Relationship Between the Altered Expression and Epigenetics of \textit{GSTM3} and Age-Related Cataract

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\textbf{PURPOSE.} Glutathione S-Transferase Mu 5 (\textit{GSTM3}) protects the lens from oxidative stress that contributes to age-related cataract (ARC) formation. We examined the expression and epigenetics of \textit{GSTM3} in lens epithelial cells (LECs) and lens cortex of ARC, and investigated the potential role of molecular changes in ARC pathogenesis.

\textbf{METHODS.} This study included 120 ARCs and 40 controls. Expression of \textit{GSTM3}, DNA methylation, and histone modification were assessed by quantitative real-time PCR, Western blot, bisulfite-sequencing PCR, pyrosequencing, and chromatin immunoprecipitation assay. Human lens epithelial (HLE) cell lines, SRA01/04 and HLEB3, were served as an in vitro model to observe the relationship between epigenetic status and \textit{GSTM3} expression. Potential transcription factors binding to \textit{GSTM3} promoter were detected by electrophoretic mobility shift assay.

\textbf{RESULTS.} Expression of \textit{GSTM3} decreased in ARC lens tissues compared to that in the controls, which correlated with the hypermethylation of \textit{GSTM3} promoter. Lower level of \textit{GSTM3} was detected in HLEB3 than in SRA01/04, while HLEB3 displayed hypermethylation of \textit{GSTM3} and SRA01/04 did not. Compared to SRA01/04, HLEB3 displayed lower acetylated H3 and higher trimethylated H3K9 levels. After treatment with DNA methyltransferase inhibitor or histone deacetylase inhibitor, HLEB3 had an increased \textit{GSTM3} expression. Methylation of \textit{GSTM3} promoter abrogated the potential transcription factor binding. The \textit{GSTM3} expression declined in hydrogen peroxide-treated HLE cell lines.

\textbf{CONCLUSIONS.} Expression of \textit{GSTM3} might be regulated by epigenetic changes in lens tissue. Hypomethylation in \textit{GSTM3} promoter and altered histone modification might have a role in the ARC formation. The results provided a potential strategy of ARC management by manipulating epigenetic changes.

\textbf{Keywords:} age-related cataract (ARC), \textit{GSTM3}, epigenetics, methylation, oxidative stress

\textbf{A}ge-related cataract (ARC) is one of the leading causes of visual impairment and blindness worldwide among the elderly. It has become an increasing vision burden in China.\textsuperscript{1–3} Age-related cataract can be classified as cortical, nuclear, and posterior subcapsular according to the location of the opacity within the lens.\textsuperscript{1} Although surgery is an effective management for cataract, it is associated with high cost and inevitable risks. The disease is complex with multiple genetic and environmental risk components.\textsuperscript{5} The pathogenesis of ARC is not completely understood, but oxidative stress with the accumulation of reactive oxygen species (ROS) in the lens is considered to be a major initiating factor in the formation of ARC.\textsuperscript{6–10} Oxidative stress may result from an imbalance between the production of the ROS and the cellular antioxidant defense mechanisms.\textsuperscript{11} The imbalance is due to the lack of antioxidant capacity and induced oxidative DNA damage. In our previous studies, an oxidative DNA damage marker, 7,8-dihydro-8-oxoguanine (8-oxoG), was significantly increased in ARCs compared to controls.\textsuperscript{12,15}

Reactive oxygen species induced by hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) change the oxidant/antioxidant balance in the aqueous humor. Reactive oxygen species decrease antioxidant levels in the lens, and accelerate the damage to the lens epithelial cells (LECs), resulting in subsequent cataract development.\textsuperscript{14–16} When the lens is exposed to H\textsubscript{2}O\textsubscript{2}, a vast amount of ROS, which exceeds the self-scavenging ability of the lens, is adequately generated, resulting in oxidative stress and DNA damage.\textsuperscript{17} Glutathione (GSH) functions as an important intracellular radical scavenger, and protects cells against ROS. Under normal conditions, the lens contains high levels of reduced GSH,\textsuperscript{18} which is likely to have an important role in defending the lens against oxidative stress. The levels of GSH in the LECs of patients with pseudoexfoliation syndrome were significantly lower than those in the LECs of nonpseudoexfoliation patients.\textsuperscript{19} Glutathione also functions as a coenzyme in glutathione S-transferases (GSTs).\textsuperscript{20–22} Glutathione S-transferases belong to an almost universal superfamily of enzymes that is present and has evolved in all aerobic organisms,\textsuperscript{23,24} and are considered major players in the phase II detoxification of endogenous oxidative stress products and exogenous electrophilic chemical compounds. Glutathione S-transferases are a family of detoxification enzymes classified as α, μ, π, θ, or 0 according to their sequence homologies and substrate specificities.\textsuperscript{25} Certain types of GSTs have a key role in regulating mitogen-activated protein kinase pathways involved in the cellular response to oxidative stress. It is believed that GSTs are...
involved in the transport and biosynthesis of endogenous compounds and cellular defense mechanisms against xenobiotics and oxidative damage by catalyzing the conjugation of reduced glutathione through its cysteine thiol.24 Cellular detoxification mechanisms are of critical importance in maintaining health by providing protection against numerous noxious agents in the environment. They also allow the cell to withstand harmful endogenously produced substances, such as ROS that arise through normal metabolic processes.25 The GSTs activity was detected in the central cortex; however, there was no measurable activity in the nucleus.26 Thus, supplementation with antioxidant nutrients is one reasonable approach to prevent cataract development.

Previous studies have elaborated that the human lens expresses only μ and π classes of GST isoenzymes.26 Glutathione S-Transferase Mu 3 (GSTM3), which belongs to the μ-class subfamily, has a strong antioxidant function.29 It has been reported that GSTM3 polymorphism is a risk factor for many oxidative-stress-related and age-related diseases.27–31 However, a former study had informed that the polymorphism of the GSTM3 may not be a genetic risk factor for the development of cortical cataract.32 We have found that copy number variation of glutathione S-Transferase Mu 1 (GSTM1) was involved in ARC mechanism, but copy number variations of GSTM3 were not associated with increased risk of ARC.33 Loss of functional GSTM3 is involved in the pathogenesis of many oxidative-stress-related and age-related diseases.34,35 Previous studies have shown that the methylation of GSTM3 may be associated with oxidative stress in liver failure.36

Reactive oxygen species produced by oxidative stress stimulate the alterations in DNA methylation patterns, without changing the DNA base sequence.37 Such alterations in DNA methylation patterns are known to strongly control the expression of genes.38 It has been demonstrated that aberrant DNA methylation reduces GSTM3 expression in Barrett’s adenocarcinoma.39 Previously, we also have illustrated that methylation of many DNA repair genes are associated with an increased risk of ARC.40–42

DNA methylation is an important epigenetic mechanism in gene regulation. It is usually associated with transcriptional silencing of genes without altering the DNA sequence. Methylation at the C5 position of cytosine is a common epigenetic modification, and cytosine methylation in CpG dinucleotides can occur. Such methylation most often appears in clusters throughout the genome known as CpG islands.43 Most CpG islands are located in the promoters or first exons of genes.44 Previous reports have shown that differential DNA methylation regulates gene expression of O-6-methylguanine-DNA methyltransferase (MGMT), 8-oxoguanine DNA glycosylase 1 (OGG1), and Werner syndrome gene (WRN) in cataract lenses.40–42

We were interested in assessing whether epigenetic events have a crucial role in the formation of ARC. The present study was done to test the hypothesis that hypermethylation of CpG islands in promoter of GSTM3 and histone modification are associated with the expression of the gene in lens tissues from ARC patients. The study might provide a proof of concept for the intervention of epigenetic factors, such as DNA methylation and histone modification in ARC therapy and prevention.

Materials and Methods

Study Participants

The research followed the tenets of the Declaration of Helsinki. All participants had signed the informed consent forms. The study was approved by the Ethics Committee of Affiliated Hospital of Nantong University. We enrolled 120 ARC patients, consisting of three subgroups: age-related cortical cataract (ARC-C, n = 40), age-related nuclear cataract (ARC-N, n = 40), and age-related posterior subcapsular cataract (ARC-P, n = 40). The inclusion criteria for ARC groups were as follows: (1) opaque ocular lenses, (2) ≥50 years of age, and (3) C ≥ 4, N ≥ 4, or P > 4 according to the lens opacity classification system III (LOCSIII).45 The exclusion criteria were: (1) complicated cataract due to high myopia, uveitis, ocular trauma, or other known causes and (2) hypertension, diabetes, or other systemic diseases. We enrolled 40 patients with vitreoretinal diseases who received transparent lens extraction as control group. The inclusion criteria for the control group were: (1) transparent ocular lenses and (2) ≥50 years of age, while the exclusion criteria were: (1) other major eye diseases, such as glaucoma, myopia, diabetic retinopathy, and uveitis, and (2) hypertension, diabetes, or other systemic diseases. There were no statistically significant differences between the ARCs and controls regarding the variable “age” (P > 0.05). The demographic information was listed in the Table.

LEC2s and Lens Cortex Preparation

The LECs were collected by anterior continuous curvilinear capsulorrhexis during cataract surgery. Lens cortex was collected from the transparent cortex of controls and opaque cortex of ARC patients. The samples were rapidly frozen in liquid nitrogen, and then stored at −80°C for later extraction of genomic DNA, mRNA, and protein detection.51

Cell Culture and Treatment

As previously described,12,46 human lens epithelial (HLE) cell lines, SRA01/04 and HLEB3, originated from human lens epithelium which was maintained in Dulbecco’s modified eagle medium (DMEM) with 10% fetal bovine serum (FBS), and streptomycin (100 µg/mL). All cells were maintained at 37°C in a humidified incubator of 95% air and 5% CO2. When grown to 80% to 90% confluence, the cells were maintained at 37°C in a humidified incubator of 95% air and 5% CO2. When grown to 80% to 90% confluence, the cells were exposed to H2O2 (Sigma-Aldrich Corp., St Louis, MO, USA) for oxidative stress. The cells were cultured overnight in DMEM with 2% FBS, followed by serum-free DMEM for 30 minutes. Then the cells were collected for different assays after treatment with H2O2 (50, 100, 150, 200, and 300 µM) for 24 hours. The cells were demethylated by incubation in medium containing 3 µM of 5-Aza-2′-deoxycytidine (5-Aza-dC; the DNA methyltransferase inhibitor; Sigma-Aldrich Corp.) for 24 hours. For trichostatin A (TSA; the inhibitor of histone deacetylases; Sigma-Aldrich Corp.) treatments, the cells were grown in six-well plates and treated with 500 nM of TSA for 24 hours. At the end of the experiment, the cells were harvested and used for different assays. Parallel cultured control cells were grown in the similar media without treatment as controls in all experiments.

To authenticate the lens epithelial cell lines, we conducted Western blot assays of 3 crystallin proteins of cell lysis using rabbit anti-human α A crystallin antibody (1:1000, Abcam Ltd., Cambridge, UK), rabbit anti-human α B crystallin antibody.

### Table. Demographic Information of Study Participants

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Aberrant Epigenetic Modifications of GSTM3 in ARC

Cell Proliferation Assays
Cell viability was examined by Cell Counting Kit 8 (CCK8; Doino Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions. Briefly, cells were plated at a density of 0.5 × 10^4 cells/well in 96-well plates. After being treated or untreated with H_2O_2 for 24 hours, cells were added with 10 μl CCK8 solutions for 2 hours, and then the absorbance at 450 nm was calculated. The experiment was performed at least three times.

RNA Isolation and cDNA Preparation
Total RNA of LECs, lens cortex, SRA01/04, and HLEB3 cells were isolated from the tissues and cells 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, Foster City, CA, USA) were used for GSTM3 mRNA quantification (assay ID: Hs00954695_g1). β-Actin (Hs01060665_g1) was used as an internal control. Quantitational real-time PCR (qRT-PCR) was performed using ABI 7500 real time PCR system (Applied Biosystems). The fold change of gene expression was determined using the comparative CT (2^-△△CT) method.

Western Blot Assay
The protein of LECs, lens cortex, and the treated or untreated SRA01/04 and HLEB3 cells were extracted separately in lysis buffer. Equal amounts of proteins were size fractionated by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels. Proteins then were transferred onto polyvinylidene difluoride filter membranes (Millipore, Bedford, MA, USA). Nonspecific protein binding to the membrane was blocked by blocking buffer (5% nonfat milk). The blocked membrane then was incubated with rabbit anti-human GSTM3 antibody (1:800; Sigma-Aldrich Corp.) and mouse anti-human β-actin antibody (1:1000; Abcam) at 4°C for 12 hours. After washing three times with TBS-T (20 mM Tris, 500 mM NaCl, 0.1% Tween 20) for 5 minutes each time, the membrane was incubated with an alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse IgG antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 hours. An enhanced chemiluminescence detection system was used to read the Western signals (Pierce Chemical Co., Rockford, IL, USA).

Genomic DNA Isolation
The isolation of genomic DNA from the LECs, lens cortex, SRA01/04 and HLEB3 cells was performed by standard phenol-chloroform extraction. DNA concentration and purity were determined by comparing the ratio of optical density measurements at 260 and 280 nm.

Measurement of DNA Methylation
Transcription start site (TSS) of GSTM3 was predicted by online software (available in the public domain at http://dbtss.hgc.jp/). The CpG islands of the gene were predicted by online software (available in the public domain at http://www.urogene.org/methprimer/). The parameters used were 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 (2 μg) was treated with sodium bisulfite using the Epitector Bisulfite Kit (Qiagen, Frederick, MD, USA). Epitector Control DNA (Qiagen) was used as positive and negative controls in all experiments.

Bisulfite-sequencing PCR (BSP) was conducted using the primers designed by web-based Meth Primer software (available in the public domain at http://www.urogene.org/methprimer/) to cover a CpG island near GSTM3. The primers used for region 1 (R1) were: 5'-AATTTTTTTTTTGGTTGTTTAGAG-3' (forward) and 5'-AAACCAAACTCATTACTAATTACC-3' (reverse). The primers used for region 2 (R2) were: 5'-TTTTTATTGGTATTTAATGGAAGT-3' (forward) and 5'-ATCCCCAAAATACAAAAAATCAACCTTA-3' (reverse). The PCR products were gel-extracted and cloned into the pMD-20-T vector (Takara). Plasmid-transformed bacteria DH5α were grown for 14 hours and the plasmid DNA was isolated. At least 50 clones were chosen for sequence analysis. The degree of methylation was presented as mC/CpG. Each circle graph represented each numbered CpG site and the percentage of methylated clones (number of methylated clones/50 analyzed clones × 100).

Quantitative DNA methylation analysis of the bisulfite-treated DNA was performed by pyrosequencing.47,48 Regions of interest were amplified using 20 ng of bisulfite-treated genomic DNA and 5 pmol of forward and reverse primers, one of them being biotinylated. Primers for PCR amplification and pyrosequencing were used to cover regions of interest (biotin: forward primer, 5'-TGTTGTTAGGTTGTTTTGATG-3'; reverse primer, 5'-CAACAACAAAATAACCCCTA-3'; sequence to analyze: AACAAAAAACATTCCCTCCTTTAACCTTACCACCCAAACAAAC).

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According to the manufacturer's instructions, the PCR program consisted of a denaturing step of 3 minutes at 95°C followed by 35 cycles of 25 seconds at 94°C, 25 seconds at 60°C, and 25 seconds at 72°C, with a final extension of 5 minutes at 72°C. Quantitative DNA methylation analysis was done on a PyroMark Q96 ID pyrosequencer (Qiagen) with the PyroMark Gold Q96 Reagent (Qiagen). For each locus, methylation status was analyzed individually as a T/C single nucleotide polymorphism using PyroMark CpG Software 1.0.11 (Qiagen).

Chromatin Immunoprecipitation (ChIP) Assay
Chromatin immunoprecipitation assay was performed using Tissue Acetyl-Histone H3 ChIP kit, and Tri-Methyl-Histone H3K9 ChIP kit (Epigentek, Farmingdale, NY, USA) according to the manufacturer’s instructions. Briefly, the SRA01/04 and HLEB3 cells were cross-linked with 1% formaldehyde for 8 minutes and then homogenized. The homogenate was sonicated for 4 pulses of 15 seconds each at level 2, using the microtip probe of a Branson Digital Sonifier (Model 450; Branson Ultrasonics Corporation, CT, USA), with a 40-second interval on ice between each pulse, to generate fragments of genomic DNA ranging from 200 to 800 base pairs (bp) in length. For the ChIP assays, equal amounts of treated chromatin were added to microwells containing immobilized antibody for the targeted protein or a negative control normal rabbit IgG antibody. In addition, a small portion of treated chromatin, which was equal to 5% of the extracted genomic DNA, was used as the Input DNA to calculate the enrichment of the leptin promoter DNA after the immunoprecipitation of the targeted proteins. After incubating for 90 minutes at 65°C to reverse the crosslinks and elute the DNA, Fast-Spin columns were used for DNA purification, and then were detected by SYBR Green-based real time PCR.
**Electrophoretic Mobility Shift Assay (EMSA)**

Nuclear extracts were prepared from HLEB3 cells with the Nuclear Extraction Kit (Thermo-Fisher Scientific, Waltham, MA, USA). Electrophoretic mobility shift assay was performed according to the manufacturer’s instruction of LightShift Chemiluminescent kit (Thermo-Fisher Scientific). A completely unmethylated (UnM) oligonucleotide (Sangon, Shanghai, China) was used: 5'-GTCGTACTTCCGGCTTGGCCCA-3' located at R1. The completely methylated (M) oligonucleotide was prepared by incubating 1 μg of unmethylated probe with 10 units Hpall Methyltransferase (New England Biolabs, Inc., Ipswich, MA, USA), and 10 μl 1× Hpall Methyltransferase Buffer, supplemented with 80 μM S-adenosylmethionine at 37°C for 1 hour, followed by 15 minutes at 65°C to inactivate the methylase, purified by polyacrylamide gel electrophoresis. The biotin-labeled double-stranded oligonucleotides were incubated with 10 μg nuclear extracts for 20 minutes at room temperature and were subjected to electrophoresis on 6% polyacrylamide gels, then transferred to a nylon membrane and results were visualized using enhanced chemiluminescence (ECL kit; Pierce Chemical Co.). In the competition group, a 50,100 and 200-fold molar excess of unlabeled oligonucleotides was added to the reaction mixture before the addition of biotin labeled probe.

**Statistical Analysis**

A 1-way ANOVA analysis was used to determine the difference in averages between the groups. P value < 0.01 was considered statistically significant. Statistical analyses were performed with SPSS software (SPSS 17.0; SPSS, Inc., Chicago, IL, USA).

**RESULTS**

**Expression of GSTM3 in LECs and Lens Cortex**

Lower GSTM3 mRNA (Fig. 1A) expression in LECs, lens cortex was detected in all three subtypes of ARCs compared to the controls (all P < 0.01). To confirm the change of GSTM3 in protein level between the controls and ARCs, Western blot analysis was performed. As shown in Figures 1B and 1C, the expression pattern of GSTM3 protein in LECs, lens cortex was lower in all three subtypes of ARCs compared to the controls (all P < 0.01).

**Methylation Status of GSTM3 in LECs and Lens Cortex**

To analyze the relationship between methylation status and expression of GSTM3, we detected the methylation rate of GSTM3 promoter in the DNA extracted from the LECs and lens cortex of the controls and ARCs using BSP. Bioinformatic analysis indicated CpG islands in the promoter of GSTM3 (Fig. 2A; relative to the TSS). Figures 2B and 2C showed a representative result of BSP of the R1 fragment. As shown in Figure 2D, the methylation rate of all three subtypes of ARCs was higher than that of the controls at R1 in GSTM3 promoter (all P < 0.01). The methylation rates at R2 in GSTM3 promoter had no statistical significance in the controls and ARCs (data not shown).

To obtain better quantitation, pyrosequencing, a quantitative DNA sequencing method, was used to determine single-base variations caused by CpG methylation of the GSTM3 promoter in each group. Figure 3 showed the methylation status of the GSTM3 promoter in the controls, ARC-C, ARC-N, and ARC-P groups. The results were consistent with BSP. The six selected CpG sites in the ARC-C, ARC-N, and ARC-P groups displayed hypermethylation in comparison with those in the controls (all P < 0.01). The HLEB3 cells displayed hypermethylation in comparison with SRA01/04 cells (Figs. 4A–C, P < 0.01). These results indicated that DNA hypermethylation in promoter of GSTM3 reduced the expression of GSTM3.
FIGURE 2. Methylation status at R1 in GSTM3 promoter in LECs and cortex of controls and ARCs. (A) The positions of CpG islands within GSTM3 promoter. (B) Methylation status at R1 in GSTM3 promoter in LECs of controls (n = 20) and ARC-C (n = 20), ARC-N (n = 20), and ARC-P (n = 20). (C) Methylation status at R1 in GSTM3 promoter in lens cortex of controls and ARCs. (D) Methylation status at R1 in GSTM3 promoter in LECs and cortex of controls and ARCs. Values presented as mean ± SD. *P < 0.01 versus controls.
FIGURE 3. CpG island methylation of GSTM3 promoter in controls and ARCs. (A) Representative pyrosequencing results of controls (n = 10), ARC-C (n = 10), ARC-N (n = 10), ARC-P (n = 10). (B) The six CpG sites in controls and ARCs, presented as mean ± SD. *P < 0.01 versus controls.
Figure 4. Relative expression and epigenetic status of GSTM3 in SRA01/04 and HLEB3 cells. (A) Quantitative RT-PCR analysis of the expression of GSTM3 in SRA01/04 and HLEB3 cells. The mRNA levels measured were normalized to β-actin levels. Values represent mean ± SD. *P < 0.01 versus SRA01/04 cells. (B) Protein levels of GSTM3 in SRA01/04 and HLEB3 cells were detected using Western blotting. (C) Relative GSTM3 protein level to β-actin is presented as mean ± SD. (D) Representative pyrosequencing results of SRA01/04 and HLEB3 cells. (E) The six CpG sites in SRA01/04 and HLEB3 cells. Values represent mean ± SD. (F) Histone modifications of GSTM3 promoter in HLE cell lines. *P < 0.01 versus SRA01/04 cells.
Histone Modifications Around the CpG Island of the \textit{GSTM3} in HLE Cell Lines

Hypermethylation of H3K9 exhibits the silencing of gene expression, whereas acetylation of H3 is associated with activation of gene expression. Chromatin immunoprecipitation analysis depicted that acetylated H3 (H3ac) levels were lower while trimethylated H3K9 (H3K9me3) levels were higher in HLEB3 cells than those in SRA01/04 cells (Fig. 4F, \( P < 0.01 \)).

Protein Expression of \textit{GSTM3} in HLEB3 Cells After Treatment With 5-Aza-dC or TSA

To test the effects of epigenetic changes on the expression of \textit{GSTM3}, an in vitro study was performed. After the HLEB3 cells were treated with 3 \( \mu \)M 5-Aza-dC or 500 nM TSA for 24 hours, the \textit{GSTM3} protein levels increased in the cells. After the HLEB3 cells were treated with 5-Aza-dC, \textit{GSTM3} in the cells displayed hypomethylation compared to \textit{GSTM3} in untreated cells (Fig. 5, \( P < 0.01 \)).

In Vitro Methylation of the \textit{GSTM3} Promoter Abrogated the Binding With Potential Transcription Factors

We performed EMSA with an UnM or M probe of the \textit{GSTM3} promoter. A strong complex was observed when nuclear extracts from HLEB3 cells were incubated with the UnM probe (Fig. 6, lane 3). In contrast, no nucleoprotein complex was observed when the M-probe was used (Fig. 6, lane 1). The complex formation was significantly suppressed by the addition of a 200-fold molar excess of unlabeled probes to the incubation mixtures (lanes 4–6). Results are representative of at least three independent experiments.

Oxidative Stress by H\(_2\)O\(_2\) Treatment Decreased Cell Proliferation and \textit{GSTM3} Expression in Cultured HLE Cell Lines

The CCK8 assay indicated that cell viability was significantly lower, compared to that in the controls, in SRA01/04 and HLEB3 cells when they were treated with 150 and 100 \( \mu \)M H\(_2\)O\(_2\), respectively (Fig. 7A, all \( P < 0.01 \)). These findings suggested that oxidative stress decreased cell proliferation of HLE cell lines.

The qRT-PCR and Western blot revealed significant downregulation of \textit{GSTM3}, in SRA01/04 cells treated with 150 and 100 \( \mu \)M H\(_2\)O\(_2\) for 24 hours (Figs. 7B, 7C, all \( P < 0.01 \)). Meanwhile, after treatment with H\(_2\)O\(_2\), methylation of \textit{GSTM3} in SRA01/04 and HLEB3 cells was higher than the control cells (Fig. 7D, all \( P < 0.01 \)). Hence, the oxidative stress decreased the expression of \textit{GSTM3}.
FIGURE 7. Relative expression and promotor methylation status of \( \text{GSTM3} \) in HLE cell lines by treatment with H\(_2\)O\(_2\). (A) HLE cell lines were treated with H\(_2\)O\(_2\), and the cell viability (represented by A\(_{450}\) value) was measured using CCK8 assay. Values represent mean ± SD. **\( P < 0.01 \) versus control cells, & \( P < 0.01 \) HLEB3 cells versus SRA01/04 cells. (B) Quantitative RT-PCR analysis of the expression of \( \text{GSTM3} \) in SRA01/04 and HLEB3 cells treated or untreated with H\(_2\)O\(_2\). The mRNA levels measured were normalized to \( \beta\)-actin levels. Values represent mean ± SD. **\( P < 0.01 \) versus control cells. (C) GSTM3 protein levels in SRA01/04 and HLEB3 cells treated or untreated with H\(_2\)O\(_2\). Relative GSTM3 protein level to \( \beta\)-actin is presented as mean ± SD. **\( P < 0.01 \) versus control cells. (D) Methylation status at R1 in \( \text{GSTM3} \) promotor in SRA01/04 and HLEB3 cells after treatment with H\(_2\)O\(_2\). Values represent mean ± SD. **\( P < 0.01 \) versus control cells. & \( P < 0.01 \) HLEB3 cells versus SRA01/04 cells.
DISCUSSION

Epigenetic changes have been associated with age-related macular degeneration (AMD) and ARC. Recent advances in epigenetics and epigenomics have provided evidence that epigenetic mechanisms may function as an interface between environmental factors and the genome. In this study, we found that the mRNA and protein levels of GSTM3 were significantly reduced in LECs and lens cortex of ARCs compared to the controls, which corresponded to hypermethylation of the GSTM3 promoter CpG islands.

DNA methylation is stably maintained, inheritable, and regarded as an epigenetic marker. DNA methylation has a crucial role in a spectrum of physiologic processes. A previous study has reported that methylation changes are susceptible to nongenetic factors known to contribute to AMD development and progression. Hypermethylation of promoter CpG islands and histone H3 methylated at lysine 9 of relevant genes have been linked to heterochromatin and gene silencing, whereas histone H3 acetylated is enriched in euchromatic domains and correlates with active gene expressions. Changes in histone modifications also have been observed in experimental models of diabetic retinopathy and glaucoma. We have previously reported that the altered expression of MGMT, OGG1, and WRN might be regulated by epigenetic changes and involved in the development of ARC.

In this study, we demonstrated the presence of GSTM3 promoter hypermethylation in ARC patients. The aberrant DNA hypermethylation of gene promoter regions downregulating the expression of genes, even silencing several genes, is an important epigenetic mechanism. In our study, the methylation of GSTM3 promoter may be associated with gene silencing. Glutathione-S-transferase M3 is an antioxidative enzyme, so the dysfunction of GSTM3 caused by promoter methylation may result in the dysfunction of the cellular antioxidative system and contribute to oxidative stress-associated lens damage. Impaired LECs could fail to correct the exogenous and endogenous oxidative stresses, and, thus, ROS occurs. In this case, the accumulation of ROS will produce oxidative DNA damage which has an important role in DNA mutagenesis and cell death. Oxidative damage induces formation and relocalization of a silencing complex which may explain aberrant DNA methylation and transcriptional silencing. Our result presented higher frequency of GSTM3 promoter methylation in ARCs than that in the controls. We realized that the methylation was increased regardless of the type of cataract. Although the data largely support our research hypothesis, we could not rule out the possibility that GSTM3 promoter methylation could be a consequence of cataractogenesis.

We used the in vitro models to further characterize the interaction of the transcriptome and epigenetic charges of GSTM3. The intention was to find cell lines with different levels of transcriptome and epigenetic charges of GSTM3 to observe the correlation of the molecular parameters. SRA01/04 and HLEB3 cell lines have been used extensively for in vitro studies of lens epithelial biology. Our results indicated a strong link between the DNA methylation/histone modification and GSTM3 expression. SRA01/04 cell line was derived from a single human lens epithelial cell immortalized using the SV40 gene and HLEB3 cell line was derived from human lens epithelial cells immortalized with adenovirus. The human cell line HLEB3 was described to express β-crystallin, while a second human cell line SRA01/04 was reported to express crystallin α-A (CRYAA) and crystallin β2 (CRYBB2). Though both cell lines are supposed to represent healthy human lens epithelial cells, the two cell lines might have different genomic characteristics, such as microRNAs (miRNAs) or aging status; which might lead to the different molecular events observed in this study. In our results, we found that GSTM3 showed different levels of expression and epigenetic status in the two cell lines. After H2O2 treatment, methylation of GSTM3 promoter increased in HLEB cell lines.

Using EMSA, the protein-DNA complex was detected as preferentially binding to UnM-probe but not the complex was observed when the M-probe was used. It would be logical that methylation of the GSTM3 promoter abrogated the potential transcriptional factors binding to the region. Transcriptional factors upregulate a diversity of genes expression including GSTM3. However, the question of which transcriptional factors are crucial for the expression of GSTM3 needs further investigation.

Although the exact function and mechanism of GSTM3 in the lens must be elucidated further, the downregulation of GSTM3 in LECs not only reduced the cell viability, but also made LECs more susceptible to oxidative stress, which could induce the formation of cataract. There are some limitations in our study. For instance, the study does not have adequate evidence of a cause-effect relationship between GSTM3 methylation and cataract onset. The exact mechanism of GSTM3 promoter methylation and oxidative stress in the prognosis of cataract should be explored in a multicenter, large, perspective cohort.

In conclusion, our results demonstrated that altered epigenetic events in GSTM3 promoter in lens of ARC. Therefore, it is conceivable that GSTM3 promoter epigenetic might contribute to oxidative stress-associated lens damage in ARC. The results highlighted the importance of the protection of oxidative stress in healthy lens. However, the molecular mechanism of GSTM3 promoter methylation and histone modification and their specificity to ARC pathogenesis remain questionable and require further investigation.

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References

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