Original Article

Alteration of non-coding RNAs expression pattern in metastasis process of esophageal squamous cell carcinoma

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Background: Esophageal squamous cell carcinoma (ESCC) is a primary subtype of esophageal cancer (EC), with high morbidity and mortality. This study aimed to identify aberrant expression profiling of non-coding RNAs in ESCC metastasis.

Methods: The expression profiling of lncRNA/miRNA/mRNA was measured by RNA-sequencing in primary tumor loci of ESCC patients with metastasis (metastasis group) and without metastasis (primary group). Differentially expressed lncRNA/miRNA/mRNA (DELs/DEMIs/DEMs) were identified in metastasis group. DEMIs-DEMs interaction network, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment, Gene Ontology annotation of DEMs were conducted to predict the potential functions of DELs and DEMIs. Quantitative real-time polymerase chain reaction (qRT-PCR) and the Cancer Genome Atlas (TCGA) illumine hiseq data was used to validate the expression level of DELs/DEMIs/DEMs in metastasis and primary group.

Results: Respective 43 DELs, 128 DEMIs and 205 DEMs were identified in ESCC metastasis group compared to primary group. DEMIs-DEMs interaction network was constructed, which consisted of 145 nodes and 214 edges involved in 88 DEMIs, furthermore, *miR-495-3p*, *miR-200b-5p* and *miR-200a-5p* had the highest connectivity with DEMs. DEMs were significantly enriched in MAPK signaling pathway, pathways in cancer, and cell adhesion molecules (CAMs). Digestion, cell fate commitment and positive regulation of cell proliferation were three significant enrichment of biological process in GO annotation. *AKR1B10* was significantly up-regulated; *KRT19* and *XIST* were significantly down-regulated; *SLC7A11* and *hsa-miR-224-5p* had the up-regulated tendency in metastasis group compared with primary group.

Conclusions: Our work might provide useful information for exploring the metastasis mechanism in ESCC and benefit to identification of potential therapeutic targets in ESCC metastasis.

Keywords: RNA; long noncoding; microRNAs; gene expression profiling; esophageal squamous cell carcinoma (ESCC); neoplasm metastasis

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Introduction

Esophageal cancer (EC) is the common malignancy in digestive tract and ranks the sixth leading cause in male and ninth leading cause of cancer-related mortality in female in 2012 worldwide (1). Patients with EC are frequently diagnosed at advanced stages, with 5 years survival rate less than 10%, deprived of the chance of surgical resection for long-term survival (2,3).

Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) are the two histological

Table 1 Characteristics of patients for RNA-sequencing

Index	Case 1	Case 2	Case 3	Control 1	Control 2	Control 3
Age (years)	58	77	52	63	42	54
Gender	Male	Male	Male	Male	Female	Male
Type	ESCC	ESCC	ESCC	ESCC	ESCC	ESCC
TNM stage	T3N1M0	T3N1M0	T2N1M0	T3N0M0	T3N0M0	T2bN0M0
Tumor differentiation	Well	Moderate	Well	Moderate	Moderate	Poor

Case was indicated the patients of ESCC patients with metastasis. Control was indicated the patients of ESCC patients without metastasis. ESCC, esophageal squamous cell carcinoma.

subtypes of EC. Smoking, alcohol drinking and low intake of fruits and vegetables increase the occurrence risk of ESCC (4).

In currently, the etiological factors of ESCC are unclearly. It is reported that aberrant expression of non-coding RNAs, such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) regulate multiple tumorigenic processes, including proliferation, invasion, metastasis and prognosis in ESCC. Decreased *miR-630* induces ECCC cell proliferation, invasion, metastasis, EMT and poor overall survival of patients with ESCC (5). High plasma concentration of oncogenic *miR-21* is an independent risk factor of chemo-resistance in ESCC (6). *miR-143-3p* is decreased in ESCC tissues and ectopic expression of *miR-143-3p* inhibits ESCC cell proliferation and induces cell apoptosis (7). Except of miRNAs, lncRNAs also play vital roles in ESCC tumorigenesis. Up-regulation of lncRNA *BANCR*, *PCAT-1* and *BC200* is correlated with advanced TNM stage, lymph node metastasis and shorter survival (8-10). *LOC100130476* is significantly down-regulated in ESCC cell lines and tissues; up-regulation of *LOC100130476* inhibits ESCC cell proliferation and invasiveness (11). lncRNA POLR2E rs3787016 C/T and HULC rs7763881 A/C polymorphisms are associated with a significantly decreased risk for ESCC (12).

At present, the tumorigenesis and metastasis mechanism of ESCC is largely unknown. In this study, dysregulated lncRNAs, miRNAs and mRNAs were identified in ESCC metastasis. Our study investigated non-coding RNA expression pattern and might provide valuable information for exploring pathogenesis and metastasis mechanism in ESCC.

Methods

Patients and samples

Seven ESCC patients with metastasis and seven ESCC patients without metastasis in the Daping Hospital were enrolled in our study. Among which, three ESCC patients with metastasis and three ESCC patients without metastasis were enrolled for RNA-sequencing; four ESCC patients with metastasis and four ESCC patients without metastasis were enrolled for quantitative real-time polymerase chain reaction (qRT-PCR). Primary tumor tissues of ESCC patients with metastasis and non-metastasis were obtained from esophagectomy. All these patients were diagnosed as ESCC based on postoperative pathology and none of them, received chemo- or radiotherapy before esophagectomy. The detailed information of patients was displayed in *Tables 1* and *2*.

Ethics

This work was approved by the Ethics Committee of the Daping Hospital (2015-035) and informed written consent was obtained from all patients. The research complied with the principles of the Declaration of Helsinki.

Library preparation and high-throughput sequencing

Three primary tumor loci of ESCC patients without metastasis (primary group) and three primary tumor loci of ESCC patients with metastasis (metastasis group) were pooled for RNA-sequencing, respectively. Total RNA of collected specimens was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then cDNA libraries were constructed according to the manufacture instruction. Likewise, cDNA libraries of small RNA were constructed. Lastly, each library was loaded into one lane of the illumine hiseq 4,000 for sequencing.

Data preprocessing

The raw image data obtained from high-throughput RNA-

Table 2 Characteristics of patients for qRT-PCR

Index	Case 4	Case 5	Case 6	Case 7	Control 4	Control 5	Control 6	Control 7
Age (years)	58	65	58	58	58	56	61	68
Gender	Female	Male	Male	Female	Female	Male	Female	Male
Type	ESCC	ESCC	ESCC	ESCC	ESCC	ESCC	ESCC	ESCC
TNM stage	T3N1M0	T3N1M0	T3N3M0	T3N1M0	T3N0M0	T2N0M0	T3N0M0	T3N0M0
Tumor differentiation	Poor	Moderate	Moderate	Well	Well	Moderate	Moderate	Moderate

Case was indicated the patients of ESCC patients with metastasis. Control was indicated the patients of ESCC patients without metastasis. qRT-PCR, quantitative real-time polymerase chain reaction; ESCC, esophageal squamous cell carcinoma.

sequencing was translated into raw FASTQ sequence data by Base Calling. Nucleotides with a quality score <20 were trimmed from the end of the sequence and N base rate of raw reads more than 10% were discarded using Cutadapt 1.9.1. Finally, clean reads were obtained (13). TopHat was used to align the clean reads with the human reference genome, Ensemble GRCh38 *vs.* 84 (hg19) by using (14). Fragments per kilobase of exon per million fragments mapped (FPKM) was used to decipher the transcription abundance of long non-coding RNA (lncRNA) and protein coding mRNA (mRNA), which was quantified by cuffquant and cuffnorm. miRDeep2 was used to quantified the transcription abundance of miRNAs (15).

Differentially expressed genes analysis

The differentially expressed lncRNAs (DELs) and differentially expressed mRNA (DEMs) were identified in metastasis group compared with primary group via Cuffdiff. lncRNAs and mRNAs with FDR <0.05 and $|\log_2 FC >1|$ was selected as DELs and DEMs. In addition, differentially expressed miRNAs (DEMIs) in metastasis group compared with primary group were identified using DEGseq package in R language, with the threshold of FDR <0.001 and $|\log_2 FC >1|$.

Identification of target genes of DEMIs

miRWalk database (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/), was used to predict the target genes of DEMIs (16). In our study, 6 algorithms including RNA22, miRanda, miRDB, miRWalk, PICTAR and Targetscan in miRWalk was used. Moreover, the genes, predicted by more than 4 out of 6 algorithms, were as considered as the target gene of the DEMIs. DEMIs-target gene interacting pairs in a negative manner were subjected to construct DEMI-target gene interaction network, visualized by Cytoscape (17).

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis

In order to obtain insight into the biological functions and signaling pathways of dysregulated mRNAs in metastasis group involved in, GeneCoDis3 (http://genecodis.cnb.csic.es/analysis) analysis (18), including GO function and KEGG pathway enrichment were conducted. Items with FDR <0.05 was filed as significant enrichments.

qRT-PCR

Total RNA of metastasis group and primary group tissues were extracted by using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacture instructions.

FastQuant cDNA and miRcute Plus (Tiangen, Beijing, China) and miRNA First-Strand cDNA Synthesis Kit (Tiangen, Beijing, China) was used to synthesize the cDNA of mRNA and miRNA, respectively. qRT-PCR reactions were performed by using SuperReal PreMix Plus SYBR Green Kit (Tiangen, Beijing, China) and miRcute Plus miRNA qPCR Detection Kit (Tiangen, Beijing, China) on Applied Biosystems 7500 (Applied Biosystems, Foster City, CA, USA). *GAPDH* and *U6* were used as internal control for mRNA and miRNA detection, respectively. The relative expression of candidate genes was calculated by using the $2^{-\Delta\Delta CT}$ equation methods (19). The PCR primers used in our study were shown in *Table S1*. At least triple experiments were subjected to qRT-PCR verification.

Validation the expression of selected DELs, DEMIs and DEMs in the Cancer Genome Atlas (TCGA) database

As a public funded project, TCGA (https://tcga-data.nci.

Table 3 The top 10 up- and down-regulated DELs in metastasis group compared with primary group

Gene	Chromosome	FDR	Log_2(FC)
Up-regulation			
LOC105378306	chr10	0.003766	6.55743
SMIM2-AS1	chr13	0.024283	2.768527
LINC01348	chr1	0.003766	2.586664
LOC105369777	chr12	0.003766	2.240836
LOC105377734	chr5	0.003766	1.84847
LINC00668	chr18	0.003766	1.791835
LOC101927189	chr6	0.009723	1.379205
LOC101928978	chr4	0.003766	1.260869
LOC101929260	chr2	0.003766	1.258483
Down-regulation			
LINC01206	chr3	0.003766	−6.52182
XIST	chrX	0.043908	−5.7486
LOC102724702	chr12	0.003766	−5.727
SNHG5	chr6	0.003766	−4.42145
LOC105374264	chr3	0.003766	−3.70496
LOC102723828	chr4	0.012426	−2.99813
LOC101928599	chr14	0.003766	−2.60887
LOC105374020	chr3	0.003766	−2.55265
LOC101927522	chr19	0.003766	−2.49207
FIRRE	chrX	0.003766	−2.35665

Metastasis group indicated primary tumor loci of ESCC patients with metastasis; primary group indicated primary tumor loci of ESCC patients without metastasis. DELs, differentially expressed lncRNAs; ESCC, esophageal squamous cell carcinoma; FC, fold change; chr, chromosome; FDR, false discovery rate.

nih.gov/tcga/) stores multidimensional data of various human tumors at the DNA, RNA and protein levels. A TCGA illumine hiseq data consisted of 32 metastasis ESCC tissues and 40 primary ESCC tissues were used to validate the expression of three DELs (*LINC00668*, *XIST* and *SNHG5*) and nine DEMs (*AKR1B10*, *SOX5*, *CYP4F11*, *SLC7A11*, *HLA-C*, *ICAM2*, *CLDN8*, *CTLA4* and *KRT19*) between metastasis and primary ESCC tissues. A TCGA illumine hiseq data consisted of 38 metastasis ESCC tissues and 48 primary ESCC tissues were used to validate the expression of selected three DEMIs (*miR-224*, *miR-229* and *miR-200a*) between metastasis and primary ESCC tissues.

Statistical analysis

Mean ± standard deviation and independent-samples *t*-test was used in the statistical analysis. P<0.05 was considered as significant difference. * indicated P<0.05; ** indicated P<0.01 and *** indicated P<0.001.

Results

Identification of DELs and DMEs in metastasis group tissues compared with primary group tissues

Total of 43 DELs (9 up- and 34 down-regulated) and 205 DEMs (49 up- and 156 down-regulated) were identified in metastasis group tissues based on the threshold of FDR <0.05 and abs (log_2FC)≥1. *LOC105378306* and *LINC01206* were the most obviously up- and down-regulated DEL in metastasis group tissues, respectively (*Table 3*). In addition, *AKR1B10* and *SOX5* were the most obviously up- and down-regulated DEM in metastasis group tissues, respectively (*Table S2*).

In our study, 43 identified DELs were distributed in chromosomes X and 17 autosomes, apart from chromosome 7, 17, 20, 21 and 22; 205 DEMs were distributed in all autosomes and chromosomes X (*Figure 1*).

Identification of DEMIs in metastasis group tissues

Total of 128 DEMIs (9 up- and 119 down-regulated) were identified in metastasis group tissues compared with primary group tissues based on the threshold of FDR <0.001 and abs (log_2FC)1.5. The *hsa-miR-508-3p*, *hsa-miR-224-5p* and *hsa-miR-514a-3p* were the three significantly up-regulated DEMIs in metastasis group tissues; *hsa-miR-3664-3p*, *hsa-miR-449b-5p* and *hsa-miR-548ah-3p* were the three highly significantly down-regulated DEMIs in ESCC (*Table 4*).

DEMIs-DMEs interaction network

The target genes of 128 identified DEMIs, predicted through miRWalk database, were overlapped with 205 DEMs in metastasis group tissues and DEMIs-DMEs interaction pairs were distinguished, which were visualized using Cytoscape. As shown in *Figure 2*, DEMIs-DEMs interaction network consisted of 145 nodes and 214 edges, which involved in 88 DEMIs and 57 DEMs. The

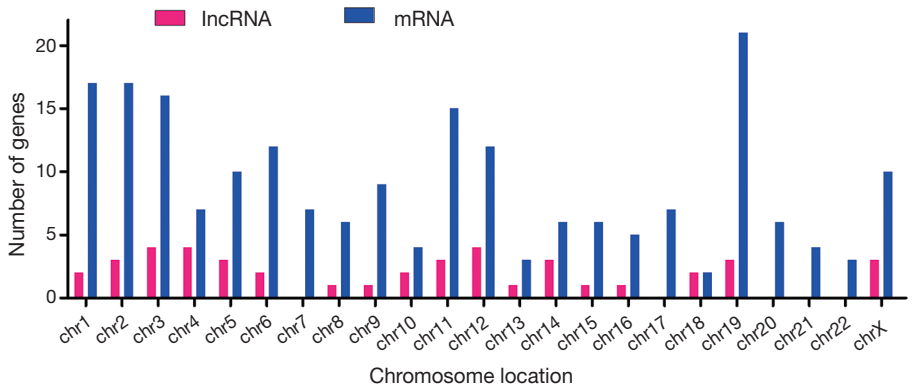

Figure 1 The distribution of DELs/DEMs in ESCC on chromosomes. Rose color and blue color indicated DELs and DEMs. The height of the bar represented the number of genes in each of chromosome. DELs, differentially expressed lncRNAs; DEMs, differentially expressed mRNAs; ESCC, esophageal squamous cell carcinoma.

Table 4 The top 10 up- and down-regulated DEMIs in metastasis group compared with primary group

Gene	Log_2(FC)	FDR
Up-regulation		
hsa-miR-508-3p	3.586963414	4.30×10^{-6}
hsa-miR-514a-3p	2.473143514	1.31×10^{-17}
hsa-miR-509-3p	2.22374711	9.86×10^{-8}
hsa-miR-889-3p	1.620048047	1.45×10^{-15}
hsa-miR-365a-5p	1.236777059	2.84×10^{-5}
hsa-miR-584-5p	1.142855985	2.48×10^{-18}
hsa-miR-337-3p	1.11854923	2.91×10^{-6}
hsa-miR-495-3p	1.116005542	4.56×10^{-5}
hsa-miR-224-5p	1.078239414	1.28×10^{-147}
Down-regulation		
hsa-miR-5683	–3.375997249	0
hsa-miR-9-5p	–2.931774354	0
hsa-miR-615-3p	–2.838946885	3.89×10^{-74}
hsa-miR-4488	–2.633253665	5.24×10^{-100}
hsa-miR-4417	–2.620426413	2.43×10^{-43}
hsa-miR-196b-5p	–2.611069026	0
hsa-miR-4454	–2.523096738	8.22×10^{-254}
hsa-miR-625-5p	–2.444349184	2.47×10^{-32}
hsa-miR-200a-3p	–2.387075711	0
hsa-miR-155-5p	–2.312662463	0

Metastasis group indicated primary tumor loci of ESCC patients with metastasis; primary group indicated primary tumor loci of ESCC patients without metastasis. DEMIs, differentially expressed microRNAs; ESCC, esophageal squamous cell carcinoma; FC, fold change; FDR, false discovery rate.

hsa-miR-495-3p, *hsa-miR-200b-5p* and *hsa-miR-200a-5p* had the highest connectivity with DEMs, regulated 8, 6 and 6 DEMs, respectively. *SLC7A11*, *GDNF* and *SRXN1* were regulated by 37, 15 and 13 DEMIs, respectively.

GO and KEGG pathway enrichment

To predict the functions of DEMs targeted by DEMIs, GO and KEGG pathway were conducted to demonstrate it. 205 DEMs were significantly enriched in 14 signaling pathways (*Table 5*), such as cytokine-cytokine receptor interaction (KEGG: 04060), MAPK signaling pathway (KEGG: 04010), pathways in cancer (KEGG: 05200), chemokine signaling pathway (KEGG: 04062) and cell adhesion molecules (CAMs, KEGG: 04514). In addition, 205 DEMs were significantly enriched in cell-cell signaling, digestion, cell fate commitment, positive regulation of cell division and positive regulation of cell proliferation of biological process (*Table S3*).

qRT-PCR validation of the expression level of representative genes

In order to validate the expression level of dysregulated genes in metastasis group tissues based on bioinformatics analysis, qRT-PCR was applied. Five dysregulated genes, including 1 lncRNA *XIST*, 3 DEMs (*AKR1B10*, *KRT19*, *SLC7A11*) and 1 DEMIs *hsa-miR-224-5p*, were chose for qRT-PCR verification in 4 metastasis group tissues and 4 primary group tissues. As *Figure 3A*,*B* shown, *AKR1B10* and *KRT19* were significantly up- and down-regulated in metastasis group compared with primary group; *SLC7A11* had the up-regulated tendency in

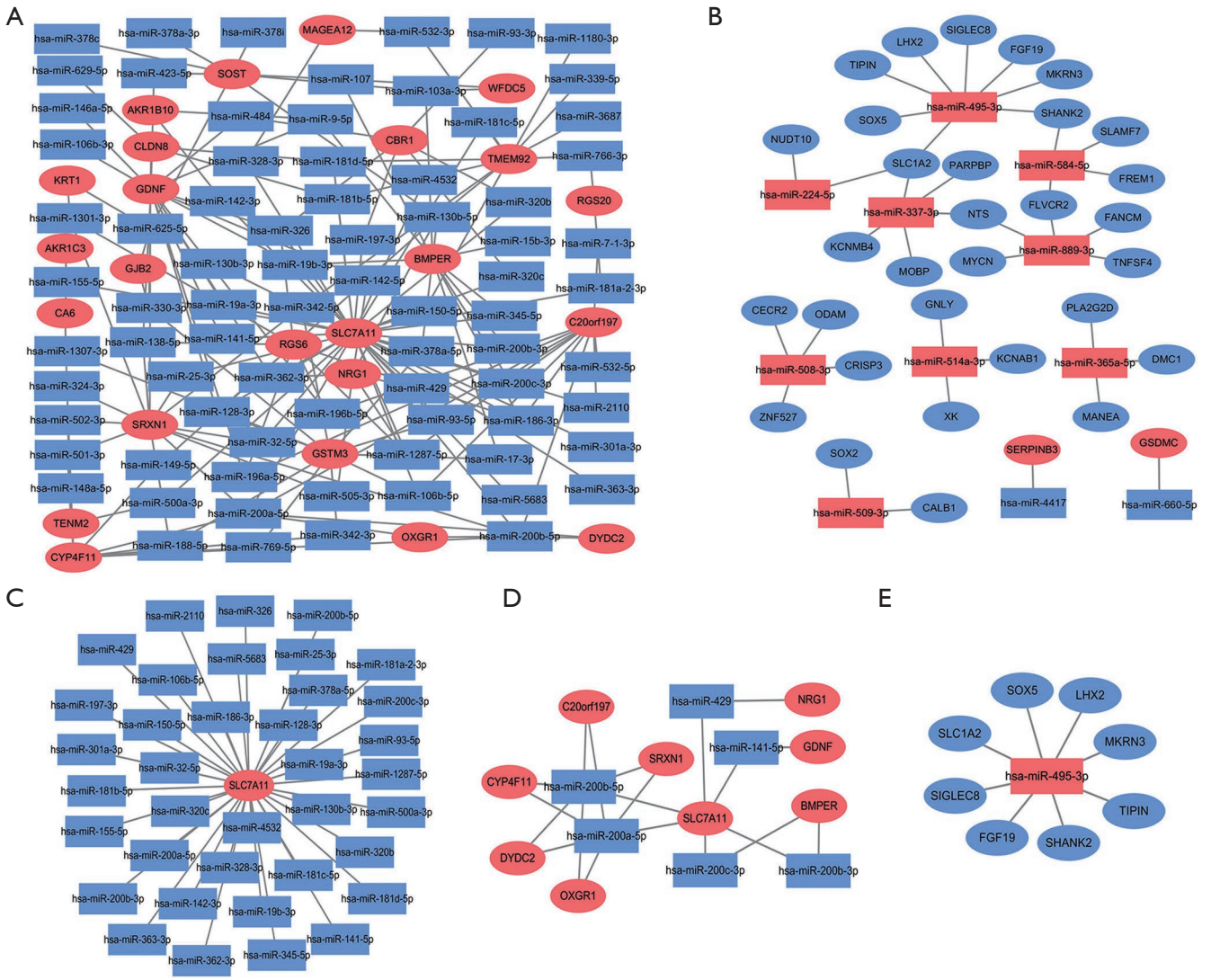

Figure 2 DEMIs-DEMs interaction network in ESCC metastasis. (A) The interaction network between down-regulated DEMIs and up-regulated DEMs; (B) the interaction network between up-regulated DEMIs and down-regulated DEMs; (C) the sub-network of SLC7A11; (D) the sub-network of miR-200 family; (E) the sub-network of hsa-miR-495-5p. The rectangle node and circular node indicated DEMIs and DEMs; the red color represented up-regulation; the blue color represented down-regulation. DEMIs, differentially expressed miRNAs; DEMs, differentially expressed mRNAs; ESCC, esophageal squamous cell carcinoma.

metastasis group (*Figure 3C*). In *Figure 3D,E*, lncRNA *XIST* was significantly down-regulated and DEMIs *hsa-miR-224-5p* had the up-regulated tendency in metastasis group compared with primary group.

Validation the expression of selected DELs, DEMIs and DEMs in TCGA database

The relative expression of selected DEMs, DEMIs and DELs between metastasis and primary ESCC tissues in the TCGA illumine hiseq data were shown in *Figure 4*. Four DEMs (*AKR1B10*, *CYP4F11*, *SLC7A11* and *CLDN8*), lncRNA *LINC00668* and *miR-224* were up-regulated while five DEGs (*SOX5*, *HLA-C*, *ICAM2*, *CTLA4* and *KRT19*), two DELs (*XIST* and *SNHG5*) and two DEMIs (*miR-429* and *miR-200a*) were down-regulated in metastasis ESCC tissues compared to primary metastasis. Except for *HLA-C*, *SNHG5*, *miR-429* and *miR-200a*, the expression of other 11 one was

Table 5 KEGG enrichment of dysregulated DEMs in metastasis group compared with primary group

Items	Items details	Gene	FDR	Genes
KEGG: 00590	Arachidonic acid metabolism	5	0.000263	*PLA2G2D, AKR1C3, CYP4F11, CBR1, ALOX12B*
KEGG: 00980	Metabolism of xenobiotics by cytochrome P450	4	0.006335	*GSTM3, AKR1C3, GSTM1, ALDH3A1*
KEGG: 03320	PPAR signaling pathway	4	0.004713	*FABP5, SLC27A2, PPARG, FABP6*
KEGG: 04810	Regulation of actin cytoskeleton	3	0.006262	*FGF19, FGF4, FGF3*
KEGG: 04010	MAPK signaling pathway	3	0.006262	*FGF19, FGF4, FGF3*
KEGG: 05200	Pathways in cancer	3	0.006262	*FGF19, FGF4, FGF3*
KEGG: 05218	Melanoma	3	0.006262	*FGF19, FGF4, FGF3*
KEGG: 00982	Drug metabolism—cytochrome P450	3	0.018244	*GSTM3, GSTM1, ALDH3A1*
KEGG: 04060	Cytokine-cytokine receptor interaction	3	0.018331	*CXCR3, CCL24, CCR5*
KEGG: 04062	Chemokine signaling pathway	3	0.018331	*CXCR3, CCL24, CCR5*
KEGG: 04514	Cell adhesion molecules (CAMs)	4	0.016798	*ICAM2, CLDN8, HLA-C, CTLA4*
KEGG: 04640	Hematopoietic cell lineage	3	0.030646	*CD3G, CD3D, CD33*
KEGG: 04970	Salivary secretion	3	0.029045	*MUC5B, BEST2, ADRA1B*
KEGG: 04660	T cell receptor signaling pathway	3	0.048031	*CD3G, CD3D, CTLA4*

Metastasis group indicated primary tumor loci of ESCC patients with metastasis; primary group indicated primary tumor loci of ESCC patients without metastasis. DEMIs, differentially expressed microRNAs; ESCC, esophageal squamous cell carcinoma; FDR, false discovery; KEGG, Kyoto Encyclopedia of Genes and Genomes.

consistent with our RNA-sequencing results, generally.

Discussion

Dysregulated DELs, DEMIs and DEMs were identified in ESCC metastasis group compared with primary group. In order to validate our bioinformatics analysis, the expression of selected DEMs, DEMIs and DELs were validated by qRT-PCR and TCGA illumine hiseq data. The validated expression results of qRT-PCR and TCGA illumine hiseq data were generally in accordance with our RNA-sequencing results which suggested that our RNA-sequencing results were convincing.

The *miR-200* family of miRNAs (*miR-141*, *200a*, *200b*, *200c* and *429*) are key regulators/inhibitors of epithelial to mesenchymal transition, which is involved in cancer cell behaviors including cell proliferation, cell cycle and apoptosis (20-22). In our study, all of *miR-200* family number including *miR-200a-5p*, *miR-200b-3p*, *miR-200b-5p*, *miR-200c-3p*, *miR-141-3p* and *miR-429* were significantly down-regulated in metastasis group compared with primary group.

Whereby, *miR-200b-5p* and *miR-200a-5p* were the hubs in DEMI-DEM interaction network. It is reported that the expression level of *miR-200a*, *miR-200b*, *miR-200c*, *miR-141* and *miR-429* are significantly lower in esophageal adenocarcinoma compared with Barrett's esophagus epithelium (23). The *miR-200b* suppresses cell invasiveness, cell growth and induces cell cycle arrest in ESCC; whereas, loss of *miR-200b* promotes cell invasiveness through activating Kindlin-2/integrin β1/AKT pathway in ESCC (24-26). Higher expression of *miR-200c* in serum patients with ESCC is significantly associated with TNM stage and worse response to platinum-based chemotherapy (27). Increased *miR-429* suppressed cell invasiveness and induces cell apoptosis by targeting *Bcl-2* and *SP-1* in esophageal carcinoma (28). A published article demonstrates *miR-141* is significantly over-expressed in ESCC compared with normal tissues, which is disharmony with our in silicon analysis, and over-expression *miR-141* contribute to an acquired chemo-resistance in EC-cells (29). Currently, the functions of *miR-141* in ESCC were unknown. The expression status of it need to be validated d in ESCC tissues by qRT-PCR in a large sample size of patients with ESCC, in addition, the roles of *miR-141* in cell behaviors of ESCC need to be explored in the future work. *SLC7A11*, with obviously significant up-regulation in metastasis group (*Table S2*), was

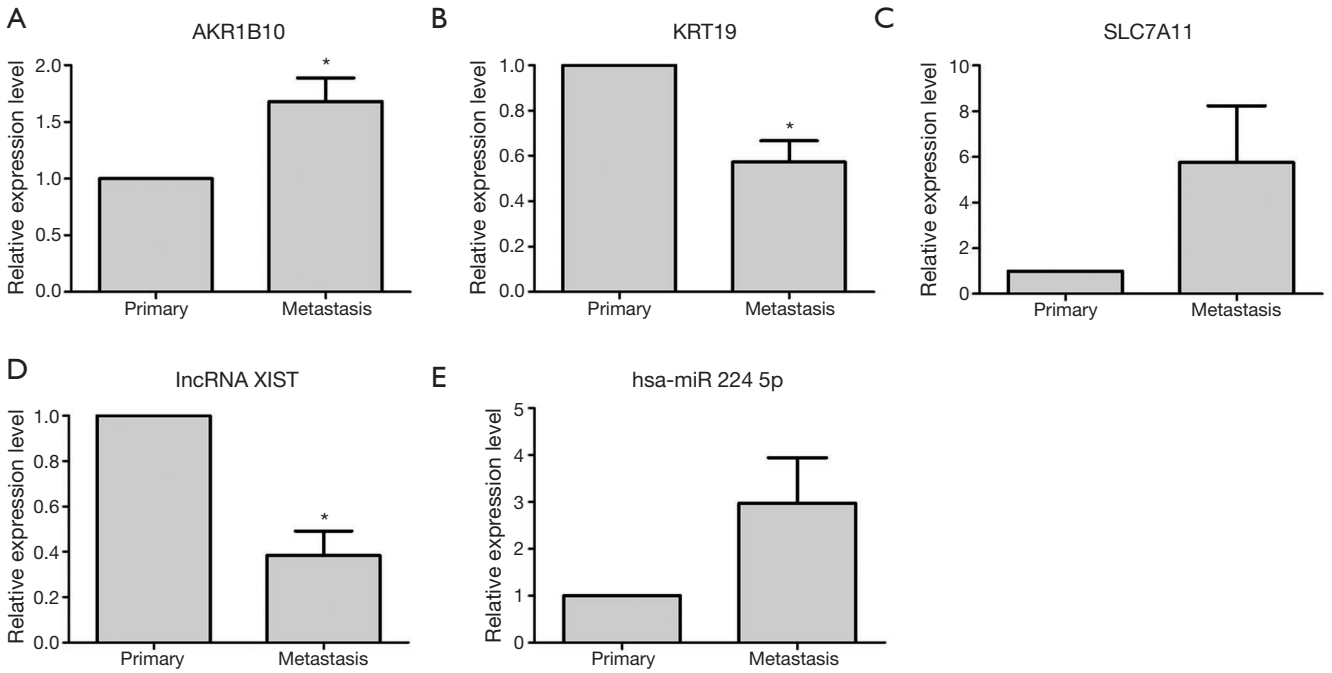

Figure 3 qRT-PCR validation of representative dysregulated DELs, DEMs and DEMIs in metastasis group tissues compared with primary group tissues. (A) The expression level of AKR1B10; (B) the expression level of KRT19; (C) the expression level of SLC7A11; (D) the expression level of lncRNA XIST; (E) the expression level of hsa-miR-224-5p. *, represented P<0.05. Metastasis group indicated primary tumor loci of ESCC patients with metastasis; primary group indicated primary tumor loci of ESCC patients without metastasis. qRT-PCR, quantitative real-time polymerase chain reaction; DELs, differentially expressed lncRNAs; DEMs, differentially expressed mRNAs; DEMIs, differentially expressed miRNAs; ESCC, esophageal squamous cell carcinoma.

targeted by 37 DEMIs, such as 6 abovementioned members of *miR-200* family. Our bioinformatics analysis and qRT-PCR verification indicated *SLC7A11* was up-regulated in metastasis group compared with primary group. Increased *SLC7A11* induces cell growth and is positively correlated with tumor invasiveness and shorter overall survival in patients with glioblastomas (30,31). Along with *SCL7A11*, *CYP4F11*, belonged to top 10 up-regulated DEMs in metastasis group (*Table S2*), was targeted by *miR-200b-5p*, respectively. *CYP4F11* is highly expressed in breast cancer than in adjacent normal tissues, which is associated with Ki67 protein expression (32). However, the biological function of *CYP4F11* in ESCC has not been demonstrated. Based on aforementioned information, *miR-200* family might play essential roles in tumorigenesis and metastasis process of ESCC by regulating the expression of their target DEMs.

LncRNA *LINC00668*, *XIST* and *SNHG5* were significantly aberrant expression in metastasis group compared with primary group in ESCC. *LINC00668* was ectopically expressed in metastasis group (*Table 3*). *In vivo* and *in vitro* experiments decipher that over-expression of *LINC00668*, induced by *E2F1* transcription factor, enhance cell proliferation and predicts a poor prognosis of patients with gastric cancer (33). The expression level of *XIST* and *SNHG5* was significantly decreased in metastasis group (*Table 3*). *SNHG5* over-expression inhibits cell growth, migration, invasion and metastasis of gastric cancer *in vivo* and *in vitro* experiments (34). Moreover, the serum level of *SNHG5* is significantly higher in the patients with melanoma than in the normal subjected, which might be a new tumor marker of malignant melanoma (35). *XIST* is found to be up-regulated and acts as oncogene in a number of cancer types, including non-small cell lung cancer (NSCLC), gastric cancer and glioblastoma (36-38). In NSCLC, increased *XIST* predicts shorter overall survival and poor prognosis of patients; knockdown of *XIST* impedes cell proliferation, migration and invasion (36). In gastric cancer, over-expression of *XIST* is markedly associated with lymph node invasion, distant metastasis

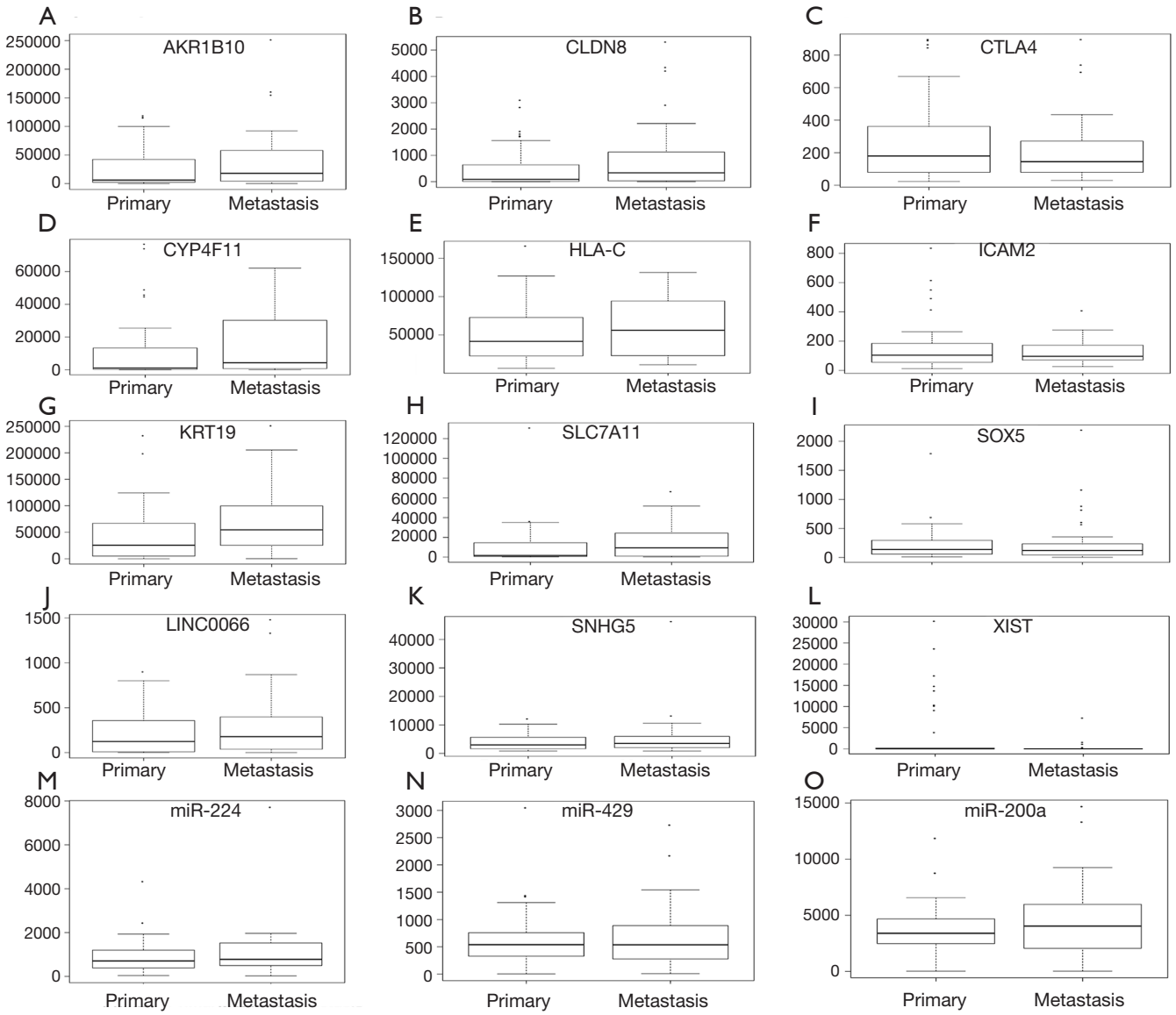

Figure 4 TCGA validations of selected DELs, DEMs and DEMIs in metastasis group tissues compared with primary group tissues. The X-axis shows primary and metastasis ESCC groups and Y-axis shows expression reads counts. (A) AKR1B10; (B) CLDN8; (C) CTLA4; (D) CYP4F11; (E) HLA-C; (F) ICAM2; (G) KRT19; (H) SLC7A11; (I) SOX5; (J) LINC0066; (K) SNHG5; (L) XIST; (M) miR-224; (N) miR-429; (O) miR-200a. DELs, differentially expressed lncRNAs; DEMs, differentially expressed mRNAs; DEMIs, differentially expressed miRNAs.

and TNM stage in patients; silencing *XIST* inhibits cell growth and cell metastasis (37). However, the functions of *LINC00668*, *XIST* and *SNHG5* have not been elucidated in ESCC, which might exert essential functions in tumorigenesis and metastasis of ESCC.

AKR1B10 and *SOX5* was the significantly up- and down-regulated DEMs in metastasis group (*Table S2*). A previous study reports that *AKR1B10* is dramatically up-regulated during chemo-resistant induction in gastric cancer cell, which facilitates cell migration and invasiveness, through down-regulating PPARγ and reducing the sensitivity to the chemotherapy (39,40). Protein *AKR1B10*, a member of aldo keto reductases family, is involved in digestion and is highly expressed in the gastrointestinal tract. *AKR1B10* was significantly enriched in digestion of GO terms. *SOX3*, *SOX5*, *SOX8*, the member of SRY-related HGM-box

family of transcription factors, were all down-regulated in metastasis group. *SOX5* promotes epithelial-mesenchymal transition and cell invasion through regulation of Twist1 in hepatocellular carcinoma (41). In addition, *SOX5* and *SOX8* were significantly enriched in cell fate commitment in our work. In our study, *HLA-C* was significantly down-regulated in metastasis group. It is reported that *HLA-C* is down-regulated at both the protein and mRNA levels in human ESCC, which might attributes to DNA hypermethylation of the promoter region of *HLA-C* (42). In currently, the mechanisms of *AKR1B10*, *SOX5* and *HLA-C* in ESCC tumorigenesis and metastasis are ambiguous.

Dysregulated DEMs in metastasis group were highly enriched in 13 KEGG signaling pathways including MAPK signaling pathway, pathways in cancer and CAMs; and significantly enriched in digestion, cell fate commitment, positive regulation of cell division and positive regulation of cell proliferation of biological process. In our work, along with *HLA-C*, identified DEMs of *ICAM2*, *CLDN8* and *CTLA4* were enriched in CAMs pathway, which is involved in a series of biologic processes, including cellular adhesion, cell fate and cell metastasis in cancer (43,44). CAMs are composed of 4 main groups, such as integrin family, the immunoglobulin super family, selectins, and cadherins. In esophageal carcinoma, higher expression of *CTLA-4* in the tumor environment is associated with shorter overall survival, which predicts poor prognosis (45). In colon cancer, CAMs pathway associated genes (*ICAM2*) is modulated by down-regulation *WISP1*, and protein β-catenin binds with *WISP1*, exerts the functions of promoting cell proliferation and cell invasiveness (46). *CLDN8*, an integral membrane protein, forms tight junctions together with occludin. *CLDN8* was downregulated in metastasis group in our analysis and it is found to be down-regulated in colorectal tumors compared to adjacent non-tumor tissues (47,48).

Conclusions

In conclusion, we identified the expression profiling of abnormally expressed lnRNAs, miRNAs and mRNAs in ESCC metastasis. Our study indicated that those dysregulated genes including *XIST*, *SNHG5*, *miR-200* family, *AKR1B10* and *SOX5* might synergistically contributes to tumorigenesis and metastasis process in ESCC via complex interactions between each other through MAPK signaling pathway, pathways in cancer and CAMs. Our study might lay the foundation for illumination of tumorigenesis and metastasis mechanisms and discovery of potential therapeutic targets in ESCC metastasis. However, pooled RNA-sequencing in our study is a limitation. Although pooled RNA-sequencing is reliable and cost-effective approach to obtain genome-wide information (49), analysis of variation in expression level between individuals was lacked. Sequencing using RNAs from individual ESCC tissues and compare the expression profiling between primary and metastasis ESCC tissues with larger sample size were need.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2017.10.35). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This work was approved by the Ethics Committee of the Daping Hospital (2015-035) and informed written consent was obtained from all patients.

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Supplementary

Table S1 The primers for qRT-PCR

Primers	Sequence
XIST	Forward: 5' GCATAACTCGGCTTAGGGCT 3'
XIST	Reverse: 5'TCCTCTGCCTGACCTGCTAT3'
AKR1B10	Forward: 5'TCAGAATGAACATGAAGTGGGG3'
AKR1B10	Reverse: 5'TGGGCCACAACTTGCTGAC3'
KRT19	Forward: 5'ACCAAGTTTGAGACGGAACAG3'
KRT19	Reverse: 5'CCCTCAGCGTACTGATTTCCT3'
SLC7A11	Forward: 5'GGTCCATTACCAGCTTTTGTACG3'
SLC7A11	Reverse: 5'AATGTAGCGTCCAAATGCCAG3'
hsa-miR-224-5p	Forward: 5'CAAGUCACUAGUGGUUCCGUU3'
GAPDH	Forward: 5'GGAGCGAGATCCCTCCAAAAT3'
GAPDH	Reverse: 5'GGCTGTTGTCATACTTCTCATGG3'
U6	Forward: 5'CTCGCTTCGGCAGCACA3'
U6	Reverse: 5'AACGCTTCACGAATTTGCGT3'

qRT-PCR, quantitative real-time polymerase chain reaction.

Table S2 The top 10 up- and down-regulated DEMs in metastasis group tissues compared with primary group tissues

Gene	Chromosome	FDR	Log_2(FC)
Up-regulation			
AKR1B10	chr7	0.00376565	7.057264
RGS6	chr14	0.00376565	6.824392
LGALS7B	chr19	0.00376565	6.810906
SLC7A11	chr4	0.00376565	5.382058
GSDMC	chr8	0.00376565	5.336748
CYP4F11	chr19	0.0304908	5.288782
MUC5B	chr11	0.0173033	4.890905
GKN1	chr2	0.00376565	4.88301
ATP4B	chr13	0.00376565	4.806546
ALDH3A1	chr17	0.00688717	4.632386
Down-regulation			
SOX5	chr12	0.0263028	–8.38081
EFCAB6	chr22	0.00376565	–6.31789
HLA-C	chr6	0.00376565	–6.24172
FREM1	chr9	0.00972307	–5.96312
FDCSP	chr4	0.045714	–5.52268
CECR2	chr22	0.0404465	–4.99631
FGF19	chr11	0.00376565	–4.96977
KRT4	chr12	0.00376565	–4.90891
CDKL3	chr5	0.00688717	–4.85269
NUDT10	chrX	0.00376565	–4.71575

Metastasis group indicated primary tumor loci of ESCC patients with metastasis; primary group indicated primary tumor loci of ESCC patients without metastasis. DEMs, differentially expressed mRNAs; ESCC, esophageal squamous cell carcinoma; FC, fold change; chr, chromosome.

Table S3 GO terms enrichment of dysregulated DEMs in metastasis group compared with primary group

Items	Items details	Gene	FDR	Genes
GO: 0007267	Cell-cell signaling (BP)	10	5.91×10^{-5}	*BARX1, SIRPG, FGF3, CCL24, ADRA1B, EFNA2, FGF4, GJB2, CCR5, CD33*
GO: 0007586	Digestion (BP)	6	6.90×10^{-5}	*TFF2, CCKAR, AKR1B10, GKN1, AKR1C3, CHIA*
GO: 0045165	Cell fate commitment (BP)	3	0.000105	*PPARG, SOX5, SOX8*
GO: 0007399	Nervous system development (BP)	3	0.000371	*GDNF, GBX2, FGF19*
GO: 0045892	Negative regulation of transcription	11	0.000436	*NR0B1, TBX20, PPARG, SOX5, NRG1, ZNF256, RCOR2, SOX8, L3MBTL1, MYB, TNFSF4*
GO: 0008206	Bile acid metabolic process (BP)	4	0.000565	*FABP6, SLC27A2, SLCO1B3, AKR1C3*
GO: 0001755	Neural crest cell migration (BP)	4	0.000577	*GDNF, SOX8, GBX2, FGF19*
GO: 0007275	Multicellular organismal development (BP)	4	0.000577	*BARX1, FGF3, ADRA1B, FGF4*
GO: 0015721	Bile acid and bile salt transport (BP)	3	0.000615	*FABP6, SLCO1B3, AKR1C3*
GO: 0042493	Response to drug (BP)	8	0.002486	*GAD1, PPARG, CARD9, APOC2,EMX1, CPS1, ALDH3A1, SLC1A2*
GO: 0008543	Fibroblast growth factor receptor signaling pathway (BP)	3	0.002593	*FGF3, FGF4, FGF19*
GO: 0008286	Insulin receptor signaling pathway (BP)	3	0.002593	*FGF3, FGF4, FGF19*
GO: 0045892	Negative regulation of transcription, DNA-dependent (BP)	5	0.002599	*TBX20, PPARG, SOX5, SOX8, MYB*
GO: 0051781	Positive regulation of cell division (BP)	3	0.003262	*FGF3, GKN1, FGF4*
GO: 0008284	Positive regulation of cell proliferation (BP)	3	0.003262	*FGF3, GKN1, FGF4*

Metastasis group indicated primary tumor loci of ESCC patients with metastasis; primary group indicated primary tumor loci of ESCC patients without metastasis. FDR, false discovery rate; ESCC, esophageal squamous cell carcinoma; BP, biological process; GO, Gene Ontology.