

Research Paper

# Epigenetic alterations of a novel antioxidant gene *SLC22A3* predispose susceptible individuals to increased risk of esophageal cancer

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## Abstract

Esophageal squamous cell carcinoma (ESCC) occurs with the highest frequency in China, especially in the high-risk Northern Chinese. Recent studies have reported that *SLC22A3* is significantly downregulated in non-tumor (NT) esophageal tissues from familial ESCC patients compared with those from sporadic ESCC. However, the mechanism of how *SLC22A3* regulates familial ESCC remains unknown. In this study, post hoc genome-wide association studies (GWAS) in 496 cases with a family history of upper gastrointestinal tract cancers and 1056 controls were performed and the results revealed that *SLC22A3* is a novel susceptibility gene for familial ESCC. Reduced expression of *SLC22A3* in NT esophageal tissues from familial ESCC patients significantly correlates with its promoter hypermethylation. Moreover, case-control study of Chinese descendants from different risk areas of China revealed that the methylation of the *SLC22A3* gene in peripheral blood leukocyte (PBL) DNA samples could be a risk factor for developing ESCC in this high-risk population. Functional studies showed that *SLC22A3* is a novel antioxidant gene, and deregulation of *SLC22A3* facilitates heat stress-induced oxidative DNA damage and formation of  $\gamma$ -H2AX foci in normal esophageal epithelial cells. Collectively, we show that epigenetic alterations of *SLC22A3* predispose susceptible individuals to increased risk of esophageal cancer.

Key words: Familial ESCC - *SLC22A3* - DNA methylation - antioxidant gene -oxidative DNA damage

## Background

Esophageal carcinoma (EC) is ranked as the sixth leading cause of cancer death [1]. Esophageal squamous cell carcinoma (ESCC) is the predominant histological type of esophageal carcinoma [1, 2], affecting more than 450 000 people worldwide. Despite recent development in diagnosis and treatment, the overall 5-year survival rate for patients with ESCC remained 15% to 25% [3]. ESCC occurs with the highest frequency among the Chinese,

especially the high-risk Northern Chinese, where the incidence rate is 121 per 100,000 people, which is over 20 times higher than that in low-risk regions [4]. Although environmental factors are critical to the varied distribution of ESCC, in a high-incidence area, only a minor proportion of the population develops ESCC, suggesting the importance of host susceptibility factors for those individuals who are at high risk to develop this disease [5-7]. Recently, the

potential for individual susceptibility was further supported by genome-wide association studies (GWASs) on ESCC in Chinese populations [8-11], in which several genetic susceptibility loci for ESCC have been identified. Further, epidemiological surveys from China have shown that a family history of esophageal cancer significantly increased the risk of developing ESCC [6, 12], indicating there might be differences in genetic susceptibility between familial aggregated and sporadic cancer patients.

Recently, our group found that *SLC22A3* is significantly downregulated in non-tumor (NT) esophageal tissues from familial ESCC patients compared with those from sporadic ESCCs [13], indicating that *SLC22A3* may serve as a potential tumor susceptibility gene in familial ESCC. The *SLC22A3* gene encodes a cation transport protein belonging to the SLC22A family (SLC22A1-3 or OCT1-3), which is critical for drug transportation and cellular detoxification [14, 15]. Gene structure analysis found that the promoter region of *SLC22A3* is located within a CpG island [16], suggesting that regulation of this gene may largely depend on methylation. More recent studies in prostate cancer revealed that the 6q risk variant is significantly associated with decreased *SLC22A3* transcript levels; however, functional assays showed that *SLC22A3* repression leads to reduced viability in both normal prostate and tumor prostate-derived cells [17]. Interestingly, unlike other prostate cancer risk variants, the association between the 6q risk locus and decreased *SLC22A3* transcription is only observed in populations of European ancestry but not of Japanese and African ancestries, suggesting gene-gene or gene-environment interactions underlie this observation [17].

In the present work, we aimed to explore whether *SLC22A3* promoter methylation is associated with its transcription and confers susceptibility to ESCC by performing BGS and analyzed the promoter region of the *SLC22A3* gene in peripheral blood leukocyte (PBL) DNA samples from healthy volunteers and patients with ESCC. We also investigated the function of *SLC22A3* in normal esophageal cells and its potential role in ESCC initiation.

## Methods

### Clinical Specimens

A case-control cohort from the Linzhou People's Hospital in Henan (ESCC High-risk Cohort) and the Sun Yat-Sen University Cancer Center in Guangzhou (ESCC Low-risk Cohort) were collected between 2005 and 2011. All participants provided informed consent. The High-risk Cohort consisted of peripheral blood

leukocyte (PBL) DNA samples from healthy volunteers (n = 115) and patients with ESCC (n = 133, including 49 FH+ and 84 FH- cases). High-risk cases were chosen for this study based on three criteria: (a) the patient had resided in one of four geographic regions close to the Linzhou cancer hospital (Linzhou, Anyang, Xinxiang, and Hebi); (b) the patient had newly diagnosed cancer of the esophagus without previous treatment (i.e., no surgery, chemotherapy, or radiotherapy); and (c) histological diagnosis of ESCC was confirmed by pathologists. The Low-risk Cohort consisted of PBL DNA samples from healthy volunteers (n = 100) and patients with ESCC (n = 112). We also included low-risk cases in this study based on three criteria similar to those for high-risk cases, except Guangdong was the ancestral home for these patients. However, descendants from Chaoshan, a high-risk region in Guangdong province, were excluded from this study. The controls were matched to the ESCC cases by age, sex, and geographic region (Table S1). We also obtained snap-frozen specimens of tumors and surrounding non-tumorous esophageal tissues from patients with ESCC as described previously [13].

### RNA extraction and qPCR

Total RNA was extracted by Trizol (Invitrogen) from ESCC cell lines. cDNA was produced using a PrimeScript RT Master Mix (TaKaRa), following the standard protocols provided by the manufacturer. Real-time quantitative PCR was performed using the SYBR-Green Prime Script RT-PCR kit (Takara). PCR was performed at 95°C for 15 s, 60°C for 1 min, and 72°C for 1 min for 40 cycles. Quantification was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Housekeeping gene *GAPDH* was used as an internal control and mRNA expression was calculated using the comparative  $C_T$  method ( $\Delta\Delta C_T$  method). Primer sequences for qPCR are listed in Table S2.

### Post hoc GWAS study

Based on the dataset of a previous esophageal cancer GWAS [8], we conducted genotyping data analyses by selecting 114 SNPs in a ~500-kb region covering the *SLC22A3* gene on 6q25 from 496 cases with a family history of upper gastrointestinal tract cancers and 1056 controls. We then examined potential genetic relatedness based on pairwise identity by state for all the successfully genotyped samples using PLINK 1.07 software. The original script from EIGENSTRAT was modified to extract principal components for plotting, as described previously [18]. The association results for selected 6q25 SNPs are summarized in Table S3.

## Bisulfite treatment and promoter methylation analysis

Bisulfite genomic sequencing (BGS) and real-time quantitative MSP (qMSP or MethyLight assay) were conducted to analyze the promoter region of the *SLC22A3* gene. For BGS, at least 8-10 clones from each sample were selected for sequencing analysis using the ABI310 automatic sequencer (Applied Biosystems). The PCR for BGS was performed using an AmpliTaq Gold 360 PCR Master Mix Kit (Applied Biosystems). For the MethyLight assay, CpGenome Universal Methylated DNA (Chemicon) was used as a positive control. Quantitative MSP with a TaqMan probe was performed on an ABI7700 Sequence Detector System (Applied Biosystems) using FastStart Taq DNA Polymerase (Roche). Duplex PCR with a  $\beta$ -actin (*ACTB*) primer and probe sequences containing no CpGs was performed for normalization. A methylation index (*MI*), representing the ratio of densely methylated DNA in the sample at the target sequence relative to the fully methylated positive control, was calculated according to Mori et al. [19]:  $MI = (Ts/Tc)/(As/Ac)$ , where *Ts* and *Tc* represent the levels of *SLC22A3* methylation in the sample and the control DNA, respectively, and *As* and *Ac* correspond to the amplified *ACTB* level in the sample and the control DNA, respectively. Samples containing > 5% ( $MI > 0.05$ ) fully methylated molecules were designated as methylated, and samples containing  $\leq$  5% were designated as unmethylated. The 0.05 cutoff gave the best discrimination between case and control. Primer sequences for BGS and MethyLight analyses are listed in Table S2.

## Lentiviral transduction

Plasmids reconstructed with *SLC22A3* shRNA (Sigma Aldrich NM\_021977) and shRNA NTC were purchased from sigma Aldrich. Lenti-virus were produced using transfection reagent Lipofectamine® 3000 (Thermo) and were applied to infect ESCC cells according to the manufacturer's instructions. ESCC cells were seeded in antibiotic-free growth medium before infection with lenti-virus. When the cells reached 70–80% confluence, they were infected with the harvested lentiviral media. Polybrene (Sigma-Aldrich) was added in the lentiviral media to increase the infection efficiency. The stable cell lines were screened with puromycin in proper concentration. qPCR was used to confirm the transfection efficiency.

## Immunofluorescence staining (IF)

Cells were cultured on coverslips to 80% confluence, and fresh medium was added before the

experiments. Cells received heat-shock treatment at 43°C for 45 min, followed by recovery at 37°C for 30 min. Immunofluorescence staining was performed according to the standard protocol with primary antibodies (Anti- $\gamma$ -H2AX abcam, 1:100) overnight at 4°C, and then incubated with Alexa fluor 594 goat anti-mouse IgG (Life, 1:500). Images were captured using a *Leica DMRA* fluorescence microscope (Rueil-Malmaison). Images were acquired by ZEN 2012 software. The  $\gamma$ -H2AX-positive cells refer to cells with  $\geq 5$   $\gamma$ -H2AX foci. The data represent the means  $\pm$  SD ( $n \geq 50$ ) from three independent experiments.

## MitoSOX Red Staining

Oxidative stress in esophageal cells was assessed by staining with the mitochondrial superoxide indicator, MitoSOX Red (Molecular Probes). Briefly, cells were cultured on coverslips to 60-80% confluence, and fresh medium was added before the experiments. After heat-shock treatment (43°C for 45 min), MitoSOX Red was added to a final concentration of 2.5  $\mu$ M, according to the manufacturer's recommendation. The cells were allowed to load MitoSOX for 15 min in a CO<sub>2</sub> incubator at 37°C and were washed twice with Hank's Buffered Salt Solution (HBSS) containing calcium and magnesium before analysis. Slides were counterstained with DAPI in anti-fade solution. Images were captured using a *Leica DMRA* fluorescence microscope (Rueil-Malmaison).

## Comet DNA damage assay

Cells received heat-shock treatment at 43°C for 45 min, followed by recovery at 37°C for 30 min. Cells were then collected and prepared using the OxiSelect Comet Assay Kit (Cell Biolabs). Comets were imaged by fluorescence microscopy. Percentage of Tail DNA, Tail moment (fraction of DNA in the tail  $\times$  tail length) and Olive Tail Moment were analyzed with CASP software. The data were calculated from the measurement of 50 Comets for each sample.

## Western blotting

Western blot analyses were performed according to the standard protocol with the following antibodies: anti-*SLC22A3* (Abmart, 1:1000) anti- $\gamma$ -H2AX (Abcam, 1:1000) and GAPDH (Abcam, 1:1000).

## Statistical analysis

The statistical analyses were performed with SPSS standard version 17.0 (SPSS Inc), and  $P < 0.05$  was considered as statistically significant. The *SLC22A3* methylation levels in the cases and controls were compared using the Wilcoxon rank sum test with continuity correction. Correlations among

*SLC22A3* expression, DNA methylation was analyzed using Spearman correlation.

## Results

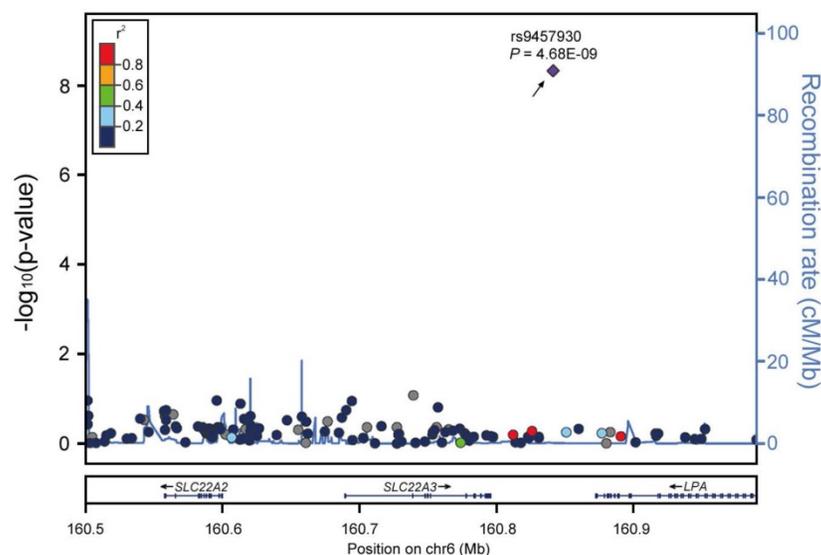
### *SLC22A3* is a candidate susceptibility gene for familial ESCC

To test the association between *SLC22A3* and ESCC in familial ESCC high-risk individuals, we performed a post hoc GWAS analysis to investigate the influence of common genetic variants around the *SLC22A3* gene region on ESCC risk. We selected 114 genetic variants in a ~500-kb region surrounding the *SLC22A3* gene on 6q25 from the dataset of a previous esophageal cancer GWAS in 496 cases with a family history of upper gastrointestinal tract cancers and 1056 healthy controls [8]. One SNP (rs9457930), located at ~45-kb downstream of the *SLC22A3* gene, was found to be significantly associated with reduced ESCC risk in high-risk individuals with a family history of ESCC [ $P = 4.68 \times 10^{-9}$ , odds ratio (OR) = 0.508, 95% confidence interval (CI) 0.504-0.512; Figure 1], indicating that *SLC22A3* could be a protective factor and that deregulation of this gene may confer susceptibility to familial ESCC.

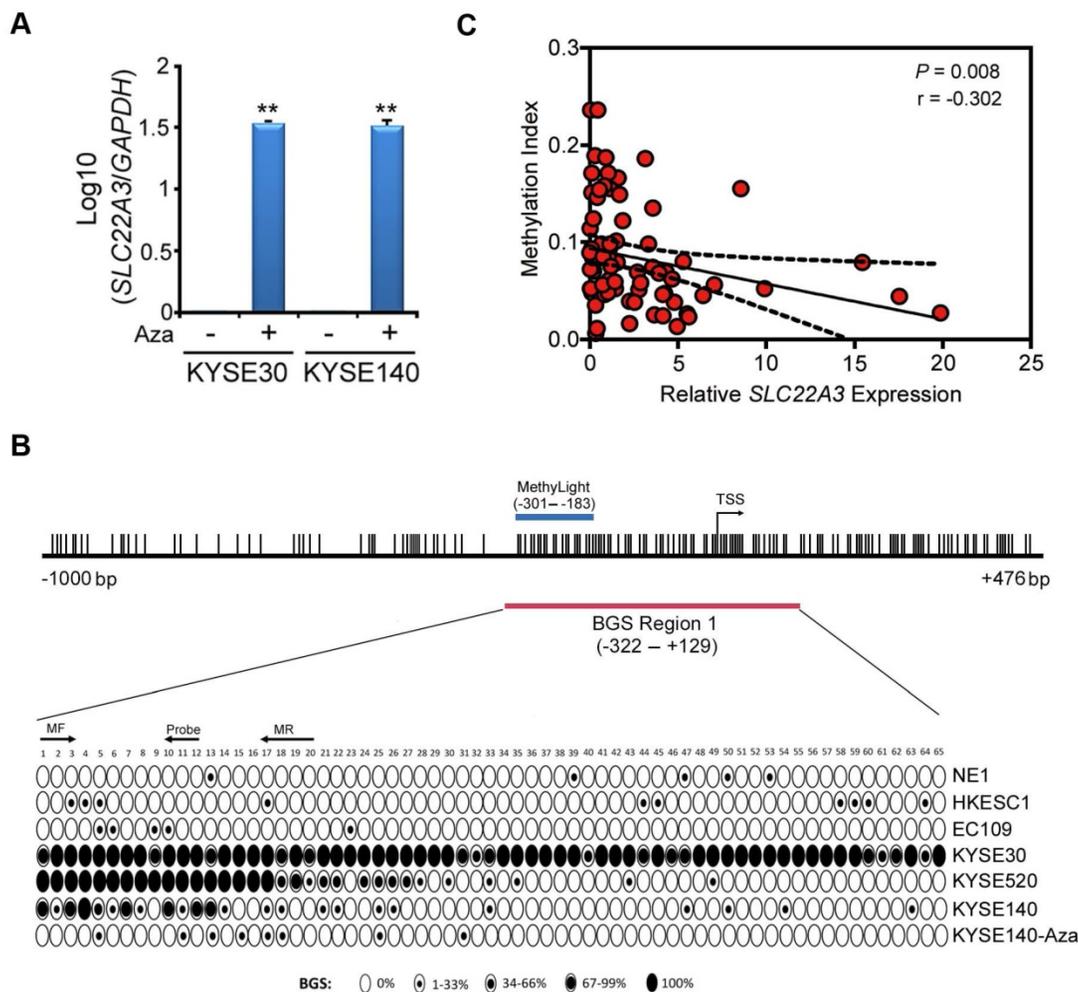
### *SLC22A3* downregulation was associated with its promoter methylation in ESCC

We have previously reported that *SLC22A3* expression was downregulated in 7 ESCC cell lines compared to normal esophageal epithelial cell line NE1 [13]. Promoter hypermethylation, especially in tumor suppressor genes, is an important mechanism of transcript silencing in tumourigenesis [20, 21]. As

*SLC22A3* promoter region contains a CpG island, we speculated that *SLC22A3* downregulation in ESCC is associated with its promoter methylation. Two ESCC cell lines (KYSE30 and KYSE140) without *SLC22A3* expression were treated with 5-Aza-2'-deoxycytidine (5-Aza), a DNA methyltransferase inhibitor. After 5-Aza treatment, *SLC22A3* expression was dramatically restored, suggesting that *SLC22A3* methylation was associated with its downregulation in ESCC (Figure 2A). We next used a CpG-island searcher to study the 5'-region of the *SLC22A3* gene (-1000 to +476) and found it was located right in a CpG island (Figure 2B). The methylation pattern of *SLC22A3* was further analyzed in more detail, using high-resolution bisulfite genomic sequencing (BGS). A 451-base pair BGS region (BGS region 1: -322 to +129 relative to the transcription start site) composed of 65 CpG sites were analyzed (Figure 2B). In all cell lines studied, *SLC22A3* BGS region 1 (proximal promoter) showed varying degree of methylation density and intra-sample heterogeneity of methylation (Figure 2B). Next, we designed a MethyLight assay overlaid on the 5' regions of the BGS region 1 (-301 to -183) and assessed the CpG sites from 1 to 20, as indicated in Figure 2B. A positive result would be obtained if there were methylation at the 3' ends of each primer (CpGs 3 and 17) and the probe binding site covering CpGs 10, 11, and 12. Intriguingly, *SLC22A3* promoter methylation showed a significantly negative correlation with its mRNA level in 76 family history FH+ NT tissues (Spearman  $r = -0.302$ ,  $P = 0.008$ ; Figure 2C) [13], suggesting that promoter hypermethylation of *SLC22A3* contributes to its gene silencing in familial ESCC.



**Figure 1.** *SLC22A3* is a novel tumor susceptibility gene in familial ESCC. Regional association plots for the *SLC22A3* gene at 6q25.3. The  $-\log_{10} P$  values (y axis) of 114 SNPs in 496 FH+ ESCC cases and 1056 controls are presented according to their chromosomal positions (x axis). Genetic recombination rates, based on CHB (Han Chinese in Beijing, China) and JPT (Japanese in Tokyo, Japan) samples from the HapMap Project, are represented by light-blue lines, and genes within the regions are depicted by arrows. The top genotyped SNP is labeled by rs ID, and the  $r^2$  values of the rest of the SNPs with the top genotyped SNP are indicated by different colors.



**Figure 2.** *SLC22A3* promoter methylation correlates with its reduced expression in ESCC cell lines and familial ESCC. (A) Restoration of *SLC22A3* expression was observed in KYSE30 and KYSE140 cells after Aza-treatment.  $\beta$ -actin was used as an internal control. (B) A 1476-bp region containing the putative *SLC22A3* promoter and its first exon was analyzed, and the region was found to be located within a CpG island (CGI). The locations of the two bisulfite genomic sequencing (BGS) regions and one quantitative methylation-specific PCR (MethylLight) region are indicated. The transcription start site (TSS) is denoted by an arrow. The CpG sites within the CGI are shown by vertical bars. For each sequenced cell line, the percentage methylation of each CpG was determined as the percentage of methylated CpGs from 10 randomly sequenced clones. Black or white circles, completely methylated or completely unmethylated CpGs, respectively; Concentric circles, partially methylated CpGs. (C) Correlations among *SLC22A3* expression and DNA methylation in familial ESCC. Correlations between the relative expression levels of *SLC22A3* and promoter methylation in NT specimens from 76 FH+ ESCCs. The data are expressed using Spearman correlation coefficients, linear regression lines (solid) and 95% CI lines (dotted).

### ***SLC22A3* methylation confers susceptibility to ESCC in a high-risk population**

Our previous study showed that the *SLC22A3* mRNA level was significantly lower in FH+ NT tissues compared with that in FH- NT tissues [13], indicating that methylation-mediated downregulation of *SLC22A3* could be an earlier event in ESCC high-risk individuals. We first used BGS to obtain a detailed analysis of methylation in NT esophageal epithelia, which were isolated from randomly selected FH+ (n = 10) and FH- ESCC cases (n = 10). Interestingly, the *SLC22A3* proximal promoter showed a distinctly different pattern of methylation in FH+ NT esophageal epithelia compared with that in FH- NT tissues (Figure S1). The entire BGS region 1 was largely lack of methylation in all ten FH- NT tissues, but heterogeneous methylation was found in 5 of 10 FH+ NT tissues. To determine whether this

type of methylation is an early event, we next sequenced bisulfite-modified peripheral blood leukocyte (PBL) DNA from FH+ (n = 10) and FH- (n = 10) ESCC patients (Figure S2). Similar to the methylation pattern observed in NT esophageal epithelia, the blood DNA samples also showed inter-individual variations of *SLC22A3* methylation, of which FH+ blood DNA samples showed a higher degree of methylation than FH- samples (Figure S2). To further prove that *SLC22A3* hypermethylation is an early event that predisposes high-risk individuals to develop esophageal cancer, we extended the PBL DNA methylation study to case-control sample sets, including 133 cases and 115 controls recruited from an ESCC high-risk region of North China (high-risk cohort), as well as 112 cases and 100 controls from an ESCC low-risk region of South China (low-risk cohort). Initially, we conducted bisulfite sequencing

to reveal the detailed methylation profile of *SLC22A3* in high- and low-risk cohorts (n = 10 for each group). As shown in Figure 3, the highest degree of *SLC22A3* methylation was found in the FH+ cases, while the lowest degree of *SLC22A3* methylation was detected in the healthy controls from the low-risk region. From the results of the MethyLight assay, we found that the methylation level of *SLC22A3* was significantly higher in ESCC cases than that in healthy controls from the high-risk region [Median Methylation Index (MI): 0.065 vs. 0.040; P = 1.12e-08; Figure 4A]. However, the methylation level of *SLC22A3* showed no difference between cases and controls from the ESCC low-risk region (Median MI: 0.030 vs. 0.027; P = 0.475; Figure 4A). We further stratified the high-risk ESCC cases into FH+ (n = 49) and FH- (n = 84); the results showed that the methylation level of *SLC22A3* was significantly higher in FH+ cases than that in FH- cases (Median MI: 0.080 vs. 0.056; P = 0.0025; Figure 4B). Moreover, a conditional logistic regression model revealed that *SLC22A3* promoter hypermethylation was significantly associated with ESCC risk in the high-risk cohort (OR per 1% increment in *SLC22A3* methylation = 1.352, 95% CI = 1.208-1.514) but not in the low-risk cohort (OR = 1.070, 95% CI = 0.9364-1.223; Table 1), indicating that early *SLC22A3*

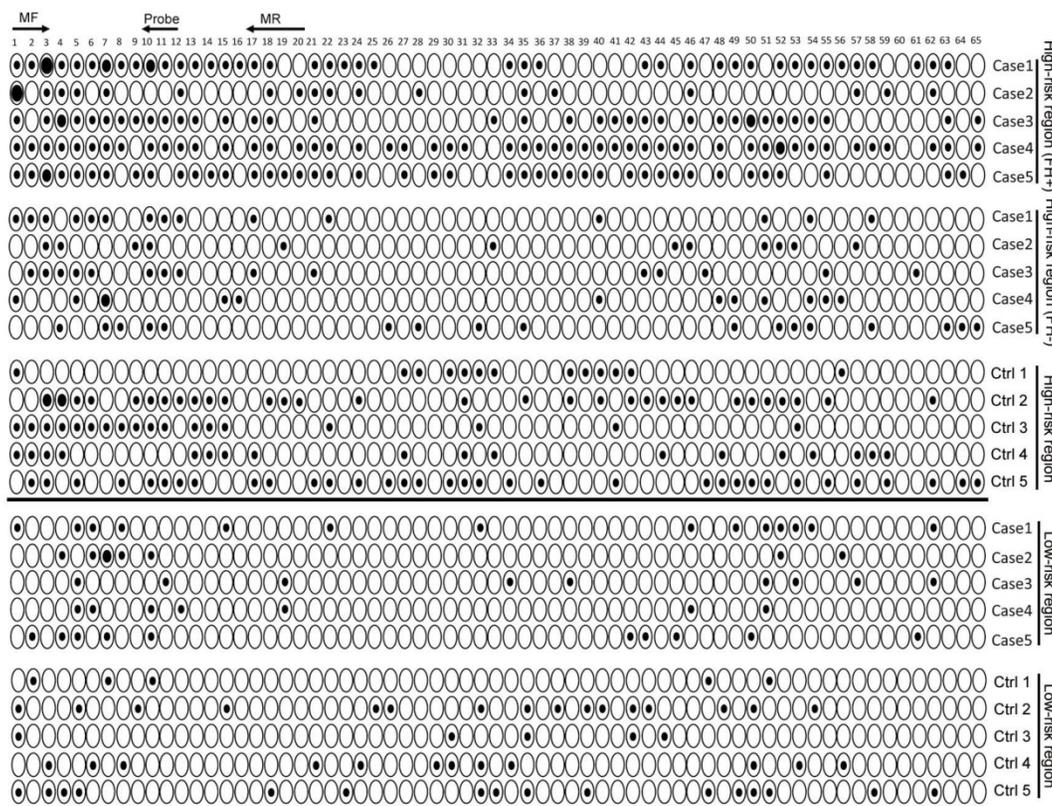
hypermethylation contributes to ESCC susceptibility and may serve as an effective marker for earlier screening.

**Table 1.** Association between *SLC22A3* methylation and risk of ESCC

Group	<i>SLC22A3</i> Methylation (1% increment)	
	OR(95% CI)	AdjOR*(95% CI)
<b>High-risk region</b>		
Cases/Controls 133/115	1.335(1.198-1.489)	1.352(1.208-1.514)
<b>P-value</b>		
Likelihood ratio	1.05E-09	2.06E-11
Wald test	1.94E-07	4.88E-08
Score test	2.89E-08	1.59E-09
<b>Low-risk region</b>		
Cases/Controls 112/100	1.058(0.9274-1.208)	1.070(0.9364-1.223)
<b>P-value</b>		
Likelihood ratio	0.397	0.089
Wald test	0.399	0.098
Score test	0.398	0.092

***SLC22A3* protects normal esophageal cells from heat stress-induced reactive oxygen species (ROS) and DNA damage**

*SLC22A3* is reported to be a heat stress responsive gene and plays a critical role in cellular



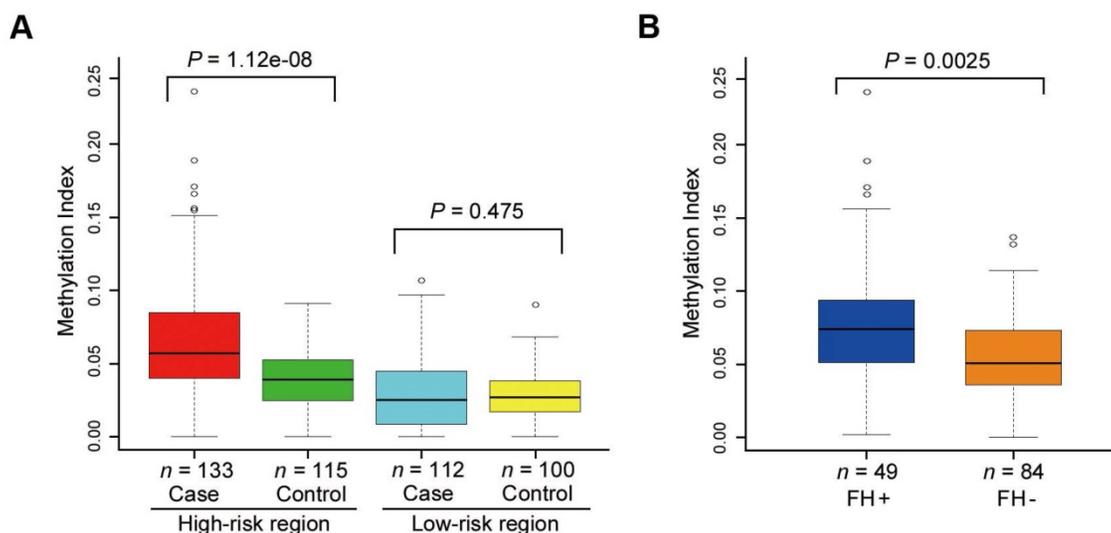
**Figure 3.** *SLC22A3* promoter methylation status in ESCC high- and low-risk cohorts. Representative high-resolution methylation profiles obtained by bisulfite sequencing of BGS region I (-322 to +129), comprised of 65 CpG sites in the *SLC22A3* promoter, in randomly selected cases and controls from ESCC high- and low-risk regions. For each sequenced blood leukocyte (PBL) DNA sample, the percentage methylation of each CpG was defined as the percentage of methylated CpGs from 8-10 randomly sequenced clones. Black circle, completely methylated CpG; White circle, completely unmethylated CpG; Concentric circle, partially methylated CpG.

detoxification [15, 22]. We explored whether *SLC22A3* could protect normal esophageal cells from heat stress-induced ROS and DNA damage. To verify the antioxidant function of *SLC22A3* in normal esophageal cells, we knocked down the endogenous *SLC22A3* expression in NE1 and NE3 cells (NE1/NE3-sh*SLC22A3*) by lentiviral transfection. Non-template short hairpin RNA-transfected cells (NE1/NE3-NTC) were used as control (Figure 5A). MitoSOX Red dye staining was performed to examine intracellular ROS levels in NE1/NE3-sh*SLC22A3* and NE1/NE3-NTC after heat shock. As shown in Figure 4B, after heat shock, a more significant increase in the mitochondrial ROS was detected in *SLC22A3*-repressed NE1/NE3 cells compared with control cells, evident by more mitochondrial fluorescence in the cell bodies. We next investigated whether the oxidative stress-induced DNA damage is enhanced in *SLC22A3*-repressed NE1/NE3 cells. Alkaline Comet Assay revealed that NE1/NE3-sh*SLC22A3* exhibited higher levels of DNA damage in response to heat-shock than the control cells (Figure 5C, D). In addition, the formation of  $\gamma$ -H2AX foci, a marker of DNA double strand breaks, was increased in heat-shock treated NE1/NE3-sh*SLC22A3* cells compared with control cells (Figure 5E, F). Western blot demonstrated that *SLC22A3* expression was negatively correlated with the expression of DNA damage marker  $\gamma$ -H2AX in these cells (Figure 5G). All these data indicated that *SLC22A3* may act as an antioxidant molecule and protect normal esophageal cells from heat stress induced ROS and DNA damage.

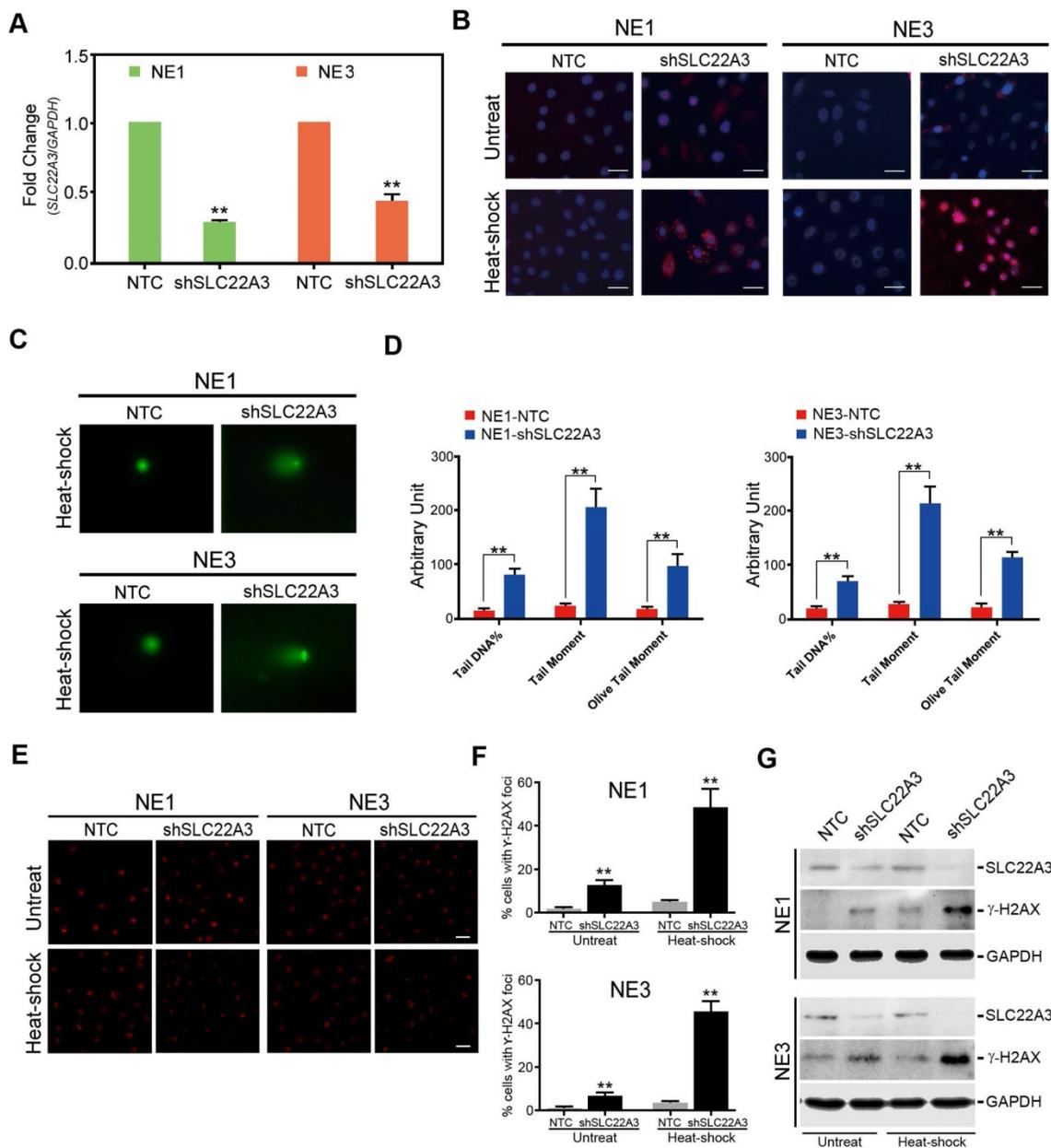
Collectively, we propose a disease model in which the epigenetic alterations of *SLC22A3*, predisposed in high-risk individuals with a family history, could accelerate the malignant transformation of normal esophageal epithelial cells under heat stress, leading to the development of ESCC (Figure 6).

## Discussion

Multiple evidence has established *SLC22A3* as a novel tumor susceptibility gene in high-risk individuals with a family history of ESCC in northern China. First of all, we identified *SLC22A3* as one key gene that is associated with familial ESCC. A post hoc GWAS analysis revealed that one SNP (rs9457930), located at ~45-kb downstream of the *SLC22A3* gene, is significantly associated with reduced ESCC risk in familial ESCC cases. This result is in line with our previous finding that *SLC22A3* is downregulated in familial ESCC cases, indicating that *SLC22A3* could be a protective factor and downregulation of this gene may confer susceptibility to ESCC. Secondly, epigenetic alterations of the *SLC22A3* gene contribute to its significantly lower expression in FH+ ESCCs. Finally, population methylation analyses demonstrate that a gain of *SLC22A3* promoter methylation is associated with an increased risk of developing ESCC in a northern Chinese population. Emerging evidence has shown that CpG island promoter hypermethylation of tumor suppressor genes is an early event in tumorigenesis, suggesting that epigenetic signatures are promising biomarkers for cancer risk and early neoplasia [23].



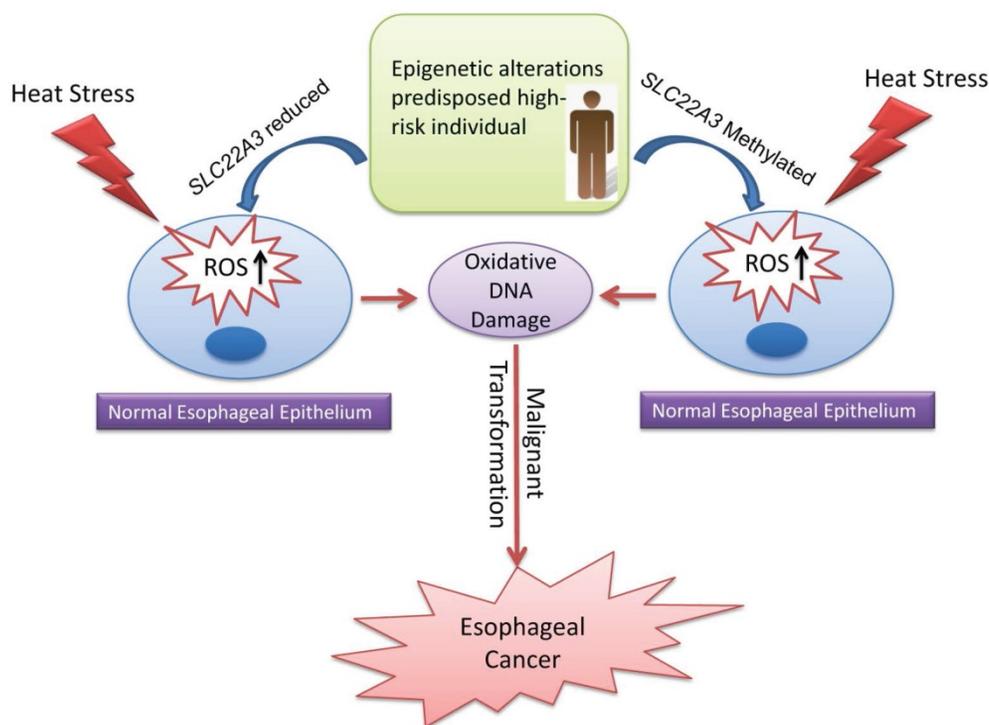
**Figure 4.** *SLC22A3* promoter methylation levels in ESCC high- and low-risk cohorts. (A) Tukey boxplots represent the *SLC22A3* promoter methylation indexes (MIs) for 133 ESCC cases and 115 age/sex matched controls from the high-risk region, as well as 112 ESCC cases and 100 matched controls from the low-risk region. Open circles, outliers; Rectangular box, range for the 25th to 75th percentile; Black line, median value. The measurement of the MIs was performed using real-time quantitative MSP (MethyLight), and the CpG-free ACTB genomic sequence was used for normalization. P values were calculated by the Wilcoxon rank sum test with continuity correction. (B) Tukey boxplots represent the *SLC22A3* promoter MIs for the 133 high-risk ESCCs further stratified into cases with family history (FH+; n = 49) and cases without family history (FH-; n = 84). P values were calculated by the Wilcoxon rank sum test with continuity correction.



**Figure 5.** *SLC22A3* protects normal esophageal cells from heat stress-induced ROS and DNA damage. **(A)** Expression of *SLC22A3* in shSLC22A3 stably transfected NE1 cells and NE3 cells were confirmed by qPCR. Nontemplate shRNA-transfected cells (NE1-NTC and NE3-NTC) were used as controls. \*\*,  $P < 0.001$  by Student's *t*-test. **(B)** Representative images of MitoSOX Red dye staining in *SLC22A3* knockdown NE1 cells and NE3 cells with and without heat-shock treatment. Scale bar = 20  $\mu$ m. **(C)** Alkaline Comet assay showing DNA break formation after heat-shock treatment shSLC22A3 transfected NE1 cells and NE3 cells. NE1-NTC and NE3-NTC were used as controls. **(D)** Percentage of Tail DNA, Tail moment (fraction of DNA in the tail  $\times$  tail length) and Olive Tail Moment were analyzed with CASP software. The data were calculated from 50 comets for each sample. \*\*,  $P < 0.001$  by Student's *t*-test. **(E)** Cells (NE1-shSLC22A3, NE1-shSLC22A3, NE1-NTC and NE3-NTC) that were treated with or without heat-shock were fixed and immunolabeled with an anti- $\gamma$ -H2AX antibody. (Magnification: 40 $\times$ ). Images were performed by ZEN 2012 software. **(F)** Quantification of  $\gamma$ -H2AX-positive cells in (E). The  $\gamma$ -H2AX-positive cells refer to the cells with  $\geq 5$   $\gamma$ -H2AX foci. The data represent the means  $\pm$  SD ( $n \geq 100$ ) from three independent experiments. **(G)** Expression of *SLC22A3* and  $\gamma$ -H2AX of these cells were examined by Western blotting.

With the rapidly developed methylation analysis technologies, cancer-specific hypermethylation events could be detected in more easily accessible biological material, such as sera, feces, urine, and sputum [24-27]. To date, several studies have reported that the epigenetic alterations detected in peripheral blood cell DNA could be a valuable predictive marker for people with a high risk for cancer [28-30]. Here, we also provide the first evidence that DNA methylation

of the *SLC22A3* gene in peripheral blood cells is associated with an increased esophageal cancer risk. However, to determine the diagnostic value of *SLC22A3* methylation in an ESCC high-risk population, large-scale case-control cohorts need to be further investigated. Moreover, our methylation study also shows pronounced inter-allelic heterogeneity of methylation with varying degrees of methylation density at the *SLC22A3* promoter in the



**Figure 6.** Proposed model illustrating the epigenetic alterations of *SLC22A3* predispose in high-risk individuals, accelerate malignant transformation of normal esophageal epithelia under heat stress and lead to the development of ESCC.

NT tissues from ESCC patients. In line with previous observations, methylation heterogeneity or epigenetic mosaicism observed in cancer precursor tissues suggests that this epigenetic variability could reflect a type of genomic instability in the early development of cancer [31].

Numerous evidence has proved that long-term consumption of hot food and drink was associated with an increased risk of esophageal cancer [32, 33]. In response to frequent heat stress, normal esophageal epithelia may accumulate DNA damage and become carcinogenic. In the *SLC22A3* knockdown stable cell line (NE1/NE3-sh*SLC22A3*), its intracellular ROS level increased leading to higher level of DNA break formation after heat shock, compared to that in NE1/NE3-NTC. ROS generation is often triggered by various forms of stress, including oxidative stress, heavy metal stress and heat stress [34, 35]. The unfavorable effects of ROS include oxidative damage to DNA, which is now generally believed to be a risk factor in carcinogenesis [36, 37]. DNA damage-related mutagenesis occurs in a wide range of human precancerous lesions, and is associated with early tumorigenesis [38, 39]. A recent study revealed that intraepithelial neoplasia (IEN) expressed high levels of  $\gamma$ -H2AX and shared similar genetic variant spectrum with ESCC, implying that defects in DNA damage repair and accumulation of mutation burden could contribute to the progression from IEN to ESCC [40]. Intriguingly, a previous epidemiological study

found that frequent precursor lesions detected in an ESCC high-risk population in China are significantly associated not only with the consumption of burning hot beverages but also with a family history of esophageal cancer [41]. Taken together, these results demonstrated that *SLC22A3* promoter hypermethylation may directly suppresses its gene transcription, resulting in increased susceptibility to ESCC in high-risk individuals. With the rapid development of methylation analysis technologies, detecting DNA methylation of *SLC22A3* in sera, feces, urine, and sputum could be novel diagnostic and prognostic method for familial ESCC in the future.

## Abbreviations

ESCC: esophageal squamous cell carcinoma  
*SLC22A3*: solute carrier family 22 member 3  
 GWAS: post hoc genome-wide association studies  
 EC: esophageal carcinoma  
 NT: non-tumor  
 FH: family history  
 PBL: peripheral blood leukocyte  
 BGS: bisulfite genomic sequencing  
 IF: immunofluorescence staining  
 MI: methylation index  
 OR: odds ratio  
 CI: confidence interval  
 ROS: reactive oxygen species

$\gamma$ -H2AX: phosphorylated (serine 139) H2A histone family, member X  
CpG: 5'-C-phosphate-G-3'

## Supplementary Material

Supplementary figures and tables.

<http://www.ijbs.com/v14p1658s1.pdf>

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## Ethics approval and consent to participate

This study was approved by the institutional review board of Shenzhen University. Informed consents were obtained for the original human work that produced the tissue samples.

## Competing Interests

The authors have declared that no competing interest exists.

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