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Promoter demethylation of *Keap1* gene in human diabetic cataractous lenses

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ABSTRACT

Age-related cataracts (ARCs) are the major cause of visual impairments worldwide, and diabetic adults tend to have an earlier onset of ARCs. Although age is the strongest risk factor for cataracts, little is known how age plays a role in the development of ARCs. It is known that oxidative stress in the lens increases with age and more so in the lenses of diabetics. One of the central adaptive responses against the oxidative stresses is the activation of the nuclear transcriptional factor, NF-E2-related factor 2 (Nrf2), which then activates more than 20 different antioxidative enzymes. Kelch-like ECH associated protein 1 (*Keap1*) targets and binds to Nrf2 for proteosomal degradation. We hypothesized that hyperglycemia will lead to a dysfunction of the Nrf2-dependent antioxidative protection in the lens of diabetics. We studied the methylation status of the CpG islands in 15 clear and 21 diabetic cataractous lenses. Our results showed significant levels of demethylated DNA in the *Keap1* promoter in the cataractous lenses from diabetic patients. In contrast, highly methylated DNA was found in the clear lens and tumorized human lens epithelial cell (HLEC) lines (SRA01/04). HLECs treated with a demethylation agent, 5-aza-2′deoxycytidine (5-Aza), had a 10-fold higher levels of *Keap1* mRNA, 3-fold increased levels of *Keap1* protein, produced higher levels of ROS, and increased cell death. Our results indicated that demethylation of the CpG islands in the *Keap1* promoter will activate the expression of *Keap1* protein, which then increases the targeting of Nrf2 for proteosomal degradation. Decreased Nrf2 activity represses the transcription of many antioxidant enzyme genes and alters the redox-balance towards lens oxidation. Thus, the failure of antioxidant protection due to demethylation of the CpG islands in the *Keap1* promoter is linked to the diabetic cataracts and possibly ARCs.

1. Introduction

The theory for the development of age-related cataracts (ARCs) is similar to the "free-radical theory of aging" [1]. Increases in oxidative stress during chronological aging is at the center of the pathogenesis of ARCs [2,3]. Oxidative stress is composed of three basic elements; (1) generation of reactive oxygen species (ROS), (2) scavenging of ROS, and (3) removal of oxidized macromolecules. ARCs including diabetic cataracts are the major visual impairment in the world, and more than 50 million ARC patients undergo cataract surgery every year. The cost to Medicare for the cataract surgery is one of the highest expenses in the USA (Global initiative for the elimination of avoidable blindness. Fact sheet number 213, Geneva: WHO, 2011, available from, http://www.who.int/mediacentre/factsheets/fs213/en/).

The development of ARCs is closely associated with many environmental stresses such as electrophilic reactive species, xenobiotics, drugs, inflammation, ionizing radiation, sunlight, atomic oxygen, and diabetes [4,5]. Among the ARCs, the diabetic cataracts have been extensively studied, and the ROS levels or glycoxidation can increase markedly under hyperglycemia stress [6]. These changes can also induce the unfolded protein response (UPR) [7,8]. We realized that most stresses known to cause cataracts in humans also induce the UPR [9]. The UPR is a protective mechanism that eliminates toxic ROS in most organisms. Interestingly, chronic ER stress also generates the UPR and production of ROS, and cell death. In such chronic stress antioxidant defense protective mechanisms must be failed.

One of the main antioxidant protection mechanisms is the activation of NF-E2-related factor 2 (Nrf2). Nrf2 is a transcriptional activator, and it binds to the antioxidant response element (ARE).

Abbreviations: ARCs, age-related cataracts; ARE, antioxidant response element; 5-Aza, 5-aza-2′ deoxycytidine; BGS, bisulfite genomic DNA sequencing; EthD, ethidium homodimer-III; ER, endoplasmic reticulum; FCS, fetal calf serum; HLECs, human lens epithelial cells; *Keap1*, Kelch-like ECH associated protein 1; Nrf2, NF-E2-related factor 2; PBS, phosphate buffered saline; ROS, reactive oxygen species; UPR, unfolded protein response; H_2-DCFH-AD, 2′,7′-dichlorodihydrofluorescein diacetate.

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This then leads to the transcription of approximately 200 protective genes including 20 antioxidant associated enzymic genes [10,11].

There is growing evidence that oxidative stress stimulates DNA modification including DNA methylation [12–15]. We hypothesize that the Nrf2-dependent antioxidant protection is dysfunctional in diabetic lenses under chronic UPR. We initially studied the status of DNA methylation of the Nrf2 and *Keap1* genes. We found significant levels of demethylation in the *Keap1* gene in diabetic cataractous lenses but not in the clear lenses. We shall present evidence that the lenses of diabetic subjects can become cataractous induced by a failure of antioxidative processes against oxidation in the lens.

2. Materials and methods

2.1. Human lens epithelial cell (HLECs) culture

HLECs were cultured in high glucose, Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) and antibiotics at 37 °C under 20% atmospheric oxygen. For the experiments, HLECs were pre-cultured overnight in low glucose DMEM (Invitrogen, Grand Island, NY) with 10% FCS and antibiotics under 1% of atmospheric oxygen.

2.2. Bisulfite conversion

The genomic DNA of epithelial cells from clear human lenses and from diabetic cataractous lenses (NDRI, Philadelphia, PA) was subjected to bisulfite conversion by EZ DNA Methylation-Direct™ kit (Zymo Research Corporation, Orange, CA). HLECs from the area of capsulotomy were isolated from the center of the anterior surface of clear lenses and of diabetic cataractous lenses under a dissection microscope. The human lenses were fixed in 4% formaldehyde for 1 h at room temperature and then stained with Coomassie blue for 10 min. The bisulfite converted DNA was then used for bisulfite genomic DNA sequencing (BGS).

2.3. Bisulfite genomic DNA sequencing

The bisulfite-modified DNAs were amplified by bisulfite sequencing PCR using Platinum® PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA) with primers specific to human *Keap1* promoter (Table S1). The primers were designed using the Methyl Primer Express Software v1.0 (Applied Biosystems Inc. Foster City, CA). The PCR products were purified by gel extraction using the Zymoclean™ Gel DNA recovery kit (Zymo Research Corporation, Orange, CA), then cloned into pCR®4-TOPO vectors using TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA). The recombinant plasmids were transformed into One Shot® TOP10 chemically competent *Escherichia. coli* (Invitrogen, Carlsbad, CA) using the regular chemical transformation method as described in the manufacturer's instructions. Plasmid DNA were prepared from about 10 independent clones of each amplicon with PureLink™ Quick Plasmid Miniprep kit (Invitrogen, Carlsbad, CA) and sequenced (High-Throughput DNA Sequencing and Genotyping Core Facility, University of Nebraska Medical Center, Omaha, NE) to determine the status of CpG methylation. Clones with an insert with > 99.5% bisulfite conversion, i.e., non-methylated cytosine residues to thymine were included in this study, and the remaining was excluded. Then the sequenced data of each clone was compared with the in silico reference human *Keap1* promoter bisulfite converted DNA sequence derived from methyl primer express software (version 1.0), and a schematic diagram of CpG methylation status was produced.

2.4. 5-Aza-2′-deoxycytidine treatment

HLECs were cultured in low glucose DMEM (Invitrogen, Grand Island, NY) along with 10 μmole/L 5-Aza-2′-deoxycytidine (5-Aza; Sigma–Aldrich, St. Louis, MO) for 7 days. The culture medium was changed every 2 days. At the end of the experiment, the cells were harvested and used for cell death assay, intracellular ROS production, bisulfite genomic DNA sequencing, real-time RT-PCR and western blotting.

2.5. Cell death staining

HLECs treated with/without 5-Aza were stained with ethidium homodimer-III (EthD) as included in the Cytotoxicity Assay kit (Biotium Inc., Hayward, CA) for 30 min as described in the manufacturer's protocol. Then the cells were washed twice with phosphate buffered saline (PBS) and examined under a fluorescent microscope (Nikon, Eclipse TE2000-U) with a red filter (for dead cells).

2.6. Intracellular ROS staining

HLECs treated with/without 5-Aza were stained by adding 1 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCFH-AD) (Invitrogen, Carlsbad, CA) in PBS for 30 min at 20 ° C. Then the cells were washed twice with PBS, and examined under a fluorescent microscope with a green filter (Nikon, Eclipse TE2000-U).

2.7. Real-time PCR

Total RNA was extracted from the HLECs treated with/without 5-Aza with Quick-RNA™ MicroPrep solution (Zymo Research Corporation, Orange, CA) following the manufacturer's instructions. Then the purified total RNA was reverse transcribed by iScript™ Reverse Transcription Supermix for real-time PCR (Bio-Rad, Hercules, CA) following the manufacture's protocol. The reverse transcribed RNA was analyzed by real-time PCR using the SsoFast™ EvaGreen® supermix (Bio-Rad, Hercules, CA). The primer sequences were designed using the OligoPerfect™ Designer software with the instructions of Invitrogen for optimal primer design and were synthesized commercially. The primer sequences for *Keap1* and *β-actin* are given in Table S2. Each reaction was carried out in triplicate and three independent experiments were run. A standard curve was prepared using a serial dilution of a reference sample and was included in each real-time run to correct for possible variations in product amplification. The relative copy numbers were obtained from the standard curve and were normalized to the values obtained for *β-actin*, the internal control. The fold change in expression was then obtained by the $2^{-\Delta\Delta CT}$ method.

2.8. Protein blot analysis

At the conclusion of the experiment, HLECs were harvested and lysed with 200 μl of RIPA buffer (Cell Signaling Technology, Inc. Beverly, MA). The lysates were centrifuged (12,000 × *g*; 10 min, 4 °C), and the protein content of the supernatant was determined by the Bradford method [16]. The soluble proteins, 10–20 μg, were loaded and separated by 10% SDS–PAGE and blotted onto a polyvinylidene fluoride membrane. Then, the membranes were blocked with 5% nonfat dry milk (in 1× TTBS buffer) for 1 h at room temperature before an overnight incubation with primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C. After rinsing the membranes, they were incubated with secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature and the immunostained bands was made more visible by enhanced chemiluminescence (Pierce, Thermo, Rockford, IL). The

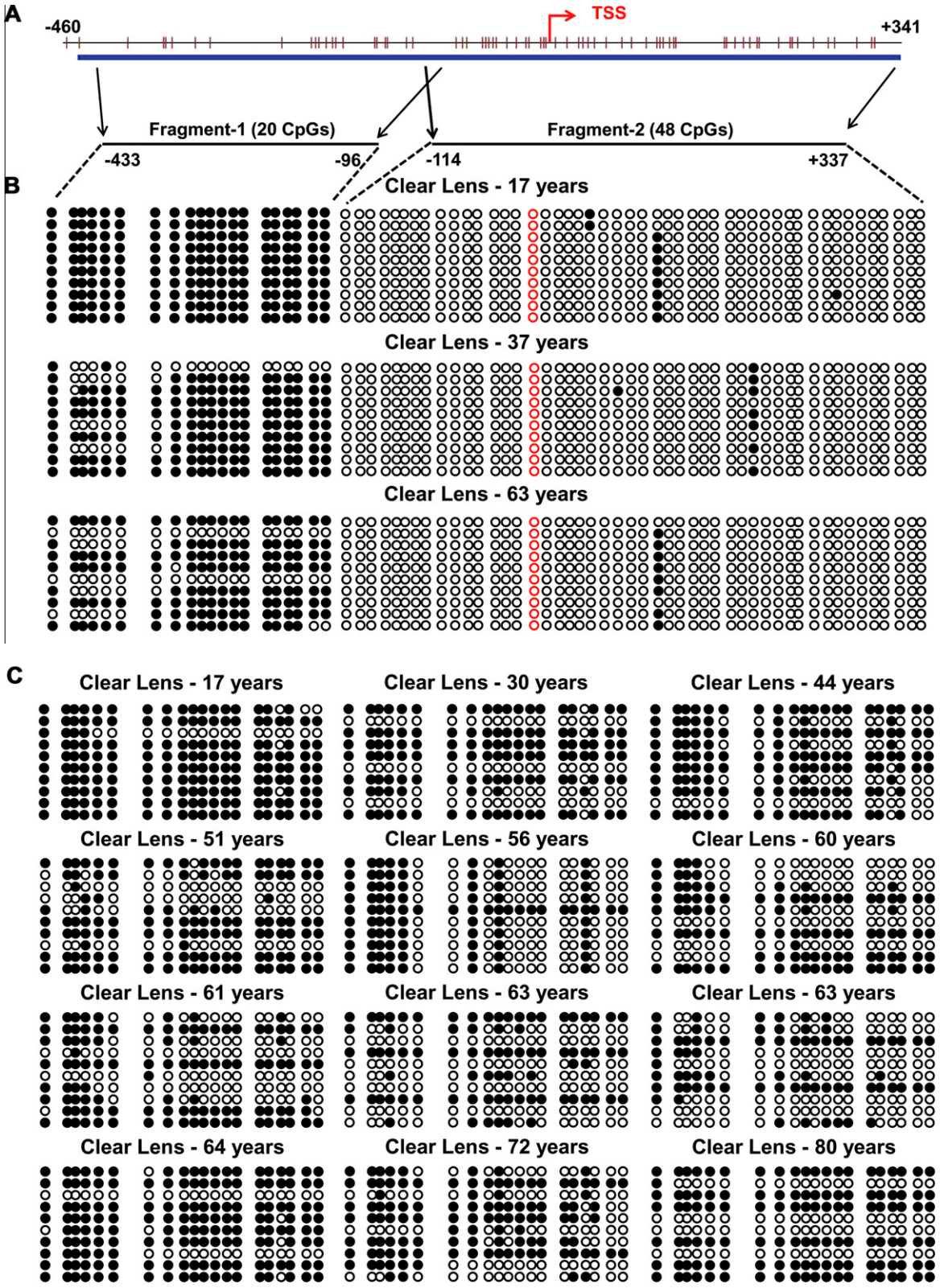

Fig. 1. CpG islands and DNA methylation in the Keap1 promoter of the fragments-1 and -2 in the clear and diabetic cataractous lenses. (A) The CpG islands were found in the 5′-flanking (between -460 and 0), the 1st exon regions (between 0 and +341) of human Keap1 gene. Here '0' refers the transcriptional start site (TSS). The blue underline indicates the CpG islands. (B) Ten individual clones of the bisulfite converted DNA sequences of LECs from a single anterior center of the clear lenses were analyzed in the two DNA fragments; fragment-1 (between −433 and −96) and fragment-2 (between −114 and +337). (C) Representative of DNA sequences in fragment-1 of the 12 individual clear lenses from ages from 17 to 80 years. The clear lenses of all ages have highly methylated DNA. Each row of circles represents a DNA sequence of single plasmid clone. Open circle represent cytosines, black circle represent methylcytosines. Red circle represents the nearest CpG dinucleotides to the transcriptional start site.

intensity of each band was normalized to that of *β-actin* and quantified using the ImageJ analysis software [17].

3. Results and discussion

3.1. In silico methylation analysis of the human Keap1 gene

The genomic DNA sequence of the human *Keap1* gene (NM_203500.1) was downloaded from the NCBI genome database. The CpG islands of the entire *Keap1* gene was predicted by using the online CpG island searcher (http://cpgislands.usc.edu) with the setting of the confidence intervals; minimum CG content –50%, ratio between observed and expected CpG – 0.6, minimum CpG island length – 200 bp, and gap between adjacent CpG island – 100 bp. We found a CpG island predominantly located in 5′-flanking region and the 1st exon of the *Keap1* gene (between –460 and +341) with a GC content of 66.3%, observed and expected CpG ratio of 0.60, and a total of 68 CpGs (Fig. 1A).

3.2. Analysis of methylated CpGs in human Keap1 promoter by bisulfite genomic DNA sequencing

The bisulfate-converted DNA was amplified by bisulfite sequencing PCR with specific primers as shown in Table S1. The amplicons between –433 bp and –96 bp (fragment-1) and –114 bp and +337 bp (fragment-2) (Fig. 1A) were sub-cloned into pCR®4-TOPO vectors and chemically transformed into One Shot® TOP10 chemically competent *E. coli*. Then, we isolated the plasmid DNA from at least 10 transformed bacterial colonies to determine the CpG methylation by DNA sequencing. We found that fragment-1 consisted of 20 CpG dinucleotides and the fragment-2 consisted of 43 CpG dinucleotides. The number of methylated CpGs in fragment-1 was significantly higher than that in fragment-2 in the 30 individual clones from the clear lens (Fig. 1B) and 30 individual clones from the diabetic cataractous lenses (Fig. 2). The difference in the degree of demethylation of *Keap1* promoter of fragment-1 in the clear and diabetic cataractous lenses was significant. These results clearly indicated that cytosine in the CpG dinucleotides of the *Keap1* promoter was highly methylated in the clear lenses obtained from individuals ages 17, 37, and 63 years. On the other hand, the CpG was significantly demethylated in the diabetic cataractous lenses obtained from individuals ages 84, 59, and 79 years. However, fragment-2 was not significantly methylated in both clear and diabetic cataractous lenses (Fig. 1B and Fig. 2).

Next, we sequenced the bisulfite genomic DNA of fragment-1 in another 12 individual clear lenses (Fig. 1C) and 18 individual diabetic cataractous lenses (Fig. 3). All of the DNA sequences from diabetic cataractous lenses showed a significant loss of methylated cytosine in the CpG dinucleotides of the *Keap1* promoter than clear lenses. The methylated cytosines in fragment-1 of clear lenses were 64% whereas the methylated cytosine in fragment-1 of diabetic cataractous lenses (three samples from Fig. 2 and 18 samples from Fig. 3) was only 12%. Thus, diabetic cataractous lenses had significantly reduced levels of methylated cytosine in the CpG dinucleotides of the *Keap1* promoter.

Chronological aging might have an effect on the *Keap1* promoter CpG dinucleotide demethylation in the clear lenses, because the 17 year-old clear lenses had more methylated cytosines than that of the 80 year-old clear lens. In the diabetic cataractous lenses, however, there was no significant increase in the methylation of the CpG dinucleotide of the *Keap1* promoter from ages 48 years to 86 years. Because cataracts are slowly developed for several years, we thought that DNA demethylation might be gradually changed parallel to the progression of lens opacity. Contrary, all

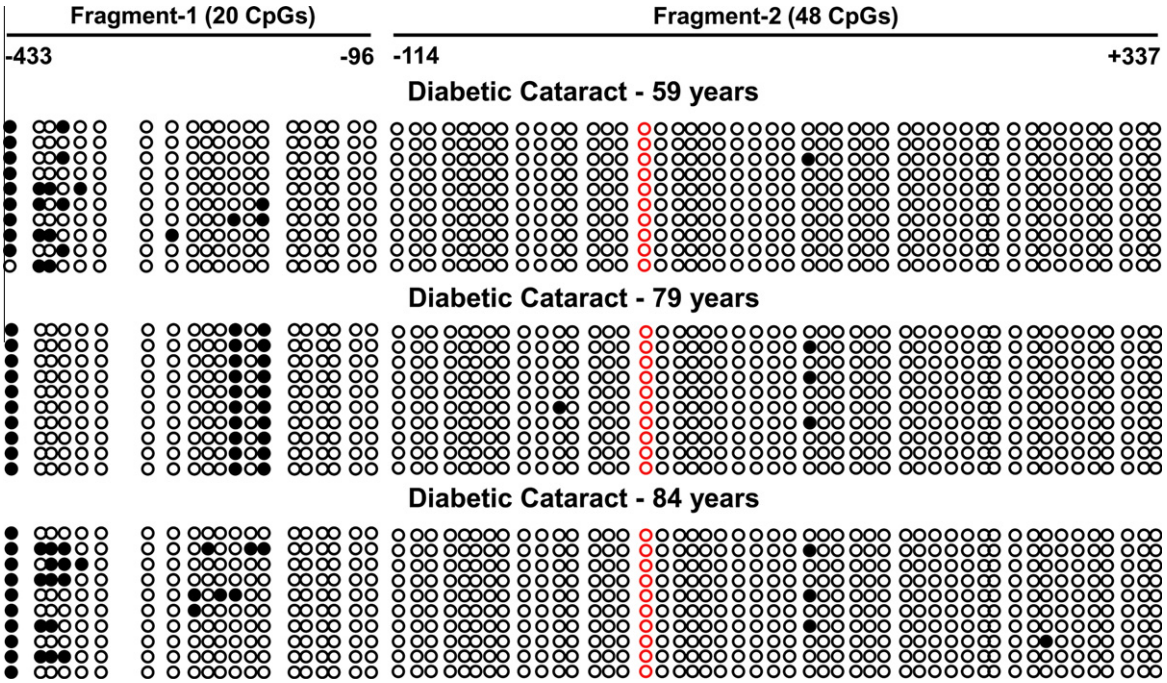

Fig. 2. DNA methylation status in the CpG islands of the fragment-1 and -2 in diabetic cataractous lenses. Ten individual clones of the bisulfite converted DNA sequences of LECs from a single anterior center of the diabetic cataractous lenses were analyzed in the two DNA fragments; fragment-1 (between -433 and -96) and fragment-2 (between -114 and +337). The diabetic cataractous lenses from age 59-, 79-, and 84 year-old diabetic donors have significantly reduced levels of DNA methylation in both fragments-1 and -2. Each row of circles represents a DNA sequence of single plasmid clone. Open circle represent cytosines, black circle represent methylcytosines. Red circle represents the nearest CpG dinucleotides to the transcriptional start site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

diabetic cataractous lenses had relatively high levels of demethylated *Keap1* promoter regardless of chronological age. These results support the idea that diabetic stress might play a greater role than chronological aging.

It is well-known that DNA methylation in the promoter region hinders the binding of a variety of regulatory proteins, and the variations in the methylation frequency might provide some insight on the regulation of gene expression by the binding of transcriptional factors on the promoter region of a gene. We used Transcription Element Search System (TESS) (http://www.cbil.upenn.edu/cgi-bin/tess/tess), an online computer program, to predict the crucial transcriptional binding elements in the 5′-flanking promoter region of *Keap1*. We found one methylated CpG for Sp1 and one methylated CpG for AP2 binding sites in the clear lens (data not shown). The AP2 transcription factors recognize sequences that contain CpG residues, and their binding to each has been shown to be inhibited by methylation. In contrast, another transcription factor, Sp1, was not sensitive to a methylation of their binding sites [18]. However, the Sp1 transcription factor is a universal guardian for unmethylated CpGs, and it does this by impeding DNA methyltransferases activity to newly synthesized DNA strands.

AP2 is expressed in selected tissues and is involved in the expression of epidermis-specific genes [19] including the major intrinsic proteins [20]. The expression of the *Gelatinase A* gene is mediated by a unique interaction of two developmentally regulated transcription factors, AP2 and YB-1, within a discrete 40-base pair enhancer element (RE-1) located in the 5′-flanking region [21]. Thus, a demethylation in the AP2 element might enhance the transcription of the *Keap1* gene.

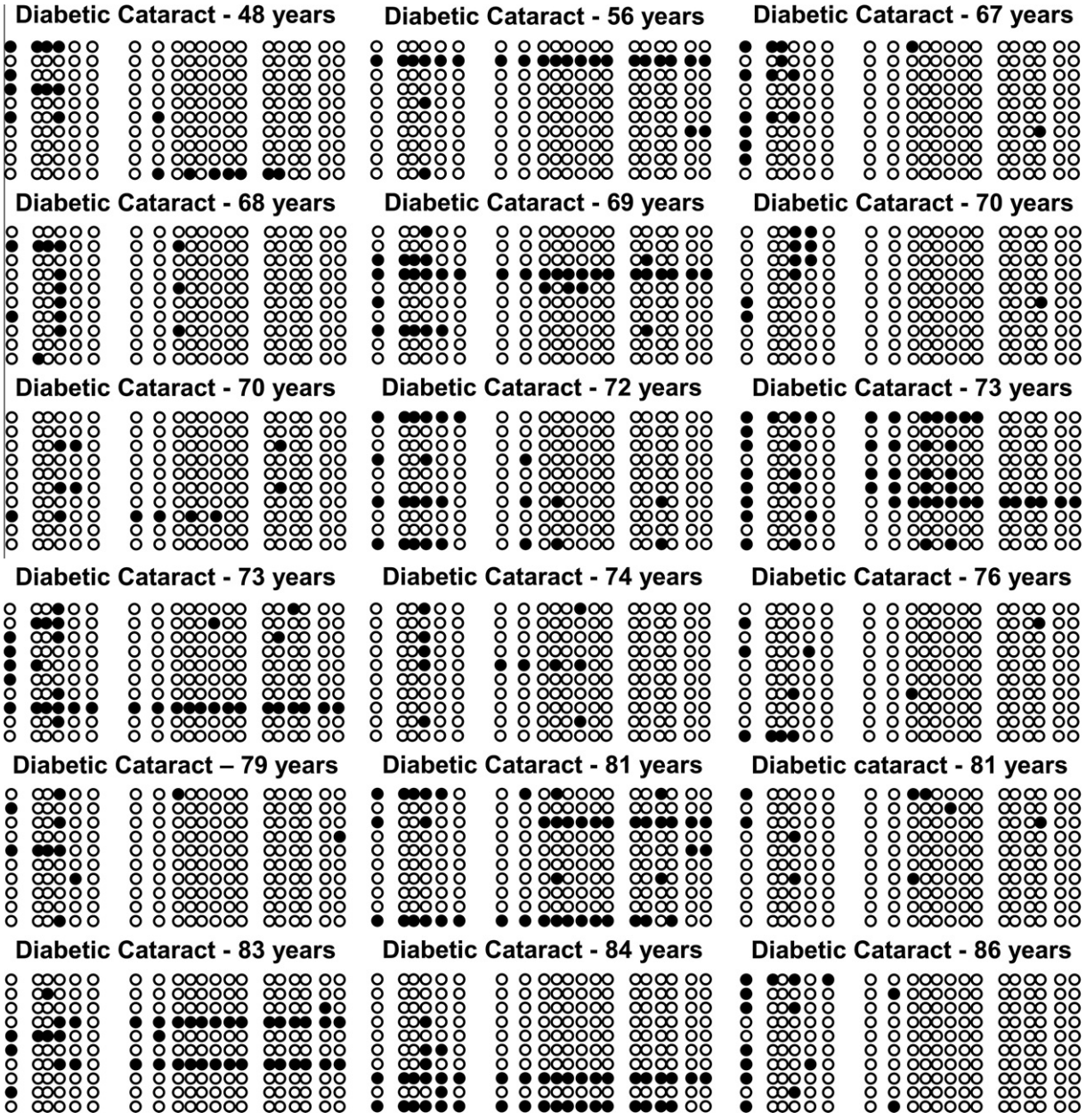

Fig. 3. Representative of DNA sequences in the CpG islands of the fragment-1 of 18 individual diabetic cataractous lenses. Each row of circles represents a single plasmid clone. Open circle represent cytosines and black circle represent methylcytosines. The DNA sequence of the same age represents the DNA isolated from different individual with same age.

3.3. Assay of ROS production and cell death in HLECs treated with 5-Aza

About two-thirds of HLECs treated with 5-Aza for 7 days died, and many of the surviving cells appeared to be multi-nucleated. Thus, this exposure was highly toxic for HLECs. In addition, the amount of protein in the 5-Aza treated cells was 50% of that in the control HLECs. Approximately 10–15% of various shaped HLECs treated with H_2-DCFH-AD had a higher fluorescence which suggested that these cells produced much higher levels of ROS. HLECs treated with 5-Aza induced much higher levels of ROS induced cell death than that of control (Fig. S1).

3.4. Analysis of Keap1 promoter methylation status in HLECs treated with 5-Aza

Numerous studies have reported that the degree of methylation of CpG dinucleotides is inversely correlated with the gene expression in the mammalian genome [12,15,22]. To investigate whether demethylated CpGs in the promoter DNA activates and methylated CpGs in the promoter DNA suppresses gene transcription in HLECs, we analyzed the degree of methylation of HLECs. We found almost all of the cells contained methyl cytosine in the CpG dinucleotides of the *Keap1* promoter fragment-1 (Fig. 4A). In contrast, HLECs treated with 5-Aza, an irreversible inhibitor of DNA methyltransferase, for 7 days, had 144 demethylated cytosines among the 200 methylated cytosines (72% decrease) in the CpG dinucleotides of fragment-1.

3.5. Analysis of Keap1 mRNA levels in HLECs treated with 5-Aza

Next, we quantified the level of the mRNA of *Keap1* in the HLECs treated with 5-Aza by real-time PCR. The results showed that the mRNA level of *Keap1* was increased by 10-fold more than that of control HLECs (Fig. 4B). These results suggested that methylated CpGs suppresses and demethylated CpGs up-regulates the *Keap1* mRNA levels in HLECs. The half-life of the mRNAs of the house keeping genes is generally short and they do not survive for a long time [23]. We suggest that the 10-fold increase in the mRNA of *Keap1* might be due to an activation of the transcription due to CpG demethylation of the *Keap1* promoter and not inhibition of *Keap1* mRNA degradation in the HLECs treated with 5-Aza.

The DNA methylation status of the *Keap1* gene in the 5-Aza treated HLECs lost only 72% of the methylated cytosine even though the mRNA increased by 10-fold. The fact that the diabetic cataractous lenses also lost 64% of methylated cytosine in the CpG island of fragment-1 suggests that the levels of *Keap1* mRNA must be significantly higher in the HLECs of diabetic cataractous lenses than that of clear lenses.

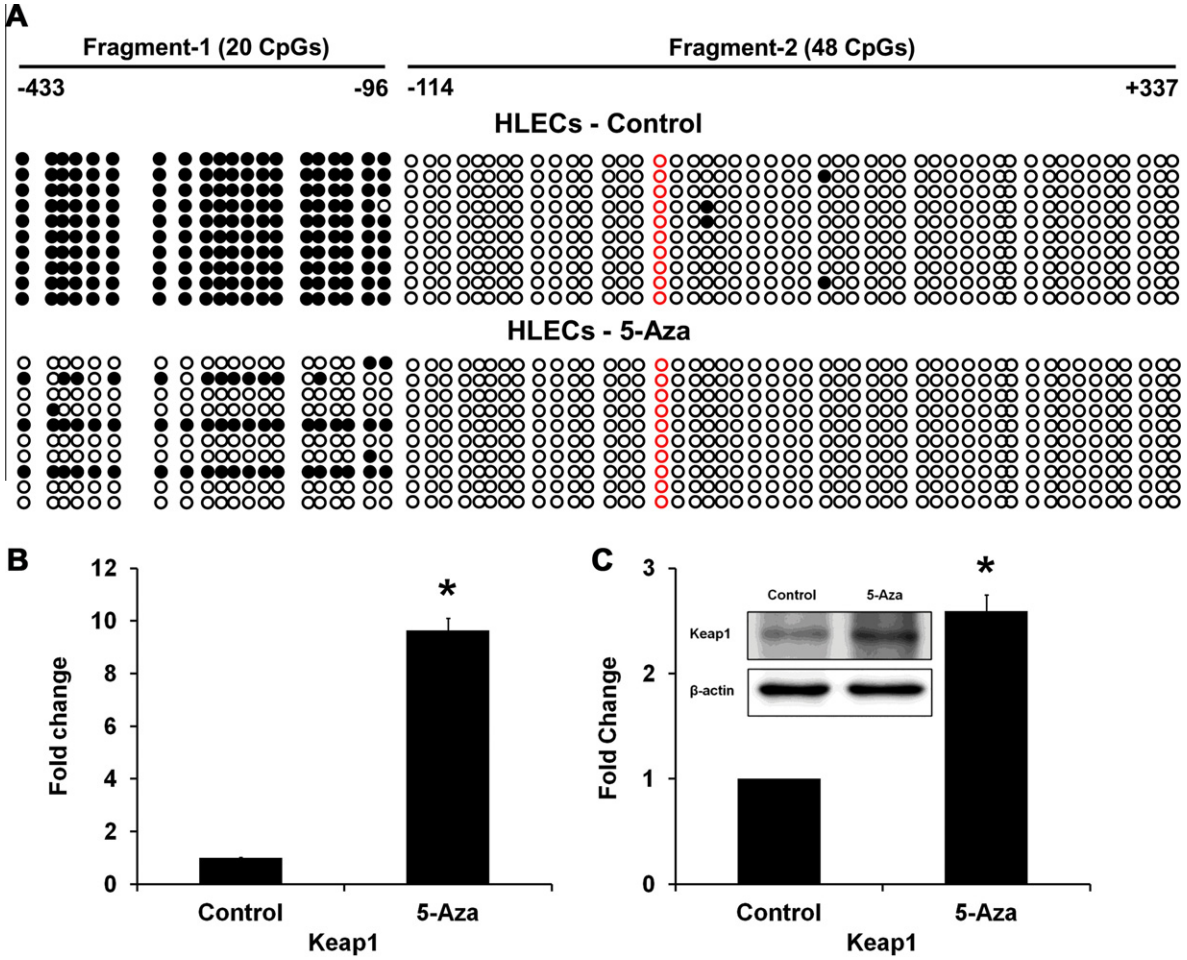

Fig. 4. DNA methylation analysis in the CpG islands of the *Keap1* promoter and the levels of *Keap1* mRNA and *Keap1* protein in HLECs treated with/without 5-Aza. (A) Each row of circles represents a single plasmid clone in fragments-1 and -2. Open circles represent cytosines and black circles represent methylcytosines. Red circle represent the nearest CpG dinucleotides to the transcriptional start site. (B) *Keap1* mRNA is increased by 10-fold in the HLECs treated with 5-Aza than that of untreated HLECs. (C) Protein blot analysis of *Keap1* showing a three-fold increase in the HLECs treated with 5-Aza than that of untreated HLECs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Analysis of Keap1 protein levels in HLECs treated with 5-Aza

The results of protein blot analysis indicated that the levels of *Keap1* is increased by approximately 3-fold in the HLECs treated with 5-Aza for 7 days (Fig. 4C). Because the mRNA of *Keap1* was increased by 10-fold, the level of *Keap1* protein might be much higher in these HLECs. However, it is well known that the levels of the mRNA are not always correlated with levels of protein [24,25]. A recent report showed that exposure to 5-Aza induced proteosomal degradation of chromatin-associated DNA methyltransferase [26]. We suggest that the proteosomal degradation reduces the levels of *Keap1* protein in HLECs treated with 5-Aza. Protein blot analysis of HLECs treated with 10 μM 5-Aza for 1–10 h showed that the level of *Keap1* was increased significantly at 3 h, but then it was significantly decreased at 5 h, and decreased further to undetectable levels by 10 h (data not shown). These results strongly suggest that 5-Aza activates the degradation of *Keap1* protein prior to the demethylation of *Keap1* promoter CpG island in the HLECs treated with 5-Aza. At present, we do not know whether the *Keap1* mRNA has aberrant splicing or posttranscriptional mechanisms [14] or loss of the translational factors or proteosomal degradation [26]. It is interesting that 5-Aza activates the proteolysis of *Keap1* in HLECs in such a short time.

Finally, the *Keap1* promoter is methylated in cancerous cells but it was demethylated in diabetic cataractous lenses. CpG islands in the promoter region of house-keeping genes are usually unmethylated in all normal tissues, regardless of their transcriptional activity [27]. The human lens is only organ in which cancer is never found. Although, the *Keap1* gene was highly methylated in the clear lens, it was highly demethylated in the diabetic cataractous lenses. The HLECs is considered to be tumor cell line because it was generated by the transfection of a plasmid vector DNA containing a large T antigen of SV40 [28]. Agreeably, the *Keap1* gene in the HLECs was highly methylated (Fig. 4), which is similar to DNA methylation in the lung cancer cell lines, lung cancer cells [12,15], or prostate cancer cells [14]. Because *Keap1* is methylated and its expression is suppressed, Nrf2 is constitutively stabilized in the cancer cells. This is one of hypothesis for the stabilization of Nrf2 by promoter DNA methylation in the *Keap1* gene [12,15]. However, the findings in control lung cells suggest that the methylation might not be a cancer specific event [12] indicating that further studies are required. We are interested in how methylation is induced in tumor cells and demethylation in the diabetic cataractous lenses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.164.

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