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ORIGINAL RESEARCH

Epigenetic Study of Esophageal Carcinoma Based on Methylation, Gene Integration and Weighted Correlation Network Analysis

Yanzhao Xu[1]
Na Wang[2]
Rongfeng Liu[3]
Huilai Lv[1]
Zhenhua Li[1]
Fan Zhang[1]
Chunyue Gai[1]
Ziqiang Tian[1]

[1]Department of Thoracic Surgery, [2]Department of Cancer Institute, [3]Department of Oncology, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei, 050011, People's Republic of China

Purpose: Esophageal carcinoma is a common and highly metastatic malignant tumor of the digestive tract. The aim of the present study was to identify potential molecular markers of esophageal carcinoma that may help its diagnosis and treatment.

Materials and Methods: First, mRNA and DNA methylation data were downloaded from The Cancer Genome Atlas (TCGA) database for the identification of differentially expressed genes (DEGs) and DNA methylation analysis. Secondly, Weighted Gene Co-Expression Network Analysis (WGCNA) was used to identify important modules and hub genes. In addition, correlation analysis between DNA methylation genes and DEGs was performed. Thirdly, the GSE45670 dataset was used to validate the expression of the diagnostic and survival ability analysis of genes in TCGA data. Finally, reverse transcription-quantitative PCR and immunohistochemical analysis of genes were performed.

Results: A total of 2408 DEGs and 5134 differentially methylated sites were obtained. In the WGCNA analysis, the royal blue module was found to be the optimal module. In addition, hub genes in the module, including ESRRG, MFSD4, CCKBR, ATP4B, ESRRB, ATP4A, CCKAR and B3GAT1, were also differentially methylated genes and DEGs. It was found that CCKAR, MFSD4 and ESRRG may be diagnostic gene biomarkers for esophageal carcinoma. In addition, the high expression of MFSD4 was significantly correlated with patient survival. Immunohistochemistry analysis results showed that the gene expression levels of ATP4B, B3GAT1, CCKBR and ESRRG were decreased in esophageal carcinoma tissues, which was in line with the bioinformatics results.

Conclusion: Therefore, these identified molecular markers may be helpful in the diagnosis and treatment of esophageal carcinoma.

Keywords: esophageal carcinoma, DNA methylation, Weighted Gene Co-Expression Network Analysis; differentially expressed genes, diagnosis, prognosis

Introduction

Esophageal carcinoma is a common type of cancer of the digestive tract with a high morbidity and mortality, and its morbidity varies significantly among different regions and ethnic groups.[1,2] In China, the morbidity and mortality rates of esophageal carcinoma are higher in men than in women.[3] Patients with esophageal carcinoma have no obvious symptoms in the early stage, and present with typical dysphagia in the middle and late stages.[4] To date, the most common treatment for esophageal carcinoma is surgical resection. However, most patients with esophageal carcinoma are already in the middle and advanced stages of the disease upon

Correspondence: Ziqiang Tian
Department of Thoracic Surgery, The Fourth Hospital of Hebei Medical University, No. 12, Jiankang Road, Shijiazhuang, 050011, People's Republic of China
Email tianziqianghebei@163.com

admission.[5] In these patients, tumor metastasis leads to the recurrence of esophageal carcinoma, ultimately resulting in the failure of surgical treatment.[6]

Although the specific pathological mechanisms of esophageal carcinoma are unclear, certain studies have reported various genes involved in the progression of the disease. Previous studies have found that solute carrier family 52 member 3 (SLC52A3) was up-regulated in esophagus dysplasia and esophageal squamous cell carcinoma (ESCC), and can therefore be used as a biomarker for the prediction and prognosis of esophageal carcinoma.[7] The expression levels of phospholipase C epsilon 1 (PLCE1) and protein kinase C alpha (PRKCA) are up-regulated in esophageal carcinoma, an increase that is significantly associated with poor prognosis.[8] In addition, insulin-like growth factor binding protein like 1 (IGFBPL1)[9] SHANK associated RH domain interactor (SHARPIN)[10] and TNF alpha induced protein 8 like 2 (TIPE2),[11] among others, are involved in the regulatory mechanism of esophageal carcinoma.

Epigenetic modifications, such as DNA methylation, can change gene expression patterns and play an important role in cancer formation.[12] DNA methylation has been extensively investigated and is used for the diagnosis and treatment of tumors.[13] Abnormal hypermethylation is associated with the inactivation of tumor-related genes in multiple tumor types.[14] Previous studies have reported that, compared with healthy controls, patients with esophageal carcinoma exhibit abnormal p16 gene methylation. In the case analysis based on p16 methylation, the mutation/polymorphism of p53 was found to be significantly associated with the risk of esophageal carcinoma.[15] Moreover, the cyclin-dependent kinase inhibitor 2A (CDKN2A) is a tumor suppressor gene that significantly increases the methylation frequency during esophageal carcinoma carcinogenesis, and may be a biomarker for the early diagnosis of esophageal carcinoma.[16] In addition, one-carbon metabolism-related B vitamins may interact to affect esophageal carcinoma and dysplasia risks, since vitamin B2, B6, B12 and folate play important roles in DNA synthesis, repair and methylation.[17]

Therefore, analyzing the correlation between gene transcription sequencing data and DNA methylation modification data is important for elucidating the epigenetic regulation mechanism of esophageal carcinoma. In the present study, the DNA methylation and gene expression data of esophageal carcinoma were analyzed, and Weighted Gene Co-Expression Network Analysis (WGCNA) was performed to identify important modules and hub genes. Finally, 8 candidate genes (ESRRG, MFSD4, CCKBR, ATP4B, ESRRB, ATP4A, CCKAR and B3GAT1) were identified as potential diagnostic and treatment targets in esophageal carcinoma.

Materials and Methods

Datasets

In the present study, mRNA expression (IlluminaHiSeq_RNASeqV) and DNA methylation data (JHU_USC_HumanMethylation450) were downloaded from The Cancer Genome Atlas data portal (TCGA, https://tcga-data.nci.nih.gov/tcga/). The data were from primary solid tumor tissue samples of patients with esophageal carcinoma and normal tissue samples of a normal control group. The information of 185 samples was used for mRNA expression and DNA methylation data analysis. Among them, there were 170 common samples (161 cases and 9 normal controls) between mRNA expression and DNA methylation data. The clinical information of 185 patients with esophageal carcinoma from TCGA database was analyzed. Chi-square test was used to verify the significance of the difference. The results showed no significant difference in disease stage or outcome between the ESCC and esophageal adenocarcinoma (EAC) groups (Table S1). Therefore, ESCC and EAC were grouped together into a disease group for unified analysis.

Differential Analysis of Genes and DNA Methylation

The differentially expressed genes (DEGs) were screened out using the R-Bioconductor package DESeq.[18] First, the genes (count=0) with a distribution of >20% in the sample were filtered. Next, false discovery rate (FDR)<0.05 and |log2FoldChange|>1 were used to identify DEGs.

The COHCAP package in R was used to screen differentially methylated sites.[19] The Δβ is the value of differentially methylated sites. First, the methylated sites without a β-value and a distribution of >20% in the sample were filtered. Next, Δβ>0.2 and FDR<0.05 were used to obtain differentially methylated sites and regions. Subsequently, a Fisher's Exact test (odds ratio≠1, and P<0.05 or P<0.01) was used for functional enrichment analysis of the genome in the differentially methylated sites.

Construction of the WGCNA Co-Expression Network

First, the WGCNA package in R (http://www.r-project.org/) was used to identify significant modules and genes in esophageal carcinoma under the threshold of β=5. Next, |correlation coefficient|>0.2 and P<0.05 were used to explore the correlation between modules and tumor-paracancer. A Fisher's Exact test (P<0.05) was then used for enrichment analysis, to explore the relationship between modules and DEGs. Finally, Cytoscape software (http://www.cytoscape.org) was used for the visualization of the optimal module with a threshold of >0.1. The selection criteria for hub genes in the optimal module was |correlation. module module| (|Cor.MM|), which was used to rank top 10 genes.

Functional Analysis of Genes in the Optimal Module

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of genes in the optimal module were performed using the GeneCodis 4.0 (https://genecodis.genyo.es/). FDR<0.05 was set as the threshold of statistical significance.

Correlation Analysis of DNA Methylation Genes and DEGs

Correlation analysis between DNA methylation gene and gene expression profile was mainly to analyze the correlation between differentially methylated sites and corresponding gene expression differences. First, the up/down-regulation relationship between differentially methylated gene CpG islands and DEGs was integrated. Next, P<0.05 and |correlation| (|cor|)>0.5 were used to analyze the correlation between the differentially methylated sites and the DEGs. Finally, P<0.05 was used to identify the DEGs that were significantly negatively correlated with differentially methylated regions/sites.

Expression Validation, and Diagnostic and Prognostic Analysis of Identified Genes

The expression validation of identified genes was performed in the GSE45670 data set (involving 28 cases and 10 normal controls) which was obtained from the Gene Expression Omnibus (GEO) database.[20] In addition, TCGA data were used to analyze the diagnostic and survival ability of the identified DEGs.

In vitro Validation of Identified DEGs

The detailed inclusion criteria for patients with esophageal carcinoma were as follows: (1) Patients who had received simple surgical treatment as initial treatment; (2) patients had complete preoperative imaging data of computed tomography (CT), esophagoscopy and esophagus; (3) patients were diagnosed with esophageal carcinoma by histopathological or cytological examination; (4) patients aged ≤80 years (to avoid confusion with natural death); (5) patients had no history of other malignant tumors. The detailed exclusion criteria for patients with esophageal carcinoma were as follows: (1) Patients with other tumors; (2) patients did not meet the diagnostic and inclusion criteria; (3) patients had a metastatic malignant tumor of the esophagus; (4) patients presented with recurrence or received secondary treatment; (5) patients died during hospitalization or discharge within 30 days (during this period, the cause of death of patients was mostly surgical wounds, not the disease itself); (6) incomplete basic information of patients on admission; (7) patients who suffered from cognitive impairment and multiple organ failure.

According to the above criteria for esophageal carcinoma, 6 patients with esophageal carcinoma were selected for the present study. The tumor and adjacent normal tissue samples were subjected to reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using a TRIzol (tissue sample) kit (Invitrogen; Thermo Fisher Scientific, Inc.). A FastQuant cDNA synthesis kit (KR106; Tiangen Biotech Co., Ltd.) was used for mRNA reverse transcription. RT-qPCR was performed using SuperReal PreMix Plus (SYBR Green), which was carried out using SuperReal reagent (FP205; Tiangen Biotech Co., Ltd.) manual. Each experiment was repeated three times. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin beta (ACTB) were used as internal control for gene detection. The gene expression levels were calculated as fold-changes using the $2^{-\Delta\Delta Ct}$ method.[21]

All experimental procedures were approved by the Clinical Research Ethics Committee of The Fourth Hospital of Hebei Medical University (NO.2020KY183). All participants were informed as to the purpose of this study, and that this study complied with the Declaration of Helsinki.

Immunohistochemical Analysis

Tumor and adjacent normal tissue samples were obtained for immunohistochemical analysis. A total of 4 genes

(ATP4B, B3GAT1, CCKBR and ESRRG) were randomly selected from the candidate genes for validation. Immunohistochemistry was performed using the streptavidin-peroxidase (SP) method. Paraffin sections of tissue samples (4μm) were dewaxed routinely and washed with phosphate-buffered saline (PBS) for three times (5 min/time). These sections were repaired with pH 6.0 citric acid at high pressure for 3 min, kept warm for 30 min and cooled to room temperature, and then washed with PBS three times (5 min/time). The slides were immersed in 3% H2O2 for 30 min to block endogenous peroxidase activity and were then washed with PBS three times (5 min/time). The slides were sealed with goat serum for 30 min to block natural or impure antibodies. After adding the primary antibodies (ATP4B, B3GAT1, CCKBR and ESRRG), the slides were incubated overnight at 4°C, washed and rewarmed for 30 min for the next day, washed with PBS three times (5 min/time). The secondary antibody was added dropwise and the sections were incubation at room temperature for 30 min, and washed with PBS three times (5 min/time). Finally, 3,3′-diaminobenzidine (DAB) staining, hematoxylin restaining and microscopic observation were performed. Brown-yellow coloring was defined as positive expression.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc.). For the RT-qPCR verification assay, one-way analysis of variance method was used to statistically analyze the difference in gene expression. $P<0.05$ was considered to indicate a statistically significant difference. All analyses were repeated at least three times independently.

Results

Identification of DEGs and DNA Methylation Analysis

According to the screening criteria of FDR<0.05 and |log2FoldChange|>1, a total of 2408 DEGs were obtained. Among them, 1311 were up-regulated and 1097 were down-regulated. The top 10 up-regulated genes were MMP11, TPX2, ESM1, BIRC5, NUF2, TDO2, KIF18A, KIF23, NEK2 and UBE2T. The top 10 down-regulated genes were TMED6, AQP4, PGA3, SIGLEC11, CKMT2, GPR155, CKM, ESRRB, STX12 and SLC1A2.

Following the pre-treatment of methylation chip data without a β-value, 395,516 sites were obtained. A total of 5134 differentially methylated sites (4151 hypermethylated and 983 hypomethylated sites) were obtained under the screening criteria of Δβ>0.2 and FDR<0.05. The Manhattan figure and volcano map of these differentially methylated sites are presented in Figure 1. The heatmap of the top 200 differentially methylated sites in terms of tumor stage, histological diagnosis and sex is shown in Figure 2. The enrichment results of genomic features of differentially methylated sites are shown in Table 1. In addition, 2940 differentially methylated CpG islands were obtained using Δβ>0.2 and FDR<0.05. Among them, there were 2637 hypermethylation and 303 hypomethylation regions.

Analysis of the WGCNA Co-Expression Network

A total of 49 modules were identified by WGCNA co-expression network analysis (β=5; Figure 3). According to the correlation analysis between modules and tumor-paracancer, modules and DEGs, the royal blue module was identified as the optimal module (Tables 2 and 3). The visualization of the royal blue module, which consisted of 88 nodes, 1211 edges and 10 hubs, is shown in Figure 4. The details of these 10 hub genes are shown in the Table 4.

Functional Analysis of Genes in the Royal Blue Module

According to KEGG enrichment analysis, “gastric acid secretion” and “protein digestion and absorption” were the only two significantly enriched signaling pathways (Table S2). In addition, on the basis of GO enrichment analysis (Tables S3–S5), “ion transport” and “transmembrane transport” were the significantly enriched biological processes; “integral component of membrane” and “membrane” were the significantly enriched cellular components; “voltage-gated ion channel activity” and “chloride channel activity” were the significantly enriched molecular functions.

Correlation Analysis of DNA Methylation Genes and DEGs

A total of 97 differentially methylated regions-differentially expressed genes relationship pairs (DMRs-DEGs, involving 85 DEGs) were identified, which included 96 pairs of hypermethylated and down-regulated

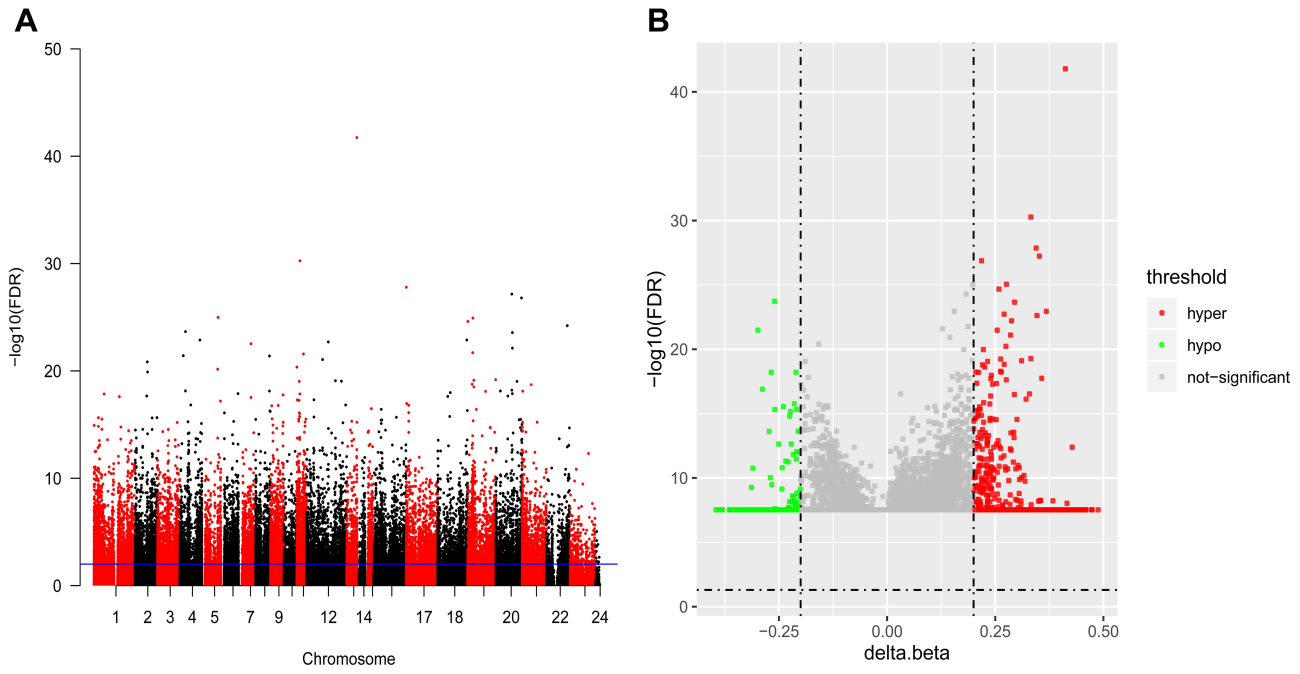

Figure 1 Manhattan figure and volcano map of differentially methylated sites. (**A**) Manhattan figure of differentially methylated sites in the chromosome. The X-axis and Y-axis represent the chromosome and the -log10 (FDR) of differentially methylated sites, respectively. (**B**) Volcano map of differentially methylated sites. The X-axis and Y-axis represents Δβ and -log10 (FDR), respectively. Green represent hypomethylation and and red represent hypermethylation. FDR, false discovery rate.

genes and 1 pair of hypomethylated and up-regulated genes. Based on correlation analysis (|cor|>0.5, P <0.05), 57 pairs of differentially methylated sites-DEGs (19 positive correlation and 38 negative correlation) were obtained (Table 5). The enrichment results of the above 85 DEGs in modules is presented in Table 6. The results showed that these DEGs were significantly enriched in the royal blue, yellow and turquoise modules (P<0.05). Moreover, it was found that ESRRG, MFSD4, CCKBR, ATP4B, ESRRB, ATP4A, CCKAR and B3GAT1 in hubs were also differentially methylated genes and DEGs.

Expression Verification, and Diagnostic and Prognostic Analysis

ATP4A, ATP4B, CCKAR, MFSD4 and ESRRG were randomly selected for expression verification (Figure 5). The expression levels of all aforementioned genes, except CCKAR, were down-regulated in esophageal carcinoma tissues. Among them, MFSD4 and ESRRG were the most significantly down-regulated (P<0.05). Simultaneously, diagnostic and prognostic analysis of these genes was performed (Figure 6). In the receiver operating characteristic (ROC) curve analysis, the area under curve (AUC) of ATP4A, ATP4B, CCKAR, MFSD4 and ESRRG was 0.605, 0.645, 0.805, 0.761 and 0.747, respectively (Figure 6A–E). It was indicated that the CCKAR, MFSD4 and ESRRG genes may be potential biomarkers for the diagnosis of esophageal carcinoma. Of note, the high expression of MFSD4 was found to have a prognostic value (Figure 6F), which showed that MFSD4 was significantly actively correlated with survival.

RT-qPCR Verification

The clinical information of enrolled individuals is presented in Table 7. GPR155, ESRRB, ESRRG, CCKAR, MMP11, TPX2, BIRC5, NUF2 and ANO1 were the top-ranking or reported genes selected for RT-qPCR verification. Among them, GPR155 (hypermethylated), ESRRB (hypomethylated), ESRRG (hypermethylated), CCKAR (hypermethylated) and MMP11 (hypermethylated) were differentially methylated genes, while TPX2, BIRC5, NUF2 and ANO1 were DEGs. All primers used are shown in Table 8. The results showed that MMP11, TPX2, BIRC5, NUF2 and ANO1 were up-regulated and ESRRG down-regulated (Figure 7), which was consistent with the results of the bioinformatics analysis. However, the results regarding GPR155, ESRRB and CCKAR were in contrast with those of the bioinformatics analysis. The small sample size may account for the inconsistent results, and so, further research is required.

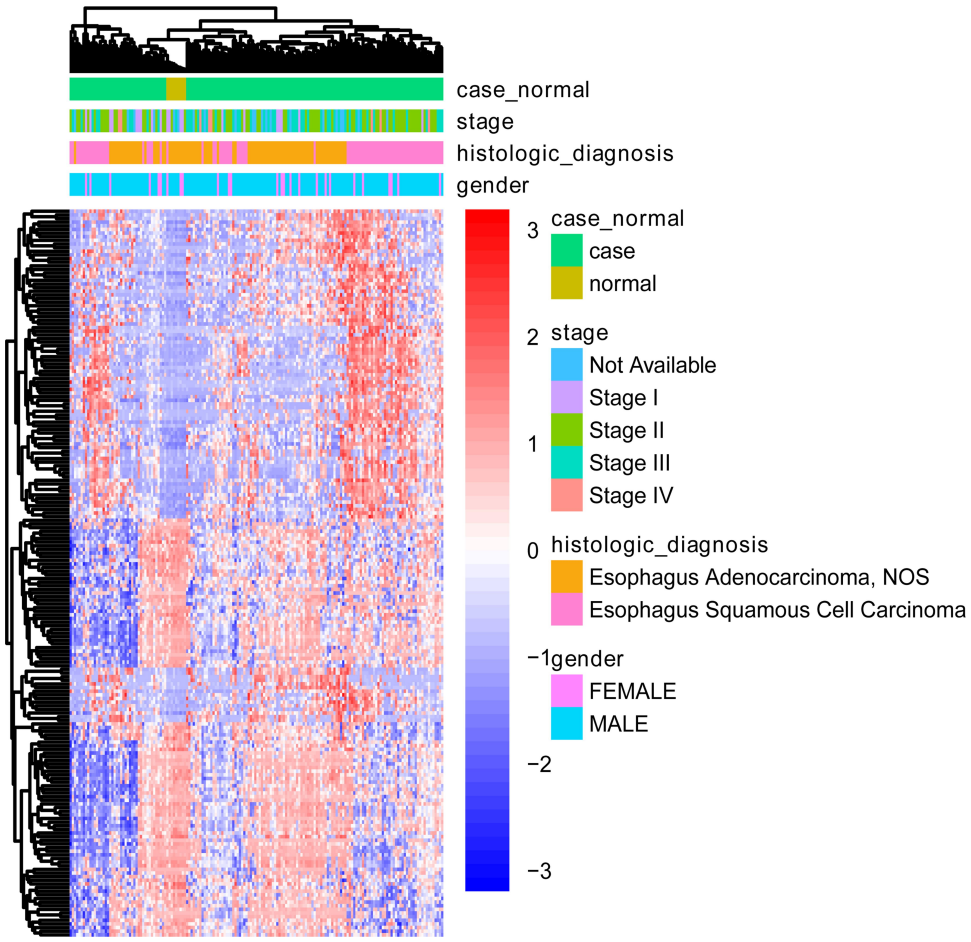

Figure 2 Heat map of the top 200 differentially methylated sites in terms of tumor stage, histologic diagnosis and sex. The figure shows the two-way hierarchical clustering results of the top 200 differentially methylated sites and samples. The clustering is constructed using the full chain method together with the Euclidean distance. Each row and column represents a differentially methylated sites and a sample, respectively. Differentially methylated sites clustering tree is shown on the right. Red indicates below the reference channel. Blue indicates the above reference.

Immunohistochemical Analysis of ATP4B, B3GAT1, CCKBR and ESRRG

During immunohistochemistry, cytoplasmic staining was observed in cells with positive staining. In addition, the gene expression of ATP4B, B3GAT1, CCKBR and ESRRG was negatively correlated with the occurrence and development of esophageal carcinoma. The number and degree of cells staining positive for ATP4B, B3GAT1, CCKBR and ESRRG genes

Table 1 Enrichment Results of Genomic Features of Differentially Methylated Sites

Feature	Genomic Context	Odds Ratio	95% CI_Lower	95% CI_Upper	P value
Gene feature	TSS200	1.308234	1.22967	1.391213	3.26E-17
	TSS1500	1.308234	1.22967	1.391213	3.26E-17
	3′UTR	1.308234	1.22967	1.391213	3.26E-17
	5′UTR	1.308234	1.22967	1.391213	3.26E-17
	1stExon	1.308234	1.22967	1.391213	3.26E-17
	Body	1.308234	1.22967	1.391213	3.26E-17
CpG island (CGI) feature	Island	0.525387	0.489485	0.563533	3.54E-82
	Shelf	1.032788	0.940418	1.132242	0.491955
	Shore	1.308234	1.22967	1.391213	3.26E-17

Abbreviations: CI, confidential interval; TSS, transcriptional start site.

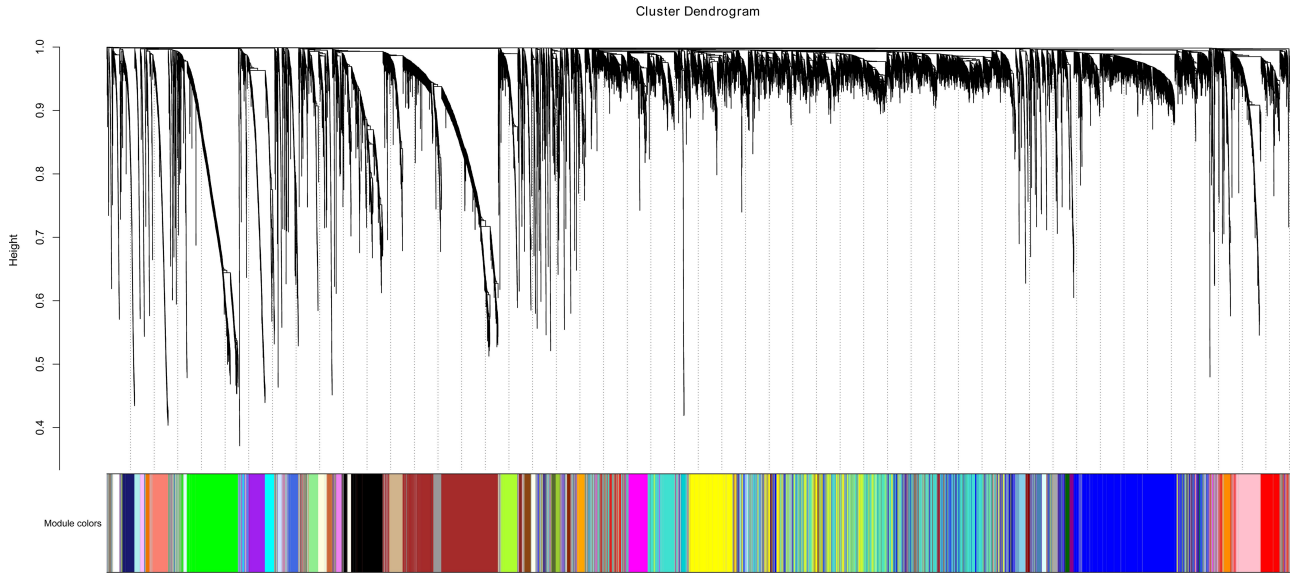

Figure 3 Clustering results of mRNA modules according to WGCNA analysis. The genes in the modules were marked with different colors, and the lower panel presents the color assigned to each module. WGCNA, Weighted Gene Co-Expression Network Analysis.

were significantly reduced in esophageal carcinoma tissues compared with adjacent normal tissues (Figure 8).

Discussion

ATPase H^+/K^+ transporting subunit alpha (ATP4A) encodes H^+, K^+-ATPase which plays an essential role in the secretion of gastric acid.[22] Previous studies have shown that ATP4A is associated with atypical familial type I gastric neuroendocrine tumors.[23] A homozygous missense mutation in the 14th exon of the ATP4A gene triggers changes in the transmembrane region and prevents the release of protons from cells to the stomach, causing a lack of stomach acid.[23] ATP4A is completely or partially methylated in tumor tissues. Following the use of a demethylating agent, the expression of ATP4A can be reactivated to inhibit the occurrence of gastric tumors.[24] In addition, ATP4A was identified as a significant gene associated with Barrett's esophagus.[25] ATPase H^+/K^+ transporting subunit beta (ATP4B) is an important tumor suppressor gene, whose down-regulation is associated with gastric cancer.[22,26] A notable consistency was identified between the downregulated and methylated states of ATP4B and ATP4A in gastric cancer tissues.[22] These studies indicated that ATP4A and ATP4B play an important role in the inhibition of cancer. In the present study, ATP4A and ATP4B were down-regulated and hypomethylated in esophageal carcinoma tissues. Therefore, we hypothesized that the down-regulation of ATP4A and ATP4B may be associated with DNA methylation. This further suggested that epigenetic changes of ATP4A and ATP4B may play an essential role in esophageal carcinoma.

Cholecystokinin A receptor (CCKAR) is a G protein-coupled receptor that can be activated by cholecystokinin

Table 2 Correlation Analysis Results Between the Modules and Tumor-Paracancer

Module	Coefficient	P value
Royalblue	−0.63	2.80E-20
Violet	0.26	0.00053
Diumpurple3	0.21	0.0059
Darkolivegreen	0.33	9.50E-06
Yellow	0.39	1.10E-07
Darkturquoise	0.26	0.00053
Turquoise	0.34	4.90E-06
Darkred	0.27	0.00032
Grey	0.34	7.80E-06

Table 3 Enrichment of Modules in DEGs

Module	Odds Ratio	95% CI_Lower	95% CI_Upper	P value
Royalblue	1115.129	192.8794	4.5E+15	4.54E-138
Violet	2.735852	1.720684	4.253173	2.30E-05
Yellow	0.357309	0.279922	0.45057	1.26E-23
Turquoise	0.494915	0.422081	0.577697	3.60E-22
Darkred	0.083847	0.010054	0.308444	9.07E-08

Abbreviation: CI, confidence interval.

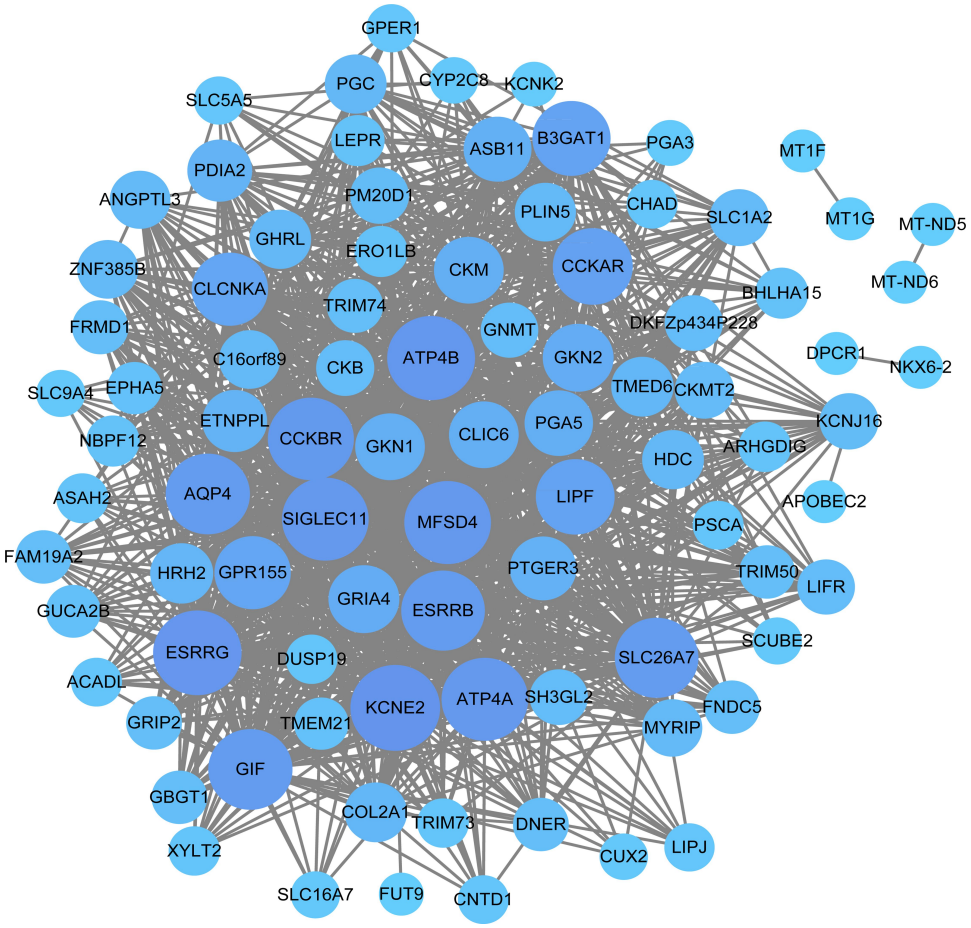

Figure 4 Visualization of the royal blue module in the WGCNA co-expression network. The circle and gray edge line represents the genes in the modules and the edge, respectively. The larger circle represent the hub genes. WGCNA, Weighted Gene Co-Expression Network Analysis.

(CCK), whose mutation can affect the risk of gallbladder cancer in women.[27] Previous studies have found that the expression level of CCKAR in a gallbladder with gallstones was significantly lower than that in a gallbladder without gallstones.[28] In addition, CCKAR has been found to be associated with tumor suppression and is used as a candidate gene for studying ESCC.[29] The expression level of CCKBR is decreased in breast cancer. A higher protein expression of CCKBR is found in gastric cancer tissue compared with normal mucosal tissue.[30] In addition, CCKAR and CCKBR have been shown to be up-regulated and down-regulated, respectively, in pancreatic cancer, suggesting that CCKAR and CCKBR may play different roles in different cancer types.[31] In the present study, CCKAR and CCKBR were down-regulated in esophageal carcinoma. In addition, CCKAR and CCKBR were hypermethylated and hypomethylated, respectively. We therefore hypothesized that the down-regulation of CCKAR and CCKBR may be associated with DNA methylation. This further suggested that epigenetic changes in CCKAR and CCKBR may play an essential role in esophageal carcinoma.

The expression level of major facilitator superfamily domain containing 4A (MFSD4) mRNA is lower in gastric cancer tissues than that in the paracancerous tissues.[32]

Table 4 Details of Hub Genes in the Royal Blue Module

Hubs	Module	Coefficient	P value
KCNE2	Royalblue	0.968426	2.34996E-13
ESRRG	Royalblue	0.968071	2.59365E-16
MFSD4	Royalblue	0.956811	2.35772E-15
CCKBR	Royalblue	0.950419	1.07104E-10
ATP4B	Royalblue	0.944469	2.41009E-17
SIGLEC11	Royalblue	0.944115	7.91562E-23
ESRRB	Royalblue	0.93753	7.82567E-21
ATP4A	Royalblue	0.936846	4.9997E-18
CCKAR	Royalblue	0.911212	2.05779E-08
B3GAT1	Royalblue	0.910831	1.56673E-15

Table 5 Correlation Analysis Between Differential Methylated Sites and DEGs

Gene	Site ID	Correlation	P value
B4GALNT1	cg01723148	0.586028	4.65E-17
B4GALNT1	cg12230728	0.558113	2.64E-15
B4GALNT1	cg25392692	0.580127	1.13E-16
DLX1	cg01244270	0.519615	3.85E-13
DLX1	cg15552158	0.503489	2.59E-12
FBP2	cg01197831	−0.60906	1.23E-18
FBP2	cg20356482	−0.56327	1.29E-15
GPT	cg11617144	−0.51193	9.68E-13
GPT2	cg05380921	−0.52082	3.32E-13
HOXD10	cg06005169	0.590076	3.87E-17
HOXD9	cg13158481	0.51537	6.42E-13
HYAL1	cg06360465	−0.567	7.59E-16
HYAL1	cg10580282	−0.50645	1.84E-12
HYAL1	cg12930727	−0.65804	1.85E-22
HYAL1	cg14943722	−0.56014	1.99E-15
IGFBP2	cg23059946	−0.55095	7.00E-15
IQGAP2	cg08356262	−0.64251	3.58E-21
KALRN	cg21425296	−0.58269	7.69E-17
KCNQ1	cg02180189	−0.54064	2.74E-14
LRRC2	cg10031651	−0.52858	1.28E-13
MAPK12	cg13963658	0.562568	1.42E-15
ME3	cg02493602	−0.69321	1.13E-25
ME3	cg14883291	−0.69388	9.67E-26
ME3	cg15698545	−0.70097	1.90E-26
MECOM	cg00024967	−0.63607	1.16E-20
MECOM	cg00988247	−0.58743	3.76E-17
MECOM	cg01277372	−0.57962	1.22E-16
MECOM	cg03679456	−0.59565	1.06E-17
MECOM	cg06201393	−0.52954	1.13E-13
MECOM	cg20096204	−0.55583	3.61E-15
MFSD7	cg02096793	−0.50801	1.54E-12
MFSD7	cg13656831	−0.52078	3.34E-13
MFSD7	cg17952262	−0.53049	1.00E-13
MKRN3	cg00215587	−0.8241	2.52E-43
MKRN3	cg05952543	−0.65901	1.53E-22
MKRN3	cg11100640	−0.83807	4.51E-46
MKRN3	cg23234999	−0.81036	7.52E-41
NXPH4	cg08711175	0.561717	1.60E-15
PHYHD1	cg14299940	−0.54099	2.62E-14
POU6F2	cg03962846	−0.60838	1.37E-18
PRDM16	cg18381051	−0.53476	5.84E-14
PTPRN2	cg03899215	0.605294	2.27E-18
PTPRN2	cg04237822	0.537075	4.34E-14
PTPRN2	cg06413087	0.574856	2.45E-16
PTPRN2	cg08514594	0.568674	5.98E-16
PTPRN2	cg10288307	0.50932	1.32E-12
PTPRN2	cg10879854	0.53169	8.63E-14
PTPRN2	cg11111139	0.591528	2.00E-17
PTPRN2	cg27096172	0.525207	1.94E-13
RORC	cg18149207	−0.60897	1.25E-18
RPH3AL	cg18222500	0.69607	5.88E-26
SAMD13	cg03204322	−0.55147	6.52E-15
SELENBP1	cg18515587	−0.5324	7.88E-14
TMEM220	cg26234644	−0.53839	3.67E-14
VAX1	cg03851159	0.568126	6.47E-16
VSIG2	cg02082342	−0.63226	2.31E-20
VSIG2	cg23730696	−0.70122	1.79E-26

(*Continued*)

Table 5 (Continued).

Table 6 Enrichment of Differentially Methylated and DEGs in Modules

Module	Odds Ratio	95% CI_Lower	95% CI_Upper	P value
Royalblue	18.01649	8.445462	35.03891	1.97E-10
Violet	0	0	7.914082	1
Diumpurple3	0	0	Inf	1
Darkolivegreen	0	0	8.075999	1
Yellow	0	0	0.510528	0.001837
Darkturquoise	0	0	5.941329	1
Turquoise	0.147352	0.017565	0.549924	0.000464
Darkred	0	0	5.12711	1
Grey	0.985924	0.383056	2.134444	1

Abbreviation: CI, confidence interval.

Furthermore, the hypermethylation of the MFSD4 promoter was detected in cells with a low MFSD4 expression.[32] In the present study, MFSD4 was down-regulated and hypermethylated, which suggested that the down-regulation of MFSD4 may be associated with hypermethylation. Beta-1, 3-glucuronyltransferase 1 (B3GAT1), also known as CD57 or HNK1, which have found frequent losses in ESCC cells.[33] Previous studies have reported high B3GAT1 expression in benign prostate tissue, prostate intraepithelial neoplasia and early low-grade prostate cancer.[34] However, as the tumor grows, the expression of B3GAT1 gradually and locally disappears.[34] In addition, the expression level of B3GAT1 is reduced in the T lymphocytes of patients with chronic fatigue syndrome and the brains of patients with Alzheimer's disease.[35,36] In the present study, B3GAT1 was down-regulated and hypomethylated in esophageal carcinoma tissues. We hypothesized that the down-regulation of B3GAT1 was associated with DNA methylation. In conclusion, we proposed that epigenetic modifications of MFSD4 and B3GAT1 play an important role in the pathology of esophageal carcinoma.

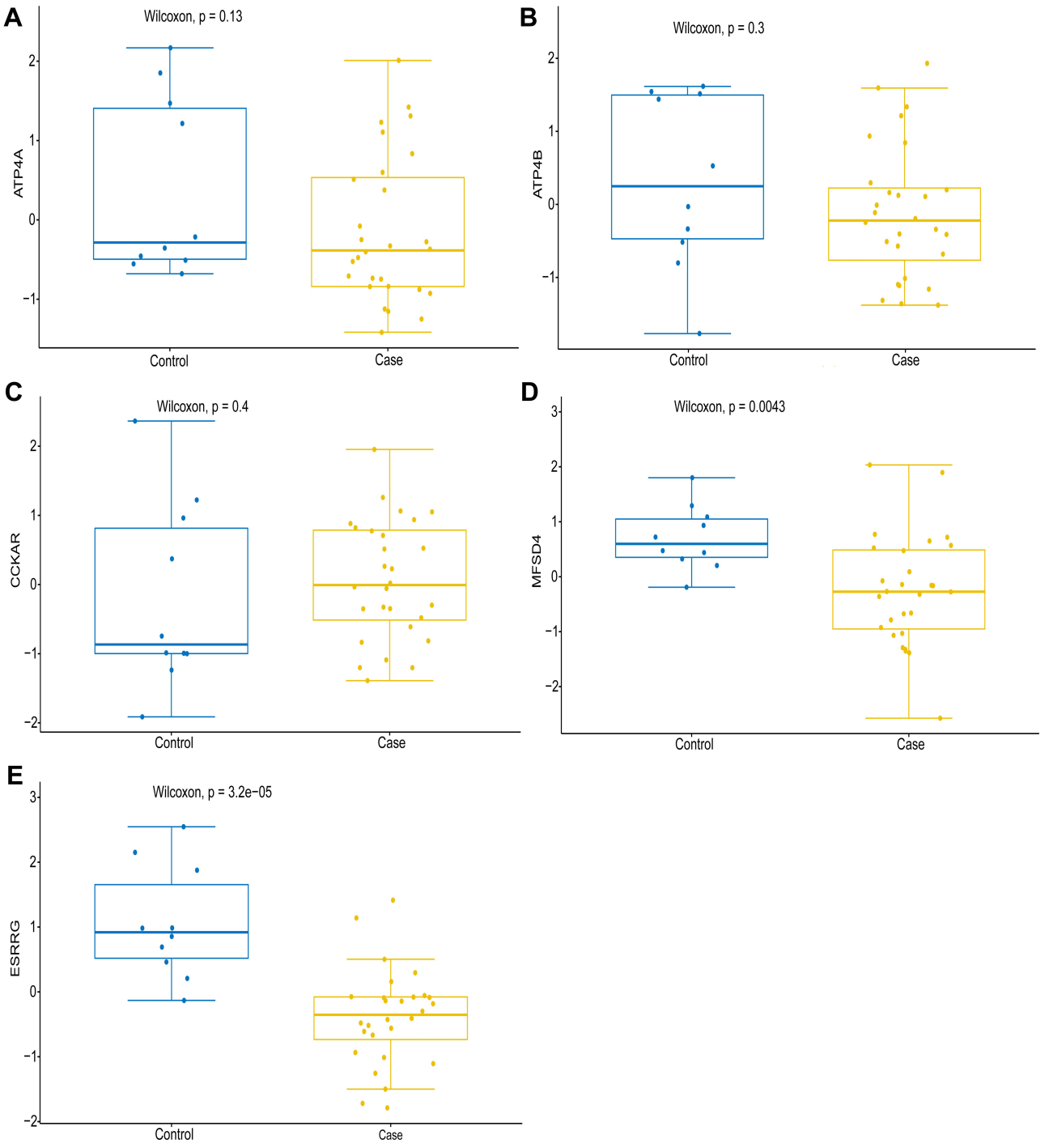

Figure 5 Electronic verification of ATP4A, ATP4B, CCKAR, MFSD4 and ESRRG at the mRNA level. (**A**) Electronic expression verification of ATP4A; (**B**) Electronic expression verification of ATP4B; (**C**) Electronic expression verification of CCKAR; (**D**) Electronic expression verification of MFSD4; (**E**) Electronic expression verification of ESRRG. P<0.05 was considered to indicate a statistically significant different. Blue color and yellow color represent the control group and the case group, respectively.

This provides a potential direction for further research on the molecular mechanism of esophageal carcinoma.

It has been found that mice lacking estrogen related receptor beta (ESRRB) in heart cells develop dilated cardiomyopathy (DCM) in middle age, leading to defects in calcium processing and cell contractility.[37] The over expression of ESRRB protein can inhibit the proliferation of prostate cancer cells, revealing the anti-proliferative effect of ESRRB in prostate cancer.[38] Recent studies have found that the expression of ESRRB protein is missing in breast cancer cells, indicating that ESRRB also plays a tumor suppressor role in breast cancer.[39,40]

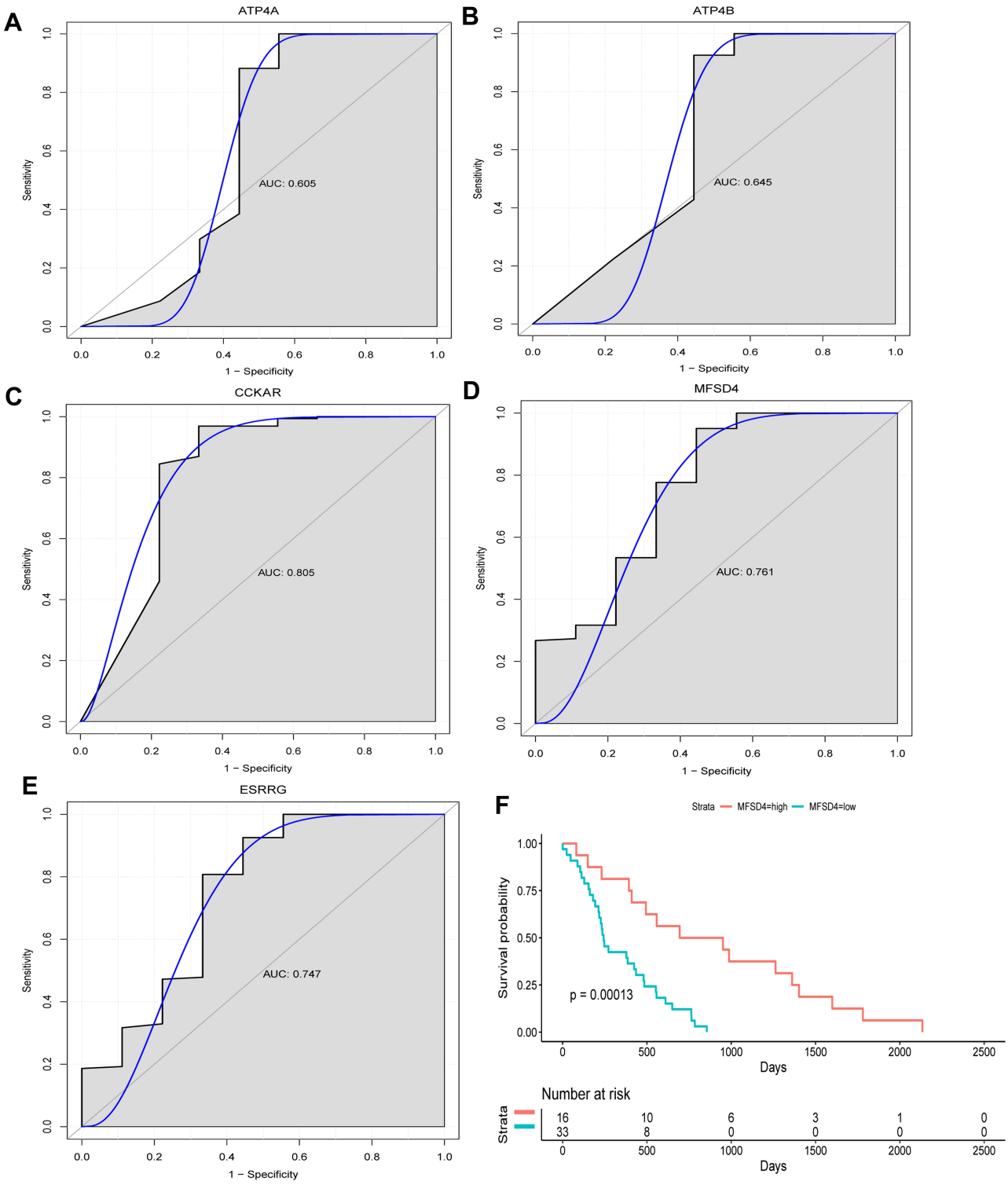

Figure 6 ROC curve of ATP4A, ATP4B, CCKAR, MFSD4, ESRRG (**A**–**E**) and prognostic analysis of MFSD4 (**F**).
Abbreviations: ROC, receiver operating characteristic; AUC, area under curve

In addition, the analysis of exon data from esophagogastric junctional adenocarcinoma revealed mutations in ESRRB, suggesting that ESRRB may be a potential therapeutic target.[41] Estrogen related receptor gamma (ESRRG) also encodes a protein similar to estrogen receptors.[42] Previous studies have shown that ESRRG is a type of tumor suppressor that can inhibit Wingless/Integrated (Wnt) signaling in gastric cancer.[43] A recent

Table 7 Clinical Information of Patients and Normal Controls in the RT-qPCR

Number	Gender	Age	BMI	Symptom	Tumor Location	Pathological Type	Clinical Stages	Onset Time (Day)	Smoking History	Drinking History	Family History of Cancer	Other Tumors	First Diagnosed	Radiotherapy and Chemotherapy	Medication History (Within 1 Month)
1	Male	62	19.8	Dysphagia	From the incisors 29–34cm	Squamous carcinoma	IIIA	180	Yes	Yes	No	No	Yes	No	No
2	Male	76	29	Swallowing discomfort	From the incisors 25–30cm	Squamous carcinoma	IIA	35	No	Occasional	No	No	Yes	No	No
3	Male	56	25.4	Dysphagia	From the incisors 33–36cm	Squamous carcinoma	IIIA	30	Yes	Occasional	No	No	Yes	No	No
4	Male	65	23.6	Dysphagia	From the incisors 26cm	Squamous carcinoma	IIA	90	Yes	Occasional	No	No	Yes	No	No
5	Female	63	28.4	Dysphagia	From the incisors 29–36cm	Squamous carcinoma	I	15	No	No	No	No	Yes	No	No
6	Female	72	17.8	Dysphagia	From the incisors 20–25cm	Squamous carcinoma	IIIB	150	No	No	No	Early cardia adenocarcinoma	Yes	No	No

Abbreviation: BMI, body mass index.

Table 8 Primer Sequences in the RT-qPCR

Primer Name	Primer Sequence (5′ to 3′)
GAPDH-F (internal reference)	5-CTGGGCTACACTGAGCACC-3
GAPDH-R (internal reference)	5-AAGTGGTCGTTGAGGGCAATG-3
ACTB-F (internal reference)	5-TCCGCAAAGACCTGTACGC-3
ACTB-R (internal reference)	5-CTGGAAGGTGGACAGCGAG-3
GPR155-F	5-AGCAAAGCTGGACTATTCCCT-3
GPR155-R	5-GCCACCAAATAAATGTACTGGA-3
ESRRB-F	5-GCAGCCCTGAACACCCATT-3
ESRRB-R	5-AAGGGCGTGGATTGGGGAT-3
ESRRG-F	5-GCCCTGCCACGAATGAATGT-3
ESRRG-R	5-TGGGCTGTTCTCCGCATCT-3
CCKAR-F	5-GTGATCCGCATGCTCATCGT-3
CCKAR-R	5-TGACGCAGGAGGAGGTGTAG-3
MMP11-F	5-CCCTGTGAAGGTGAAGGCTC-3
MMP11-R	5-GATGGCCATGGGTCTCTAGC-3
TPX2-F	5-ATGGAACTGGAGGGCTTTTTC-3
TPX2-R	5-TGTTGTCAACTGGTTTCAAAGGT-3
BIRC5-F	5-AGGACCACCGCATCTCTACAT-3
BIRC5-R	5-AAGTCTGGCTCGTTCTCAGTG-3
NUF2-F	5-TGCCTGCCTTCATGTCAGT-3
NUF2-R	5-GGTCCTCCAAGTTCAGGCTC-3
ANO1-F	5-GAGAGATCGGTTCCCAGCCT-3
ANO1-R	5-TGTGACTGTGACCCGGATGT-3

study showed that ESRRG promoter hypermethylation was associated with the occurrence of laryngeal squamous cell carcinoma (LSCC), which suggested that ESRRG can serve as a biomarker for the diagnosis and prognosis of LSCC.[44] Furthermore, the expression level of ESRRG was found to be reduced in prostate cancer tissues, indicating that ERRG may play an important regulatory role in prostate cancer.[45] ERRG also plays an essential role in the pathophysiology of breast and endometrial cancer.[46,47] In the present study, ESRRB and ESRRG were down-regulated in esophageal carcinoma tissues. Furthermore, ESRRB and ESRRG were hypomethylated and hypermethylated, respectively. However, the action mechanism of ESRRB and ESRRG in esophageal carcinoma remains unclear. It was found in the present study that ESRRB and ESRRG were differentially methylated genes, which provides a potential direction for further research on esophageal carcinoma.

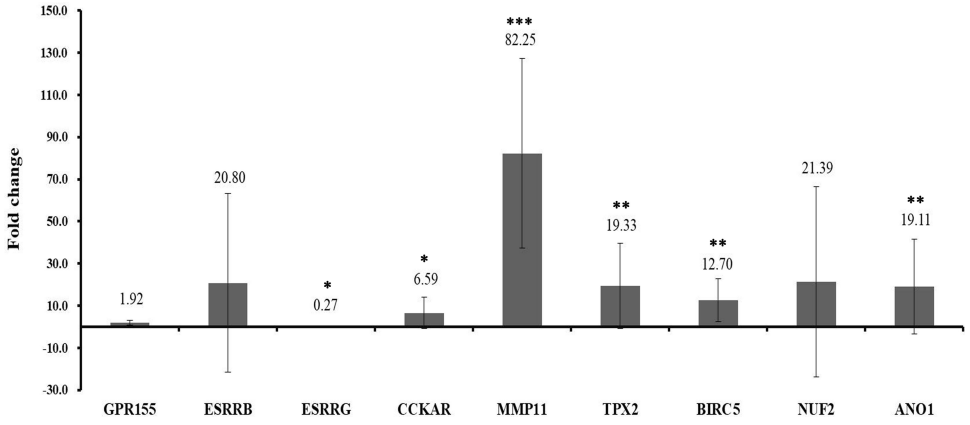

Figure 7 RT-qPCR validation of GPR155, ESRRB, ESRRG, CCKAR, MMP11, TPX2, BIRC5, NUF2 and ANO1 in tissue samples. *Represent p<0.05, **Represent p<0.01, ***Represent p<0.001. P<0.05 was considered statistically significant. Fold change >1 represent up-regulation, Fold change <1 represent down-regulation. RT-qPCR, reverse transcription PCR.

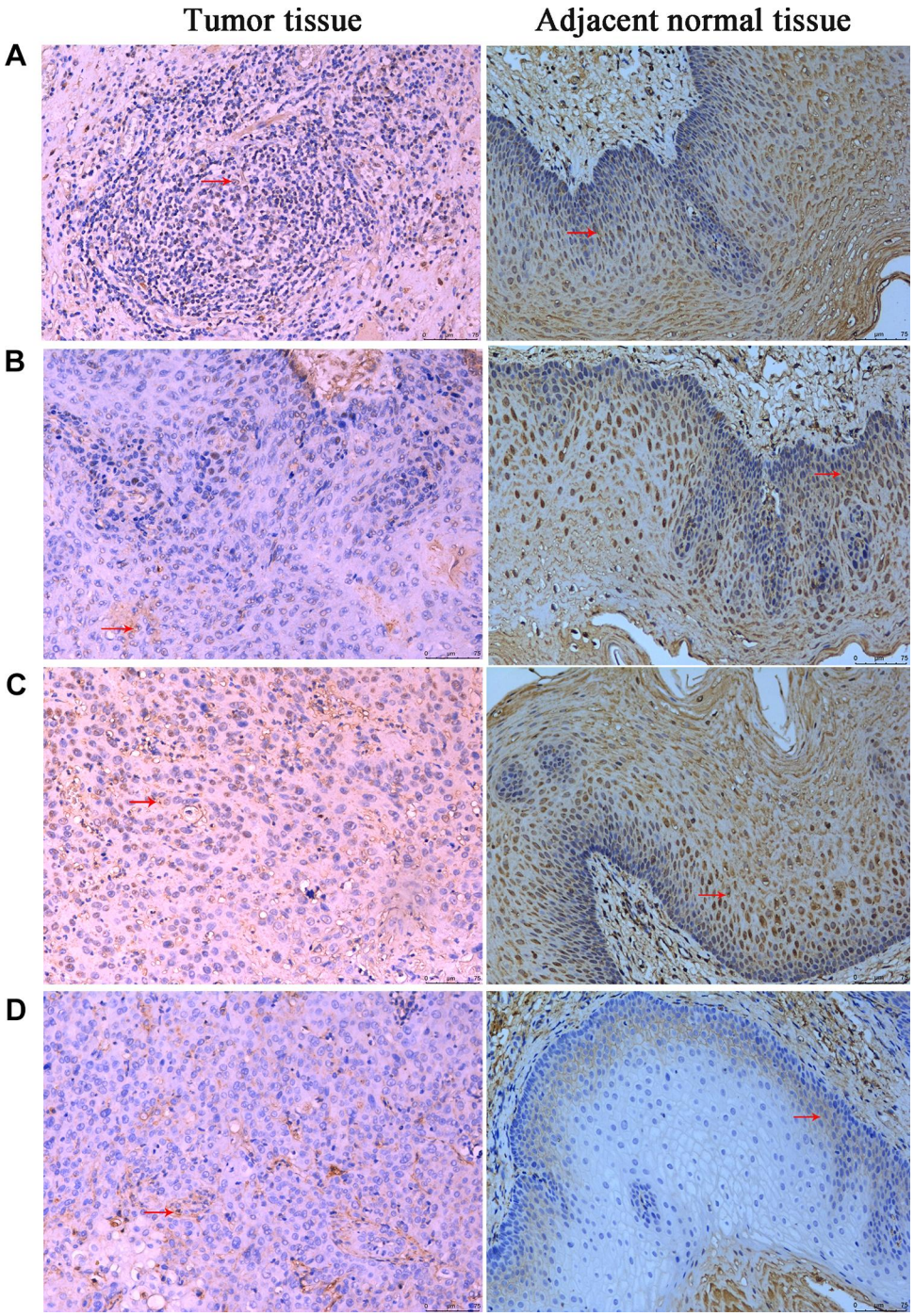

Figure 8 Immunohistochemical analysis of ATP4B (**A**), B3GAT1 (**B**), CCKBR (**C**) and ESRRG (**D**). The arrow represents positive ATP4B, B3GAT1, CCKBR and ESRRG staining. The length of scale bars in (**A**–**D**) was 75 μm. Magnification, 200x.

In addition to the aforementioned 8 genes, several DEGs, including G protein-coupled receptor 155 (GPR155), matrix metallopeptidase 11 (MMP11), TPX2 microtubule nucleation factor (TPX2), were found to play important regulatory roles in cancer. In a previous study, the mRNA expression of GPR155 was decreased in the hepatocellular carcinoma cell line compared with the control non-cancerous cell line.[48] A different study showed that the mRNA expression level of MMP11 in pancreatic cancer tissues was significantly higher than that of normal tissues.[49] In addition, reducing the expression of MMP11 can inhibit tumor growth and the invasion of gastric cancer cells.[50] Recent research has found that TPX2 is up-regulated in a variety of malignant tumors,

such as ESCC,[51] hepatocellular carcinoma,[52] cervical cancer.[53] GPR155 was found to be down-regulated, and MMP11 and TPX2 to be up-regulated in esophageal carcinoma. The expression trend was the same as that in other cancer types, which suggests that these three genes may play an important regulatory role in esophageal carcinoma.

The present study had certain limitations. First, the sample size of in vitro verification was too small, which led to a certain degree of error between the validation and bioinformatics analysis results. Thus, future studies with a larger sample size are required. Secondly, the molecular mechanism of the identified genes in esophageal carcinoma remains unclear and should be further studied. In combination, the results of the present study demonstrated that, compared with the paracancerous tissues, ESRRG, MFSD4, CCKBR, ATP4B, ESRRB, ATP4A, CCKAR and B3GAT1 were significantly down-regulated in esophageal carcinoma tissues, suggesting that they may play an essential role in the physiopathology of esophageal carcinoma. The identification of these genes may prove helpful in the development of early diagnosis and treatment options for esophageal carcinoma.

Conclusion

In the present study, ESRRG, MFSD4, CCKBR, ATP4B, ESRRB, ATP4A, CCKAR and B3GAT1 were differentially methylated genes and DEGs. CCKAR, MFSD4 and ESRRG may serve as diagnostic gene biomarkers in esophageal carcinoma. The high expression level of MFSD4 was significantly correlated with patient survival. Therefore, it was suggested that epigenetic changes of hub genes may play an essential role in esophageal carcinoma. Immunohistochemical analysis showed that the gene expression levels of ATP4B, B3GAT1, CCKBR and ESRRG were decreased in esophageal carcinoma tissues, which was in line with the bioinformatics results. The identified molecular markers may therefore be helpful in the diagnosis and treatment of esophageal carcinoma.

Data Sharing State

All data generated or analyzed during this study are included in this published article.

Ethics Approval and Consent to Participate

This study was approved by the ethics committee the Fourth Hospital of Hebei Medical University (NO.2020KY183). All participants were informed as to the purpose of this study, and that this study complied with the Declaration of Helsinki.

Consent for Publication

All of the authors have agreed to the publication of the work.

Author Contributions

Conception and design: Y.X. and Z.T.

Execution: Y.X., H.L. and Z.L.

Data analysis and interpretation: N.W., R. L., F.Z. and C.G.

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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