

# Human age-related cataracts: Epigenetic suppression of the nuclear factor erythroid 2-related factor 2-mediated antioxidant system

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**Abstract.** Human aging is a multifactorial process. The most prominent effects of aging include visual impairments, particularly age-related cataracts (ARC). Several studies have reported that oxidative stress and failure of the antioxidant system are the major factors contributing to lens oxidation. The present study focused on the nuclear factor erythroid 2-related factor 2 (Nrf2)/kelch-like ECH-associated protein 1 (Keap1)-mediated antioxidant system and its failure in aging lenses. The protein levels, gene expression and methylation status of Nrf2/Keap1 were investigated in human lenses from different age groups. Human lens epithelial cells were collected from different age groups ranging between 15 and 80 years and cataract lenses were also collected for the comparative study. The results demonstrated significantly lower protein and gene expression levels of Nrf2 in lenses of increasing age; however, a significant increase in the expression of the Nrf2 regulator, Keap1, was observed. Based on these results, the present study then aimed to investigate the underlying mechanisms. A gene specific DNA methylation study was performed in cataractous lenses of different ages, which revealed significantly increased levels of demethylated DNA in the Keap1 promoter with increasing age. Notably, the results from cataract lenses demonstrated significant demethylation of the Keap1 promoter, which was also reflected in the results of clear lenses aged between 66 and 80 years. These results suggested that demethylation in the Keap1 promoter region activated the expression of the Keap1 protein, which then increased the targeting of Nrf2 for proteasomal degradation. Therefore, decreased activity of Nrf2 restrained the transcription of its downstream antioxi-

dant enzyme and led to the failure of the antioxidant system, ultimately leading to the formation of ARCs.

## Introduction

A study from the American Federation for Aging Research states that as normal cells become senescent, whether due to ongoing cell division, direct DNA damage, activated oncogenes or other causes, they incur hundreds of biological alterations that affect several of their activities (1). A number of these changes are similar, if not identical, to the types of changes that are observed in aging humans. It is hypothesized that the numerous losses in function that occur in cells as they approach senescence leads to an increased vulnerability to age-related diseases or pathologies. Therefore, the study of cellular senescence continues to provide important insights into the aging process at the level of the cell and the pathways within the cell.

An imbalance in antioxidants and pro-oxidants results in oxidative stress, thus, the destruction of toxic free radicals, produced as a result of normal metabolism, is essential. Cataracts are the leading cause of blindness worldwide and opacity of the lens is a direct result of oxidative stress (2). Visual dysfunction and blindness induced by age-related cataracts (ARCs) increases as the world's population ages. Environmental components and genetic predisposition contribute to the development of ARC. In addition, increased age and being female are associated with an increased risk for ARC (3).

One of the main antioxidant protective mechanisms is the activation of nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 is a transcriptional activator, which binds to the antioxidant response element. This leads to the transcription of ~200 protective genes, including numerous antioxidant-associated enzymatic genes (4,5). Previous studies have investigated the antioxidant components sulforaphane, curcumin and acetyl-L-carnitine for the prevention of cataracts (6-9). However, the molecular mechanism underlying the failure of the antioxidant system in human lenses remains to be elucidated. Several studies have reported that oxidative stress stimulates the modification of DNA, including DNA methylation (10-13).

The present study aimed to investigate the underlying mechanism of the Nrf2-mediated suppression of the

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antioxidant system in lenses with age-associated cataracts by comparing them with normal clear lenses of different age.

## Materials and methods

**HLECs and bisulphite conversion.** Two different types of sets of lenses were used in the present study: Sets of healthy clear lenses from donors aged between 15-80 years were obtained from the eye bank of Shandong Eye Institute (Shandong, China) and the other sets of lenses were obtained from patients aged between 45-90 years who had undergone cataract surgery. These patients participated in the present study at the Department of Ophthalmology, Linyi People's Hospital (Linyi, China). Patients with other systemic disease, including diabetic cataracts, were excluded in order to avoid misleading parameters. In total, ~120 samples with at least one sample from each subject were used. The Institutional Review Board of Linyi People's Hospital approved the study and written informed consent was obtained from each participant.

**Detection of Nrf2/Keap1 protein levels.** HLECs were harvested and lysed with 200  $\mu$ l immunoprecipitation assay buffer (Pierce IP Lysis Buffer; Thermo Fisher Scientific Inc., Rockford, IL, USA). The lysates were centrifuged at 13,500  $\times$  g for 15 min at 4°C and the protein content of the supernatant was determined using the Bradford method (14). The soluble proteins (10-20  $\mu$ g) were loaded and separated by 10% SDS-PAGE (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and blotted onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Inc.). Subsequently, the membranes were blocked with 5% non-fat dry milk powder solution for 1 h at room temperature prior to overnight incubation with Nrf2 and Keap1 rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C. Following rinsing the membranes, the membranes were incubated with a secondary antibody for 1 h at room temperature and the bands were made more visible using enhanced chemiluminescence. The intensity of each band was normalized to that of  $\beta$ -actin and quantified using Image J analysis software (National Institutes of Health, Bethesda, MD, USA).

**Evaluation of Nrf2/Keap1 gene expression.** Total RNA was extracted from the HLECs using a Quick-RNA MicroPrep solution (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. The purified total RNA was reverse transcribed using iScript Reverse Transcription Supermix for quantitative PCR (qPCR; Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The reverse transcribed RNA was analyzed by qPCR using the SsoFast EvaGreen supermix (Bio-Rad). Roche's ProbeFinder designed the optimal qPCR assay for the Nrf2 and Keap1 genes. The primer sequences used were as follows: *Nrf2*, forward 5'-ACACGGTCCACAGCTCATC-3' and reverse 5'-TGCCTCCAAAGTATGTCAATCA-3' with a product size of 96 bp; *Keap1*, forward 5'-GGGTCCCCTACAGCCAAG-3' and reverse 5'-TGGGGTCCAGAAGATAAGC-3' with a product size of 66 bp and  $\beta$ -actin, forward 5'-CCACCGCGAGAAGATGA-3' and reverse 5'-CCAGAGGCGTACAGGGATAG-3' with a product size of 97 bp. Each reaction was performed in triplicate and a standard curve was prepared using a serial dilution of a reference

sample. The relative copy numbers were obtained from the standard curve and were normalized to the values obtained for  $\beta$ -actin as the internal control.

**Sample preparation bisulfite conversion.** The genomic DNA of HLECs was subjected to bisulfite conversion using an EZ DNA Methylation-Direct™ kit (Zymo Research). HLECs from the area of capsulotomy were isolated from the center of the anterior surface of lenses and of age-related cataractous lenses under a dissection microscope (Carl Zeiss AG, Oberkochen, Germany). The bisulfite converted DNA was then used for bisulfite genomic DNA sequencing.

**Genomic DNA bisulfite sequencing.** The bisulfite-modified DNA was amplified by bisulfite sequencing PCR using Platinum PCR SuperMix High Fidelity (Invitrogen Life Technologies, Carlsbad, CA, USA) with primers specific to the human Keap1 promoter region 1 with 330 bp (forward 5'-TTAGTTATTTAGGAGGTTGAGGTT-3' and reverse 5'-AACCCCCCTTCTCACTA-3') and region 2 with 448 bp (forward 5'-AGTGAGAAGGGGGGTTTGG-3' and reverse 5'-CCAAAATAAAATAAACACC-3'). The primers were designed using the Methyl Primer Express software from Applied Biosystems (Foster City, CA, USA). The PCR products were purified by gel extraction using the Zymoclean™ Gel DNA recovery kit (Zymo Research) and then cloned into the pCR4-TOPO vectors using a TOPO TA Cloning kit (Invitrogen Life Technologies). The recombinant plasmids were transformed into One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen Life Technologies) using the regular chemical transformation method, according to the manufacturer's instructions. Plasmid DNA was prepared from ~12 independent clones of each amplicon using a PureLink Quick Plasmid Miniprep kit (Invitrogen Life Technologies) and sequenced to determine the status of cytosine-phosphate-guanine methylation. The sequenced data of each clone was then compared with the *in silico* reference human Keap1 promoter bisulfite converted DNA sequence, derived using the Methyl Primer Express software (Applied Biosystems).

## Results

**Detection of protein levels.** Initially, the protein levels of Nrf2 and Keap1 from human clear lenses of different ages (15, 25, 35, 45, 55, 65, 70, 75 and 80 years) were examined. An age-dependent decrease in the level of Nrf2 was detected by protein immunoblot analysis (Fig. 1). A significant decrease in the protein level of Nrf2 was detected in lenses from the group aged between 65 and 80 years compared with that observed in the group aged between 15 and 45 years. However, a contrast in results was observed in the Keap1 immunoblots. A significant increase in the protein level of Keap1 was detected in lenses of group aged between 65 and 80 years compared with the group aged between 15 and 45 years (Fig. 1). An increase in the protein level of Keap1 may explain the decrease in the level of Nrf2, as Keap1 is a regulator protein, which binds to Nrf2 and targets it for proteasomal degradation. Therefore, the mechanism underlying this marked increase in the protein levels of Keap1 with increasing age was next investigated.

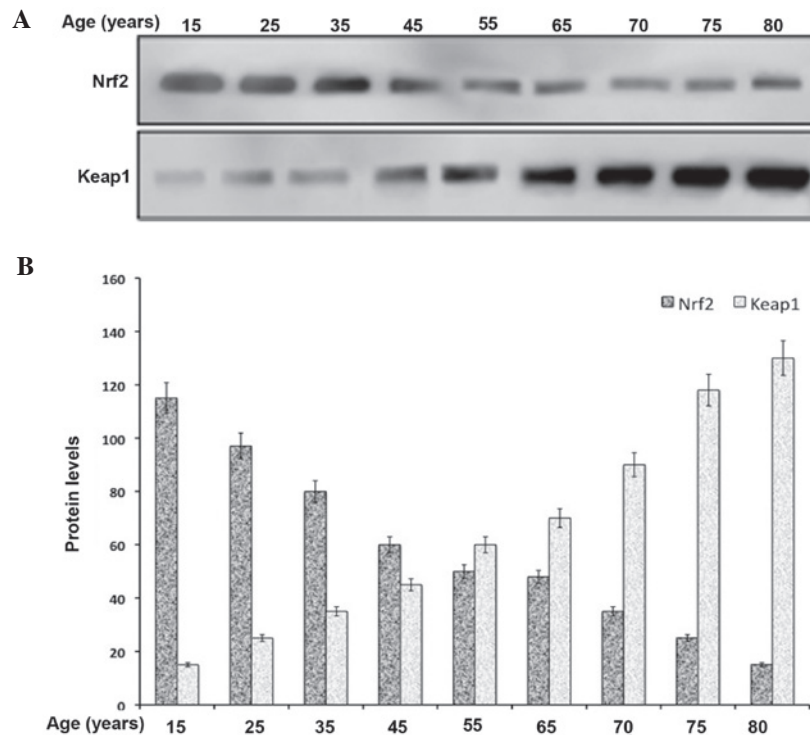


Figure 1. Age-dependent differential expression of lens antioxidant proteins Nrf2 and Keap1. (A) Representative western blot analysis of Nrf2 and Keap1 of human clear lenses from different age groups (15-80 years). (B) Level of protein (intensity) expression, normalized with the corresponding intensity of GAPDH expression in respective samples. Nrf2, nuclear factor erythroid 2-related factor 2; Keap1, kelch-like ECH-associated protein 1.

**Gene expression study.** Following the results of the protein blotting, the mRNA and gene expression levels of *Nrf2* and *Keap1* were examined in human clear lenses from different age groups (15-80 years). qPCR was performed to analyze or quantify the level of mRNA transcription, the results of which reflected the protein immunoblotting results. A gradual decrease was observed in the level of *Nrf2* gene expression as age increased (Fig. 2). In addition, a significant decrease in the expression of *Nrf2* was observed in lenses from the group aged between 65 and 80 years compared with those <40 years old. However, a gradual increase was observed in the gene expression of *Keap1* in lenses from the group aged between 15 and 65 years, while a significant increase was observed after 65 years (Fig. 2). This result suggested that the marked increase in the gene expression of *Keap1* with increasing age may have been regulated by epigenetic modification, including DNA methylation or demethylation.

**Gene specific DNA methylation study.** The present study aimed to investigate whether DNA methylation was involved in *Keap1* gene expression. Consequently, the gene specific DNA methylation in the promoter region of the *Keap1* gene was examined by implementing the genomic DNA Bisulfite sequencing method. The *keap1* promoter region was analyzed using two sets of primers as region 1 (-430 to -110) and region 2 (-115 to +335; Fig. 3A). The clear lenses from different age groups (15-80 years) and the age-related cataract lenses from patients aged between 45 and 90 years were selected for the investigation. Complete methylation was identified in the clear lenses from the group aged between 15 and 45 years (Fig. 3B). However, gradual demethylation was observed in the group aged between 45 and

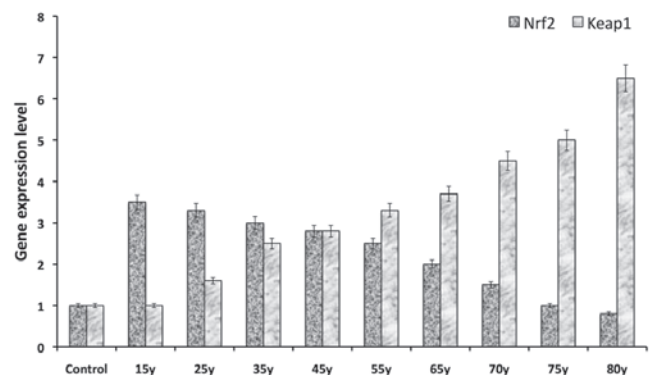


Figure 2. Age-dependent differential expression of lens antioxidant genes *Nrf2* and *Keap1*. The graph shows quantitative polymerase chain reaction of *Nrf2* and *Keap1* mRNA in human lens epithelial cells from different age groups (15-80 years). Nrf2, nuclear factor erythroid 2-related factor 2; Keap1, kelch-like ECH-associated protein 1.

65 years (Fig. 3C) and significant demethylation was found in lenses from the group aged between 65 and 80 years (Fig. 3D). Notably, similar significant demethylation was observed in the age-related cataract lenses from patients aged between 45 and 90 years (Fig. 3E). These results demonstrated that gradual demethylation occurred throughout aging, which was due to the exposure of oxidative stress in the cells. Depending on the level of exposure, demethylation of the *Keap1* promoter DNA or increase in the gene expression of *Keap1* occurred and suppressed the protein levels of Nrf2. This led to the failure of the antioxidant system in the lenses and ultimately to cataract formation. The methylation study revealed the percentage of demethylation in different age groups, which was estimated as 3%



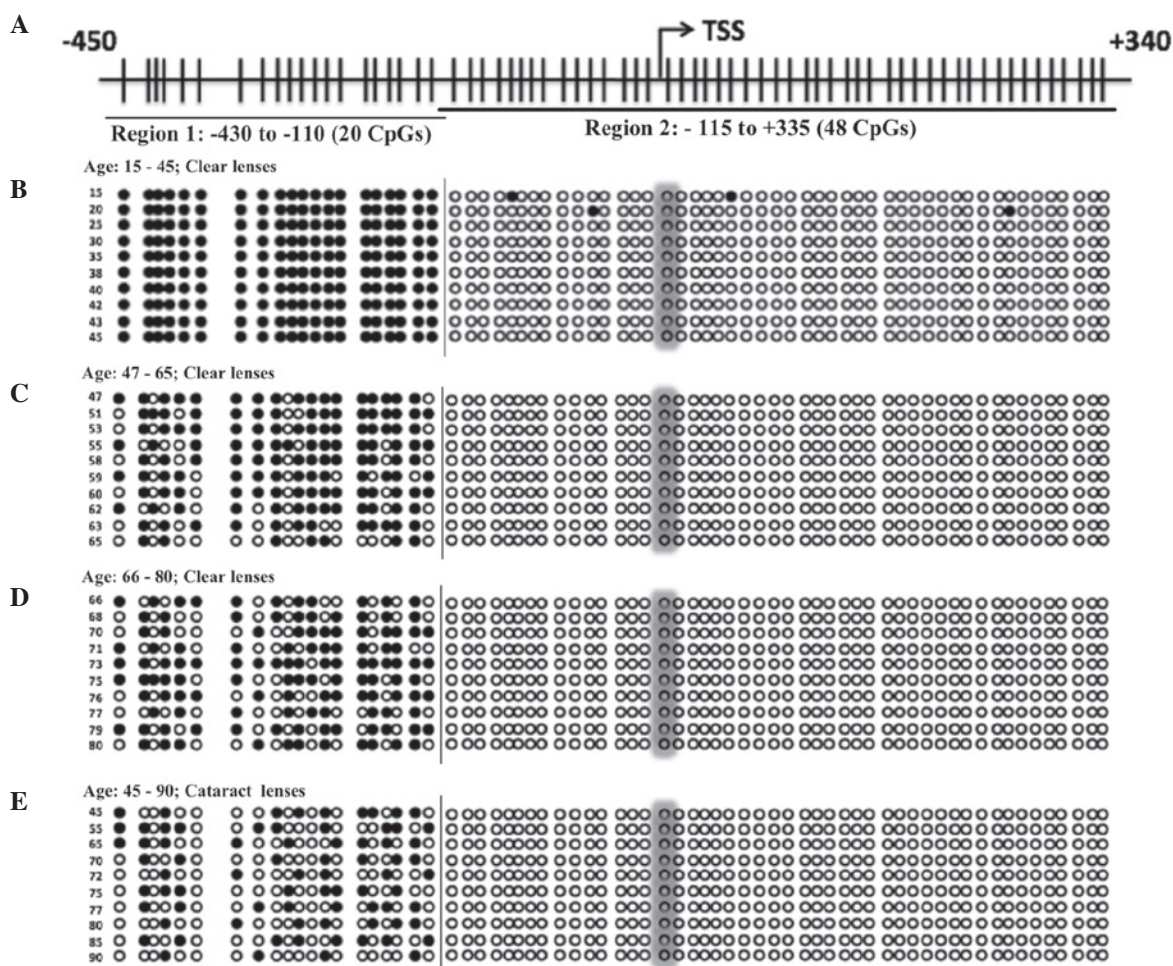


Figure 3. Age-dependent DNA methylation status in the promoter region of the Keap1 gene in human clear and cataract lenses. (A) Presence of CpG islands in the DNA promoter region of the Keap1 gene. TSS denotes the transcription start site. This region was analyzed using two sets of primers by splitting the region as region 1 with 20 CpG islands (-430 to -110) and region 2 with 48 CpG islands (-115 to +335). Representative bisulfite converted DNA sequence analyzed from two promoter regions in clear lenses from age groups between (B) 15 and 45 years, (C) 47-65 years, (D) 66-80 years and (E) cataract lenses from patients aged between 45 and 90 years. Each row of circles indicates the sequence results of two regions of promoter DNA in a specific age of human lenses (ages presented at the left end of the row). Black circle: methylated DNA (methylcytosines); open circle: unmethylated DNA (cytosines); shaded open circle: CpG island near the transcriptional start site. CpG, cytosine-phosphate-guanine; Keap1, kelch-like ECH-associated protein 1.

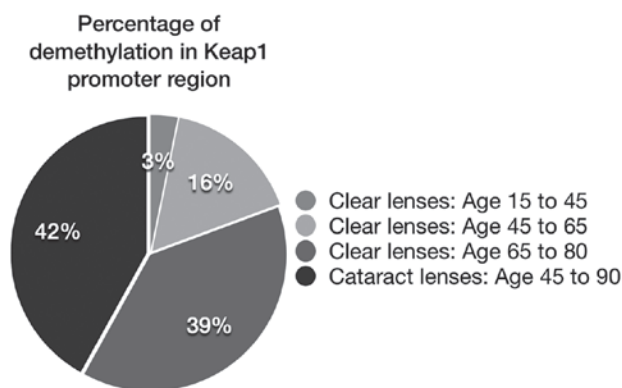


Figure 4. Percentage of demethylation in human clear and cataract lenses from different age groups between 15 and 90 years. Keap1, kelch-like ECH-associated protein 1.

(age 15-45 years), 16% (age 45-65 years), 39% (age 65-80 years) and 42% in cataract lenses from patients aged between 45 and 90 years (Fig. 4). This result clearly indicated that demethylation

of the Keap1 gene led to lens oxidation or cataract formation, which was observed in aging lenses. The percentage of demethylation in lenses from the group aged between 65 and 80 years (39%) was almost equal to the percentage observed in cataract lenses (42%). Therefore, due to Keap1 demethylation, the aged lenses were susceptible to cataract formation.

## Discussion

The combination of aging with environmental and genetic stresses is considered to be the main factor contributing to oxidation, modification and aggregation of lenticular proteins. The human lens grows throughout life by generating new fiber cells on old lens fiber cells. The lens epithelial cells in the germinative zone, which differentiate into the cortical fiber cells, are more prone to reactive oxygen species (2). This suggests that these lens fiber cells have less Nrf2-dependent antioxidant protection and the changes result in oxidation and crystallin aggregation in the lens cortical and posterior regions (15). In the present study, the level of antioxidant system proteins was investigated and the results demonstrated that the protein level of Nrf2 decreased as

the age of the human lens increased. However, the protein level of Keap1 increased upon aging (Fig. 1), which is consistent with previous studies demonstrating that Keap1 is a negative regulatory protein of Nrf2; increased Keap1 stimulates the degradation of Nrf2 due to proteasomal degradation and a reduction in Nrf2 suppresses Nrf2-dependent antioxidant protection (16).

The gene expression of *Nrf2* and *Keap1* was then determined in human clear lens epithelial cells from different age groups (15-80 years). A similar pattern of expression to the protein blotting was observed. The results revealed an age-dependent increase in *Keap1* gene expression but an age-dependent decrease in *Nrf2* gene expression (Fig. 2). These results are consistent with those of a previous study demonstrating that increased activation of *Nrf2* increases the expression of cytoprotective genes that detoxify electrophiles to a greater extent than those that detoxify reactive oxygen species in livers from Keap1-knockdown mice (17). This previous study supports the observation in aging human lens in the present study, with gene expression indicating the impact of epigenetic modification in the DNA promoter region of these genes. It may be either gene specific methylation or demethylation that occurs during aging. This transcriptional regulation of *Keap1* and the proteasomal degradation of *Nrf2* leads to the failure of *Nrf2*-dependent oxidative stress protection, which may result in the oxidation of HLECs and formation of cataracts.

The present study also investigated the gene specific DNA promoter methylation/demethylation for Keap1 gene expression. The present study examined the normal aging of clear lenses in from different age group ranging between 15 and 80 years and the age-related cataract lenses from patients aged between 45 and 90 years. The clear lens from the group aged between 15 and 45 years demonstrated complete methylation (3%), the group aged between 45 and 65 years demonstrated mild demethylation (16%) and the group aged between 65 and 80 years demonstrated significant demethylation (39%) of the Keap1 promoter DNA (Fig. 4). The normal aging lenses from the group aged between 65 and 80 years revealed almost equal demethylation status with the cataract lenses from patients aged between 45 and 90 years old (Fig. 4). This is consistent with previous studies investigating the occurrence of DNA hypomethylation in cancer and age-dependent increases in demethylation in normal tissues in vertebrates, including humans (18-20). The present study revealed the age-dependent epigenetic modification of the Nrf2-mediated antioxidant system in the human lens. These results are also consistent with previous data suggesting that the development of age-related inflammatory diseases, including rheumatoid arthritis and polymyalgia rheumatica, in which TNF is an important mediator, may be affected by changes in DNA methylation (21).

The present study demonstrated that the Nrf2/Keap1-mediated antioxidant system has an important role in the lens antioxidant system. ARCs may be caused by an imbalance in the Nrf2 and Keap1 regulatory mechanism. Imbalance in the gene expression of Keap1 directly affects Nrf2-mediated protection in the lens. The present study revealed that the age-dependent gene specific DNA promoter demethylation of *Keap1* gene expression was the key mechanism underlying the formation of human ARCs. However, a previous study demonstrated that hypermethylation

of the Keap1 promoter region suppressed the mRNA expression of *Keap1* and increased the expression of nuclear Nrf2 and the downstream antioxidant response element gene in colorectal cancer cells and tissues (22). In conclusion, the present study provided a greater understanding of the novel mechanism underlying the development of human ARCs. Further detailed studies in this area may provide support for the development of treatments.

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