational protocol. *S100A4* primers were: F 5'-CCCTGGA TGTGATGGTGTC-3' and R 5'-CTCGTTGTCCCTGTTGC TG-3', and the amplified fragment was 185 bp in length. *ß-actin* primers were: F 5'-CCAGATCATGTTTGAGA CCT-3' and R 5'-TTGAAGGTAGTTTCGTGGAT-3', and the amplified fragment was 480 bp. The PCR reaction was performed in a 25-μl reaction system, starting with denatur-ation at 94°C for 4 min, then 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 45 sec, followed by an extra extension at 72°C for 5 min. The PCR product was examined by 1.5% agarose gel electrophoresis and ultraviolet transmission lamp photo-graphy. With the aid of Fluorchem v2.0 Stand Alone, the gel image scanning analytical system was able to provide the photo density of the electrophoresis band of *S100A4* and *ß-actin*, which was used as the internal control. Relative quantitative analysis was carried out based on the photo density ratio (*S100A4/ß-actin*).

Western blotting. For sample preparation, 100 mg of tissue was taken from each sample and ground to a powdery preparation with liquid nitrogen. The sample then underwent a process of homogenizing for 10 min in the presence of tissue protein extracting fluid, ice-bath for 30 min, and centrifugation at 10,000 x g for 5 min at 4°C. The supernatant fluid was then collected. The sample concentration was determined by an ultraviolet spectrophotometer. Proteins (60 μg/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on precast 12% polyacrylamide gels. Following electrophoretic transfer onto PVDF membranes, blots were incubated in blocking buffer for 1 h at room temperature, incubated with *S100A4* monoclonal antibody (Santa Cruz, CA, USA, 1:2000) and then with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (ZhongShan, China, 1:5000). The blot was treated with ECL reagent and exposed to film. The image was captured by transmission scanner with the internal control of the tubulin protein level, and relative quantitative analysis was carried out based on the photo density ratio (S100A4/ tubulin).

Tissue DNA extraction and methylation-specific PCR (MSP) of bisulfate-modified DNA. Genomic DNA was isolated from the frozen tissues using a DNA isolation kit (Tiangen, Beijing, China) and subjected to bisulfite treatment. DNA (2 μg) in a volume of 50 μl was denatured by 5.5 μl NaOH (3 mM) for 10 min at 37°C. Hydroquinone (10 mM) (30 μl) (Sigma) and 520 μl of 3.6 M sodium bisulfate (Sigma) at a pH 5.0, both freshly prepared, were mixed with denatured DNA. The samples were subsequently incubated under mineral oil at 50°C for 16 h. Modified DNA was purified using Wizard DNA purification resin according to the
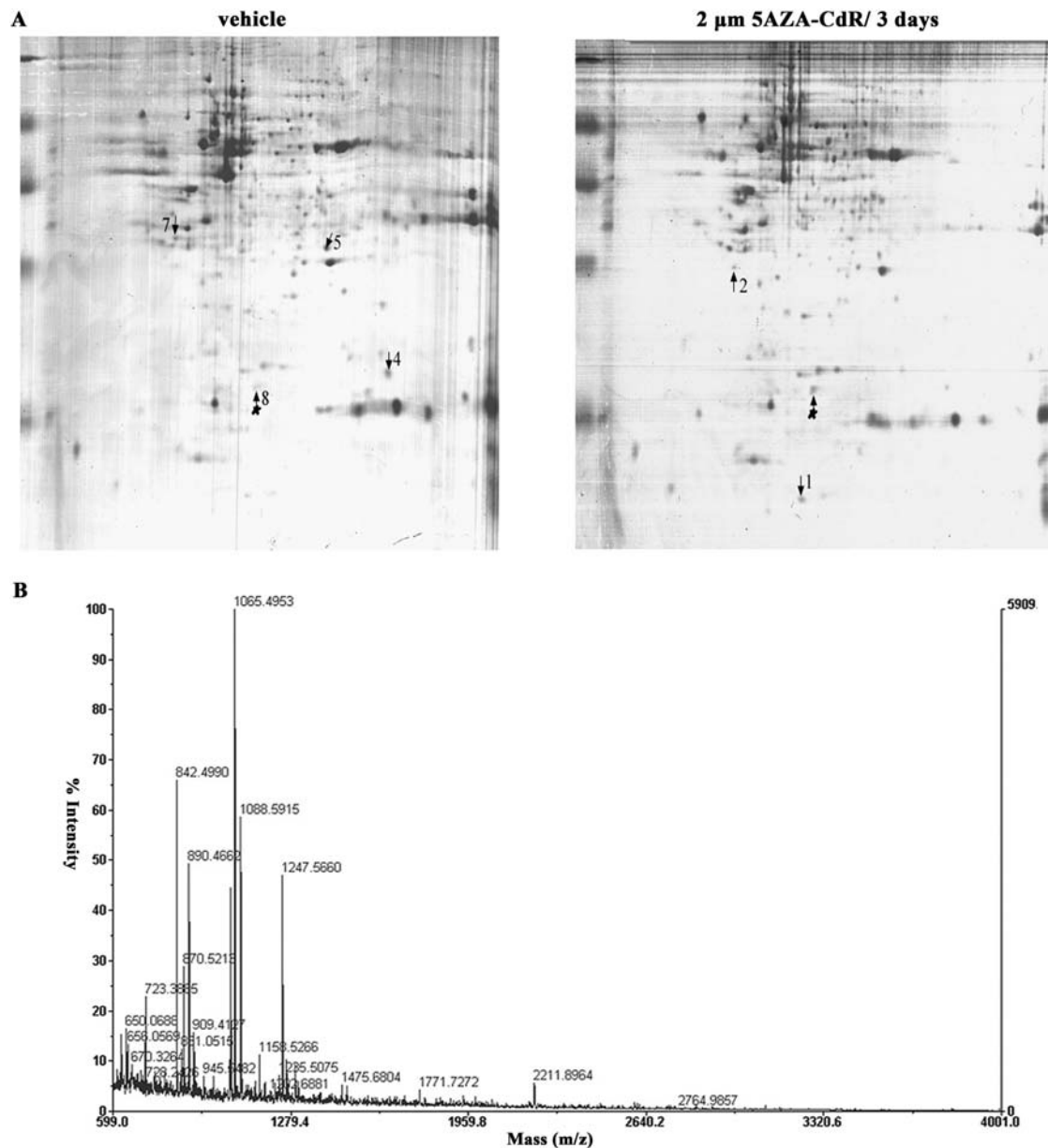
Figure 1. 2-D-PAGE of Hep-2 cells treated with 5AZA-CdR. (A) 5AZA-CdR-associated protein profiles in Hep-2 cells. The image of 2-D-PAGE gels stained by Coomassie Blue was obtained from 2 µM 5AZA-CdR-treated Hep-2 cells for 3 days, compared to the vehicle. Protein spots expressed differentially were marked with Arabic numbers on the maps and further identified by MALDI-TOF MS. (B) The peptide mass fingerprinting (PMF) of protein spot 1.

manufacturer (Promega) and eluted into 50 µl of water. Modification was completed by NaOH treatment for 5 min at 37˚C, followed by ethanol precipitation. DNA was re-suspended in distilled water and used immediately or stored at -20˚C. The methylation status of *S100A4* was determined by MSP which takes advantage of DNA sequence differences between methylated and unmethylated alleles after bisulfite modification. The genome DNA of peripheral blood from normal humans and the DNA digested with *Sss*I methylase, which make most of the CpG methylated, were regarded as negative and positive controls, respectively. Primer sequences were: *S100A4*-M F 5'-TATACGTTGTTGTTATAGTACG-3', *S100A4*-M R 5'-ACTTCCTACTCCCGAATACG-3' (product size 96 bp), *S100A4*-U F 5'-ATATGTTGTTGTTA TAGTATGTG-3' and *S100A4*-U R 5'-CTTCCTACTCCCA AATACAC-3' (product size 94 bp). Both pairs of primers

were reacted in one 25-µl reaction system under proper conditions (denaturation at 94˚C for 45 sec, annealing at 58˚C for 45 sec and extension at 72˚C for 60 sec, for 38 cycles) (11).

RNA interference. siRNAs were purchased from Gene Pharma (ShangHai, China). The sequences of selected regions to be targeted by siRNAs were: 5'-UCAACA AGUCAGAACUAAATT-3', 5'-UGAGCAACUUGGACAG CAATT-3', 5'-GCAUCGCCAUGAUGUGUAATT-3' for *S100A4;* and 5'-UUCUUCGAACGUGUCAC GUTT-3' for a nonsilencing siRNA (control). siRNAs were transfected into Hep-2 cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions using 20-100 nM siRNA. Total RNA was prepared 24 h post transfection, and the results of gene knockdown were determined by RT-PCR.
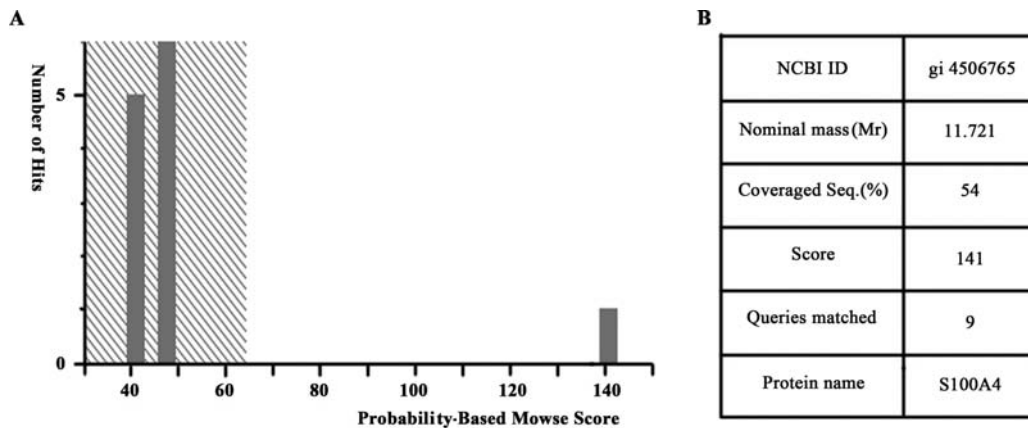
Figure 2. Identification of S100A4 from Hep-2 cells treated with 5AZA-CdR. (A) Mascot search result of spot 1. Protein score is -10*Log (P), where P is the probability that the observed match is a random event. Scores >64 are significant (P<0.05). (B) Database search results of spot 1 identified by MS in the Hep-2 cell line.

Cells were incubated for 72 h and then harvested for the following use.

Transwell chamber invasion assay. Invasion chambers (24-well) were obtained from Costar. Hep-2 cells transfected with *S100A4* siRNA were detached from the tissue culture plates, washed, resuspended in conditioned medium ($2x10^5$ cells/ml) and added to the upper compartment of the invasion chamber. Conditioned medium (500 μl) was added to the lower compartment of the invasion chamber. Then the invasion chambers were incubated at 37˚C for 48 h. After incubation, the filter inserts and the cells on the upper side of the filter were removed. The filters were fixed, mounted, and stained according to the manufacturer's instructions. The cells that invaded were counted on the underside of the filter. Three chambers were used for each experimental condition. The values obtained were calculated by averaging the total number of cells from three filters.

Statistical analysis. Statistical analysis was performed using SPSS 13.0. All data were expressed as mean ± standard deviation (SD). Statistical comparisons were carried out using one-way analysis of variance (ANOVA), randomized block analysis of variance, and the Students-Newman-Keuls (SNK) method was employed for intergroup comparison. Associations between categorical variables were verified by the Chi-square test. Statistical significance was determined as P<0.05.

Results

S100A4 identified from the Hep-2 cell line treated with 5AZA-CdR. The 2-D patterns were highly reproducible since each experiment was performed in triplicate and showed similar results. Some differentially expressed protein spots were excised from the gels and marked with numbers at the corresponding sites in Fig. 1A. The peptide mass finger-printing (PMF) maps of the distinct protein spots were obtained by MALDI-TOF MS following in-gel digestion with trypsin. One PMF data entry (Fig. 1B) was used to search NCBI nr, SWISS-PROT and MSDB databases using Mascot software (http://www.matrixscience.com). Fig. 2A

Table I. Analysis of *S100A4* mRNA and protein levels in different tissues.[a]

Tissues	*S100A4* mRNA	*S100A4* protein
PANL	0.483±0.302	0.530±0.332
Tumor	0.629±0.298	0.818±0.387
Metastatic lymph nodes	0.737±0.251	1.105±0.377
F-value	8.422	25.902
P-value	<0.001	<0.001

[a]Gray scale ratio, mean ± SD, n=73.

and B show that the protein identified was S100 calcium-binding protein A4.

Expression of the S100A4 gene in LSCC. *S100A4* mRNA and protein levels were higher in the metastatic lymph nodes than those in the paired adjacent normal laryngeal (PANL) or tumor tissues, which showed statistically significant differences among the three groups (Fig. 3 and Table I). The results of the statistical analysis also indicated that the high levels of *S100A4* were not associated with LSCC patient gender, age and TNM stage (data not shown).

DNA methylation status of the S100A4 gene in LSCC. After treatment with 5AZA-CdR, an inhibitor of DNA methyl-transferase, Hep-2 cells showed a change in DNA methylation status in *S100A4*. The DNA methylation of the first intron of *S100A4* was then detected by using MSP in 31 cases of LSCC. Twenty-six of 31 (83.9%) PANL and 17 of 31 (54.8%) cancer tissues were methylated, respectively. The results of the statistical analysis showed a significant difference between the LSCC and PANL tissues (Fig. 4) (χ^2=6.147, P<0.05).

Effects of RNA interference of S100A4. Hep-2 cells were transfected with *S100A4* siRNAs. After transfection with
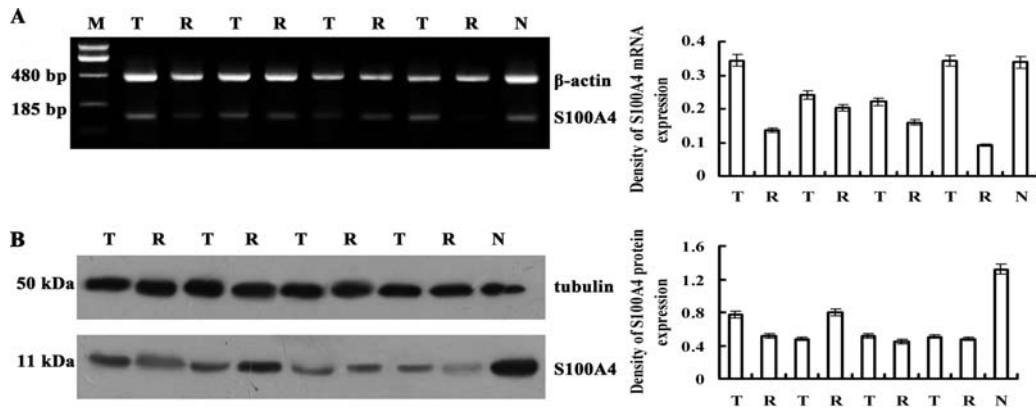
Figure 3. Expression of *S100A4* in LSCC. (A) *S100A4* mRNA levels in LSCC. (B) *S100A4* protein levels in LSCC. M, DNA marker; T, tumor; R, PANL; N, metastatic lymph node tissues.
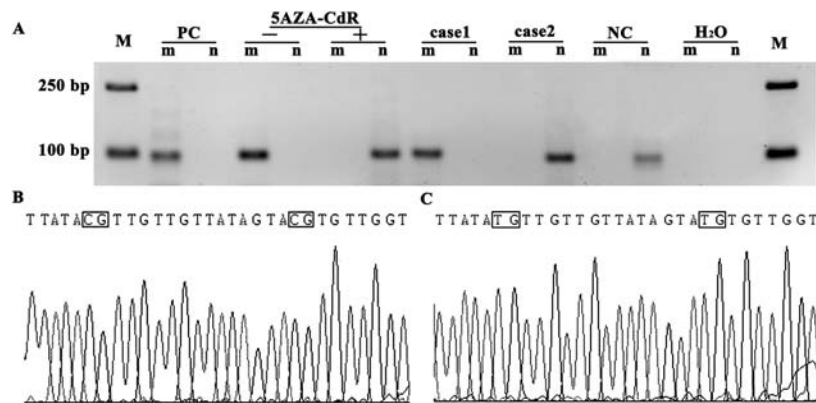

Figure 4. DNA methylation of the *S100A4* gene in LSCC. (A) MSP was used to detect unmethylated (n) and methylated (m) DNA from the first intron of the *S100A4* gene in Hep-2 cell line and LSCC tissues. PC, positive control; NC, negative control; 5AZA-CdR (-), Hep-2 cells before treatment with 5AZA-CdR; and 5AZA-CdR (+), Hep-2 cells treated with 5AZA-CdR. (B and C) Sequencing results of methylated (m) and unmethylated (n) MSP products, respectively.

S100A4 siRNA, the Hep-2 cells showed significant down-regulation of the *S100A4* gene at both the mRNA and protein levels (Fig. 5) ($P < 0.05$).

Invasiveness of Hep-2 cells transfected by S100A4 siRNA. Compared with the control Hep-2 cells (Fig. 6A) and the nonsilencing siRNA control (Fig. 6B), the number of *S100A4* siRNA group cells (Fig. 6C) migrating across the membranes decreased dramatically. The number of cells penetrating the filter membrane in the control group, nonsilencing siRNA group and *S100A4* siRNA group were 32±1.25, 30.67±0.95 and 11.58±0.52, respectively, which showed a significant difference among the different groups ($P < 0.05$) (Fig. 6D).

Discussion

5AZA-CdR, a DNA methyltransferase inhibitor, was previously used against certain anemias to induce expression of fetal globin genes. Clinical trials as an anticancer agent have been reinstated only recently because of the reversal of methylation-mediated silencing of critical genes in cancer (12). Previous clinical trials have shown that 5AZA-CdR has therapeutic potential against MDS, acute myeloid, chronic myelogenous and chronic myelomonocytic leukemias.

However, its effectiveness on solid tumors appears to be less promising (13).

From the 2-D gel electrophoresis results, we identified many differentially expressed proteins in the Hep-2 cell line treated with 5AZA-CdR. Combined with the MS analysis and database search, several significant proteins including *S100A4* were confirmed (data not shown). *S100A4* is a member of the *S100* gene family that encodes a series of calcium-binding proteins with EF-hand. In humans, overexpression of *S100A4* has been correlated with aggressiveness and a worse prognosis in different types of carcinomas, including breast, gastric, colorectal and oral cancers (14-17). *S100A4* may take part in angiogenesis, growth regulation, remodeling of the extracellular matrix, cell detachment and cell motility (18). P53, a target of *S100A4*, controls the cell cycle and apoptosis. *S100A4* regulates P53 by binding and inhibiting the phosphorylation of C-terminal peptide through protein kinase C (19). Because of its ability to activate interacting and multi-functional signaling systems, *S100A4* appears to be a new therapeutic option to offer suitable targets. Some effectors of the *S100A4*-activated pathways might also lend themselves as foci of therapeutic interest (20).

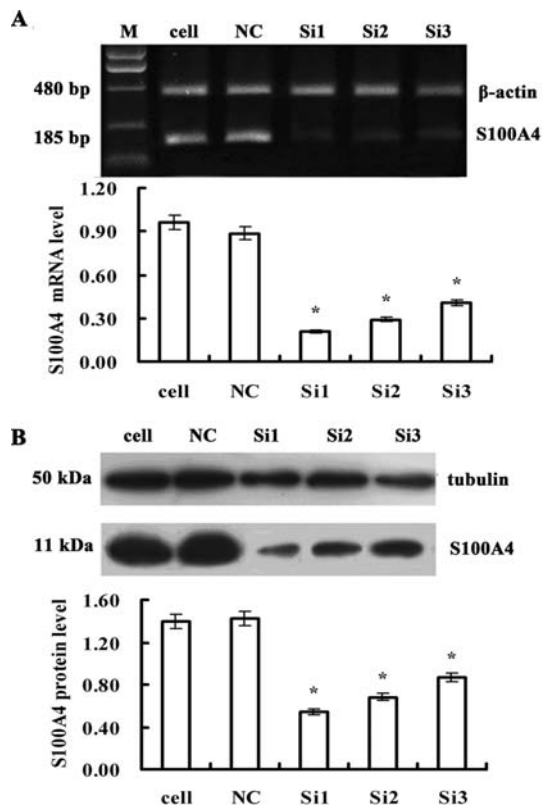At present, there is no insight concerning the role of *S100A4* in LSCC, and the exact mechanism of *S100A4* in

Figure 5. RNA interference of *S100A4* in Hep-2 cells. (A) mRNA levels of *S100A4* in Hep-2 cells interfered by siRNA. (B) Protein levels of *S100A4* in Hep-2 cells interfered by siRNA. Lanes: M, DNA marker; cell, control cells; NC, nonsilencing siRNA; Si1, siRNA1; Si2, siRNA2 and Si3, siRNA3 (*P<0.05).

promoting metastasis is also poorly understood. In this study, we found that *S100A4* displayed higher expression in metastatic lymph nodes than those in LSCC tissue and the adjacent normal mucosa. Analysis of the relationship between *S100A4* expression of LSCC tissues and patient clinico-pathological characteristics implies that *S100A4* might play a role in the metastasis of LSCC. The abnormal expression of *S100A4* has been found in many types of cancers. For example, it is over-expressed in oral, gastric and colorectal carcinomas. On the other hand, the reduction in *S100A4* expression is found in esophageal squamous cell (21) and bladder carcinomas (22). We speculate that different types of carcinomas may demonstrate different expression levels of *S100A4*. The molecular mechanism responsible for the regulation of *S100A4* gene expression is still unknown. Modulations in the translation of mRNA to protein could account for the discrepancies in expression of *S100A4* at the protein and mRNA levels. Very complex molecular mechanisms might be involved in the regulation of the aberrant expression of *S100A4*. Previous research has shown that DNA methylation may have a role in the control of *S100A4* expression (23). In certain cancer systems such as those of the lymphoma cell line and pancreas, it is believed that methylation at critical CpG sites within the first intron of the gene regulates transcription (24). The mechanism underlying the transcriptional regulation of the *S100A4* gene in LSCC or Hep-2 cells remains to be examined. Our 2-D gel electrophoresis results showed that *S100A4* is regulated by DNA methylation in Hep-2 cells, and the MSP results indicated that compared to the PANL tissues, *S100A4* mRNA up-regulated in LSCC, most of which display an unmethylation
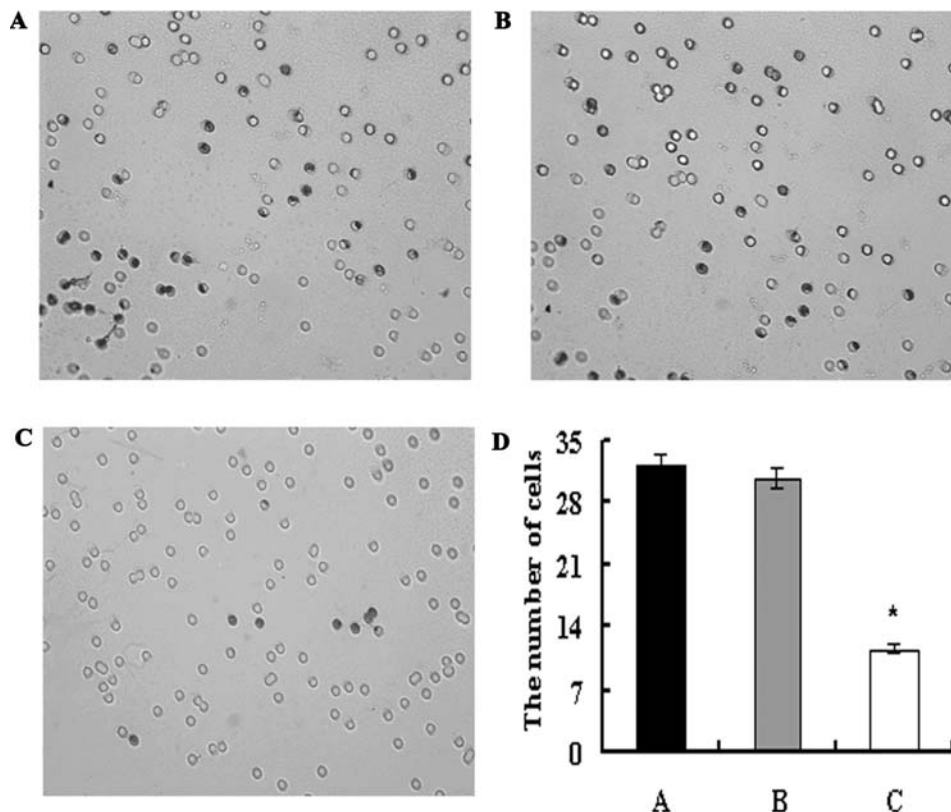

Figure 6. The invasive ability of Hep-2 cells. (A) Hep-2 cells before transfection; (B) Hep-2 cells transfected with nonsilencing siRNA; (C) Hep-2 transfected with *S100A4* siRNA. The black spots indicate the transmembrane cells. (D) The statistical analysis of transmembrane cells in the above groups (*P<0.05).

status in *S100A4* first intron. This suggests that the up-regulation of *S100A4* in LSCC might result from DNA hypomethylation. In previous studies, DNA methylation controlled the expression of *S100A4* (25,26). The results in prostate cancer, colon cancer, and pancreatic cancer revealed that *S100A4* expression was closely related to hypo-methylation (23,27,28).

The transition of tumor cells from benign tumor growth to malignancy is manifested by their ability to traverse tissue barriers and invade surrounding tissues. The event involving the traversing of basement membrane barriers is thought to be the critical one in the initiation of the metastatic cascade. Our transwell results showed that down-regulation of *S100A4* in Hep-2 cells transfected by RNAi led to a significantly lower invasiveness, which further indicates that *S100A4* participates in the aggression of LSCC. Takenaga *et al* (29) obtained similar results in which expression of *S100A4* was closely correlated with *in vitro* invasiveness of prostate cancer. Previous studies also identified that the C-terminal region of *S100A4* is a possible target for inhibitors of its metastatic action, and the amino acids of *S100A4* are involved in the induction of metastasis (30,31). *S100A4* promotes angio-genesis and extracellular matrix degradation through its up-regulation of specific matrix metalloproteinases (10), and high levels of *S100A4* expression are associated with metastatic progression in a wide range of cancers. Our current findings are therefore consistent with a role for the oncogenic activation of *S100A4* by hypomethylation in LSCC tumorigenesis.

It has been proposed that the silencing and hyper-methylation of genes that regulate proliferation are critical for the down-regulated growth early in carcinogenesis, while activation and hypomethylation of other genes may be important for metastasis. Hypermethylation of normally unmethylated tumor-suppressor genes correlates with a loss of expression in primary tumors and cancer cell lines (31,32). Results from our study on *S100A4* in LSCC provide further evidence that hypomethylation plays a role in activating genes which contribute to the invasion and metastasis of cancer.

Acknowledgements

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