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A possible gene silencing mechanism: Hypermethylation of the Keap1 promoter abrogates binding of the transcription factor Sp1 in lung cancer cells

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ABSTRACT

Hypermethylation often leads to gene silencing; however, the mechanism responsible for the low expression resulting from hypermethylation of the tumor suppressor gene Kelch-like ECH-associating protein 1 (Keap1) in human lung cancer cell lines remains unclear. In this study, using promoter deletion and site mutagenesis assays, we determined that one transcription factor stimulating protein-1 (Sp1) regulatory element in the Keap1 promoter region was important for the transcription of Keap1 in A549 cells. We demonstrated that the transcription factor Sp1 can directly bind to this element in the normal bronchial epithelial BEAS-2B cell line but not in A549 cells, as assessed with chromatin immunoprecipitation (ChIP). EMSAs and supershift assays also showed that CpG island methylation could abrogate Sp1 binding to the Keap1 promoter. Moreover, Keap1 mRNA decreased by 50% after the knock-down of Sp1 with siRNA in BEAS-2B cells, whereas the over-expression of Sp1 led to a dramatic increase in Keap1 promoter activity. The treatment of A549 cells with 5-aza-2′-deoxycytidine restored the binding of Sp1 to the promoter and Keap1 expression. Our results indicate that Sp1 is essential for Keap1 expression and that promoter methylation blocks Sp1 binding in A549 cells. These results demonstrate that hypermethylation may act as an epigenetic gene silencing mechanism, i.e., the inhibition of Sp1 binding to the hypermethylated Keap1 promoter in lung cancer cells, which suggests new approaches to lung cancer treatment.

1. Introduction

Lung cancer is the most common cause of cancer mortality and results in approximately 160,000 patient deaths each year in the US [1]. The high mortality rate associated with cancers has always driven demand for diagnostic and treatment-based research in medical science. Considerable progress has been made in understanding the biological players in cancers, such as oncogenes and tumor suppressor genes, which are the primary contributors to the occurrence and development of a tumor. Low expression or inactivation, i.e., the loss-of-function of tumor suppressor genes, may hinder tumor cell growth inhibition, which leads to malignant transformation. For example, the inactivation of p53 (deletion or mutation) often occurs during the progression from normal colon epithelium to invasive carcinoma [2,3].

Keap1 is a member of a large family of proteins containing an N-terminal BTB (broad complex, tram track, and bric-a-brac) domain and a C-terminal Kelch/DGR (double-glycine repeat) domain. Keap1 is also a newly identified nuclear factor erythroid-2 related factor 2 (Nrf2)-associated protein [4]. In recent years, studies have found that Keap1 expression is low in various cancers, such as non-small-cell lung cancer, gallbladder cancer and breast cancer. Keap1 has been identified as a novel tumor suppressor gene in prostate tumors [5–9], and the loss of Keap1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth [10]. Ohta et al. have found two nonsynonymous somatic Keap1 gene mutations and low Keap1 expression in two lung cancer lines, where this results in low Keap1 activity and leads to nuclear localization and the constitutive activation of Nrf2 [11]. Solis et al. have also found Keap1 abnormalities in non-small-cell lung carcinoma and an association between Keap1 and clinicopathologic features [13]. In our previous study, the down-regulation of Keap1 in three lung cancer cell lines and five cancer tissues was also observed [12].

Epigenetic regulation, including DNA methylation and the acetylation of histones, in contrast to gene mutation, is an established source of abnormal gene expression. Among these modifications, the hypermethylation of promoters is one of the most important forms of gene expression regulation leading to the silencing of genes. Methylation in the KEAP1 promoter region was detected in 22 out of the 47 non-small-cell lung carcinomas (47%), but in none of the normal tissues analyzed. Epigenetic mechanisms may play a pivotal role in the regulation of KEAP1 expression [12]. Our previous study revealed that the methylation pattern of CpG islands in the Keap1 promoter differed both between normal lung

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and lung cancer tissues and between human lung carcinoma cells (SPC-A1, NCI-H460 and A549 cells) and a normal bronchial epithelial cell line (BEAS-2B) [12]. However, the mechanism by which hypermethylation results in the silencing of tumor suppressor genes, specifically Keap1, remains unknown.

In light of this, the main goal of this study was to clarify the mechanism of low Keap1 expression in lung cancer cells. Our results demonstrated that Sp1 binding to the proximal region of the *cis*-acting element −160/−153 nt of the Keap1 promoter results in an up-regulation of Keap1 and that hypermethylation of the Keap1 promoter prevents Sp1 from binding to the promoter in lung cancer cells. These results demonstrate that an epigenetic gene silencing mechanism that down-regulates Keap1 expression in A549 cells might be associated with the inhibition of Sp1 binding to the promoter by hypermethylation.

2. Materials and methods

2.1. Cell culture

The human lung carcinoma A549 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, CA, USA) and supplemented with 10% (v/v) fetal bovine serum (FBS) in a humidified environment (37 °C and 5% CO_2). The human normal bronchial epithelial BEAS-2B cells (ATCC) were cultured in LHC-8 medium (Gibco). Schneider's *Drosophila* line 2 (SL-2) cells (ATCC) were cultured in Schneider's *Drosophila* medium (Gibco) and supplemented with 10% (v/v) FBS at 23 °C.

2.2. Construction of plasmids

For promoter deletion, Keap1 promoter-luciferase chimeric constructs were created through PCR amplification. The forward primer contained a *Sac*I site, and the reverse primer contained a *Hin*dIII site (Table 1). These restriction sites were used to clone the Keap1 promoter fragments into the luciferase reporter pGL3-basic (Promega, Madison, WI, USA). For site mutagenesis assays, a series of mutant plasmids was produced using the QuickChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), using a mutant primer (Table 1). All of the deletions and site-directed mutants were confirmed by DNA sequencing. The resulting constructs containing different lengths of the Keap1 promoter were termed P230-luc and P100-luc. Plasmids with mutated binding sites were termed MSp1-1 P230-luc (mutated binding site at −226/−223), MAP2 P230-luc and MSp1-2 P230-luc (mutated binding site at −158/−155) and used to identify Sp1 or Ap2 binding sites in the Keap1 promoter. The wild-type stimulating protein 1 (Sp1) expression vector (pPacSp1) and the Sp1 frameshift mutant vector that did not express Sp1 (pFXSp1) were kindly provided by Dr. Andrew D. Yurochko (Lineberger Comprehensive Cancer Center, University of North Carolina, USA) [14].

2.3. Analysis of plasmid activity with a luciferase assay

Cells were plated at a density of 4×10^4 cells/well on 96-well culture plates and transfected using Lipofectamine™ 2000 (Invitrogen, CA, USA), as described previously [15]. A549 cells were transfected with the Keap1 promoter construct and the Renilla luciferase expression vector, pRL-TK (Promega). For co-transfection experiments, the Sp1 expression plasmid was co-transfected with the Keap1 promoter construct and pRL-TK. The pcDNA3.1 vector (Invitrogen) was used as an internal control to evaluate the expression of the vector on Keap1 promoter activity. Next, the cells were assayed for both firefly and Renilla luciferase activities using a dual-luciferase system and a GloMaxTM 96-well microplate illuminometer (Promega), as described in the manufacturer's protocol.

2.4. Sp1 siRNA RNA interference

The siRNA (small interfering RNA) sequence was purchased from the Origene Company (Rockville, MD, USA). For this experiment, 2×10^6 BEAS-2B cells were transfected with 600 pmol of Sp1 siRNA with siTran 1.0 transfection reagent. After 48 h, the cells were harvested for western blotting and real-time PCR analysis.

2.5. Western blot analysis

Western blot analysis was performed using previously described methods [15]. After treatment, 2×10^7 BEAS-2B cells were harvested and the nuclear protein extracts were prepared with an NE-PER nuclear extraction kit (Pierce, Rockford, IL, USA). For electrophoresis, 50 μg of nuclear proteins was separated through SDS/PAGE, electrotransferred to a nitrocellulose membrane, and subsequently incubated with an anti-Sp1 monoclonal antibody

Table 1
List of oligonucleotides used in the present study. The mutant bases are underlined. The *Sac*I and *Hin*dIII site are shown in italics.

Name	Oligonucleotide (5′ → 3′)	Position
PCR primers		
Deletion constructs		
P230	F *AGAGCTC* CCTGC GGCCG CCGGA CCA	−230/−213
P100	F *AGAGCTC* GCCTGGCTGT GCGCCCCCGC	−100/−81
Pright	R *AAAGCTT* CCTCG GCTCC AGGGC TGCG	+86/+104
Site-specific mutagenesis		
MSp1-1P230	F CCTGATTACGCCGGACCACGAGGCCGG	−230/−204
	R CCGGCCTCGTGGTCCGGCGTAATCAGG	
MAP2 P230	F GCCGGACCACGAAATTAGCGCTGTGCGT	−221/−194
	R ACGCACAGCGCTAATTTCGTGGTCCGGC	
MSp1-2 P230	F GGCGCGCAGCTTTACGAGGAGACATCCAG	−168/−139
	R CTGGATGTCTCCTCGTAAAGCTGCGCGCC	
Real-time PCR		
Keap1 sense	GACAGCCTCTGACAACACAAC	
Keap1 antisense	GAAATCAAAGAACCTGTGGC	
β-Actin sense	ACCCACACTGTGCCCATCTA	
β-Actin antisense	GCCACAGGATTCCATACCCA	
Gel-shift oligonucleotides		
5′ biotin P168150	5′ biotin GGCGCGCAGCCCCGCGAGG 3′	−168/−150
Meth P168150	5′ biotin GGCGCGCAGCCCC^{m}GCGAGG 3′	−168/−150
P168150	5′ GGCGCGCAGCCCCGCGAGG 3′	−168/−150

(1:500) (Santa Cruz Biotech). The proteins were visualized using ECL (enhanced chemiluminescence) reagents (Pierce).

2.6. Real-time PCR analysis

Total RNA extraction was performed as previously described [15]. Reverse transcription was performed using the Super-Script™III first-strand synthesis system (Invitrogen). Real-time PCR was then performed with the ABI Prism 7300 Sequence Detection System (Applied Biosystems, CA, USA) using Power SYBR Green Master Mix (ABI, CA, USA) to quantitatively determine the mRNA level of Keap1 according to the manufacturer's protocol. The primers used are presented in Table 1. The β-actin gene was used as an internal control for all of the reactions.

2.7. EMSA and supershift assays

The EMSA was performed using the LightShift® Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's protocol with human recombinant Sp1 (Santa Cruz Biotech). The 5′-biotin end-labeled oligonucleotides, termed 5′ biotin P168150, and methylated oligonucleotides, termed Meth P168150 (Table 1), were purchased from Beijing Dingguo Biological Technology and Services Company (Beijing, China). In the competitive experiment, 4 pmol of non-labeled double-stranded oligonucleotide, termed P168150 (Table 1), was added to the reaction mixture. In the supershift assay, 1 μg of anti-Sp1 monoclonal antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) was incubated with human recombinant Sp1 for 5 min at 25 °C before the binding reaction. The biotin end-labeled DNA was detected using a streptavidin–horseradish peroxidase conjugate and LightShift chemiluminescent substrate (Pierce).

2.8. ChIP assay

ChIP was performed according to the instructions of the ChIP assay kit (Upstate, MA, USA). The nuclear DNA extracts were amplified using a pair of primers (Table 1) that spanned the Sp1 sites (−235 to −100 bp) within the Keap1 promoter region. For immunoprecipitation, 4 μg of an anti-Sp1 monoclonal antibody (Santa Cruz Biotech) was added and incubated overnight at 4 °C. As a negative control, the primary antibody was omitted or replaced with normal rabbit serum.

2.9. 5-Aza-2′-deoxycytidine treatment for Keap1 gene demethylation

To analyze whether Keap1 promoter methylation blocks the binding of the transcription factors, 5-aza-2′-deoxycytidine (5-Aza, Sigma, St. Louis, MO, USA) was dissolved in phosphate-buffered saline and used for the demethylation of the promoter region. A549 cells were incubated in culture medium with or without 10 μM 5-Aza for 5 days and then used for ChIP assays [12].

2.10. Data analysis

The data are expressed as means ± S.D. The results were evaluated using an un-paired Student's *t*-test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Functional characterization of the Keap1 promoter

In our previous study, we demonstrated that three specific CpG islands of the Keap1 promoter (the 3rd, 6th and 10th) were hypermethylated in lung cancer cells and tissue [12]. To elucidate the roles of these CpG islands in the regulation of Keap1 expression, we predicted the transcription factor-binding sites using the online Tfsitescan program. We found that the CpG islands in the region −230/−100 of Keap1 harbored crucial transcriptional regulatory elements, including AP2- and Sp1-binding sites. The 3rd and 10th CpG islands were embedded in putative Sp1-binding sites, whereas the 6th CpG island resided in the putative AP2-binding site (Fig. 1A). However, whether these transcription factor binding sites affected Keap1 promoter activity remained unknown.

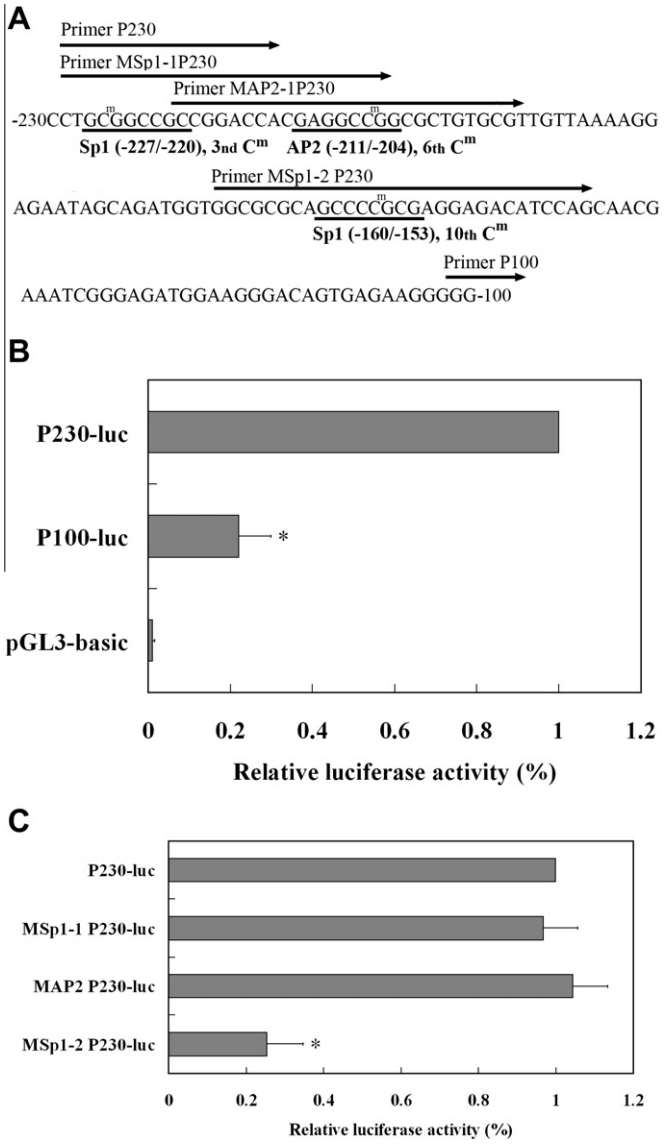

Fig. 1. Functional analysis of Keap1 promoter constructs. (A) Sequence of the Keap1 promoter from −230 to −100 bp; the numbers are relative to transcription start sites (+1). Specific protein binding sites such as AP2 and Sp1 are underlined. C^m also lies in the protein binding sites. The primers used in luciferase assay were also shown in the schematic diagram. (B) The region spanning 230 bp of the promoter and the first 100 bp, starting from the transcription start site, acquired through progressive deletion from its 5′ end, were fused to the pGL3 basic vector and transfected into A549 cells, respectively, as described in the experimental methods. Values (means ± S.D.) represent the firefly luciferase activity normalized relative to the Renilla luciferase transfection internal control. Luciferase activities are shown relative to the activity of the P230-luc, which is arbitrarily set to 1. All transfections were performed in triplicate for each of three different samples. (C) Identification of transcriptional activity at the Keap1 promoter. A549 cells were transiently transfected with the Keap1 promoter-luciferase constructs containing different site-directed mutations of the putative transcription factor binding sites.

Based upon our predictions, we cloned 230 bp in the region of the Keap1 promoter, and two fragments, P230 and P100, were generated with PCR using specific primers (Table 1). The PCR products were inserted into a vector with a luciferase reporter gene (pGL3-basic) and transiently transfected into human A549 cells (Fig. 1B). The potential binding sites for the transcription factors were identified in this region using a promoter deletion assay. As shown in Fig. 1B, the deletion from −230 to −100 bp significantly decreased the promoter activity by approximately 5-fold, suggesting that the presence of a *cis*-element strongly increased the promoter activity in this region. P230 displayed greater promoter activity than P100, indicating that the regulatory elements are located in the proximal region between −230 and −100 bp of the Keap1 promoter.

3.2. Identification of transcription factor-binding sites

We utilized three mutant promoter constructs, −227/−220 and −160/−153 of the Sp1-binding sites and −211/−204 of the AP2-binding site, to determine whether *cis*-elements were affecting Keap1 transcription in the Keap1 promoter region. As shown in Fig. 1C, the Sp1-binding site mutation −160/−153 markedly decreased Keap1 promoter activity by approximately 4-fold, similar to the decrease after deletion to −100 bp. In contrast, the putative Sp1-binding site mutation −227/−220 and AP2-binding site mutation −211/−204 had little effect on promoter activity. These results indicated that only one Sp1-binding site (−160/−153) is critical for enhancing Keap1 transcription in the −230/−100 region of the Keap1 promoter, whereas no effects of the Sp1-binding site (−227/−220) and AP2-binding site (−211/−204) in the Keap1 promoter were observed in the promoter deletion and site-specific mutagenesis assays.

3.3. Sp1 drives Keap1 promoter activity

To clarify the role of the transcription factor Sp1 in promoting Keap1 expression, we co-transfected the P230 promoter construct with either the Sp1 expression vector pPacSp1 or the Sp1 mutant vector pFXSp1 into A549 cells or SL-2 cells. The pcDNA3.1 vector was used as a control. As shown in Fig. 2A, the co-transfection of P230 with pPacSp1 enhanced luciferase gene expression by nearly 9-fold and 10-fold in the A549 and SL-2 cells, respectively, relative to the luciferase activity in untransfected cells; in contrast, the mock and Sp1 mutant vector pFXSp1 transfections exhibited minor promoter inhibition.

To further confirm the regulation of Keap1 expression by Sp1 via the −160/−153 Sp1-binding site, A549 cells were co-transfected with the mutant plasmid MSp1-2 P230-luc, which lacks Sp1

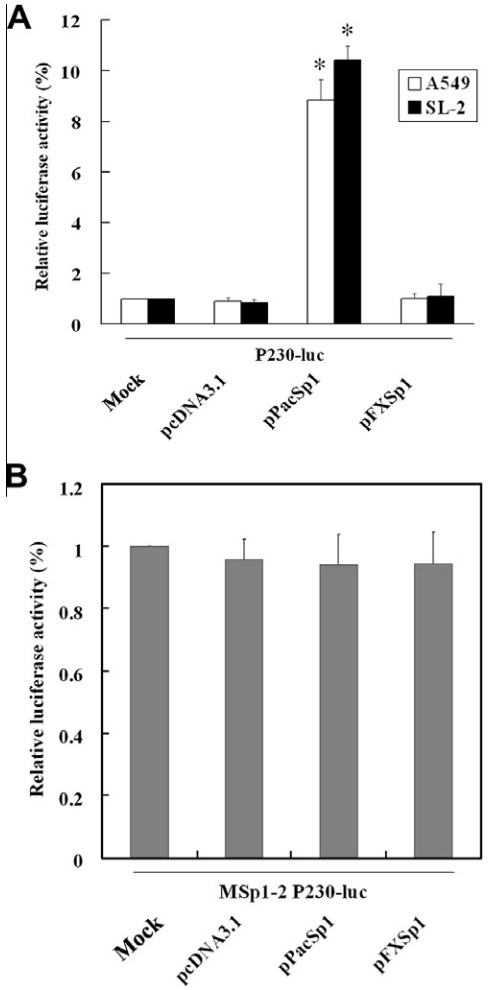

Fig. 2. Sp1-mediated transcriptional regulation of Keap1 via binding at the site −160/−153. A549 cells were transfected with (A) P230-luc or (B) MSp1-2 P230-luc, in which the −160/−153 region was mutated, in the presence or absence of the expression vector. Experiments were performed in triplicate.

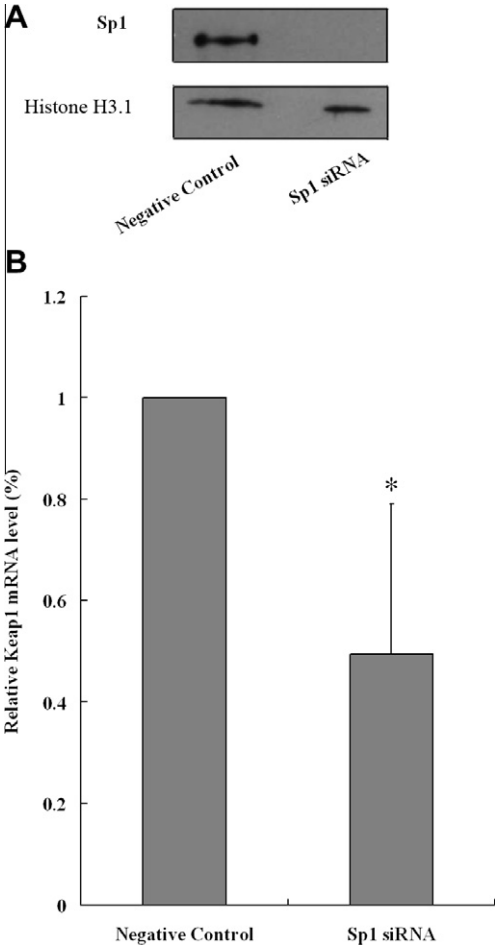

Fig. 3. Showing the effects of siRNA, which knocked down Sp1, on Keap1 gene expression. (A) Western blot analysis of Sp1 protein in nuclear extracts from BEAS-2B cells transfected with the Sp1 siRNA or the negative control. Histone H3.1 protein served as an internal control. (B) BEAS-2B cells were transiently transfected with the negative control or Sp1 siRNA. After 48 h of transfection, Keap1 mRNA was quantified by real-time PCR. Values are the means ± S.D. $^{*}p < 0.05$ indicates a significant difference between two groups.

binding at the Keap1 promoter due to a mutation of the binding site, and the Sp1 expression vector pPacSp1, the Sp1 mutant vector pFXSp1, or the pcDNA3.1 vector. Promoter activity was not enhanced in the presence of MSp1-2 P230-luc with pPacSp1 (Fig. 2B), suggesting that Sp1 plays an important role in regulating Keap1 promoter activity via its −160/−153 binding site. The Sp1 role was further demonstrated by the knockdown of the endogenous expression of Sp1 with siRNA in BEAS-2B cells. Western blot analysis showed that Sp1 protein levels were significantly decreased after 48 h of incubation with siRNA (Fig. 3A). In addition, Keap1 mRNA levels were detected using RT-PCR and, as expected, these levels had correspondingly decreased by 50% (Fig. 3B).

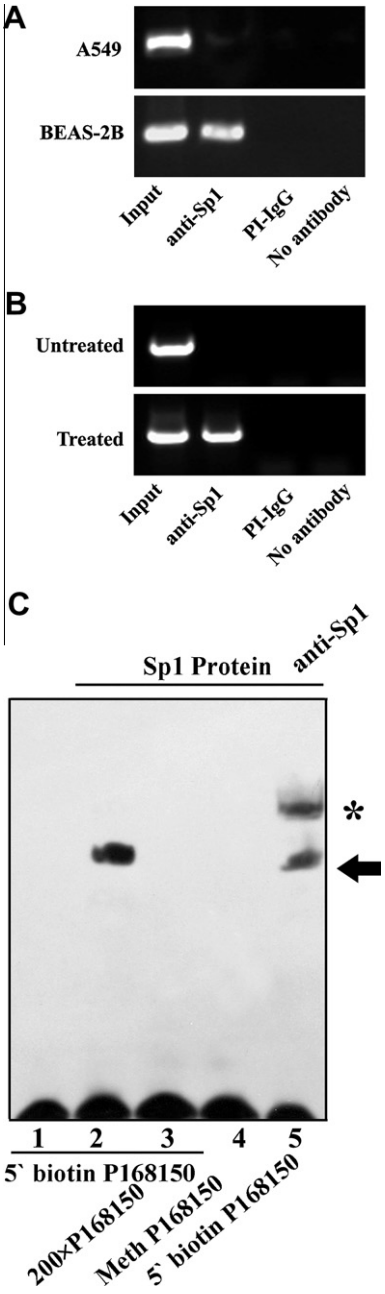

Fig. 4. ChIP and EMSA analysis showing Sp1 binding to the Keap1 promoter. (A) Soluble chromatin from A549 cells and BEAS-2B cells was immunoprecipitated with the Sp1 antibody or incubated with PI-IgG. For the control, no antibody was used. (B) The A549 cells were treated by 5-Aza for demethylation and subjected to a ChIP assay. (C) EMSA and supershift analysis show the specific binding of Sp1 to the −160/−153 Sp1-binding site in the Keap1 promoter region. The biotin-labeled P168150 probe was incubated in the absence (lane 1) or presence (lane 2) of human recombinant Sp1 protein. For the competition experiment, 200-fold molar excess of unlabeled P168150 probe (lane 3) was added to the binding reaction. The biotin-labeled methylated P168150 probe was also incubated in the presence of human recombinant Sp1 protein (lane 4). Lane 5 is the result of an anti-Sp1 antibody-mediated supershift experiment. The arrow shows a specific protein–DNA complex and the asterisk indicates the supershift.

3.4. Hypermethylation prevents Sp1 binding to the Keap1 promoter

ChIP assays were performed with an anti-Sp1 antibody to clarify whether the hypermethylation of the Sp1-binding site (−160/−153) in the Keap1 promoter affected Sp1 binding. BEAS-2B cells, which lack methylation at the Sp1-binding site in the Keap1 promoter, and A549 cells, which are hypermethylated in this region, were used in these assays. Immunoprecipitation of the BEAS-2B cells with the anti-Sp1 antibody produced a band of approximately 330 bp, indicative of bound DNA, which was amplified with PCR using primers surrounding the Sp1-binding site. When ChIP was performed with normal rabbit serum or without the anti-Sp1 antibody, a DNA band was not obtained (Fig. 4A). In contrast, no band was amplified with PCR in A549 cells, indicating that Sp1 was unable to bind to the hypermethylated Sp1-binding site in the Keap1 promoter. To further determine whether methylation affected the interaction between Sp1 and its binding site, A549 cells were untreated or treated with 10 μM 5-Aza for 5 days. The ChIP assay clearly showed a PCR-amplified band in A549 cells treated with 5-Aza (Fig. 4B).

The ChIP results were further confirmed with EMSAs and supershift assays. The human recombinant Sp1 protein was incubated with a biotin-labeled P168150 probe or a biotin-labeled P168150 methylated probe (Table 1). As shown in Fig. 4C, one specific band of a DNA–protein complex was detected (lane 2), which was outcompeted by a 200-fold molar excess of the unlabeled P168150 probe (lane 3). The binding of Sp1 disappeared after the methylation of the consensus Sp1-binding site, unlike the non-methylated fragment (lanes 4). This DNA–protein complex was clearly supershifted by the addition of the anti-Sp1 antibody (lane 5). Because DNA was immunoprecipitated with the Sp1 antibody from BEAS-2B cells and A549 cells treated with 5-Aza, which removed methylation, we concluded that Sp1 can directly bind to the −160/−153 region of the Keap1 promoter in the absence of methylation and that this binding is strongly repressed by methylation at the binding site. In other words, hypermethylation of the Keap1 promoter region blocked Sp1 binding.

4. Discussion

Keap1 was first considered as a tumor suppressor gene in 2008. It was reported that low Keap1 activity could result in nuclear localization, the constitutive activation of Nrf2 and the up-regulation of the expression of cyto-protective genes encoding multidrug resistance pumps, such as phase II detoxifying enzymes, which leads to cisplatin resistance and facilitates the occurrence and progression of lung cancer [5,6].

Recent studies have demonstrated that methylation modification is an important regulator of gene expression. DNA methylation has been shown to silence a large number of tumor suppressor genes [16,17]. Our previous study demonstrated that the low expression of Keap1 in human lung cancer tissue and cell lines was due to the hypermethylation of the Keap1 promoter [12]. However, the hypermethylation mechanism leading to the low expression of Keap1 was unclear. In the present study, we illustrated that hypermethylation of the Sp1-binding site (−160/−153) in the −230/−100 region of the Keap1 promoter abrogated the binding of transcription factor Sp1, resulting in a decrease in

promoter activity and reduced expression of Keap1 in lung cancer cells. This result may motivate future investigations into the mechanism by which hypermethylation leads to gene silencing.

Sp1 is a member of a growing family of elements that bind to and act through the GC box [18]. Sp1-binding sites have been shown to be crucial for the basal transcription of TATA-less promoters [19]. We demonstrated that Sp1 functions as an essential transcriptional activator of Keap1 and regulates Keap1 expression via binding to the SP1 site (−160/−153) in the promoter. This result was supported by the class II tumor suppressor gene H-rev107 promoter, which exhibited a high level of transcriptional activity in the non-transformed fibroblasts in which Sp1/Sp3 bound to the GC box located in its promoter [20]. The Sp1/Sp3 transcription factors are also crucial in the expression of the human protocadherin-10 gene, a novel tumor suppressor gene [21]. Previous studies have indicated that DNA methylation interferes with the binding of regulatory proteins and that the presence of methyl groups on cytosine residues inhibits interactions with transcription factors in the *cis* proximal region [22,23].

ChIP and co-transfection assays revealed that Sp1 was associated with its −160/−153 binding site in BEAS-2B cells. In contrast, a negative result was obtained in A549 cells. Using 5-Aza to demethylate the Keap1 promoter in A549 cells demonstrated that Sp1 could bind to its binding site at the Keap1 promoter in the A549 cells treated with 5-Aza via the ChIP assay. These results further indicated that methylation of the −160/−153 Sp1-binding site in the Keap1 promoter could block transcription factor Sp1 binding in lung cancer cells.

In conclusion, the present study demonstrated that the hypermethylation of Sp1-binding sites in the Keap1 promoter blocked Sp1 binding, leading to Keap1 gene silencing in lung cancer cells. Our results might further elucidate a possible hypermethylation mechanism that leads to gene silencing and low expression of its protein in an epigenetic manner in lung cancers. These results may contribute to the prediction of disease progression and therapy responses in lung cancer patients.

Conflict of interest

We confirm that there is no potential conflict of interest regarding this paper.

Acknowledgments

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