

Identification of crucial genes associated with esophageal squamous cell carcinoma by gene expression profile analysis

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Abstract. To uncover the genes associated with the development of esophageal squamous cell carcinoma (ESCC), an ESCC microarray dataset was used to identify genes differentially expressed between ESCC and normal control tissues. The dataset GSE17351 was downloaded from the Gene Expression Omnibus, containing 5 tumor esophageal mucosa samples and 5 adjacent normal esophageal mucosa samples from 5 male patients with ESCC. The differentially expressed genes (DEGs) were identified using the Linear Models for Microarray Data R package. Then, a co-expression network was constructed using the Weighted Correlation Network Analysis (WGCNA) package, and co-expression network modules were obtained with a hierarchical clustering algorithm. Additionally, functional enrichment analyses for DEGs in the top 2 modules with the highest significance were respectively conducted using the WGCNA package and the cluster Profiler package. In total, 487 upregulated and 468 downregulated DEGs were identified. A total of 24 modules were obtained from the co-expression network, and the top 2 modules with the highest significance, designated as 'blue4' and 'magenta', were further analyzed. In the module blue4, DEGs were significantly enriched in a number of Gene Ontology terms, including 'spindle organization' [e.g., ubiquitin conjugating enzyme E2 C (*UBE2C*) and SAC3 domain containing 1] and 'cell cycle process' [e.g., *UBE2C*, minichromosome maintenance complex component 6 (*MCM6*) and cell division cycle 20 (*CDC20*)]. Furthermore, a number of DEGs (e.g., *UBE2C*, *CDC20* and *MCM6*) were enriched in the 'cell cycle' and 'ubiquitin mediated proteolysis' pathways. In the module 'magenta', a number of DEGs [e.g., transferrin receptor (*TFRC*) and TEA domain transcription factor 4

(*TEAD4*)] were enriched in the primary metabolic process and intracellular membrane-bounded organelle. Additionally, 308 upregulated genes and 215 downregulated genes were differentially expressed in the same pattern in another dataset, GSE20347, including *UBE2C*, *CDC20*, *MCM6*, *TFRC*, *TEAD4*, protein phosphatase 1 regulatory subunit 3C and MAL, T-cell differentiation protein. These DEGs may function in the development of ESCC.

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of histologic subtypes of esophageal cancer, and it occurs at a relatively high frequency in China, with five-year survival rates of 14% in Hong Kong (1). However, the molecular mechanism underlying the development of ESCC remains poorly understood.

In recent years, substantial advances have been made in ESCC research. Signal transducer and activator of transcription 3 (STAT3) has been demonstrated to be upregulated by the β -catenin/T cell factor pathway in ESCC (2). STAT3 β expression is significantly associated with a shorter survival time for patients with ESCC, and it may suppress the oncogenic effects of STAT3 α in ESCC cell lines (3). Furthermore, xerophilus B can induce the G₂/M cell cycle arrest and apoptosis of ESCC cells (4). A previous study identified that extracellular matrix protein 1b is downregulated in ESCC compared with normal esophageal tissues, and that it served a potential suppressive function in tumorigenesis and metastasis (1). Additionally, plasma matrix metalloproteinase 1 was observed to be highly expressed in ESCC compared with normal esophageal tissues, and it may have contributed to the detection and survival prediction of ESCC (5). However, the pathogenesis of ESCC is not yet completely characterized.

In 2010, Lee *et al* (6) performed gene expression profiling to investigate target genes for hypoxia-inducible factor (HIF) in the esophageal tumor microenvironment; the study identified a number of HIF target genes, including prostaglandin E synthase, cyclooxygenase 2 and insulin-like growth factor binding protein-3. However, the co-expression networks and functional enrichment analysis of the differentially expressed genes (DEGs) in the ESCC samples were not investigated. In the present study, the microarray dataset of GSE17351 produced by Lee *et al* (6) was analyzed to identify DEGs in ESCC samples. Following this, the Weighted Correlation

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Network Analysis (WGCNA), a systems biology method for identifying the correlation between genes across microarray samples (7), was used to analyze co-expression networks for the upregulated DEGs. Subsequently, gene ontology (GO) and pathway enrichment analyses were performed for the DEGs in the two most significant network modules. Additionally, the DEGs were validated using another gene expression dataset, GSE20347 (8) from the Gene Expression Omnibus (GEO). These results may contribute to an improved understanding of the etiology of ESCC.

Materials and methods

Affymetrix microarray data. The GSE17351 microarray expression profile (6), based on the platform of the GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix; Thermo Fisher Scientific, Inc., Waltham, MA, USA), was downloaded from the GEO (<http://www.ncbi.nlm.nih.gov/geo/>) database. The dataset contains 5 tumor esophageal mucosa samples and 5 adjacent normal esophageal mucosa samples from 5 male patients with ESCC, with an age range of 51-76 years. All esophageal tissues were originally obtained through surgery at the Okayama University Hospital, Kitano Hospital and the Hospital of the University of Pennsylvania through the Cooperative Human Tissue Network (6).

Data preprocessing. The expression values of all probes in each sample were reduced to a single value by determining the mean expression value via the aggregate function method (9). Missing data were assigned using the k-nearest neighbor method (10). Quantile normalization for complete data was performed using the preprocess Core package in Bioconductor (11). When numerous probes were mapped to one gene, the mid-value of the data was defined as the expression level of the gene. However, when numerous genes were mapped by one probe, this probe was considered to lack specificity, and was removed from the analysis.

Identification of DEGs. The Linear Models for Microarray Data package of Bioconductor (12) was used to identify genes that were significantly differentially expressed in ESCC samples. The raw P-value was adjusted using the Benjamin and Hochberg method (13), and a \log_2 fold change (FC) > 0.585 and $P < 0.05$ were selected as the cut-off criteria.

Construction of co-expression networks and identification of co-expression network modules. The WGCNA package of R (7) was used to analyze the co-expression network for DEGs, and the co-expression networks were visualized using Cytoscape (Version 3.2.0) (14). The weighting coefficient β was set to 25. The adjacency matrix power method (15) was used to transfer matrixes to weighted co-expression networks.

Co-expression network modules were obtained using a hierarchical clustering algorithm (16). The number of genes in each module was at least 30. Then, the significant modules were identified using correlation coefficient and network significance methods included in the WGCNA package. Gene significance (GS) measure was defined as a function GS, and a module significance (MS) measure as a mean of the GS in

the module. A larger MS value indicated a greater association of a module with ESCC.

GO and pathway enrichment analyses for DEGs in the significant modules. The WGCNA package was used to obtain significant GO terms for DEGs in the significant modules, and the cluster Profiler package of R (17) was used to perform Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis for DEGs in modules. $P < 0.05$ was selected as the cut-off criterion.

Data validation of the DEGs. The gene expression dataset GSE20347 (8) from GEO was used to validate the expression of the identified DEGs. The dataset included the data from 17 micro-dissected ESCC tumor tissues and 17 matched normal adjacent tissues from patients with ESCC. The data were with the GPL571 [HG-U133A_2] Affymetrix Human Genome U133A 2.0 Array platform (Affymetrix; Thermo Fisher Scientific, Inc.).

Data preprocessing and DEG identification were performed with the same methods as for the data in GSE17351. The overlapping upregulated and downregulated DEGs between GSE20347 and GSE17351 were identified and illustrated using an online Venn diagram plotter tool (<https://omics.pnl.gov/software/venn-diagram-plotter>). The overlapping DEGs were considered to be preliminarily validated by the GSE20347 dataset.

Results

Identification of DEGs. Following data preprocessing, a total of 6,899 genes in 10 samples were excluded. Based on the cut-off criteria, 955 DEGs were identified, including 487 upregulated and 468 downregulated DEGs.

Analysis of co-expression network modules. A total of 24 co-expression network modules were identified. Of these 24, the modules 'blue4' and 'magenta' were the most significantly associated with ESCC (Fig. 1).

Construction of co-expression subnetworks for the blue4 and magenta modules. The top 30 genes with the highest connectivity degree in the modules blue4 and magenta were selected for the construction of co-expression subnetworks. In the co-expression subnetworks of the modules blue4 (Fig. 2) and magenta (Fig. 3), there were 435 gene interactions per module.

It was observed that a number of DEGs [including cysteine rich secretory protein 3 (CRISP3), neural EGFL like 2 (NELL2), MYB proto-oncogene like 2 (MYBL2) and plasminogen activator, urokinase (PLAU)] in the co-expression subnetwork of the module blue4 and a number of DEGs [including MAL, protein phosphatase 1 regulatory subunit 3C (PPP1R3C), cytochrome P450 family 3 subfamily A member 5 (CYP3A5) and biglycan (BGN)] in the co-expression subnetwork of the module magenta had a particularly high \log_2 FCI, indicating that these genes may have a greater extent of association with ESCC.

Enrichment analyses for the DEGs in the co-expression subnetworks of the modules blue4 and magenta. According to

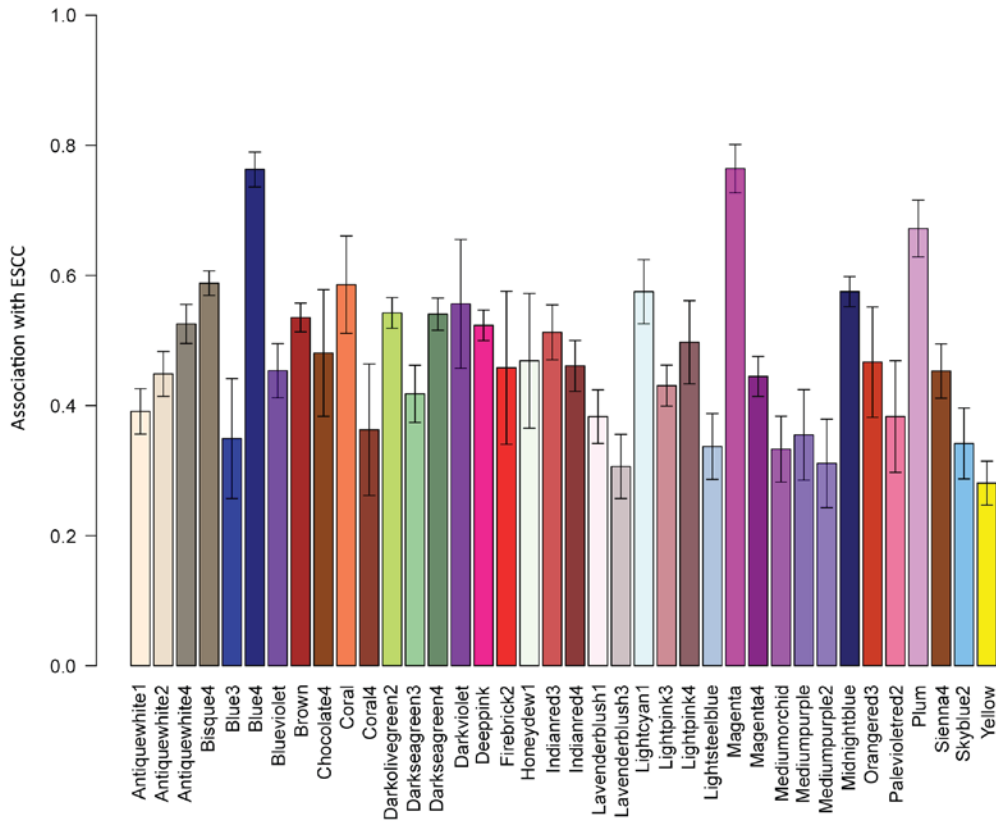


Figure 1. Relative strength of the association of co-expressed network modules with esophageal squamous cell carcinoma. The x-axis displays the names of the modules.

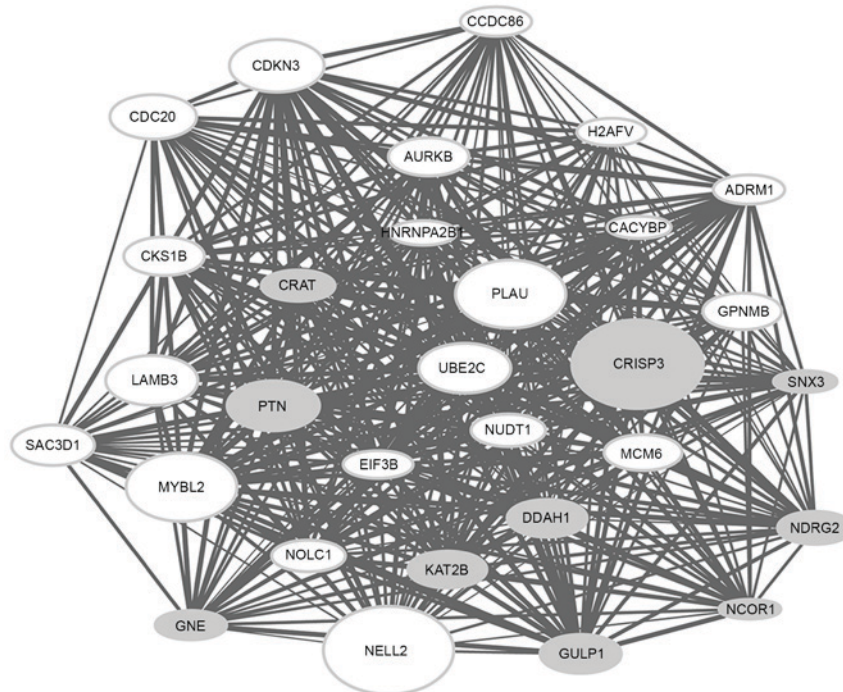


Figure 2. Interaction network for the co-expression module 'blue4'. The size of the nodes is proportional to the value of the \log_2 fold change; gray nodes represent the downregulated genes and white nodes represent the upregulated genes. The width of the lines is proportional to the co-expression coefficient.

GO enrichment analysis, DEGs in the co-expression subnetwork of the module blue4 were predominantly enriched in GO terms regarding cell division, including 'spindle organization'

[e.g., ubiquitin conjugating enzyme E2 C (*UBE2C*) and SAC3 domain containing 1 (*SAC3D1*)], 'cell cycle process' [e.g., *UBE2C*, minichromosome maintenance complex

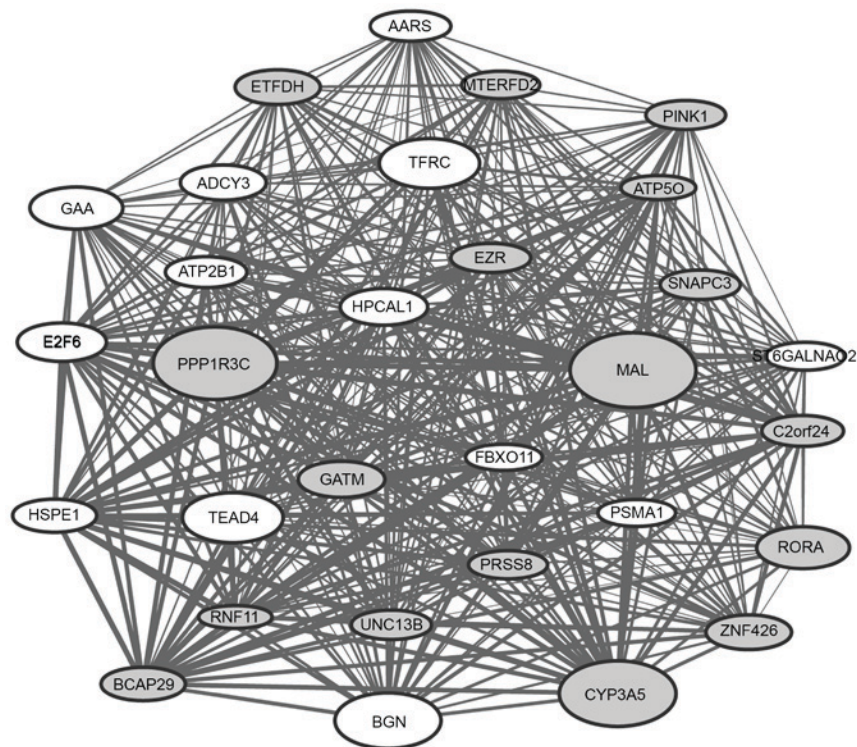


Figure 3. Interaction network for the co-expression module 'magenta'. The size of the nodes is proportional to the value of \log_2 fold change; gray nodes represent the downregulated genes and white nodes represent the upregulated genes. The width of lines is proportional to the co-expression coefficient.

component 6 (*MCM6*) and cell division cycle 20 (*CDC20*), 'protein binding' (e.g., *UBE2C*, *MCM6* and *CDC20*) and 'anaphase-promoting complex' (APC; e.g., *UBE2C* and *CDC20*; Table I). In addition, two KEGG pathways were significantly enriched, including 'cell cycle' (*CDC20* and *MCM6*) and 'ubiquitin mediated proteolysis' (e.g., *UBE2C* and *CDC20*; Table II).

DEGs in the co-expression subnetwork of the magenta module were mainly enriched in GO terms associated with metabolism, including 'energy derivation by oxidation of organic compounds' e.g., adenylate cyclase 3 (*ADCY3*), electron transfer flavoprotein dehydrogenase (*ETFDH*) and glucosidase α , acid (*GAA*), 'primary metabolic process' [e.g., *CYP3A5*, TEA domain transcription factor 4 (*TEAD4*) and transferrin receptor (*TFRC*)], and 'intracellular membrane-bounded organelle' (e.g., *TEAD4* and *TFRC*; Table III). However, no KEGG pathways were significantly enriched in the DEGs of the co-expression subnetwork in the magenta module.

Validation of DEGs. In total, 308 upregulated genes (e.g., *UBE2C*, *CDC20*, *MCM6*, *TFRC* and *TEAD4*) and 215 downregulated genes (e.g., *PPP1R3C* and *MAL*) were identified as overlapping between the GSE17351 and GSE20347 datasets (Fig. 4).

Discussion

In the present study, a total of 955 DEGs were identified, including 487 upregulated and 468 downregulated DEGs. According to WGCNA analysis, two significant co-expression network modules, blue4 and magenta, were identified. The GO

and KEGG pathway enrichment analyses for the top 30 DEGs with the highest connectivity degrees in the module blue4 revealed that a number of DEGs were associated with the 'cell cycle' and 'ubiquitin mediated proteolysis' KEGG pathways, including *UBE2C*, *CDC20* and *MCM6*.

UBE2C encodes a member of the E2 ubiquitin-conjugating enzyme family, which is involved in protein ubiquitination (18). *UBE2C* is associated with cell cycle progression and checkpoint control, as it modulates the degradation of short-lived proteins (19). A previous study reported that *UBE2C* expression was elevated in 73% (11 of 15) of esophageal adenocarcinoma samples relative to Barrett's metaplasia, and the transfection of *UBE2C* small interfering (si)RNA induced the inhibition of cell proliferation and a distortion in cell cycle distribution (20). Furthermore, key roles of *UBE2C* have been demonstrated in other cancer types, including hepatocellular carcinoma (21), cervical carcinoma (22), non-small cell lung cancer (23) and pancreatic ductal adenocarcinoma (24). *UBE2C* and *CDC20* were identified as being associated with the term 'anaphase-promoting complex' in the present study. The APC serves a crucial role in modulating cell cycle progression via forming two functionally distinct E3 ubiquitin ligase subcomplexes, APC^{Cdc20} and APC^{Cadherin1} (25). The inhibition of *CDC20* by siRNA may induce G₂/M cell cycle arrest and suppress cell growth; *CDC20* is negatively regulated by p53 (26).

Accumulating evidence has demonstrated that *CDC20* serves a notable function in the development and progression of human cancer (27). Thus, *UBE2C* and *CDC20* may be associated with ESCC. *MCM6* is essential for the initiation of eukaryotic genome replication. A previous study reported that *MCM6* is potentially associated with the lymph node metastasis of ESCC (28), indicating the crucial role of *MCM6* in

Table I. Top 5 most significantly enriched GO terms for the 30 differentially expressed genes with the highest connectivity degree in the module 'blue4' in BP and CC, and all significantly enriched terms in MF.

Category	ID	Term	P-value	Count	Genes
BP	GO:0007051	Spindle organization	0.000091	5	<i>UBE2C, SAC3D1, MYBL2, AURKB, NCOR1</i>
BP	GO:0051225	Spindle assembly	0.000131	4	<i>SAC3D1, MYBL2, AURKB, NCOR1</i>
BP	GO:0022402	Cell cycle process	0.000602	10	<i>CDKN3, UBE2C, SAC3D1, MCM6, MYBL2, KAT2B, AURKB, NOLC1, NCOR1, CDC20</i>
BP	GO:0000280	Nuclear division	0.001058	6	<i>UBE2C, SAC3D1, MYBL2, AURKB, NOLC1, CDC20</i>
BP	GO:0007067	Mitosis	0.001058	6	<i>UBE2C, SAC3D1, MYBL2, AURKB, NOLC1, CDC20</i>
MF	GO:0005515	Protein binding	0.004683	22	<i>CDKN3, GPNMB, ADRMI, UBE2C, LAMB3, MCM6, NELL2, PLAU, NDRG2, CDC20...</i>
MF	GO:0005488	Binding	0.032021	26	<i>GNE, CDKN3, GPNMB, ADRMI, UBE2C, MCM6, NELL2, PLAU, NDRG2, CDC20...</i>
CC	GO:0005680	Anaphase-promoting complex	0.013075	2	<i>UBE2C, CDC20</i>
CC	GO:0031974	Membrane-enclosed lumen	0.013075	12	<i>UBE2C, CRAT, CACYBP, HNRNPA2B1, MCM6, NUDT1, CCDC86, KAT2B, AURKB, CDC20...</i>
CC	GO:0005819	Spindle	0.013075	4	<i>SAC3D1, AURKB, NCOR1, CDC20</i>
CC	GO:0000152	Nuclear ubiquitin ligase complex	0.013075	2	<i>UBE2C, CDC20</i>
CC	GO:0044427	Chromosomal part	0.018472	5	<i>MYBL2, KAT2B, AURKB, H2AFV, NCOR1</i>

GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function.

Table II. Enriched pathways for differentially expressed genes in the module 'blue4'.

ID	Description	P-value	Count	Genes
hsa04110	Cell cycle	0.022640607	2	<i>CDC20, MCM6</i>
hsa04120	Ubiquitin mediated proteolysis	0.026418251	2	<i>UBE2C, CDC20</i>

ESCC. In the network, *UBE2C*, *CDC20* and *MCM6* were all connected with *CRISP3*, which had a high \log_2FCI . The low expression of *CRISP3* in ESCC compared with normal tissue was identified in a previous study (29), consistent with the result of the present study. The DNA copy number loss of *CRISP3* has been demonstrated in oral squamous cell carcinoma. Thus, it may be speculated that *CRISP3* is associated with the carcinogenesis of ESCC. Furthermore, *NELL2* was observed to have a higher \log_2FCI in the co-expression subnetwork of the module blue4 in the present study; it was coexpressed with *UBE2C*, *CDC20*, *MCM6* and *CRISP3*. A homolog of *NELL2*, it has been demonstrated that the promoter hypermethylation of *NELL1* is higher in ESCC than in normal esophagus tissues, and that it is associated with poor prognosis in early-stage esophageal adenocarcinoma (30). Hence, it may be speculated that *NELL2* may function in the occurrence of ESCC.

In the co-expression subnetwork of the magenta module, the upregulated *TFRC* and *TEAD4*, in addition to the down-regulated *PPP1R3C*, *MAL* and *CYP3A5* were observed to have the highest \log_2FCI . A previous study reported that the elevated expression of *TFRC* was associated with the distant metastasis of ESCC, and patients with positive results for *TFRC* mRNA expression have a notably worse prognosis (31). Additionally, *TEAD4* is a member of the transcriptional enhancer factor (TEA) family of transcription factors (32). TEAD and its coactivators may co-activate gene transcription, and it is pivotal for physiologically important processes including cell proliferation, cell differentiation and stem cell maintenance (32). One coactivator of TEAD, Yes-associated protein, is reported to be overexpressed in primary ESCC tumors (33). *PPP1R3C* catalyzes reversible protein phosphorylation, which is important in a range of cellular activities. Downregulated *PPP1R3C* was

Table III. Top 5 most enriched GO terms for differentially expressed genes in the 'magenta' module in BP and CC.

Category	ID	Term	P-value	Count	Genes
BP	GO:0015980	Energy derivation by oxidation of organic compounds	0.018735	5	<i>ADCY3, ETFDH, GAA, ATP50, PPP1R3C</i>
BP	GO:0055114	Oxidation-reduction process	0.018735	6	<i>ADCY3, CYP3A5, ETFDH, GAA, ATP50, PPP1R3C</i>
BP	GO:0006091	Generation of precursor metabolites and energy	0.027604	5	<i>ADCY3, ETFDH, GAA, ATP50, PPP1R3C</i>
BP	GO:0044238	Primary metabolic process	0.027604	23	<i>CNPPD1, HSPE1, ATP50, PPP1R3C, PRSS8, SNAPC3, TEAD4, TFRC, ZNF426, FBXO11...</i>
BP	GO:0021680	Cerebellar Purkinje cell layer development	0.032836	2	<i>AARS, RORA</i>
CC	GO:0044429	Mitochondrial part	0.022333	6	<i>MTERFD2, ETFDH, GATM, HSPE1, ATP50, PINK1</i>
CC	GO:0043231	Intracellular membrane-bounded organelle	0.022333	23	<i>PSMA1, RORA, BGN, PINK1, SNAPC3, TEAD4, TFRC, EZR, ZNF426, FBXO11...</i>
CC	GO:0043227	Membrane-bounded organelle	0.022333	23	<i>PSMA1, RORA, BGN, PINK1, SNAPC3, TEAD4, TFRC, EZR, ZNF426, FBXO11...</i>
CC	GO:0031974	Membrane-enclosed lumen	0.023484	11	<i>MTERFD2, ETFDH, GATM, HSPE1, PSMA1, RORA, BGN, SNAPC3, TEAD4, FBXO11...</i>
CC	GO:0044444	Cytoplasmic part	0.033213	18	<i>RNF11, HSPE1, MAL, ATP50, BCAP29, PSMA1, BGN, PINK1, TFRC, EZR...</i>

GO, Gene Ontology; BP, biological process; CC, cellular component.

previously observed to be associated with lymph node metastasis in ESCC (34) and was identified as potentially contributing to the development of ESCC (35). In the present study, *TFRC*, *TEAD4* and *PPP1R3C* were enriched in 'primary metabolic processes'. Iron metabolism was identified to be altered in esophageal adenocarcinoma, and the overexpression of *TFRC* was associated with increased iron deposition in esophageal adenocarcinoma (36). Thus, *TFRC*, *TEAD4* and *PPP1R3C* may serve important roles in the development of ESCC.

Downregulated *MAL* in ESCC has been identified in previous studies (37,38), which is consistent with the results of the present study. In esophageal cancer, *MAL* is able to suppress esophageal cancer cell motility, invasion and tumorigenicity and promote apoptosis via the Fas pathway (39), indicating a potential role for *MAL* in esophageal cancer. Additionally, genetic polymorphisms of *CYP3A5* in combination with the sulfotransferase family 1A member 1 *2/*2 genotype are associated with an increased risk of esophageal cancer (40), suggesting a crucial role for *CYP3A5* in esophageal cancer.

In the present study, DEGs were also validated using another ESCC gene expression dataset, GSE20347. A total of 308 upregulated genes and 215 downregulated genes were differentially expressed in the same pattern in GSE20347, including the aforementioned upregulated genes *UBE2C*, *CDC20*, *MCM6*, *TFRC* and *TEAD4*, in addition to the



Figure 4. Venn diagram of the number of overlapping up- and down-regulated genes between the datasets GSE17351 and GSE20347. The underlined numbers represent the number of downregulated genes; the numbers without an underline represent the number of upregulated genes.

downregulated genes *PPP1R3C* and *MAL*. The validation results further indicate that these genes may serve crucial functions in the progression of ESCC.

However, the present study has a number of limitations. Potential microRNAs and transcription factors targeting the identified DEGs should have been identified, and these

predictions should have been validated by experiments. In further studies, investigation of the etiology of ESCC will be performed in depth.

In conclusion, in the present study, 487 upregulated and 468 downregulated DEGs were identified. A number of DEGs (e.g., *UBE2C*, *CDC20* and *MCM6*) enriched in 'cell cycle' and 'ubiquitin mediated proteolysis', those (e.g., *TFRC* and *TEAD4*) enriched in 'primary metabolic process' and 'intracellular membrane-bounded organelle', and a number of others (e.g., *CRISP3*, *NELL2*, *PPPIR3C*, *MAL* and *CYP3A5*), may be important in the initiation and development of ESCC.

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Author's contributions

XW conceived and designed the research and drafted the manuscript. GL and QL acquired the data, analyzed and interpreted the data and performed statistical analysis. CG conceived and designed the research and revised the manuscript to present important, intellectual content.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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