Hypermethylation of the *Keap1* gene in human lung cancer cell lines and lung cancer tissues

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ARTICLE INFO

ABSTRACT

Low expression of the oxidative stress sensor Keap1 is thought to be involved in carcinogenesis. However, the mechanisms responsible for inactivation of the *Keap1* gene remain unknown. We investigated Keap1 expression using RT-PCR and found that it was downregulated in lung cancer cell lines and tissues when compared with a normal bronchial epithelial cell line. Treatment with 5-Aza-2′-deoxycytidine restored Keap1 expression in lung cancer cell lines, indicating the silencing mechanism to be promoter methylation. Moreover, we evaluated cytosine methylation in the *Keap1* promoter and demonstrated that the P1 region, including 12 CpG sites, was highly methylated in lung cancer cells and tissues, but not in normal cells. Importantly, we found evidence that three specific CpG sites (the 3rd, 6th, and 10th CpGs of P1) might be binding sites for proteins that regulate Keap1 expression. Thus, our results suggest for the first time that Keap1 expression is regulated by an epigenetic mechanism in lung cancer.

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The Nrf2/Keap1 system plays a key role in the cellular defense against oxidative stress [1]. Nuclear factor erythroid-2 related factor 2 (Nrf2) is a basic region-leucine zipper (bZIP) transcription factor that positively regulates the basal and inducible expression of a battery of detoxifying and antioxidative enzyme/protein genes [2–4]. Nrf2 protects cells against carcinogens, oxidants, and other toxic chemicals [5,6]. Kelch-like ECH-associated protein 1 (Keap1) is a cytoplasmic anchor for Nrf2 that tethers Nrf2 in the cytoplasm under basal unstressed conditions. As a result, Keap1 prevents Nrf2 from translocating to the nucleus, where it would bind to the ARE and activate gene transcription [7]. On the other hand, Keap1 also functions as a substrate adaptor for interaction of the Cul3-based E3-ubiquitin ligase complex with Nrf2, which ultimately leads to ubiquitination of Nrf2 and proteosomal degradation [8,9]. It has been reported that Nrf2 is released from Keap1 under oxidative and electrophilic stresses, resulting in increased nuclear accumulation and in turn, the transcriptional induction of target genes that ensure cell survival [10]. Two mechanisms responsible for this dissociation have been suggested: (1) reactive cysteines of Keap1 crosslink after electrophilic reaction, thereby hampering Keap1-mediated proteasomal degradation of Nrf2 [11], and (2) phosphorylation of Nrf2 by PKC promotes its dissociation from Keap1 [12].

Accumulating evidence supports the hypothesis that Keap1 is expressed at low levels in patients with cancer but is often mutated. Ohta et al. observed two lung cancer cell lines expressing Keap1 at reduced levels and elucidated two nonsynonymous somatic *Keap1* gene mutations in lung cancer tissues. They also found that loss of Keap1 function activated Nrf2 and enhanced lung cancer cell growth [13]. Singh et al. performed a systematic analysis of the Keap1 genomic locus in lung cancer patients and cell lines that revealed deletion, insertion, and missense mutations in functionally important domains of Keap1, suggesting that biallelic inactivation of Keap1 in lung cancer is a common event [14]. Another study also identified a mutation (C23Y) in the N-terminal domain of Keap1 in breast cancer [15].

However, the mechanisms responsible for low Keap1 expression have not yet been elucidated. It has been suggested that DNA methylation is the first step in epigenetic phenomena that modulate gene expression via the recruitment of transcription factors. Site-specific methylation within promoters has, in many cases, been associated with the transcriptional silencing of specifically regulated genes [16]. Therefore, our present studies focused on abnormalities of the Nrf2/Keap1 system in patients with cancer, with special attention paid to the methylation status of the *Keap1* promoter in human lung cancer cell lines and cancer tissues. Our results demonstrate that *Keap1* gene silencing may be due to aberrant DNA methylation.

Materials and methods

Cell lines and tissue samples. Human lung cancer cell lines were obtained from the Respiratory Research Department of the Third Military Medical University. A549 cells were cultured in

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Dulbecco's modified Eagle's medium (DMEM, Invitrogen, CA, USA) supplemented with 5% fetal bovine serum (FBS). SPC-A1 and NCI-H460 cells were grown in RMPI 1640 (Invitrogen) supplemented with 10% FBS. The human normal bronchial epithelial cell line (BEAS-2B) was a gift from the Academy of Military Medical Sciences and was cultured in LHC-8 medium (Biofluid, Rockville, MD). All cells were cultured in the presence of 5% CO_2 at 37 °C.

Five cancer tissue samples (from males with a mean age of 57) were obtained from patients with lung carcinoma at Xi'nan Hospital (Chongqing, China). Among them, two were squamous cell carcinoma, two were adenocarcinoma and one was adenosquamous cell carcinoma. After surgical removal, the tissues were immediately frozen in liquid nitrogen and stored at −70 °C.

DNA and RNA extraction. Genomic DNA was isolated from lung cancer cell lines and tissue samples with a Bioflux DNA Purification System (Bioflux, Japan) according to the manufacturer's instructions. The quality and integrity of the DNA were determined by the A260/280 ratio. Total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA). Purified RNA samples were suspended in DEPC water and used directly.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Reverse transcription for cDNA synthesis was performed on 2 μg total RNA using a Protoscript First Strand cDNA Synthesis Kit (New England BioLabs Inc, USA) with random $dT_{23}VN$ primers. The housekeeping gene β-actin served as an internal control to confirm the success of the reverse transcription reaction. The PCR products were subjected to 2.5% agarose gel electrophoresis. Primer sequences were as follows: Keap1 forward: 5′-GACA GCCTCTGACAACACAAC-3′, reverse: 5′-GAAATCAAAGAACCTGTG GC-3′; β-actin forward: 5′-GGAAATCGTGCGTGACATTA-3′, reverse: 5′-GGAGCAATGATCTTGATCTTC-3′. The Keap1 mRNA sequence was obtained from NCBI (GenBank Accession No. BC002930). PCR cycling conditions were 94 °C (3 min) for one cycle, 94 °C (30 s), 55 °C (45 s), and 72 °C (1 min) for 30 cycles, and a final extension of 72 °C (5 min).

Bisulfite sequencing (BSG). Denatured DNA (2 μg) was bisulfite converted using the EZDNA Methylation Kit (Zymo Research, Orange, CA) according to the manufacturer's directions. For bisulfite sequencing, PCR was performed with the following primer sets: K-F 5′-GAAAGAAAGAAAGAAAAGAAAAG-3′, K-R 5′-CACCAAAAAT AAAATAAACACCC-3′ (position shown in Fig. 2A). PCR products were cloned into a PMD-19T simple vector (Takara, Japan) after purification using a Biospin Gel Extraction Kit (Biospin, Japan). Ten clones of each specimen were sequenced by dye-terminator cycle-sequencing in both directions using an ABI 3730 DNA Analyzer (Applied Biosystems) to identify CpG methylation.

Real-time RT-PCR. Real-time PCR was carried out in a 50 μl final volume and performed in triplicates using Power SYBR Green PCR Master Mix reagents in an ABI PRISM 7300 sequence detection system (Applied Biosystems) according to the manufacturer's protocol. Primer sequences were as follows: Keap1 forward: 5′-TGGCCAAGCAAGAGGAGTTC-3′, reverse: 5′-GGCTGATGAGGGT CACCAGTT-3′; β-actin forward: 5′-TGGATCAGCAAGCAGGAGT ATG-3′, reverse: 5′-GCATTTGCGGTGGACGAT-3′. Relative transcript quantities were calculated using the comparative C_t method with β-actin as the endogenous reference gene. The conditions for real-time PCR were as follows: 95 °C (5 min) followed by 40 cycles at 94 °C (30 s), 50 °C (30 s), and 69 °C (30 s).

5-Aza-2′-deoxycytidine treatment for Keap1 induction. 5-Aza-2′-deoxycytidine (5-Aza, Sigma) was dissolved in phosphate-buffered saline. Exponentially grown cells were incubated in culture medium with and without 5-Aza at a concentration of 10 μM for two days or five days, with medium changed daily. After cells were harvested, RNA was extracted for Real-time PCR analysis.

Results and discussion

Expression of Keap1 was down-regulated in lung cancer lines and lung cancer tissues

Keap1 mRNA expression in four lung cell lines and cancer tissues was significantly decreased in three lung cancer cell lines and five cancer tissues when compared to the normal cell line BEAS-2B, where it was highly expressed (Fig. 1). To our knowledge, this is the first determination of relative Keap1 mRNA levels in lung cell lines and cancer tissues by RT-PCR. However, these results were consistent with other reports that Keap1 protein levels were dramatically decreased in lung cancer lines and cancer tissues [13,14].
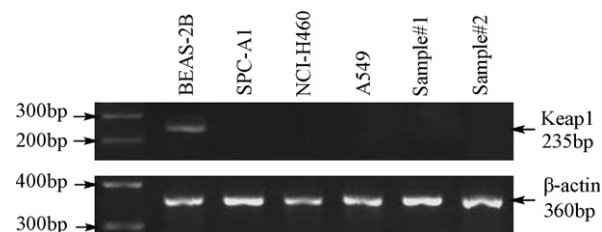
Methylation analysis of Keap1

To determine whether the decreased expression of Keap1 in lung cancer was related to cytosine methylation, we examined the methylation patterns of Keap1. The genomic sequence of human Keap1 was downloaded from the NCBI human genome database. We used Methprimer software online to identify CpG islands in the region around the transcription start site of the Keap1 gene. After setting the confidence interval for CpG location (observed/expected 0.6) and filtering the results by C+G content (50% minimum) and minimum island length (200 bp), we found a region (−252 to 277) around the transcription initiation site that fulfilled these criteria (see Fig. 2A). The sequences (−1000 to 337) including the CpG island were bisulfite-modified and sequenced (data not shown). Our results showed no obvious aberrant methylation upstream of the Keap1 gene; aberrant methylation existed only in the CpG island, which contained 62 CpG sites (CpGs).
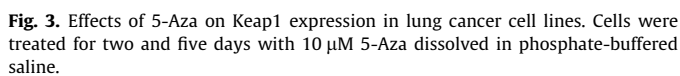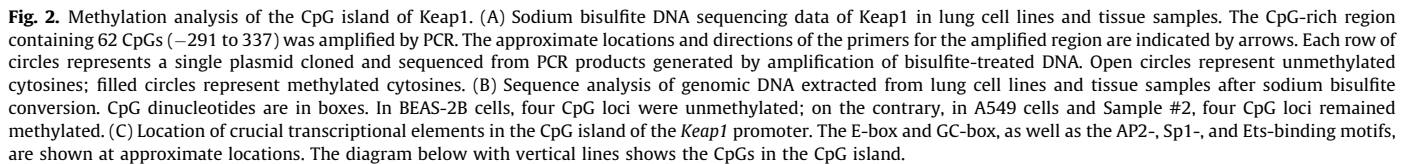
CpG island methylation analysis of the Keap1 gene

The region (−291 to 337) including the CpG island was amplified using bisulfite-modified genomic DNA as a template in lung cell lines and tissue samples. Fig. 2A shows that the P2 region including the last 50 CpGs (−88 to 337) had almost the same unmethylated status in both cell lines and tissue samples. However, the P1 region including the first 12 CpGs (−291 to −89) showed different methylation status between lung cancer and normal cells; P1 was hypomethylated in BEAS-2B cells but heavily methylated in lung cancer cell lines and cancer tissues. Overall, these results strongly suggest that reduced expression of Keap1 in lung cancer might be associated with aberrant DNA hypermethylation of CpG islands around the transcriptional start site, especially in the P1 region.

Bisulfite treatment displayed positive 5′-methylcytosines as cytosines, whereas unmethylated cytosines appeared as thymines in the final sequence. As shown in Fig. 2B, the promoter region of the Keap1 gene was largely unmethylated in BEAS-2B cells but

Fig. 1. RT-PCR analysis of Keap1 expression in lung cell lines and cancer tissues. Keap1 was highly expressed in BEAS-2B cells, but significantly downregulated in three lung cancer cell lines and five lung cancer tissue samples.

Fig. 2. Methylation analysis of the CpG island of Keap1. (A) Sodium bisulfite DNA sequencing data of Keap1 in lung cell lines and tissue samples. The CpG-rich region containing 62 CpGs (−291 to 337) was amplified by PCR. The approximate locations and directions of the primers for the amplified region are indicated by arrows. Each row of circles represents a single plasmid cloned and sequenced from PCR products generated by amplification of bisulfite-treated DNA. Open circles represent unmethylated cytosines; filled circles represent methylated cytosines. (B) Sequence analysis of genomic DNA extracted from lung cell lines and tissue samples after sodium bisulfite conversion. CpG dinucleotides are in boxes. In BEAS-2B cells, four CpG loci were unmethylated; on the contrary, in A549 cells and Sample #2, four CpG loci remained methylated. (C) Location of crucial transcriptional elements in the CpG island of the *Keap1* promoter. The E-box and GC-box, as well as the AP2-, Sp1-, and Ets-binding motifs, are shown at approximate locations. The diagram below with vertical lines shows the CpGs in the CpG island.

Fig. 3. Effects of 5-Aza on Keap1 expression in lung cancer cell lines. Cells were treated for two and five days with 10 μM 5-Aza dissolved in phosphate-buffered saline.

methylated in A549 cells and some lung cancer tissues. These findings further indicate differential *Keap1* promoter methylation between lung cancer and normal cells.

Restoration of Keap1 expression by 5-Aza-2′ -deoxycytidine treatment

Three lung cancer cell lines (SPC-A1, A549, and NCI-H460) in which CpG islands were hypermethylated and Keap1 expression was decreased were treated with 5-Aza to determine whether Keap1 downregulation occurred epigenetically. All treatments with 5-Aza restored Keap1 expression to varying degrees when compared to untreated controls (Fig. 3), suggesting that Keap1 expression might be regulated by an epigenetic mechanism in the lung cancer cell lines.

Three specific CpGs are putative binding sites for proteins that regulate Keap1 expression

DNA methylation interferes with the binding of a large number of regulatory proteins [17]. Thus, differences in methylation patterns might reveal essential binding sites for transcription factors regulating gene expression. Basing on our sequencing data, we found three specific CpGs (the 3rd, 6th, and 10th) in the CpG island that showed highly different methylation patterns between normal and cancerous lung cells. These sites were almost fully methylated in cancer cell lines and tissues, but unmethylated in the normal BEAS-2B cells. To investigate the role of these CpGs in the control of Keap1 expression, we searched for the presence of consensus

protein binding sites in the online Tfsitescan database and found that the CpG island around the transcriptional start site of Keap1 harbors many crucial transcriptional regulatory elements including the GC-box, and E-box, as well as AP2-, Sp1-, and Ets-binding motifs (Fig. 2C). It is noteworthy that the 3rd and 10th CpGs were embedded in putative Sp1 binding sites and the 6th CpG resided in the putative AP2 binding site. We therefore consider it likely that AP2 and Sp1 utilize DNA methylation for epigenetic regulation. The transcription factor AP2 is known to be a methylation-sensitive binding protein [18,19]. The transcription factor Sp1 also plays a general protector role for unmethylated CpGs by preventing access of DNA methyltransferases to newly synthesized strands [20]. Therefore, we speculate that these three CpGs might be binding sites for proteins that regulate Keap1 expression. The methylation of the 6th CpG might be involved in the down-regulation of Keap1 expression via inhibition of AP2 binding. Methylation of the 3rd and 10th CpGs might result from lost Sp1 binding. These hypotheses are supported by previous studies reporting that the presence of a methyl group in a cytosine residing in a cis-acting element inhibited interactions with transcription factors [21,22]. Further studies should be undertaken to establish the roles of these particular sites and the transcription factors AP-2 and Sp1 in *de novo* Keap1 expression in lung carcinomas.

In summary, we found that low levels of Keap1 mRNA were expressed in three lung cancer cell lines and five cancer tissues relative to normal bronchial epithelial cells. Moreover, we assessed the DNA methylation status in the 5′-upstream promoter region of the *Keap1* gene and found that the P1 region was aberrantly hypermethylated in lung cancer cell lines and tissues as compared to normal cells. More importantly, we found three specific CpGs that may be involved in the inactivation of Keap1 expression. In addition, the restoration of Keap1 expression by treatment with 5-Aza also strongly suggests that Keap1 down-regulation might be due to aberrant hypermethylation in the *Keap1* promoter.

In this study, we analyzed for the first time the epigenetic regulatory mechanism of *Keap1* gene expression in lung cancer cell lines and cancer tissues. Although our data were derived from four cell lines and five tissue samples, further investigation including more cell lines or specimens may confirm our preliminary observations. These results will be useful in clarifying the relationship between *Keap1* gene methylation and its expression, thereby providing a potential target for treatment of human lung cancer and a powerful tool for early diagnosis.

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