

# ***S100A4* is upregulated via the binding of c-Myb in methylation-free laryngeal cancer cells**

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**Abstract.** DNA hypomethylation is correlated with the over-expression of the *S100A4* gene in several types of cancers including laryngeal cancer, but the molecular mechanism is unknown. We speculated that the methylation status of the promoter affects its binding to the corresponding transcription factors. In the present study, luciferase reporter assay results indicated that the sequences -485 - +73 and -486 - -530 of the *S100A4* promoter may harbor the positive and negative *cis*-acting elements, respectively; and moreover, the luciferase activity promoted by the sequence -485 - +73 increased and the *S100A4* gene was significantly upregulated in 5-Aza-induced HEP2 cells. This implies that the methylation status of the sequence is important in regulating the expression of *S100A4*. Four transcription factor binding motifs including c-Myb, C/EBP $\alpha$ , Ap2 and Msx-1 in the region were predicted by P-Match software. c-Myb and C/EBP $\alpha$  but not Ap2 and Msx-1 were confirmed by EMSA and ChIP as transcription factors of *S100A4*. The decreased luciferase activity in methylation-free HEP2 cells transfected by the mutant c-Myb motif related to the methylated cytosine suggests that the hypomethylation of the c-Myb motif upregulates the *S100A4* expression in laryngeal cancer.

## **Introduction**

Head and neck squamous cell carcinoma (HNSCC) is the eighth leading cause of cancer-related mortality worldwide, and laryngeal squamous cell carcinoma (LSCC) is a major type of HNSCC (1). In China, the incidence of LSCC has been gradually increasing, particularly in the Northeast. LSCC is a heterogeneous disease involving deregulation of multiple pathways involved in cellular differentiation, the cell cycle,

apoptosis, angiogenesis and metastasis (2). Despite advancement in local control and overall improvement in the quality of life achieved with the use of combined modality therapies, the survival rates for LSCC patients have not improved significantly over the past 2 decades. Therefore, the current research is often focused on the identification of useful biologic and molecular markers for the diagnosis and therapy of LSCC (3,4).

As one of the epigenetic types, DNA methylation can cause the silencing of gene transcription, genetic imprinting, genomic stability and X chromosome inactivation (5-7). Aberrant methylation of genes controlling the cell cycle, proliferation, apoptosis, metastasis, drug resistance and intracellular signaling has been identified in multiple types of cancers (8-10). Tumor-suppressor genes are often inactivated in cancer tissues by hypermethylation within CpG islands (11,12). Hypomethylation usually leads to the activation of oncogenes in cancer tissues (13,14). In our previous study, we found that the *S100A4* gene is overexpressed in laryngeal cancer tissues and that RNAi *S100A4* decreased the invasiveness of HEP2 cells, which implies that *S100A4* is involved in the progression of LSCC. We also found significant hypomethylation within the promoter region of *S100A4* in LSCC tissues when compared to that in paired adjacent tissues. Statistical analysis showed the association of DNA hypomethylation and the overexpression of the *S100A4* gene in laryngeal cancer tissues (15). However, the exact mechanism remains unclear. We speculated that the methylated sites of the *S100A4* promoter affects the binding of the corresponding transcription factors to *S100A4*. In the present study, we identified the putative transcription factors that bind to *S100A4* and explored the effect of hypomethylation on the binding.

## **Materials and methods**

**Cell culture and reagents.** The human laryngeal carcinoma cell line HEP2 (Cell Biology Institute of Shanghai, Chinese Academy of Science) was maintained in RPMI-1640 (Gibco-BRL, Los Angeles, CA, USA) with 10% fetal bovine serum, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. 5'-Aza-2'-deoxycytidine (5-Aza) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cells were treated with 4  $\mu$ M 5-Aza for 72 h. PGL3-basic, pRL-TK and Dual-Luciferase reporter assay system

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Table I. Primer sequences used in the generation of the related constructs.

Primer name	Sequence: 5' to 3'
P0	TTTAAGCTTTGCTCTGGGCAGTGAACAT
P1	TTTGGTACCGCTGGGACTACAGGCTAC
P2	TTTGGTACCGTGCCCATCTCATCCAG
P3	TTTGGTACCGTGCCCACTGGGAACA
P4	TTTGGTACCTCAGCCCACAGCAGGAAG
P5	TTTGGTACCCACACACACATGCACGTAAG
P6	TTTGGTACCTGAGCAAGTGACTGAA
P7	TGGGCTTGCACATTCTGTTGCTATAGTACG
P8	TGAGATGTGGGCTTGCACATGTTGCTATAGTACGTGTTGGT
P9	ACCAACACGTACTATAGCAACATGTGCAAGCCCACATCTCA
P10	TTTGGTACCGGCTCATGTTTGCTGGGTT
P11	GGCTCATGTTTGCTGGGTTGTACACTAAGGAGCAGGAAGC
P12	ACTGGCTCATGTTTGCTGGGAGCAGGAAGCAAAGGAAAGGCA
P13	TGCCTTTCCTTTGCTTCCTGCTCCCCAGCAAACATGAGCCAGT

were obtained from Promega Corporation (Madison, WI, USA). The Plasmid Miniprep kit and Lipofectamine™ 2000 transfection reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The primary antibodies, protein A/G-agarose and the anti-IgG antibody used in the present study were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

**Plasmid construction.** Human genomic DNA was isolated from HEp2 cells using a genomic DNA isolation kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions. Over 1.2 kb fragment of the *S100A4* 5'-flanking sequence was amplified using Probest *Taq* DNA polymerase (Takara, Japan). *KpnI* and *XhoI* restriction sites were integrated into the fragment above using primers P0 and P1 (Table I). The fragment was then cloned into the plasmid PGL3-Basic, named P1283 (-1283/+73). The deletion constructs P970 (-970/+73), P912 (-912/+73), P842 (-842/+73), P530 (-530/+73) and P485 (-485/+73) were created by PCR using P1283 as a template and the oligonucleotides P2 to P6 as primers. The reporter constructs including point mutations and deletions at potential c-Myb and C/EBPα binding sites were generated using PCR-based Megaprimer technique as previously described (16). Primer P7 was used for the construction of the mutant c-Myb vector (pcMyb-M), P8 and P9 for the deletion cMyb vector (pcMyb-D), P10 for the C/EBPα wild-type vector, P11 for the mutant C/EBPα vector (pC/EBPα-M), P12 and P13 for the deletion C/EBPα vector (pC/EBPα-D). All constructs were sequenced in a capillary automatic sequencer (ABI PRISM® 3100 Genetic Analyzer; Applied Biosystems). All primer sequences are listed in Table I.

**Transient transfection and luciferase assays.** HEp2 cells were seeded in 24-well plates, grown to 60-80% confluence, and transfected by the constructs using Lipofectamine™ 2000 according to the manufacturer's instructions (Invitrogen Life Technologies). pRL-TK plasmid containing the *Renilla* lucif-

erase gene was used as the internal control in each experiment. After 24 h of transfection, the cells were incubated with 4 μM 5-Aza for an additional 3 days. Firefly and *Renilla* luciferase activities were determined using a Lumat LB 9507 luminometer (Bethold Technologies, Bad Wildbad, Germany).

**Semi-quantitative RT-PCR.** Total RNA was isolated by TRIzol reagent according to the manufacturer's instructions, and cDNA was reversibly transcribed from the isolated RNA using an AMV RNA PCR kit (Takara, Japan) in line with the standard operation protocol. The primer sequences were as follows: *S100A4* forward, 5'-CCCTGGATGTGATGGTGTC-3' and reverse, 5'-CTCGTTGTCCCTGTTGCTG-3', which were expected to produce a 185-bp DNA fragment; β-actin forward, 5'-CCAGATCATGTTTGAGACCT-3' and reverse, 5'-TTGAAGGTAGTTTCGTGGAT-3', which were expected to produce a 480-bp DNA fragment. The PCR reaction was performed in a 25-μl reaction system, starting with denaturation 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 products were run on a 1.5% agarose gel, and the band intensity was measured and normalized to the internal control β-actin.

**Western blotting.** Total protein was extracted from HEp2 cells. In brief, cells were lysed using 250 μl of RIPA lysis buffer and then underwent a process of homogenization for 10 min, ice-bath for 1 h and centrifugation at 12,000 x g for 30 min at 4°C. The supernatant was finally collected, and the protein concentration was determined by the BCA protein assay system (Pierce Biotechnology, Inc., Rockford, IL, USA). Extracts equivalent to 50 μg of total protein were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. After being blocked overnight at 4°C with 1X PBS, 0.1% Tween-20 and 5% non-fat milk, the membranes were incubated with the primary anti-

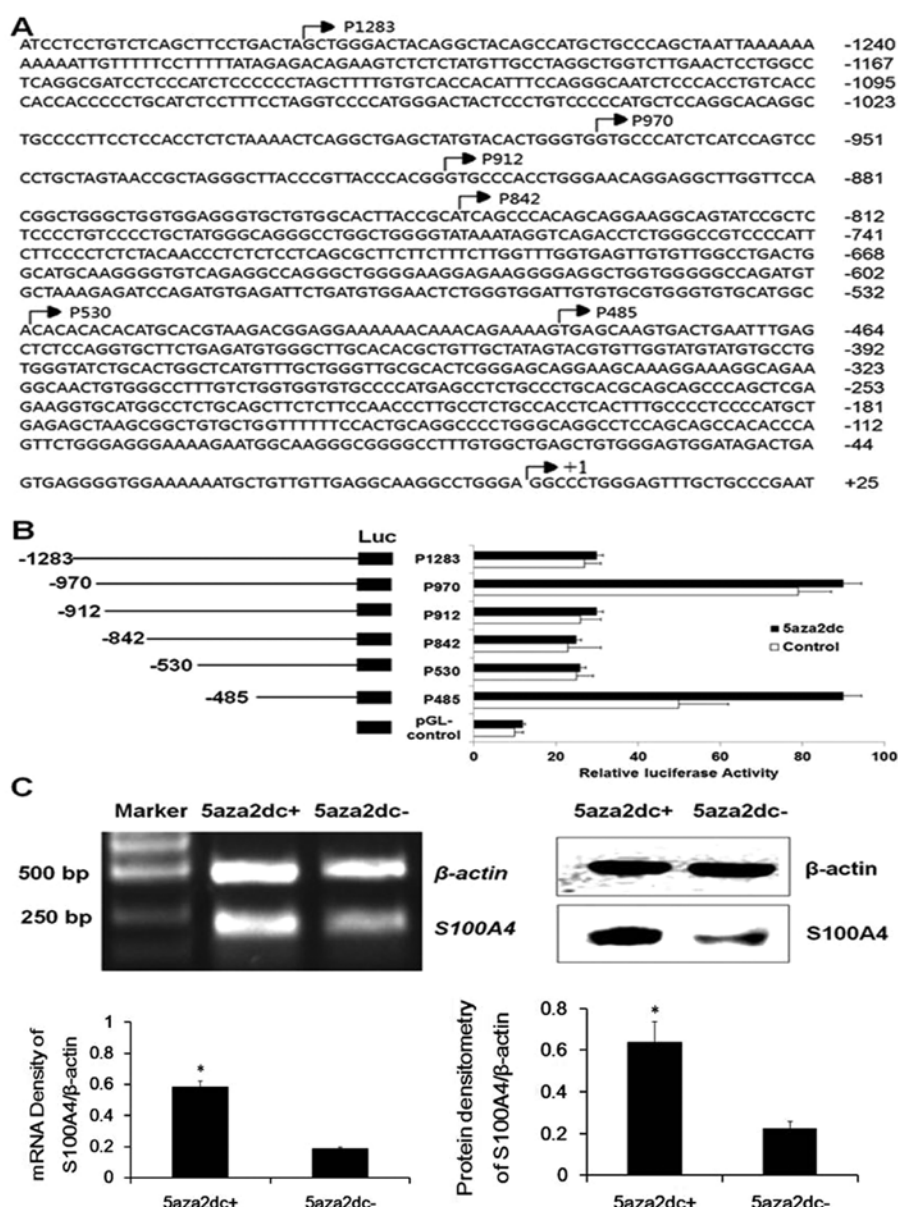


Figure 1. Analysis of the promoter activities and expression of the human *S100A4* gene. (A) Illustration of over 1.2 kb fragment of the *S100A4* 5'-flanking sequence. (B) The region -485 - +73 is responsible for the upregulation of the human *S100A4* gene in the 5-Aza-induced HEp2 cells. The pGL3-Basic-*S100A4* 5'-UTR vectors were transferred into HEp2 cells, respectively. After 72 h of transient transfection into HEp2 cells, the activities of luciferase reporter genes were detected. All values are the average of 3 independent experiments (means  $\pm$  standard deviation), and data were analyzed using SPSS 13.0 statistical software. The pGL3-Basic vector was used as control. (C) 5-Aza induces the overexpression of *S100A4* in HEp2 cells. RT-PCR and western blot methods were used to determine the mRNA and protein levels of *S100A4*, respectively.  $\beta$ -actin was used for normalization. Prior to detection, the human HEp2 cells were treated with 5-Aza for over 3 days. The data were obtained from 3 experiments. 5-Aza, 5aza2dc.

bodies against S100A4 (1:2,000) or  $\beta$ -actin (1:1,000) for 3 h at room temperature, washed twice and then incubated with the secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1:5,000; Zhongshan, China) for 2 h at room temperature. Immunodetection was performed with chemiluminescence (ECL reagent; Beyotime, China), and the membranes were exposed to film. The densities of the bands on the filters were quantified by densitometric analysis using the UVP GelWorks ID advanced version 2.5 software (Bio-Rad, USA).

**Bisulfite modification and bisulfite-specific PCR.** Genomic DNA isolated from the HEp2 cells was used to detect the methylation status of the *S100A4* promoter. In brief,  $\sim 1 \mu\text{g}$

of genomic DNA was bisulfite-modified using the EZ DNA Methylation-Gold™ kit (Zymo Research, Orange, CA, USA) according to the manufacturer's recommendation. Based on the functional promoter sequence of the *S100A4* gene, the primers (forward, 5'-TAGAAAAGTGAGTAAGTGATTGA-3' and reverse, 5'-TC TACCTTTCCTTTACTTCCTAC-3') were used in bisulfite-specific PCR detection, and the amplified fragment was 170 bp. The PCR reaction was performed in a 25- $\mu\text{l}$  reaction system, starting with denaturation at 94°C for 4 min, then 42 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec, extension at 72°C for 45 sec, followed by an extra extension at 72°C for 5 min. The BSP products were then cloned into a T-vector (Takara, Japan), and

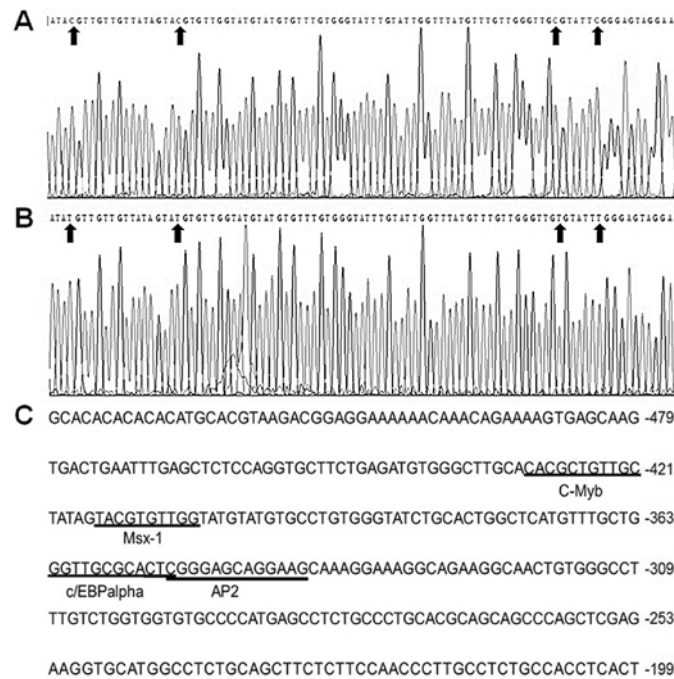


Figure 2. 5-Aza alters the methylation status of the cytosines in the region -485 - +73 which harbors several potential *cis*-acting elements in HEP2 cells. (A) The cytosines at CpG were methylated in the region -485 - +73 in the HEP2 cells prior to 5-Aza treatment. (B) The methylation-free cytosines at CpG in the region -485 - +73 in the HEP2 cells were restored by 5-Aza. BSP-based sequencing was used to detect the methylation status of the 4 CpG sites in the region -485 - +73 in the HEP2 cells prior to or after 5-Aza treatment. The arrows indicate the methylation status at the CpG sites. (C) The 4 CpGs are located in the sequences of 4 potential transcription factor binding sites predicted in the region -485 - +73. c-Myb, C/EBPα, AP2 and Msx-1 potential transcription factor binding sites were predicted using P-Match™ software.

JM109 *E. coli* competent cells (Takara, Japan) were used for transformation according to the manufacturer's instructions.

**Bioinformatics.** The human *S100A4* promoter sequence was obtained from e-Ensembl with the accession no. ENST00000368715. The binding sites were predicted by using the online P-Match™ program (<http://www.gene-regulation.com/pub/programs.html#pmatch>).

**Electromobility shift assay (EMSA).** Non-denatured cellular nuclear proteins were prepared using a nuclear protein extraction kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA, USA). EMSA was carried out using a commercially available LightShift Chemiluminescent EMSA kit (Pierce Biotechnology, Inc.). The probes used in EMSA were synthesized by Sangene (Beijing, China), and the sequences were as follows: c-Myb wild-type, 5'-tgcacacgctgttgctatagta-3' and mutant, 5'-tgcacacgctgaatctatagta-3'; cEBPα wild-type, 5'-gggttgccgactcgggagcagg-3' and mutant, 5'-gggaatcgactcgggagcagg-3'. Two micrograms of nuclear protein extracts was incubated with 3'-end-biotin-labeled *S100A4* DNA probes in binding buffer for 30 min on ice, separated on a 6% non-denaturing polyacrylamide gel in 0.5X TBE, and then transferred onto a nylon membrane and fixed by ultraviolet cross-linking. The biotin-labeled probes were detected with streptavidin-horseradish peroxidase (Pierce Biotechnology, Inc.). For the competition experiments, a 100-fold excess of unlabeled double-stranded cold probes was added to the binding reaction. To determine the effect of the antibodies on protein-DNA binding, 1 μg primary antibodies against c-Myb, C/EBPα, AP2 and Msx-1 (Santa Cruz Biotechnology, Inc.) was

incubated with the nuclear extracts for 30 min on ice prior to the addition of the biotin-labeled DNA probes, respectively.

**Chromatin immunoprecipitation (ChIP) assay.** The ChIP-IT kit was purchased from Active Motif, and the ChIP assay was carried out according to the manufacturer's protocol. One microgram of antibodies against c-Myb or C/EBPα was utilized for immunoprecipitation. After proteinase K digestion and DNA precipitation, 3 μg/l of diluted DNA was used for PCR. The primer sequences for amplifying the *S100A4* promoter and GAPDH were as follows: *S100A4* forward, 5'-CAAACAG AAAAGTGAGCAAGTGAC-3' and reverse, 5'-AGAGGCAA GGGTTGGAAGAG-3' to generate a 289-bp fragment; GAPDH forward, 5'-CGACCACTTTGTCAAGCTCA-3' and reverse, 5'-AGGGGTCTACATGGCAACTG-3' to generate a 332-bp fragment.

**Statistical analysis.** Unless otherwise stated, each experiment was performed for a minimum of 3 times. Data were subjected to statistical analysis using SPSS 13.0 software and are shown as means ± standard error of the mean (SEM). Differences in mean values were analyzed using one-way analysis of variance (ANOVA) or t-test as appropriate. Statistical significance was assumed for a two-tailed  $P < 0.05$ .

## Results

***S100A4* promoter region -485 - +73 harbors major transcription factor binding sites related to the methylation status in laryngeal cancer cells.** In search of the potential *cis*-acting elements of the *S100A4* promoter, several constructs including

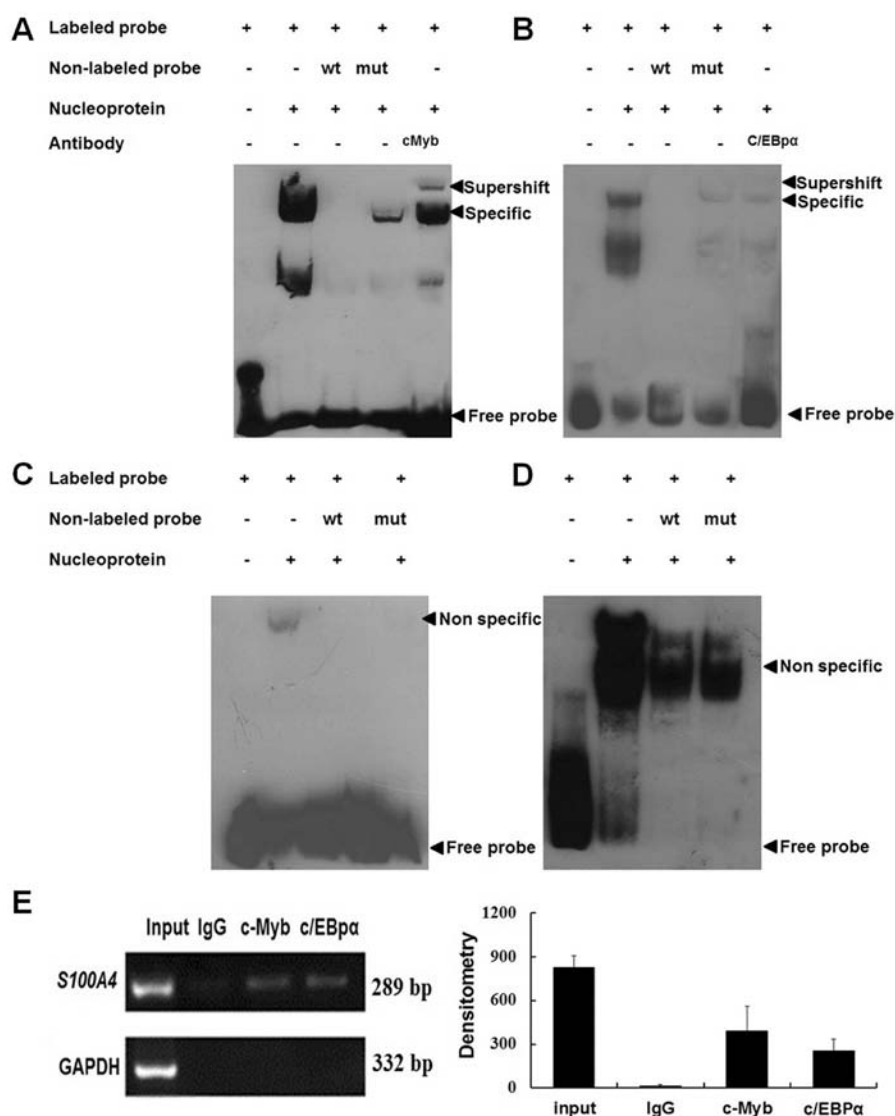


Figure 3. c-Myb and C/EBPα are novel transcription factors of *S100A4*. (A-D) c-Myb and C/EBPα, but not Ap2 and Msx-1, bind the region -485 - +73 of *S100A4* *in vitro*. The EMSA results indicated that (A) c-Myb and (B) C/EBPα could bind *S100A4* *in vitro* but not (C) Ap2 and (D) Msx-1. (E) c-Myb and C/EBPα bind the region -485 - +73 of *S100A4* *in vitro* as well. The binding of c-Myb and C/EBPα to the region -485 - +73 of *S100A4* *in vitro* was confirmed by ChIP. Input serves as the positive control. IgG and GAPDH were used as negative and internal controls in the immunoprecipitation reaction and PCR, respectively. EMSA, electromobility shift assay; ChIP, chromatin immunoprecipitation.

the region -485 - +73 of *S100A4* were generated (Fig. 1A), and transient transfection was then performed to assess their relative reporter gene activities. As shown in Fig. 1B, under basal conditions, the constructs p970-luc and p485-luc in HEp2 cells produced much higher luciferase activities than those of the other truncated vectors ( $P < 0.05$ ), indicating that the region -485 - +73 harbors the positive *cis*-acting elements, and the fragment -486 - -530 the negative *cis*-acting elements. Following a 72-h exposure to 4  $\mu$ M 5-Aza, the HEp2 cells transfected by p485-luc resulting in a significantly high level of luciferase activity when compared to the other groups ( $P < 0.05$ ). RT-PCR and western blot results also indicated that 5-Aza induced significant overexpression of *S100A4* both at the mRNA and protein levels (Fig. 1C,  $P < 0.05$ ). The MSP-based sequencing result showed that the cytosines of the dinucleotide CG at position -485 - +73 were methylated in the untreated HEp2 cells and the methyl groups of the cytosines

were removed in HEp2 cells treated with 5-Aza (Fig. 2A and B). These findings suggest that demethylation increases the expression of endogenous transcription factor binding to the area and results in the methylation-free status in the region of *S100A4* as well. Therefore, there could exist important *cis*-acting elements within the promoter region -485 - +73, which is affected by the methylation status of either the gene itself or the upstream genes regulating the transcription of *S100A4*.

**Putative *cis*-acting elements in the *S100A4* promoter region -485 - +73 are predicted.** The P-Match software was used to predict the *cis*-acting elements in the *S100A4* promoter region -485 - +73. The result indicated that this region contained the binding motifs of certain transcription factors including c-Myb, C/EBPα, Ap2 and Msx-1 (Fig. 2C). The cytosines in these binding sites were methylated in HEp2 cells (Fig. 2A and B).

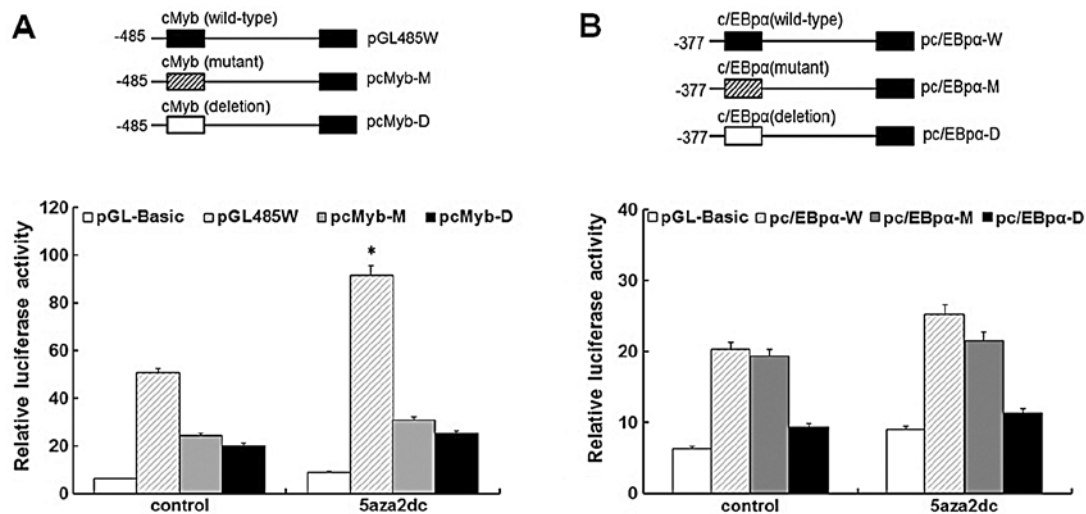


Figure 4. c-Myb is suggested to be a key transcription factor in region -485 - +73 of *S100A4* in methylation-free Hep2 cells. (A) The mutant vector (pcMyb-M) interfered with its binding to *S100A4*. (B) The mutant vector (pc/EBPα-M) did not affect its binding to *S100A4*. The c-Myb and C/EBPα wild-type, mutant and deletion luciferase reporter vectors were constructed. After transfection, the luciferase activity of the different reporter genes in Hep2 cells prior to or after 5-Aza treatment was detected. All values are the average of 3 independent experiments (means  $\pm$  standard deviation), and data were analyzed using SPSS 13.0 statistical software. 5-Aza, 5-aza2dc.

*c-Myb* and *C/EBPα* bind to the cis-acting elements in the *S100A4* promoter region -485 - +73. To confirm the binding of the *S100A4* gene to c-Myb, C/EBPα, Ap2 and Msx-1, EMSA and ChIP methods were used. As shown in Fig. 3A-D, the binding of c-Myb and C/EBPα, but not Ap2 and Msx-1, to *S100A4* was detected when the wild-type probes were used. The addition of the cold self-competitors weakened the binding, and the mutant competitor could not abolish the binding. Supershift bands were also observed when c-Myb and C/EBPα antibodies were applied. These results indicated that c-Myb and C/EBPα were able to bind the *S100A4* promoter region -485 - +73 *in vitro*. ChIP results showed that both c-Myb and C/EBPα antibodies, but not non-immune IgG, could immunoprecipitate c-Myb and C/EBPα proteins binding to the same region *in vitro* (Fig. 3E).

*c-Myb* is a major trans-acting element of *S100A4* in 5-Aza-induced Hep2 cells. To simulate the effects of demethylation on methylated sites within the c-Myb and C/EBPα response elements of *S100A4*, we constructed the mutant and deletion reporter vectors of c-Myb and C/EBPα motifs corresponding to the methylated sites, named pcMyb-M, pcMyb-D, pc/EBPα-M and pc/EBPα-D, respectively. These vectors were then transiently transfected into Hep2 cells to evaluate their relative reporter gene activities. The mutant and deletion fragments (pcMyb-M/pcMyb-D) showed a significant decrease in the reporter activities compared to the wild-type construct in the 5-Aza-treated and -untreated groups ( $P < 0.05$ ), confirming that the mutation and deletion constructs interfere with the binding of c-Myb to *S100A4* (Fig. 4A). Fig. 4A also showed the significant difference in the luciferase activities of the reported genes between 5-Aza-treated and -untreated Hep2 cells transfected by the wild-type construct, which implies that the overexpression of the endogenous c-Myb gene in Hep2 cells induced by 5-Aza may lead to the overexpression of the reporter genes in the 5-Aza-treated group. As shown in Fig. 4B, no significant

luciferase activity of the reporter genes on C/EBPα wild-type vectors was observed, which indicates that 5-Aza does not induce the overexpression of endogenous C/EBPα in Hep2 cells, even though the C/EBPα-binding sites in the *S100A4* promoter region -485 - +73 were demethylated in Hep2 cells. There was also no significant difference in luciferase activity of the reporter genes on C/EBPα wild-type and mutant-type vectors between the 5-Aza-treated or -untreated groups, which indicates that the mutant sites reflecting the methylated ones in C/EBPα do not affect the binding of C/EBPα to *S100A4* in the Hep2 cells. Yet, the C/EBPα deletion vector showed a significant decrease in the reporter activity when compared to the wild-type and mutant-type vectors in the 5-Aza-treated and -untreated groups ( $P < 0.05$ ), which suggests that the C/EBPα response element but not its cytosine methylation status plays an important role in the transcriptional regulation of the *S100A4* gene.

## Discussion

*S100A4* is overexpressed in many cancer types including laryngeal carcinoma, which indicates that this gene plays an important role in carcinogenesis. As demonstrated in our previous study, there is a strong correlation between overexpression and hypomethylation of *S100A4* in laryngeal cancer tissues (15). In addition, similar phenomena are found in endometrial, pancreatic ductal, colon and ovarian carcinomas (17-19). At present, little is known concerning the molecular mechanisms behind the transcriptional regulation of *S100A4* expression-related hypomethylation. In ovarian cancer, the upregulation of *S100A4* expression is associated with hypomethylation of CpG sites in the first intron of *S100A4*, and hypoxia can reduce the methylation of hypoxia-response elements (HRE) of the *S100A4* gene in a time-dependent fashion, in association with the increased binding of HIF-1α to a methylation-free HRE (20). Therefore, we speculate that the methylation-free sites in the *S100A4* promoter region can

increase the binding of the corresponding transcription factors to *S100A4* in laryngeal cancer.

In the present study, we cloned and characterized over 1.2 kb (-1283 to +73) region lying upstream of the human *S100A4* transcription start site to determine the major *cis*-acting elements, particularly those related to the methylation status in HEp2 cells. As a result, we revealed the existence of potential positive and negative *cis*-acting elements in the corresponding regions of the *S100A4* promoter. Further investigation was focused on the region -485 - +73 as the methylation status of the region significantly affected the expression of *S100A4*. Notably, the cytosines in the region were methylated in the HEp2 cells, and 5-Aza altered the methylation status (Fig. 2A and B). In this region, there were several putative *cis*-acting elements such as c-Myb, C/EBP $\alpha$ , Ap2 and Msx-1. EMSA and ChIP results confirmed the binding of c-Myb and C/EBP $\alpha$  to *S100A4*. Comparing the luciferase activity of the reporter genes on the mutant, deletion and wild-type vectors in the HEp2 cells treated by 5-Aza or not, we found that only c-Myb promoted *S100A4* expression in the methylation-free HEp2 cells. We thus speculated that the overexpression of endogenous c-Myb in HEp2 cells induced by 5-Aza plays a partial role in the *S100A4* expression in the methylation-free HEp2 cells. Nevertheless, the c-Myb response element in the region -485 - +73 is the major element in the regulation of *S100A4* expression in HEp2 cells when the methylation status is changed. The c-Myb proto-oncogene product (*c-Myb*) plays an important role in the proliferation of immature hematopoietic, colonic epithelial, and mammary epithelial cells and also in the differentiation of hematopoietic cells (21-24). The identified target genes of *c-Myb* include c-myc, Bcl-2 and Gbx2, which are involved in cell cycle control, blockage of apoptosis, and growth and differentiation control, respectively (25-28). Presently, no related study on the association of *c-Myb* with the development of laryngeal cancer has been reported. Similar to our study, Wang *et al* found that methylation of the CpG site in the *WNT5A* promoter influences its binding to c-Myb and 5-Aza increased the expression of *WNT5A*, which suggests that binding of c-Myb to *WNT5A* is methylation-sensitive and is inhibited by the methylation of the CpG site in prostate cancer cells (29). Campanero *et al* found that methylation of the E2F elements in the c-Myc and c-Myb promoters significantly inhibited the binding of E2F1, and suggested that methylation of the c-Myb element from the *S100A4* promoter may affect its binding to c-Myb (30). In the present study, *S100A4* was confirmed as a novel target of c-Myb and C/EBP $\alpha$ , which enriched the regulatory network in which c-Myb and C/EBP $\alpha$  were involved. Fang *et al* found that 663 transcripts including c-Myb and *S100A4* were regulated by CypB knockdown, and that many of these gene products contributed to cell proliferation, cell motility and tumorigenesis, which indicates that the co-overexpression of *c-Myb* and *S100A4* participates in the pathogenesis of breast cancer (31). Whether CypB directly regulates c-Myb and *S100A4*, respectively, or regulates *S100A4* via c-Myb is not known.

In conclusion, in the present study, we first revealed that c-Myb and C/EBP $\alpha$  can target the *S100A4* gene through binding the corresponding *cis*-acting motifs in the sequence -485 - +73. The overexpression of the *S100A4* gene is caused by demethylation of the c-Myb motif and possible overexpression of the *c-Myb* gene in methylation-free HEp2 cells. Whether the

overexpression of the *c-Myb* gene in the 5-Aza-induced HEp2 cells was caused by the demethylation of *c-Myb* gene itself or of other upstream genes regulating *c-Myb* or both warrants further investigation. The identification and functional study of potential negative response elements in the *S100A4* promoter region -486 - -530 will be the focus of subsequent research by our group.

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