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Altered Expression and DNA Methylation Profiles of *ERCC6* Gene in Lens Tissue from Age-Related Cortical Cataract

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Abstract

Ultraviolet (UV)-induced DNA damage attributes to the pathogenesis of age-related cataract (ARC) and is repaired via the nucleotide excision repair (NER). It is known that Cockayne syndrome complementation group B (CSB) protein coded by *ERCC6* is a component of NER complex. DNA methylation is one of the major epigenetic events and is catalyzed by DNA 5-cytosine-methyltransferases (DNMTs). This study was to examine the potential contribution of DNA methylation of CpG islands in *ERCC6* promoter region in lens tissues to ARC pathogenesis. Fifteen cortical type of ARC lenses and fifteen transparent lenses from human subjects were included in this study. *ERCC6* and DNMTs expression in the lenses were analyzed by qRT-PCR and western blot. Bisulfite-sequencing PCR (BSP) was performed to evaluate methylation status of *ERCC6*. An in-vitro experiment by adding a demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC) in Human lens epithelium B-3 (HLE B-3) was conducted to confirm the role of DNA methylation in *ERCC6* expression. The results show that the mRNA and protein levels of *ERCC6* were significantly reduced in the LECs and lens cortex of ARCs. DNMT3b mRNA was significantly higher in LECs of ARCs than that of the controls. In ARC group, the CpG island in the promoter region of *ERCC6* displayed hypermethylation in LECs compared to that of the controls. After treatment with 5-aza-dC, the *ERCC6* protein level increased in HLE B-3. We concluded that the overexpression of DNMT3b in lens is associated to the hypermethylation of the CpG island of *ERCC6*, which linked to the reduced *ERCC6* expression in LECs from ARC patients. This epigenetic change in *ERCC6* gene might be a factor of ARC formation that is mediated with impaired DNA repair.

Keywords: *ERCC6*; Age-related cortical cataract; Lens epithelial cells (LECs); Lens cortex; DNA methylation; DNA methyltransferase

Introduction

Age-related cataract (ARC) is one of the leading causes of visual impairment and blindness among the elderly worldwide and is an increasing vision burden in China [1,2]. Clinically, there are four common subtypes of ARC (cortical, nuclear, posterior subcapsular and mixed type) according to the location of the opacity within the lens, with cortical cataract ranking as the second common subtype. Molecular mechanism of ARC pathogenesis remains unknown [3]. Previous studies suggested ultraviolet (UV) light exposure to be a factor affecting cortical cataract development [4,5]. Exposure of the lens to UV induces DNA damage through thymine dimer formation and oxidative stress [6,7]. In cortical cataracts, the DNA damage was more abundant when compared to that of nuclear or posterior subcapsular cataracts [8,9]. There are several pathways that repair various types of DNA damage, including nucleotide excision repair (NER), double-strand break (DSB) recombinational repair, base excision repair (BER) and mismatch repair (MMR) [10]. NER is an excision mechanism that removes UV-induce DNA damage [11,12].

NER for DNA lesion removal is realized by a series of enzymatic activities. In NER process, Cockayne syndrome complementation group B (CSB) protein (coded by *ERCC6*) recruits NER repair factors to the DNA damage site. Mutations in the *ERCC6* gene cause Cockayne syndrome that often presents severe cataract [13]. It was reported that *ERCC6* polymorphism is a risk factor for age-related macular degeneration (AMD) [14,15]. Both ARC and AMD, as common age-related eye diseases, may be affected by long-term UV radiation and oxidative damage [16]. However, we found no association between selected *ERCC6* polymorphisms (rs4838519, rs4253038) and ARC [17].

Epigenetic regulation of gene expression commonly occurs at two main levels: DNA methylation and histone modification. The methylation of DNA plays a vital role in silencing gene expression through methylation of cytosines in CpG islands. CpG islands are clusters of CpG dinucleotides in CG-rich regions and often located in the promoters or first exons of gene [18]. DNA methylation is catalyzed and maintained by DNA methyltransferases DNMTs [19]. Two main types of methyltransferase activity have been found in mammals: a de novo activity and a maintenance activity. DNMT3a and DNMT3b have been identified as de novo methyltransferases. They can methylate cytosine at CpG dinucleotides on both strands. DNMT1, the principal DNA methyltransferase in mammalian cells, acts to restore methylated cytosines at CpGs on the newly duplicated strands [20,21]. Previous research had indicated the role of DNMT1 in the maintains of aberrant CpG island methylation [22].

The present study was to test the hypothesis that CpG islands methylation of *ERCC6* is associated with the expression of the gene in

lens tissues from ARC patients and that CpG islands hypermethylation of the gene is mediated with DNMTs. The result may provide new insight for the pathogenesis of ARC.

Materials and Methods

Study participants

All procedures in this human subject study conformed to the Declaration of Helsinki. The written informed consent was acquired from all participants. The study was approved by the Ethics Committee of Affiliated Hospital of Nantong University. Fifteen ARC patients (cortex opacification of lenses=C4) were selected according to the Lens Opacities Classification System III (LOCS III) [23]. The control transparent lens was obtained from fifteen age-matched subjects who had lens extraction during epiretinal membrane removal. The study excluded the patients with complicated cataract due to high myopia, uveitis, ocular trauma or other known causes; other major eye diseases such as glaucoma, diabetic retinopathy and uveitis; and systematic diseases such as hypertension, autoimmune diseases and diabetes. Each group included 8 males and 7 females respectively.

		ARC				Control	
sample	sex	age, y	LOCSⅢ	sample	sex	age, y	LOCSⅢ
1	male	60	NO1C4P0	1	female	63	NO1C0P0
2	female	62	NO2C4P0	2	female	61	NO1C0P0
3	female	65	NO1C4P0	3	male	60	NO1C0P0
4	female	67	NO2C4P1	4	male	66	NO1C0P0
5	female	64	NO1C4P0	5	male	65	NO1C0P0
6	male	65	NO2C4P0	6	female	64	NO1C0P0
7	male	67	NO2C4P1	7	female	63	NO1C0P0
8	male	63	NO1C4P0	8	female	65	NO1C0P0
9	male	65	NO2C4P0	9	male	66	NO1C0P0
10	female	63	NO1C4P0	10	male	67	NO2C0P0
11	female	62	NO1C4P0	11	male	64	NO1C0P0
12	female	62	NO1C4P0	12	male	65	NO2C0P0
13	male	62	NO1C4P0	13	male	64	NO1C0P0
14	male	66	NO2C4P0	14	female	64	NO1C0P0
15	male	61	NO1C4P0	15	female	63	NO1C0P0

Table 1: Demographic Data of the ARC Patients and Normal Controls.

The mean age for ARC patients is 63.6 years (SD=2.16 yeas) and for controls is 64 years (SD=1.85 yeas). There were no statistically significant differences between the two groups regarding the age ($p>0.05$). The basic demographic and clinical features of the study participant are listed in the Table 1.

LECs and lens cortex preparation

The LECs were collected by anterior continuous curvilinear capsulorhexis. Lens cortex was collected from transparent cortex of controls and opaque cortex of ARC patients. The sample was rapidly frozen in liquid nitrogen, and then stored at −80°C for later extraction of genomic DNA, mRNA and protein.

Cell culture and treatment

To test the relationship between the methylation and the expression of the gene of interest, an *in vitro* study of demethylation was performed. HLE B-3 were obtained from American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in Eagle's minimum essential medium (Invitrogen-GIBCO, Carlsbad, CA) with 10% fetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere. After reaching 80-90% confluency, the cells were demethylated by incubation in medium containing 3 μM of 5-aza-dC (Sigma, St. Louis, MO) for 24 hours.

RNA isolation and cDNA preparation

Total RNA of LECs and lens cortex were isolated from the frozen tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNAs were synthesized using PrimeScript® RT reagent Kit (Takara, Dalian, China).

Quantification of mRNA expression

TaqMan gene expression assay probes (Applied Biosystems, USA) were used for *ERCC6*, DNMT1, DNMT3a and DNMT3b mRNA quantification (assay ID: Hs00972920_ml, Hs00945875_ml, Hs01027166_ml, Hs00171876_ml). GAPDH (Hs99999905_m1) was used as an internal control. RT-PCR was performed using ABI 7500 real time PCR system (Applied Biosystems, USA). The fold change of genes expression was determined using the comparative CT (2^{-}CT) method.

Western blot assay

The protein of LECs, lens cortex and HLE B-3 were extracted separately in lysis buffer (1 M Tris-HCl at pH 7.5, 1% Triton X-100, 1% Nonidet p-40, 10% SDS, 0.5% sodium deoxycholate, 0.5 M EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). Equal amounts of proteins were size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 15% polyacrylamide gels. Proteins were then transferred onto polyvinylidene difluoride filter membranes (Millipore, Bedford, MA, USA). Nonspecific protein binding to the membrane was blocked with blocking buffer (5% nonfat milk, 200 mM NaCl, 50 mM Tris, 0.05% Tween 20). The blocked membrane was then

incubated with mouse anti-human-ERCC6 (Sigma) and mouse anti-human-GAPDH (1:1000; Abcam Ltd., Cambridge, UK) at 4°C for 12 hours. After the membrane was washed three times with TBS-T (20 mM Tris, 500 mM NaCl, 0.1% Tween 20) for 5 min each time, the membrane was incubated with an alkaline phosphatase-conjugated goat anti-mouse IgG antibody (1:2000; Santa Cruz) for 2 hours. An enhanced chemiluminescence detection system was used to read the Western signals (Pierce Company, USA).

Measurement of DNA methylation

Bioinformatic analysis: Transcription start site (TSS) of *ERCC6* was predicted by the online software (http://dbtss.hgc.jp/). The CpG island of the gene was predicted by the online software (http://www.urogene.org/methprimer/). The parameters used are a CG content of greater than 50%, an observed/expected ratio of greater than >0.6, and a length of CpG island exceeding 200 bp. Genomic DNA from the frozen tissues and HLE B-3 was isolated by phenol/chloroform and ethanol extraction. DNA samples were treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen, Gaithersburg,MD). EpiTect Control DNA (Qiagen, Gaithersburg, MD) was used as the positive and negative controls in all experiments.

Bisulfite-sequencing PCR cloning and sequencing: The BSP primer was designed by web-based MethPrimer software (http://www.urogene-.org /methprimer/) to cover a CpG island in promoter of the *ERCC6*. The primers for *ERCC6* were: forward 5′-TGTTTTGAATTTTTGTGTGGATATTT-3′ and reverse 5′-ACTATCCTACTTCTCTATTCCCCCTC-3′. The PCR products (208bp) were gel extracted and cloned into the pMD-20-T vector (Takara). Plasmid-transformed bacteria DH5α was grown for 14 hours and the plasmid DNA was isolated. At least 10 clones were chosen for sequence analysis. The status of CpG methylation was determined by the online tool QUMA (http://quma.cdb.riken.jp/). All sequences with a conversion rate lower than 90% were excluded by the online tool in present study. Unmethylated cytosines (Cs) change to thymines (Ts), while methylated cytosines remained as Cs. The degree of methylation was presented as mC/CpG.

Statistical analysis

Student's t-test was used to determine the difference of means between the two groups. P value <0.05 was considered for statistical significance. Statistical analyses were performed with SPSS software (SPSS 17.0; SPSS, Inc., USA).

Results

mRNA and protein expression of *ERCC6 in* the Lens epithelial cells (LECs) and cortex of lens

mRNA expression of *ERCC6* was 2.13 folds lower in LECs and 1.51 folds lower in lens cortex of ARC than that of the control group (Figure 1A). Lower protein levels of *ERCC6* were also detected in LECs and lens cortex of ARC than the control group (Figure 1B and 1C).

Methylation status of ERCC6 in the LECs and cortex of lens

Bioinformatic analysis detected a CpG island located in the promoter of *ERCC6* (Figure 2A). Figure 2B showed the representative bisulfite genomic sequencing of the target fragment (-603 to -395, relative to the TSS) of the *ERCC6* CpG islands from 10 clones in 2 representative samples. By analyzing all CpG sits of *ERCC6* from primers described, it was found that the methylation rate of *ERCC6 in* LECs of ARC group was 73.93 ± 4.95% and 23.22 ± 4.22% in the control group (Figure 2C, *P*<0.01). The methylation rate of *ERCC6* didn't differ in lens cortex between the ARC group and the control group (Figure 2C, *P*>0.05).

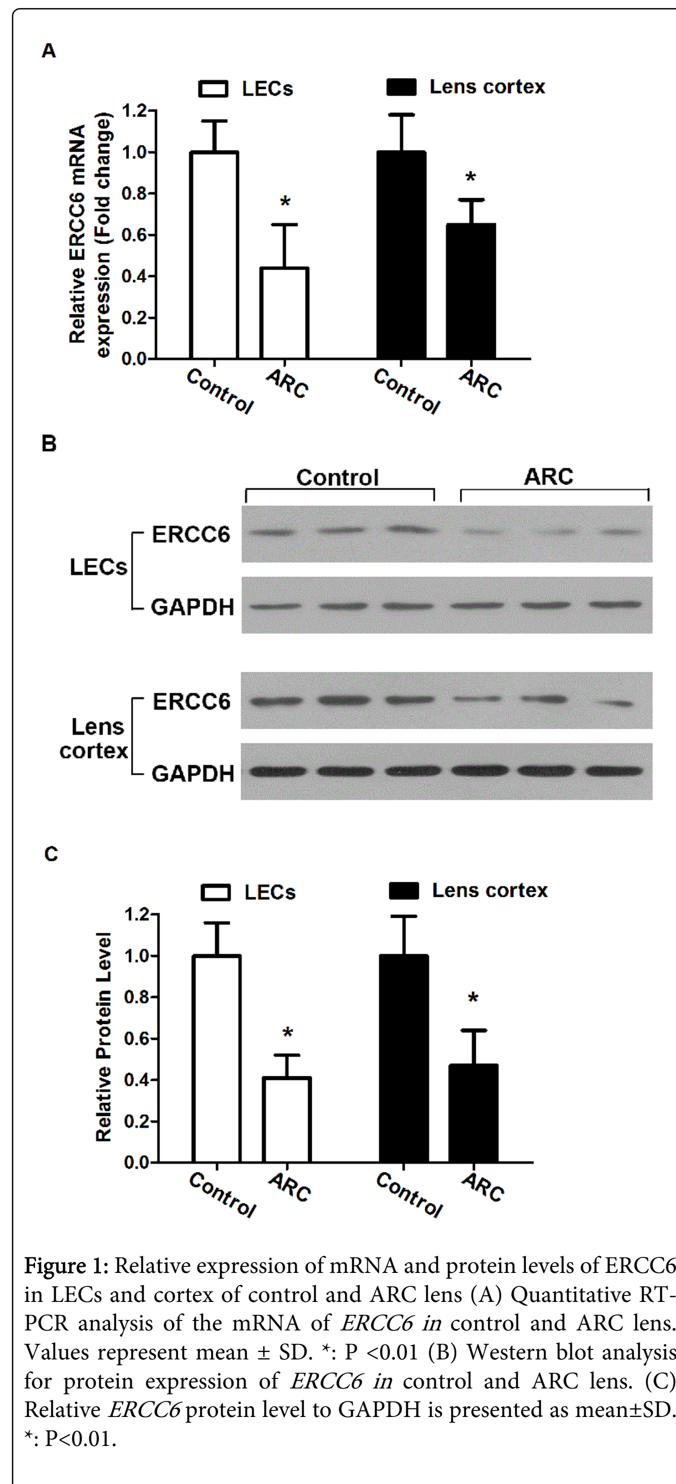

Figure 1: Relative expression of mRNA and protein levels of ERCC6 in LECs and cortex of control and ARC lens (A) Quantitative RT-PCR analysis of the mRNA of *ERCC6 in* control and ARC lens. Values represent mean ± SD. *: P <0.01 (B) Western blot analysis for protein expression of *ERCC6 in* control and ARC lens. (C) Relative *ERCC6* protein level to GAPDH is presented as mean±SD. *: P<0.01.

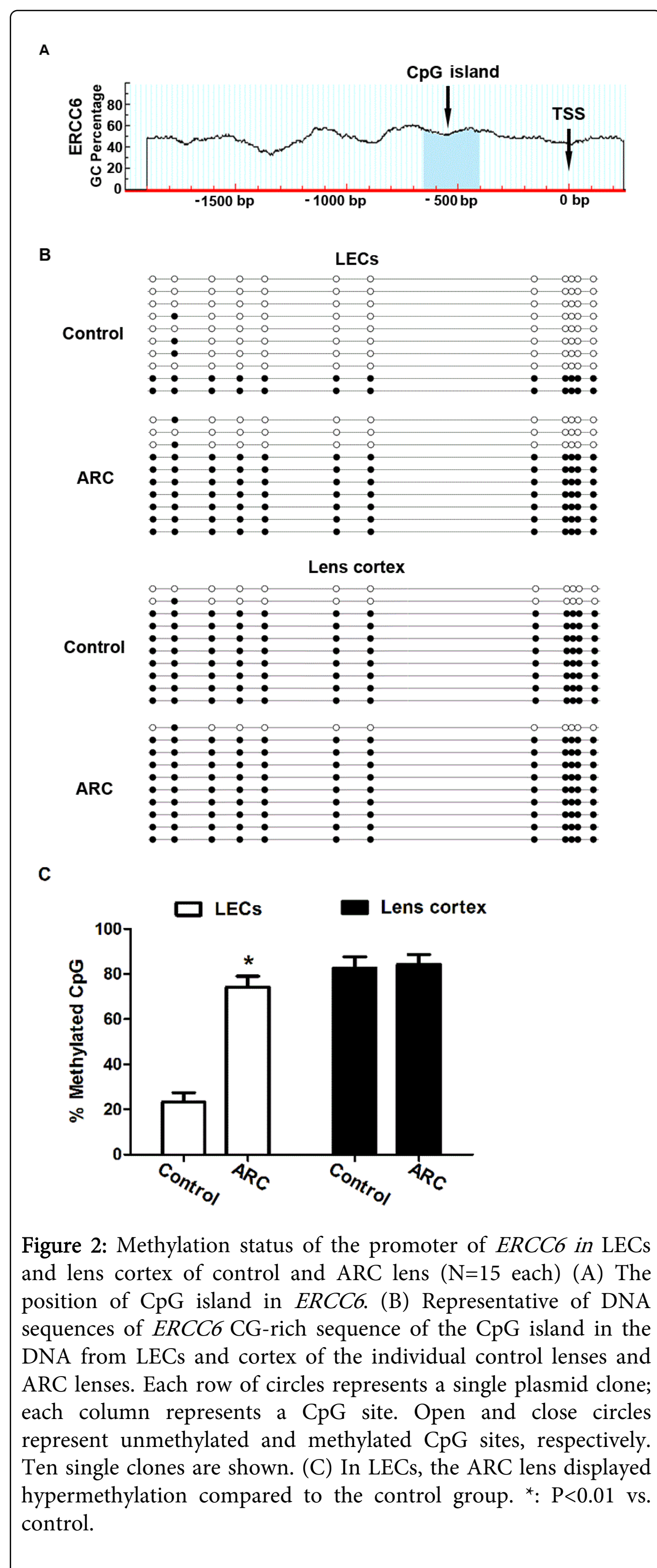

Figure 2: Methylation status of the promoter of *ERCC6 in* LECs and lens cortex of control and ARC lens (N=15 each) (A) The position of CpG island in *ERCC6*. (B) Representative of DNA sequences of *ERCC6* CG-rich sequence of the CpG island in the DNA from LECs and cortex of the individual control lenses and ARC lenses. Each row of circles represents a single plasmid clone; each column represents a CpG site. Open and close circles represent unmethylated and methylated CpG sites, respectively. Ten single clones are shown. (C) In LECs, the ARC lens displayed hypermethylation compared to the control group. *: P<0.01 vs. control.

mRNA expression of DNMTs in the LECs and cortex of lens

DNMT3b was expressed at a 2.54-fold higher in ARC LECs than that of the control (Figure 3A). However, we didn't observe differentiated expression for DNMT1 and DNMT3a in LECs between the two groups. The mRNA expression of DNMT1, DNMT3a and DNMTb didn't differ in lens cortex between the two groups (Figure 3B).

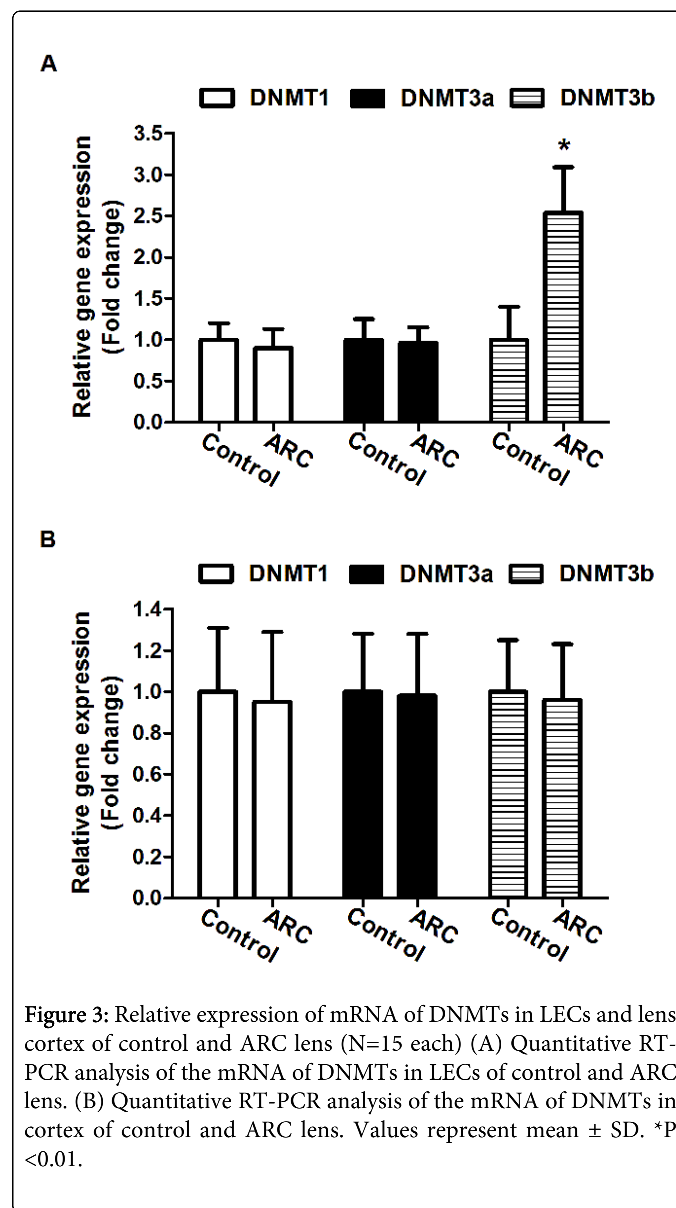

Figure 3: Relative expression of mRNA of DNMTs in LECs and lens cortex of control and ARC lens (N=15 each) (A) Quantitative RT-PCR analysis of the mRNA of DNMTs in LECs of control and ARC lens. (B) Quantitative RT-PCR analysis of the mRNA of DNMTs in cortex of control and ARC lens. Values represent mean ± SD. *P <0.01.

Protein expression and methylation status of *ERCC6 in* Human lens epithelium B-3 (HLE B-3) after treatment with 5-aza-2'-deoxycytidine (5-aza-dC)

After treatment with 3 μM 5-aza-dC for 24 h, the *ERCC6* protein level increased in HLE B-3 (Figure 4A, *P*<0.01), along with the removal of *ERCC6* loci methylation. Figure 4B showed the representative bisulfite genomic sequencing of the target fragment of the *ERCC6* CpG islands from 10 clones in HLE B-3 after treatment with 3 μM 5-aza-dC. The methylation rate of *ERCC6 in* HLE B-3 was

41.67 ± 7.65% and 5.56 ± 2.15% in the cells was demethylated by 5-aza-dC (Figure 4C, *P*<0.01).

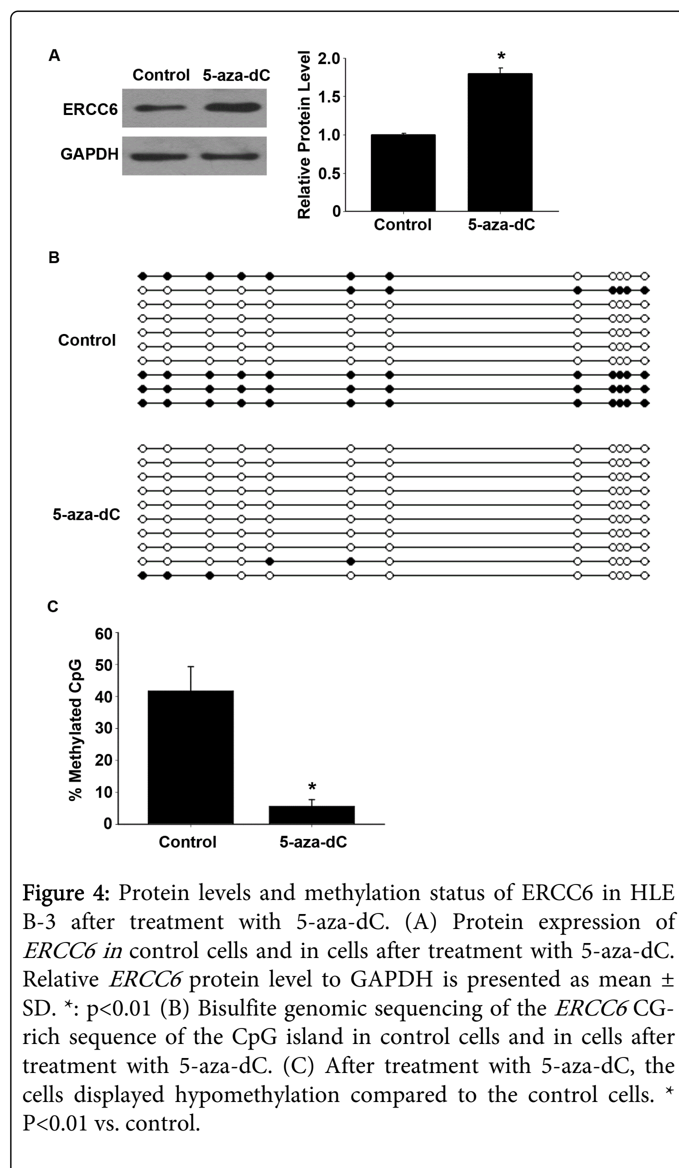

Figure 4: Protein levels and methylation status of ERCC6 in HLE B-3 after treatment with 5-aza-dC. (A) Protein expression of *ERCC6 in* control cells and in cells after treatment with 5-aza-dC. Relative *ERCC6* protein level to GAPDH is presented as mean ± SD. *: p<0.01 (B) Bisulfite genomic sequencing of the *ERCC6* CG-rich sequence of the CpG island in control cells and in cells after treatment with 5-aza-dC. (C) After treatment with 5-aza-dC, the cells displayed hypomethylation compared to the control cells. * P<0.01 vs. control.

Discussion

In the present study, we showed both the mRNA and protein expression of *ERCC6* were down-regulated in lens tissues of ARC. The mRNA of DNMT3b was significantly higher in LECs of ARC group than that of the control group. The CpG island of *ERCC6* promoter is heavily methylated in the ARC group, and treatment with 5-aza-dC had been associated with the restoration of *ERCC6* expression in HLE B-3. These findings suggest that DNA methylation associated low expression of *ERCC6* might be mediated with the formation of ARC.

The human lens consists of two metabolically different zones: the LEC and the lens cortex [24]. It is suggested that damage to the LECs may result in cataract formation [25]. Most previous studies on gene expression focused on LECs of ARC [22,26,27]. In present work, we studied the expression of *ERCC6* and DNMTs in LECs as well lens cortex from controls and ARCs.

Previous studies suggested that UV radiation exposed epidermis might be mediated with silencing of genes due to DNA methylation [28,29]. Aberrant methylation in the promoter CpG islands of DNA repair genes in NER is associated with transcriptional silencing [30,31]. In our study, low expression of *ERCC6* was detected in LECs and lens cortex of ARC group, and the CpG islands of the promoter of the gene was hypermethylated in LECs of ARC group than that of control group. Interestingly, we did not show significant differences in methylation rate of *ERCC6* between control group and ARC group in lens cortex. However, considering we did not investigate all the CpG islands in promoter of the gene. We cannot exclude differences in methylation rate could be present in CpG islands beside those analyzed portions.

The activation of DNMTs is important for methylation of the cytosine base of the CpG islands. Overexpression of these methyltransferases in tumorous region was reported possibly to result in hypermethylation of various genes [32]. In the present study, we examined the expression of DNMT1, DNMT3a and DNMT3b in lens tissues of control group and ARC group. Overexpression of DNMT3b had been correlated with DNA hypermethylation status in LECs of ARC group. A parallel analysis of methyltransferases and DNA methylation events in the same samples might not be able to conclude the sequential order of the changes in this study. However, the manipulation of methylation status by the demethylating agent in the *in vitro* model (HLE B-3) resulted in a higher expression of *ERCC6*, implying a clue of cause-effect relationship and a role of hypermethylation for the down-regulation of *ERCC6*.

In summary, we demonstrated that hypermethylation of promoter CpG island of the *ERCC6* gene links to low expression of *ERCC6 in* LECs of ARC group. Overexpression of DNMTb may be an important factor to aberrant CpG island methylation in LECs of ARC group. This finding might provide a novel avenue of study for the intervention of methylation status in ARC therapy and prevention.

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