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Original Research Article

Identification of hub genes and microRNAs with prognostic values in esophageal cancer by integrated analysis

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ABSTRACT

Esophageal cancer (EC) is the eighth most common cancer in the world, and the sixth most common cause of cancer-related mortality. The aim of the present study was to identify cell and molecular mechanisms involved in EC, and to provide the potential targets for diagnosis and treatment. Here, a microarray dataset (GSE20347) was screened to find differentially expressed genes (DEGs). Different bioinformatic methods were used to analyze the identified DEGs. The up-regulated DEGs were significantly involved in different biological processes and pathways including extracellular matrix organization and ECM-receptor interaction. FN1, CDK1, AURKA, TOP2A, FOXM1, BIRC5, CDC6, UBE2C, TTK, and TPX2 were identified as the most important genes among the up-regulated DEGs. Our analysis showed that has-miR-29a-3p, has-miR-29b-3p, has-miR-29c-3p, and has-miR-767–5p had the largest number of common targets among the up-regulated DEGs. These findings strengthen the understanding of EC development and progression, as well as representing potential markers for EC diagnosis and treatment.

1. Introduction

According to the world health organization (WHO), esophageal cancer (EC) is the eighth most common cancer in the world, and the sixth most common cause of cancer-related mortality [1]. The two main histological subtypes of EC are esophageal squamous cell carcinoma (ESCC), and esophageal adenocarcinoma (EA). The esophageal cancer belt, which includes part of northern Iran, central Asia, and China is found to have the highest prevalence of ESCC [2]. When it comes to the most prevalent type of EC in western countries, (EA) is rapidly overtaking ESCC, as the most common EC worldwide [3]. Despite advancements in surgery, radiation, and chemotherapy survival rates for ESCC have not improved considerably over the past three decades, and the clinical outcome has remained poor [4]. Resistance to radiotherapy and chemotherapy is a major issue in the treatment of ESCC nowadays [5]. EC screening is expected to become increasingly important in the future, both in terms of prevention and fatality rates [6].

In the last decade, scientists have focused on unraveling the molecular mechanisms of EC initiation, progression, and metastasis. It has been reported that the two most significant genes mutated in ESCC are *TP53*, and *NOTCH1* (92 and 33%, respectively) [7]. Moreover, it has been shown that some cellular, and molecular regulators such as microRNAs (miRNAs), signaling pathways, and transcription factors (TFs) are involved in EC development, and progression [8–10]. miR-195–5p, and miR-135b-5p were shown to be strongly correlated with tumor-node metastasis (TNM) stage, and lymphatic metastasis status in ESCC patients [11]. A new study suggests that the miR-1/FN1 axis may be an effective treatment target for ESCC, and that miR-1 is an important prognostic indicator [12]. AGE-RAGE signaling, *AKR1C1, AKR1C2, TNF*, *ICAM1*, *GPR68*, *GNB4*, *SERPINE1*, *MMP12*, and hsa-miR-34b-3p were shown to be dysregulated in ESCC patients [13] Because of the high proportion of genetic and molecular heterogeneity found in ESCC cancer, the prediction, diagnosis, and prognosis of the disease are all very important. There aren't many clinically validated

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biomarkers for early EC diagnosis. Biomarkers are worthwhile for diagnosis, monitoring disease progression, predicting disease recurrence and therapeutic treatment efficacy. Currently, molecular targeted therapies have been approved by the Food and Drug Administration (FDA) for EC treatment, including human epidermal growth factor receptor 2 (HER-2), vascular endothelial growth factor receptor (VEGFR), and programmed death ligand 1 (PD-L1) inhibitors [4]. However, ESCC, still remains susceptible to relapse, metastasis, and development of resistance to treatment, leading to poor prognosis [14]. Therefore, it is vital to discover novel biomarkers to further optimize the EC treatment regimen. Recently, some biomarkers have been suggested for ESCC prediction. For example, miR-193b has been suggested as a promising biomarker for the prediction of chemo-radiation sensitivity in ESCC patients [15]. The constant development of miRNAs as biomarkers or therapeutic targets, denotes a new field of EC therapy. Moreover, analyzing the expression profile of genes and miRNAs involved in EC development, may contribute to deciphering the molecular mechanisms of EC pathogenesis.

A distinctive feature in the development of malignant tumors is the alteration of metabolic processes and metabolic reprogramming, which drives tumor initiation, progression, and metastasis [16]. The potential role of metabolomics has been studied in various cancers such as ovarian cancer, melanoma and lung adenocarcinoma [17–19]. A recent study showed that OIP5 promotes ESCC initiation, and development via regulating intracellular lipid metabolism [20]. Therefore, targeting the metabolic differences between tumor and normal cells or the shared metabolic vulnerabilities between tumors, holds promise as a novel anti-cancer strategy. Moreover, with the interaction of metabolomics and transcriptomics a deeper comprehension of tumor pathogenesis in ESCC may be possible.

In recent years, the development of transcriptome technologies (i.e. microarray and RNA-Seq) provided promising information for the identification of molecular and cellular regulators involved in cancer development and progress, as well as for finding the association between gene expression and clinical outcomes [21]. To this end, researchers have used transcriptome data to identify cellular and molecular regulators of different cancers, as well as to identify biomarkers [22]. It has been difficult to identify biomarkers and therapeutic targets for ESCC up to this point, due to molecular characteristics, unique tumor microenvironment, tumor heterogeneity and origin [14]. Therapies targeting epithermal growth factor receptor (EGFR) or the mesenchymal-epithelial transition have not been successful, and drug resistance persists [23]. However, advancements in high-throughput genomic technologies have provided a better opportunity for understanding disease heterogeneity, and molecular mechanisms underlying ESCC development [24]. Therefore, in this study, we used ESCC microarray data and different bioinformatics analysis to find important cell and molecular regulators involved in the development, and invasion of ESCC.

2. Materials and methods

2.1. Microarray data and DEG screening

The gene expression profiles of the GSE20347 dataset were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). The GSE20347 dataset contained.

34 samples, including 17 esophageal cell carcinoma samples, and 17 normal adjacent esophageal tissue samples. All GSE series matrix files parsed by GEOquery package [25] with R version 4.0.1 and the GSE series values normalized with R. The linear models for microarray data (Limma) package [26] was used to determine the differentially expressed genes (DEGs), which were significantly up- and down-regulated in ESCC. DEGs were filtered by univariate tests according to the p-value <0.05 and log2 fold change ($|\log2\ FC| \geq 1$).

2.2. Gene ontology and functional enrichment analysis

The Gene Ontology (GO) analysis was conducted for up- and down-regulated DEGs to identify the GO categories, including biological process (BP), cellular component (CC), and molecular function (MF). Additionally, the KEGG database was used to find the DEGs-associated signaling pathways [27]. Enrichr (http://amp.pharm.mssm.edu/Enrichr) was used to determine GO and KEGG pathways.

2.3. Construction of the protein–protein interaction (PPI) network and module analysis

To determine the TFs, the identified DEG list was compared with a full list of human TFs [28]. eXpression2Kinases (X2K) was used to find protein kinases (PKs) that interact with the TFs, and intermediate proteins (IPs) [29]. Then, STRING was used to determine interactions between TFs, PKs, and IPs. Consequently, the protein interaction data were imported into the Cytoscape software [30] version 3.8.8 to obtain a network interaction map. Subsequently, the top 10 connected genes (for the up- and down-regulated DEGs) with the highest interactions were selected as hub genes for further analysis. Also, the functional module(s) of the PPI network were identified by the Molecular Complex Detection (MCODE) plug-in of Cytoscape (version 1.8.2) [31].

2.4. Hub gene analysis and validation

To confirm the obtained results, the GEPIA database (http://gepia.cancer-pku.cn/) was used to compare the expression of hub genes in cancer and normal tissues. Moreover, Receiver operating characteristic (ROC) curves were used to evaluate the performance of the hub genes as biomarkers in distinguishing between cancer and normal tissues based on GSE161533 dataset. The ROC curves were drawn using GraphPad Prism 8.0 software (GraphPad Software, lnc., La Jolla, California) and the area under the curve (AUC) values were calculated.

2.5. Prediction of miRNAs targeting cancer genes

To find the miRNAs that target the up- and down-regulated DEGs, miRTarBase was used. Cytoscape was also used to determine the degree of connections and the number of targets between the miRNAs and tumor genes.

2.6. Analysis of DEGs-associated with metabolites

To find the DEGs-related metabolites, the Enrichr dataset linked Human Metabolome Database (HMDB) was used (http://amp.pharm.mssm.edu).Top metabolites associated with up- and down-regulated DEGs were retrieved and ranked based on p-value (p-value ≤ 0.05).

3. Results

3.1. GO functional enrichment and KEGG pathway analysis of DEGs

A total of 1303 DEGs, including 597 up-regulated and 706down-regulated genes, were obtained comparing ESCC samples, and normal adjacent esophageal tissue samples (Supplementary File 1). To investigate the function of the DEGs, GO term enrichment analysis was conducted (Fig. 1). The results of GO analysis specified that the up-regulated DEGs were significantly enriched in BPs associated with extracellular matrix organization, collagen fibril organization, protein complex subunit organization, and extracellular matrix disassembly (Fig. 1A). Down-regulated DEGs mainly participated in the BPs associated with keratinocyte differentiation, peptide cross-linking, neutrophil activation involved in immune response, skin development, neutrophil degranulation, and neutrophil-mediated immunity (Fig. 1A). MF analysis displayed that the up-regulated DEGs were mainly present in collagen

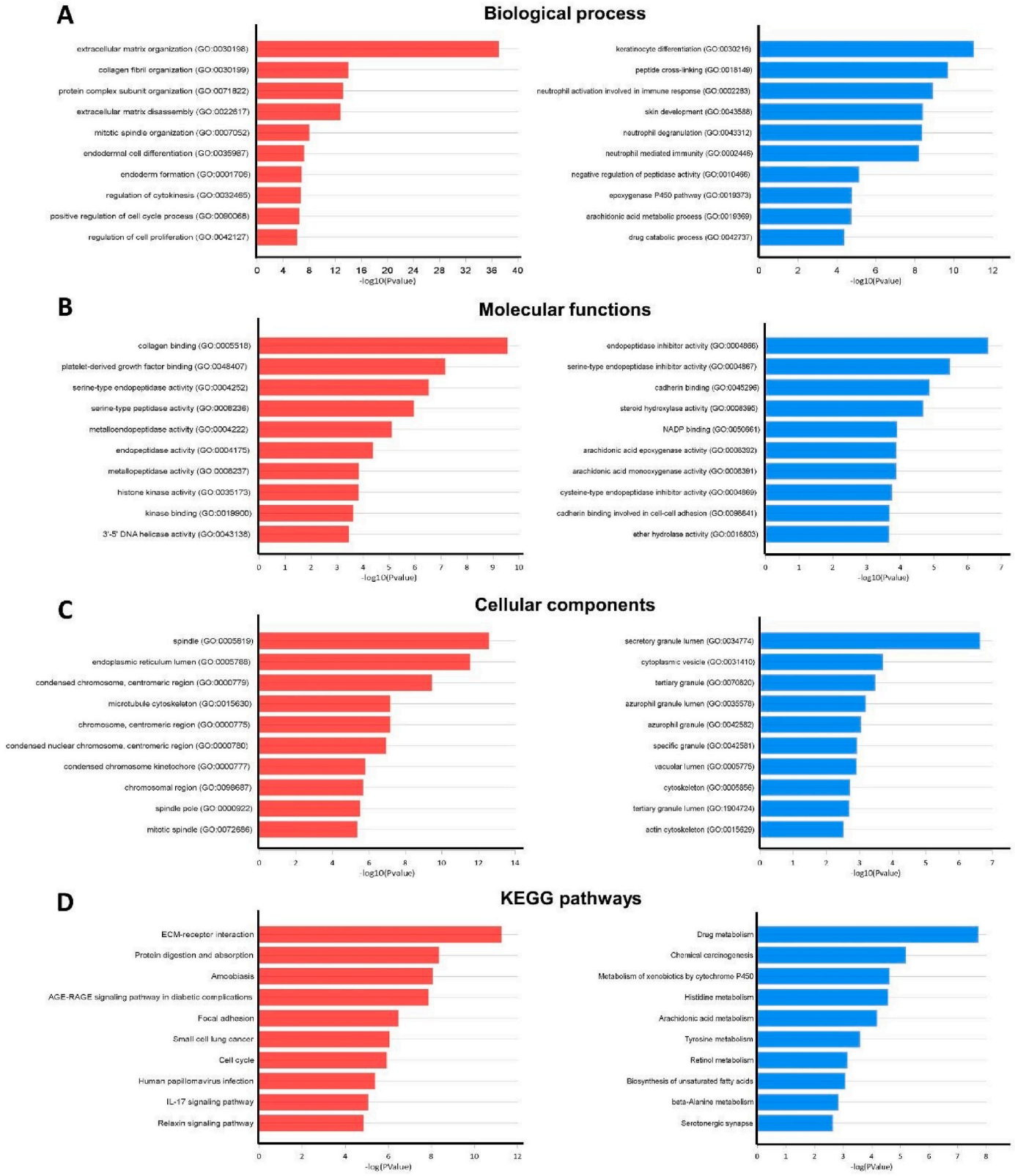


Fig. 1. Gene set enrichment and pathway analyses of the up- (red charts) and down-regulated (blue charts) genes. (A, B, C): Top 10 GO terms enriched by DEGs. Biological process (BP), Molecular Functions (MF), Cellular components (CC). **(D):** KEGG pathway enrichment analysis for the identified DEGs.

binding, platelet-derived growth factor binding, serine-type endopeptidase activity, serine-type peptidase activity, metalloendopeptidase activity, and endopeptidase activity, while the down-regulated DEGs were mainly enriched in endopeptidase inhibitor activity, serine-type endopeptidase inhibitor activity, cadherin binding and steroid hydroxylase activity (Fig. 1B). CC analysis showed that the up-regulated DEGs were mainly enriched in the spindle, endoplasmic reticulum lumen, and condensed chromosome, centromeric region, whereas the down-

regulated DEGs were mainly enriched in secretory granule lumen, cytoplasmic vesicle, tertiary granule, and azurophil granule lumen (Fig. 1C).

KEGG pathway analysis identified that the up-regulated DEGs were significantly involved in ECM-receptor interaction, protein digestion and absorption, amoebiasis, and AGE-RAGE signaling pathway in diabetic complications, whereas the down-regulated DEGs were mainly enriched in drug metabolism, chemical carcinogenesis, metabolism of xenobiotics by cytochrome P450, histidine metabolism, and arachidonic acid metabolism (Fig. 1D).

3.2. Protein-protein interaction (PPI) network construction and identification of hub genes

Analysis of the X2K for DEGs showed a list of protein kinases that are potentially involved in the expression of up- and down-regulated DEGs. The top ten protein kinases for the up-regulated DEGs were Cyclin-Dependent Kinase 1 (CDK1), Polo Like Kinase 1 (PLK1), Aurora Kinase A (AURKA), Casein Kinase 2 Alpha 2 (CSNK2A2), TTK Protein Kinase (TTK), Casein Kinase 2 Alpha 1 (CSNK2A1), Protein Kinase C Alpha (PRKCA), ATR Serine/Threonine Kinase (ATR), Glycogen Synthase Kinase 3 Beta (GSK3B), and Cyclin- Dependent Kinase 2 (CDK2). X2K analysis also identified the down-regulated DEGs associated with the top ten kinases, including Mitogen-Activated Protein Kinase Kinase 2 (MAP2K2), EPH Receptor A1 (EPHA1), Protein Kinase N3 (PKN3), Protein Kinase N1 (PKN1), SRC Proto-Oncogene, Non- Receptor Tyrosine Kinase (SRC), Platelet-Derived Growth Factor Receptor Beta (PDGFRB), Mitogen-Activated Protein Kinase Kinase 1 (MAP2K1), Mitogen-Activated Protein Kinase Kinase 3 (MAPK3), WNK Lysine Deficient Protein Kinase 4 (WNK4), and Serine/Threonine Kinase 39 (STK39).

Screening of the PPI network for up-regulated DEGs identified the important proteins with the highest number of connections ($49 \leq n \leq 67$). Accordingly, fibronectin 1 (FN1), Cyclin Dependent Kinase 1 (CDK1), Aurora Kinase A (AURKA), Topoisomerase (DNA) II alpha (TOP2A), Forkhead Box M1 (FOXM1), Baculoviral IAP Repeat-Containing 5 (BIRC5), Cell Division Cycle 6 (CDC6), Ubiquitin-Conjugating Enzyme E2 C (UBE2C), TTK Protein Kinase (TTK), and TPX2 Microtubule Nucleation Factor (TPX2) had the highest number of connections, respectively (Fig. 2A). On the other hand, the PPI network constructed for down-regulated genes revealed the proteins with the highest number of connections ($17 \leq n \leq 25$). It was identified that Filaggrin (FLG), Small Proline Rich Protein 1B (SPRR1B), Involucrin (IVL), Desmoglein 1 (DSG1), Small Proline Rich Protein 1A (SPRR1A), Small Proline Rich Protein 3 (SPRR3), Peptidase Inhibitor 3 (PI3), Periplakin (PPL), Transglutaminase 1 (TGM1), and Envoplakin (EVPL) had the highest number of connections, respectively (Fig. 2B).

3.3. Validation of the hub genes

The expression level of the top ten proteins identified using PPI network analysis was validated in The Cancer Genome Atlas (TCGA) dataset. It was identified that the expression levels of the candidate genes, extracted from the up-regulated genes PPI network, was

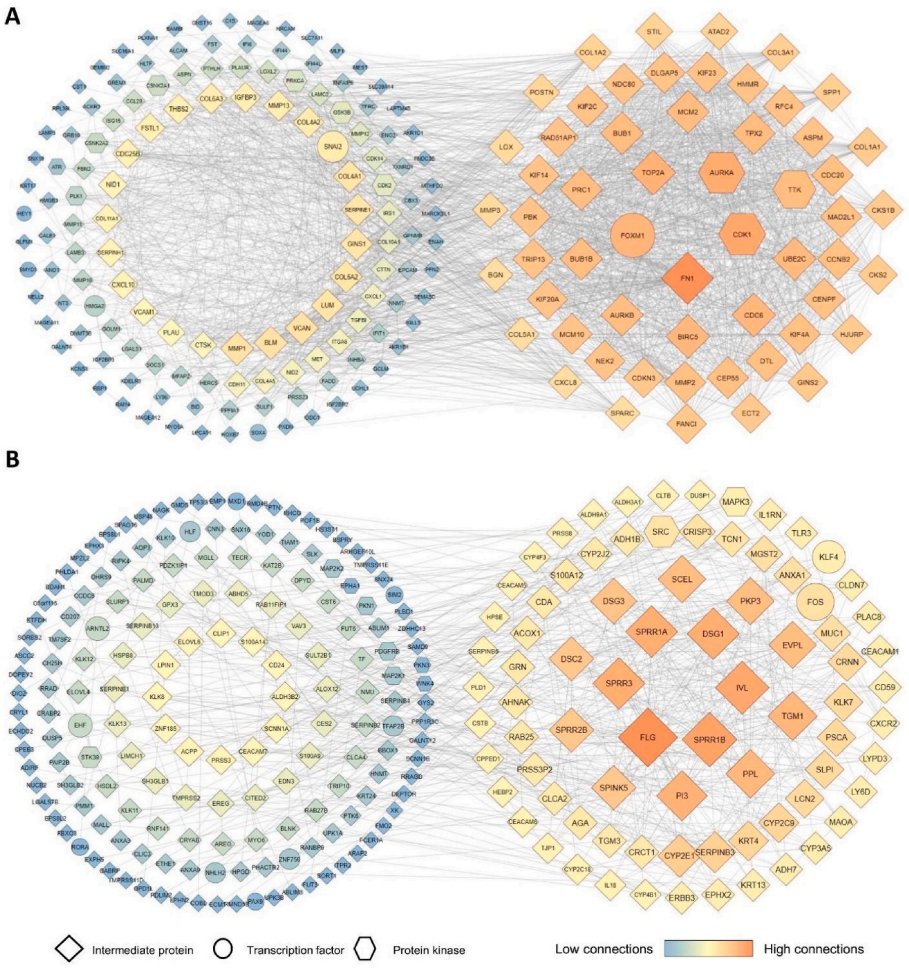


Fig. 2. Protein-protein interaction (PPI) network of DEGs in the ESCC. (A): PPI network for upregulated DEGs, **(B):** PPI network for down-regulated DEGs. Circles represent transcription factors (TFs), hexagons represent protein kinases (PIs) and diamonds are for intermediate proteins (IPs).

significantly higher in the ESCC tumor samples (Fig. 3A). In contrast, the expression levels of the candidate genes, selected from the down-regulated genes PPI network, was significantly higher in normal tissue (Fig. 3B).

To further verify our hub genes, we used an external dataset, GSE161533, consisting of tissue samples from 28 ESCC patients and 28 healthy controls. A total of 1467 genes showed significant up/down regulation in GSE161533 (710 up-regulated and 757 down-regulated). Integration of the results, showed 305 and 293 common up- and down-regulated genes, between GSE20347 and GSE161533, respectively. We found that, except for FN1, all of our identified up-regulated hub genes were among the common up-regulated genes between GSE20347 and GSE161533. Additionally, all of the identified hub genes of the down-regulated DEGs in this study, were among the common down-regulated DEGs between GSE20347 and GSE161533, apart from SPRR1B, SPRR1A and PI3. Receiver operating characteristic (ROC) curves were constructed to verify the diagnostic performance of these common hub genes based on the GSE161533 dataset. The area under the curve (AUC) values for most of the hub genes were above 0.9 (Supplementary File2, Fig. S2).

3.4. Module network analysis

The module analysis of DEGs was performed using Cytoscape. The top three significant modules were selected for more analysis. Furthermore, the BiNGO plugin is directly utilized to enrich the GO-BP process for these significant modules. The first module had an MCODE score = 43.256 and included 44 nodes with 930 edges (Fig. 4A). Functional annotation revealed that members of this module were mainly enriched in cell division related processes (Fig. 4B). The second module included 38 nodes with 248 edges and contained an MCODE score = 14.171 (Fig. 4C). Enrichment analysis showed that this module was significantly enriched in extracellular matrix-related terms (Fig. 4D). The third module cluster with an MCODE score = 9.067 included 16 nodes and 68 edges (Fig. 4E). GO analysis showed that genes in module 3 were mainly associated with extracellular matrix (Fig. 4F).

3.5. MicroRNAs target gene

The top ten miRNAs that potentially target the up- and down-regulated DEGs were recognized using miRTarBase database. hsa-miR-29b-3p, hsa-miR-192–5p, hsa-miR-215–5p, hsa-miR-29c-3p, hsa-miR-29a-3p, hsa-miR-193b-3p, hsa-miR-767–5p, has-miR1-3p, hsa-miR-145–5p, and hsa-miR-26b-5p were identified as the most important miRNAs targeting the up-regulated DEGs, respectively (Table 1). Moreover, hsa-miR-124–3p, hsa-miR-152–3p, hsa-miR-21–5p, hsa-miR-29a-3p, hsa-miR-27a-3p, hsa-miR-27b-3p, hsa-miR-34a-5p, has-miR3677–5p, hsa-miR-301a-5p, and hsa-miR-223–3p were revealed as the top 10 important miRNAs targeting the highest number of the down-regulated DEGs, respectively (Supplementary File 2, Table S1). The construction of the miRNAs-target gene network for the up and down-regulated DEGs showed interactions between candidate miRNAs and their targets (Fig. 5 and Supplementary File2, Fig. S1). Interestingly, several target genes were shared by the identified miRNAs. For example, among the up-regulated DEGs, *COL4A1*, *COL4A2*, *SPARC*, and *SERPINH1* were targeted by 5 miRNAs. On the other hand, the cocktail of 4 miRNAs involving: has-miR-29a-3p, has-miR-29b-3p, has-miR-29c-3p, and has-miR-767–5p had the largest number of common up-regulated target genes (Fig. 5).

3.6. HMDB analysis and screening

Metabolite analysis using Human Metabolome Database (HMDB) identified the top metabolites that were associated with up- and down-regulated DEGs (p-value $\leq$0.05). Ursodeoxycholic acid (UDCA), chenodeoxycholic acid (CDCA), lithocholic acid (LCA), copper, hyaluronic acid, and simvastatin, were identified as the most important metabolites associated with the up-regulated genes (Table 2). In contrast, NAP, NADPH, 3,4-Dihydroxymandelaldehyde, 5,6-Epoxy-8,11,14-eicosatrienoic acid, 8,9-EET, 14,15-Epoxy-5,8,11-eicosatrienoic acid, 11,12-EET, citalopram, acetaldehyde, and oxygen were the most important metabolites associated with the down-regulated genes (Table 2).

4. Discussion

ESCC is a tumor that grows rapidly and has a high potential for regional and distant metastasis [32,33]. So, finding effective key genes and regulators is crucial in the diagnosis and treatment of ESCC. Using GSE20347 gene expression microarray data, we identified 597 up-regulated genes and 706 down-regulated genes between ESCC and normal esophageal tissues. Here we performed a comprehensive bioinformatics analysis to identified the most important cell and molecular mechanisms and factors involved in ESCC development. We found several pathways, protein kinases, TFs and microRNAs that are involved in the regulation of ESCC-related genes. We also analyzed the metabolites associated to ESCC-related genes, to find the most important metabolites that might be used to control ESCC development and progression.

Our results showed that the up-regulated DEGs were significantly enriched in the extracellular matricx-related (ECM) processes, and pathways. It has been shown that ECM promotes tumor growth by providing biochemical, and mechanical support [34]. It seems that ECM proteins can be utilized to treat cancer patients by filling up gaps between cancer cells and changing their mechanical properties. Tumor cells must also destroy and remodel ECM components such as type I collagen meshwork. Moreover, tumor cells secrete MMPs that are important in cancer invasion and spread. It is well-known that tumor type I collagen promotes cancer cell adhesion by altering ECM [35]. Hanley et al. found that EC patients with longer collagen fibers in their Cancer-Associated Fibroblasts (CAFs) had a poorer survival rate using multi-photon laser scanning microscopy [36]. Tumor cells may be able to proliferate longer if the ECM becomes stiffer [37,38]. Extracellular matrix rigidity increases cell migration and invasion by clustering adhesion molecules, activating FAK and Rho-GTPase, and promoting the construction of cellular protrusions required for tumor cell motility through the ECM [39,40]. Most of the above mechanical concepts have been partly discovered for some cancers, but they are yet to be clarified in ESCC. Examining these qualities in EC may thus be a worthwhile endeavor. It seems that targeting both EC cells and ECM can be a promising approach for effective EC treatment.

ESCC is one of the most aggressive and deadly tumors. However, the mechanisms that produce ESCC are not well understood [23]. The expression of the HOXA13 protein is linked to a shorter median survival time in EC patients, while the functional implications are unknown. It increases keratinocyte proliferation, decreases susceptibility to chemical agents, regulates MHC class I expression and differentiation, and promotes cellular migration [41]. It has been reported that ESCC has cancer stem-like cells (CSCs) that are maintained by Fibroblast Growth Factors (FGFs) and their receptors (FGFRs). FGFR2, specifically the IIIb isoform, is highly expressed in non-CSC. In ESCC, FGFR2 loss promotes EMT and enriches CSC populations [42]. KlF5 is a key transcriptional regulator that promotes proliferation in non-transformed epithelial cells, but inhibits it in transformed cells. KLF5 has a tumor inhibitory impact in ESCC, albeit its context-dependent activity is unknown. KLf5 was proliferative when the p53 gene was wild-type, but anti–proliferativewhen the gene was mutated [43]. The deletion of the p53 mutation accelerated the cell cycle and reduced the expression of key signaling molecules like p21Waf1/Cip1 and Cip1. These signaling molecules were inhibited when mutant p53 was present or absent in cells with p53 mutations. The most common genetic mutation in ESCC is p53 [44].

The cytoplasmic domain of RAGE binds to DIAPH1 in response to AGEs, stimulating ROS production, and cellular signaling pathways that

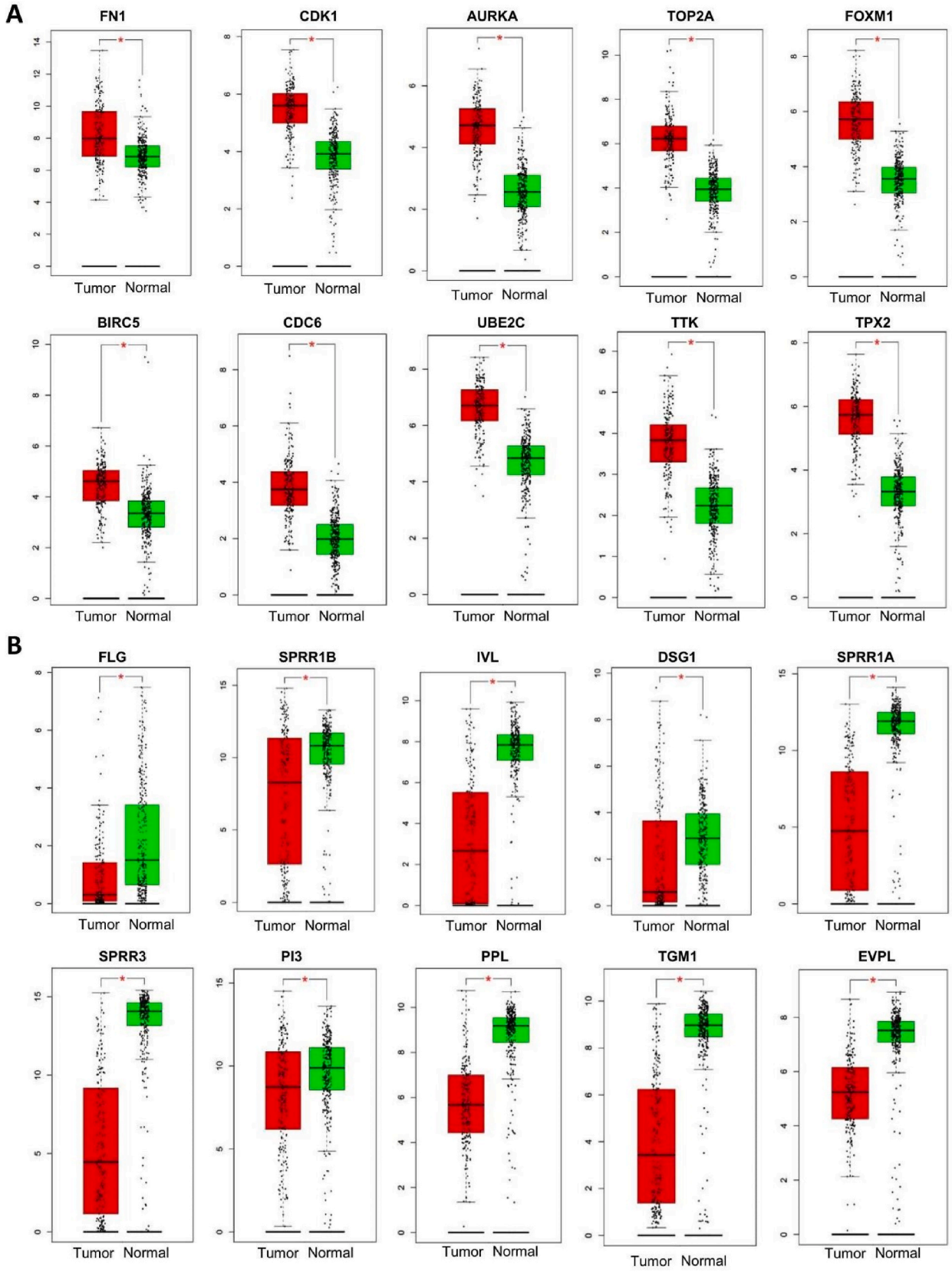
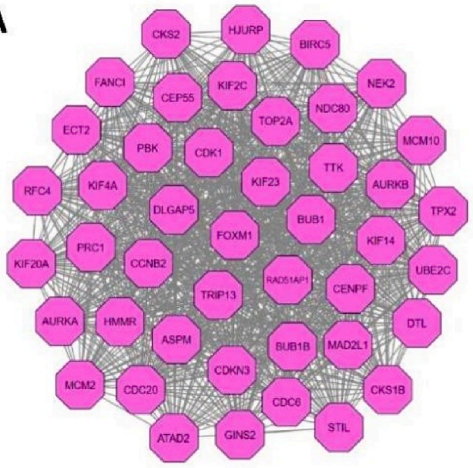


Fig. 3. The expression level of hub genes in ESCC. (A): Upregulated and **(B)** downregulated genes were identified using Gene Expression Profiling Interactive Analysis (GEPIA) based on The Cancer Genome Atlas database. Red and green boxes represent the relative expression levels of genes in the tumor and normal samples, respectively.

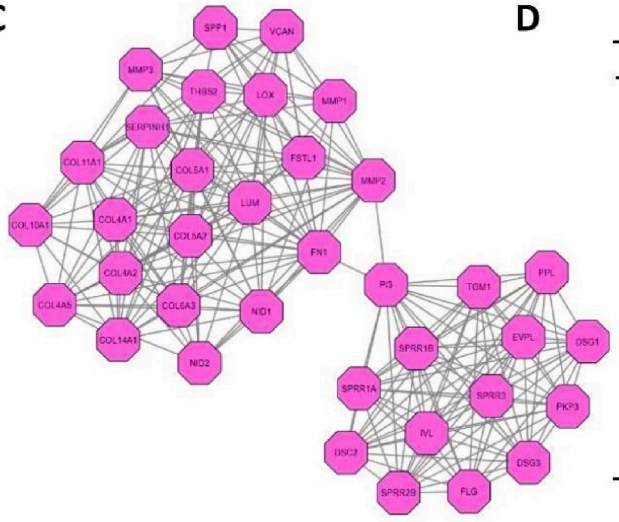
A



B

GO description	P-value	Cluster frequency	Genes
cell cycle	2.2475E-32	31/44 70.4%	HJURP, BUB1B, TTK, FOXM1, AURKB, CKS1B, AURKA, CDC20, CCNB2, PBK, NEK2, BUB1, CEP55, DLGAP5, FANCI, UBE2C, KIF23, CDC6, NDC80, ASPM, TPX2, CENPF, PRC1, CKS2, CDK1, BIRC5, KIF2C, TRIP13, MCM2, MAD2L1, CDKN3
M phase	1.0186E-31	25/44 56.8%	BUB1B, TTK, AURKB, AURKA, CDC20, CCNB2, PBK, NEK2, BUB1, CEP55, DLGAP5, UBE2C, KIF23, CDC6, NDC80, ASPM, TPX2, CENPF, PRC1, CKS2, CDK1, BIRC5, KIF2C ,TRIP13, MAD2L1
cell cycle phase	4.0835E-31	26/44 59.0%	BUB1B, TTK, AURKB, AURKA, CDC20, CCNB2, PBK, NEK2, BUB1, CEP55, DLGAP5, UBE2C, KIF23, CDC6, NDC80, ASPM, TPX2, CENPF, PRC1, CKS2, CDK1, BIRC5, KIF2C, TRIP13, MAD2L1, CDKN3,
mitotic cell cycle	4.7073E-29	24/44 54.5%	UBE2C, BUB1B, TTK, KIF23, CDC6, AURKB, NDC80, AURKA, CDC20, CCNB2, ASPM, TPX2, CENPF, PRC1, PBK, CDK1, BIRC5, NEK2, KIF2C, BUB1, CEP55, DLGAP5, MAD2L1, CDKN3
nuclear division	1.6286E-28	21/44 47.7%	UBE2C, BUB1B, KIF23, CDC6, AURKB, NDC80, AURKA, CDC20, CCNB2, ASPM, TPX2, CENPF, PBK, CDK1, BIRC5, NEK2, KIF2C, BUB1, CEP55, DLGAP5, MAD2L1

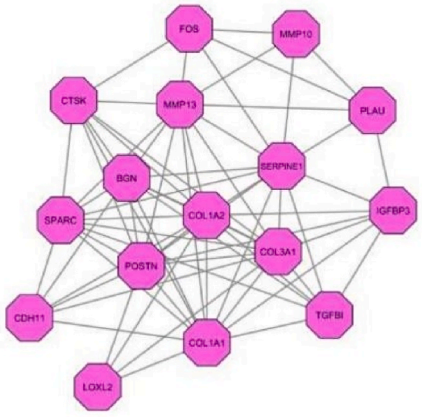
C



D

GO description	P-value	Cluster frequency	Genes
proteinaceous extracellular matrix	1.6429E-24	19/36 52.7 %	COL14A1, MMP1, LUM, COL11A1, MMP2, MMP3, FN1, NID1, NID2, VCAN, LOX, COL4A2, COL5A1, COL4A1, COL5A2, COL10A1, COL4A5, COL6A3 PI3
extracellular matrix	8.5885E-24	19/36 52.7 %	COL14A1, MMP1, LUM, COL11A1, MMP2, MMP3, FN1, NID1, NID2, VCAN, LOX, COL4A2, COL5A1, COL4A1, COL5A2, COL10A1, COL4A5, COL6A3, PI3
collagen	1.1843E-22	11/36 30.5 %	LOX, COL4A2, COL5A1, COL14A1, LUM, COL4A1, COL11A1, COL5A2, COL10A1, COL4A5, COL6A3
extracellular matrix part	2.1607E-20	13/36 36.1 %	COL14A1, LUM, COL11A1, NID1, NID2, LOX, COL4A2, COL5A1, COL4A1, COL5A2, COL10A1, COL4A5, COL6A3
extracellular region part	8.3326E-18	21/36 58.3 %	COL14A1, MMP1, LUM, COL11A1, MMP2, MMP3, FN1, NID1, FSTL1, NID2, VCAN, LOX, COL4A2, COL5A1, COL4A1, COL5A2, SPP1, COL10A1, COL4A5, COL6A3, PI3

E



F

GO description	P-value	Cluster frequency	Genes
extracellular region part	2.5174E-16	14/16 87.5 %	POSTN, SPARC, IGFBP3, SERPINE1, BGN, MMP10, LOXL2, COL1A1, COL3A1, MMP13, COL1A2, PLAU, CTSK, TGFBI
extracellular matrix	4.1215E-14	10/16 62.5 %	COL1A1, POSTN, COL3A1, SPARC, MMP13, COL1A2, SERPINE1, BGN, TGFBI, MMP10
proteinaceous extracellular matrix	1.4367E-12	9/16 56.2 %	COL1A1, POSTN, COL3A1, SPARC, MMP13, COL1A2, BGN, TGFBI, MMP10
extracellular space	2.3981E-12	11/16 68.7 %	COL1A1, COL3A1, MMP13, COL1A2, PLAU, IGFBP3, CTSK, SERPINE1, TGFBI, MMP10, LOXL2
extracellular region	5.7281E-12	14/16 87.5 %	POSTN, SPARC, IGFBP3, SERPINE1, BGN, MMP10, LOXL2, COL1A1, COL3A1, MMP13, COL1A2, PLAU, CTSK, TGFBI

Fig. 4. Module analysis of the PPI network. (A) module 1, (B) GO analysis of module 1, (C) module 2, (D) GO analysis of module 2, (E) module 3, (F) GO analysis of module 3.

ultimately lead to the activation of key transcription factors like NF-KB [45]. RAGE expression and modulation by microRNAs (miRNAs) in ESCC remain unknown. Jing et al. reported that overexpression of miR-185 results in down-regulation of RAGE in ESCC cells. Immunohistochemistry showed a strong correlation between RAGE expression and invasion depth in ESCC tissues, suggesting RAGE is implicated in ESCC. Bioinformatics and luciferase reporter assays were used to test the effect of miR-185on RAGE. miR-185 overexpression reduced RAGE expression by 27% and 49%. Both cell lines showed the effect of RAGE via immunofluorescence. Overexpressing MiR-185 and RAGE reversed the effects. The biological role of miR-185 in ESCC cell lines was investigated using viability, Ki-67 staining, migration and invasion assays, and a xenograft model. Overexpression of miR-185 inhibited ESCC cell migration, and invasion through the RAGE/HSP27 pathway. ESCC

Table 1
Top ten miRNAs targeting the up-regulated DEGs in ESCC.

miRNA name	*P*-value	Targets
hsa-miR-29b-3p	1.35E-12	SPARC; SLC16A1; MMP2; HMGA2; LAMC2; NID1; THBS2; LOXL2; COL1A1; COL3A1; COL4A2; LOX; COL5A1; COL4A1; COL5A2; SERPINH1; DNMT3B; COL10A1; COL4A5; COL6A3; ITGA6
hsa-miR-192-5p	1.55E-11	BLM; SPARC; SERPINE1; ODC1; KIF14; HJURP; RHOBTB3; BUB1B; SNX10; MCM10; TTK; SLC7A11; HMMR; NID1; PRSS23; LOXL2; CDC20; HOXA10; ALCAM; PLAU; ECT2; CEP55; DLGAP5; FANCI; STIL; RFC4; ATAD2; KIF23; ASPM; CENPF; KCNS3; PXDN; KIF20A; TRIP13; CDK14; DTL; CDKN3; MAD2L1 BLM; KIF14; HJURP; RHOBTB3; BUB1B; SNX10; MCM10; TTK; SLC7A11; HMMR; NID1; PRSS23; CDC20; HOXA10; ALCAM;
hsa-miR-215-5p	5.88E-11	PLAU; ECT2; CEP55; DLGAP5; STIL; RFC4; ATAD2; KIF23; ASPM; CENPF; KCNS3; KIF20A; TRIP13; CDK14; DTL; CDKN3; MAD2L1
hsa-miR-29c-3p	3.39E-09	SPARC; SLC16A1; MMP2; LAMC2; U2SURP; COL1A1; COL3A1; COL1A2; COL4A2; LOX; MTHFD2; COL4A1; COL5A2; SERPINH1; DNMT3B; COL10A1; ITGA6
hsa-miR-29a-3p	4.02E-08	SPARC; SLC16A1; MMP2; LAMC2; FSTL1; COL3A1; COL1A2; COL4A2; LOX; COL4A1; PXDN; COL5A2; SERPINH1; DNMT3B; COL10A1; ITGA6 TOP2A; BLM; ODC1; LPCAT1; BUB1B; MCM10; CDC20; UCHL1
hsa-miR-193b-3p	2.61E-06	PLAU; ECT2; BUB1; FANCI; GINS2; RFC4; UBE2C; ATAD2; MYO5A; CDC6; NDC80; ENAH; TPX2; ASPM; COL4A1; CDK1; TRIP13; APOBEC3B
hsa-miR-767-5p	7.43E-06	CXCL10; COL3A1; SPARC; LOX; COL4A2; COL4A1; MMP2; COL5A2; SERPINH1; COL10A1; SLC7A11 CXCL8; CXCL1; IFIT1; HERC5; NUAK1; LGALS1; FADD;
hsa-miR-1-3p	1.03E-05	FANCI; MMD; FN1; IFI44; ISG15; SLC39A14; CENPF; CTTN; MTHF2; KIF4A; PXDN; SNAI2; KIF2C; ITGA6; CDK14; MET; DFNA5; MAD2L1; MCM2
hsa-miR-145-5p	1.37E-05	MMP12; COL5A1; GOLM1; IRS1; MMP1; SERPINE1; HLTF; MYO5A; HMGA2; TGFBI; MEST; MCM2 LPCAT1; TTK; SLC7A11; FOXM1; FSTL1; LOXL2; LBH; GPNMB; LAMP3; CHST15; SERPINH1; SLC38A6; PMEPA1; BID;
hsa-miR-26b-5p	1.45E-05	ECT2; MAGEA11; GINS1; STIL; MMD; TXNRD1; FN1; IFI44; MMP10; ASPN; CDC25B; MFHAS1; ASPM; COL1A2; COL4A2; COL5A1; KIF4A; CKS2; COL4A5; TGFBI; HOXB7; CDK14; DTL; PFN2; DFNA5; MAD2L1; RAI14

patients had lower plasma miR-185 expression than healthy controls [46][30].

Analysis of the PPI network constructed based on the up-regulated DEGs identified 10 hub genes, including FN1, CDK1, AURKA, TOP2A, FOXM1, BIRC5, CDC6, UBE2C, TTK, and TPX2. Aurora kinase A (AURKA) is a serine/threonine kinase, which plays essential roles in cell division through regulation of mitosis [47]. Activation of AURKA is stimulated by TPX2, which is also implicated in cell cycle [48]. Du et al. reported that TPX2 interacts with AURKA, and their expression is correlated, suggesting their role in ESCC progression via PI3K/Akt pathway [49]. Recently, a novel role of AURKA as a non-kinase-dependent transactivating co-factor in the induction of FOXM1 expression has been reported. Nuclear AURKA is recruited by FOXM1, to transactivate its expression; whereas AURKA itself is activated by FOXM1 at the transcription level; therefore, participating in a positive feedback loop promoting breast CSCs self-renewal and drug resistance [50]. TTK protein kinase (TTK), is essential for activating the spindle assembly checkpoint (SAC); therefore, maintaining genomic integrity [51], and controlling cell fate [52]. Moreover, the cancer-testis antigen (CTA), TTK, is highly expressed in EC (>95%), but not in normal tissues except for the testis and the placenta, and can induce strong immunogenicity; therefore, it is an ideal therapeutic target for ESCC immunotherapy and development of cancer vaccination [53,54].

AURKA and TPX2 were chosen from the "Spindle Regulation by Ran" pathway by Hsu et al. cDNA microarray verification revealed that Aurora-A (88.4%) and TPX2 (90.6%) were overexpressed in ESCC tissues. High TPX2 expression predicted overall and disease-free survival (HR 1.802, p = 0.037) in univariate and multivariate analysis (HR 1.802). In growth curve investigations and clonogenic experiments, TPX2-deficient clones produced fewer colonies [55]. TPX2 protein expression was 85.5% positive in ESCC, 51.6% positive in atypical hyperplasia, and 4.83% positive in normal mucous membrane tissues. ESCC invasiveness and lymphatic metastases were associated with TPX2 protein and mRNA expression ($P < 0.01$) [56].

miRNAs are involved in numerous pathways, including angiogenesis. They are highly stable in biological fluids, and could be used as biomarkers. Increasing hsa-miR-155–5p and hsa-miR-29b-3p decreased (progression-free) PFS (HR 3.03), and overall survival (95% CI 1.06–9.09) [57]. The apoptotic miR- 29b-3p mimics SiH19 and miR-29b-3p reduces cell viability and survival [58]. Methylation of the miR29b3p gene promoter aids pancreatic cancer angiogenesis, invasion, and migration. Co-culture of human umbilical vein endothelial cells observed cell movement, and invasion (HUVECs). Tumor cell movement was inhibited by DNMT1 siRNA therapy. Methylation may slow the growth of pancreatic cancers [59].

To identify the metabolites associated with the up- and down-regulated DEGs in ESCC, we used HMDB. Ursodeoxycholic acid (UDCA), chenodeoxycholic acid (CDCA) and lithocholic acid (LCA), were identified as the top metabolites associated with the up-regulated genes. CDCA is a primary bile acid (BA); synthesized from cholesterol in the liver and subsequently secreted into the intestine as taurine or glycine conjugated form. UDCA and LCA are secondary BAs, derived from biotransformation of conjugated BAs via the gut microbiota [60]. BAs are crucial for lipid absorption in the intestines, glucose regulation and modulation of energy metabolism [61]. A previous study showed that CDCA stimulates EC angiogenesis and tumor growth through the COX-2 pathway [62]. Aberrant BA metabolism has been shown to promote hepatocellular carcinoma metastasis by inducing an immunosuppressive environment [63]. A biphasic effect of induction of apoptosis by BAs was reported in esophageal cells, depending on duration of BA exposure and concentration. According to this report, the short term effect of increased BA exposure on esophageal cells is generation of reactive oxygen species (ROS), that cause DNA damage, leading to mutation and apoptosis; while on the contrary, repeated encounters to BAs leads to the development of apoptosis-resistant esophageal cells, and further rise to tumorigenesis [64]. On the other hand, our results demonstrated that high expression of the ferroptosis-related gene, AKR1C1, is associated with BA metabolites in ESCC. Ferroptosis, is an iron-dependent, ROS-reliant, distinct form of regulated cell death, triggered by toxic accumulation of lipid peroxides on cellular membranes [65]. AKR1C1, a member of the aldo-keto reductase family, has been reported to play a pivotal role in the biosynthesis of BAs, and is closely associated with NAD(P)(H)-dependent reduction [66]. Interestingly, Huang et al. revealed that high expression of AKR1C1 is associated with poor prognosis in non-small cell lung cancer (NSCLC), and silencing AKR1C1 inhibits proliferation, and migration of NSCLC cells, and stimulates the development of ferroptosis [67]. These results may help establish a foundation for further research investigating the role of metabolites such as BAs in the development of ESCC.

The complex interplay between ROS generation and miRNAs in the pathogenesis of cancer has been shown in a number of studies, implying the prominent role of miRNAs in oxidative stress response and ferroptosis [68]. Fuschi et al. reported that miR-192–5p is up-regulated by H2O2 exposure in a p53-dependent manner, causing significantly decreased endothelial cell proliferation and inducing cell death. Moreover, this study showed that the down-regulated targets of miR-192–5p are involved in cell cycle, DNA repair, and stress response [69]. The decreased expression of miR-145 was shown to cause increased iron import via up-regulation of the iron transporter-transferrin receptor 1 (TFR1) in colorectal cancer cells, suggesting its role in iron metabolism, and thereby ferroptosis regulation [70].

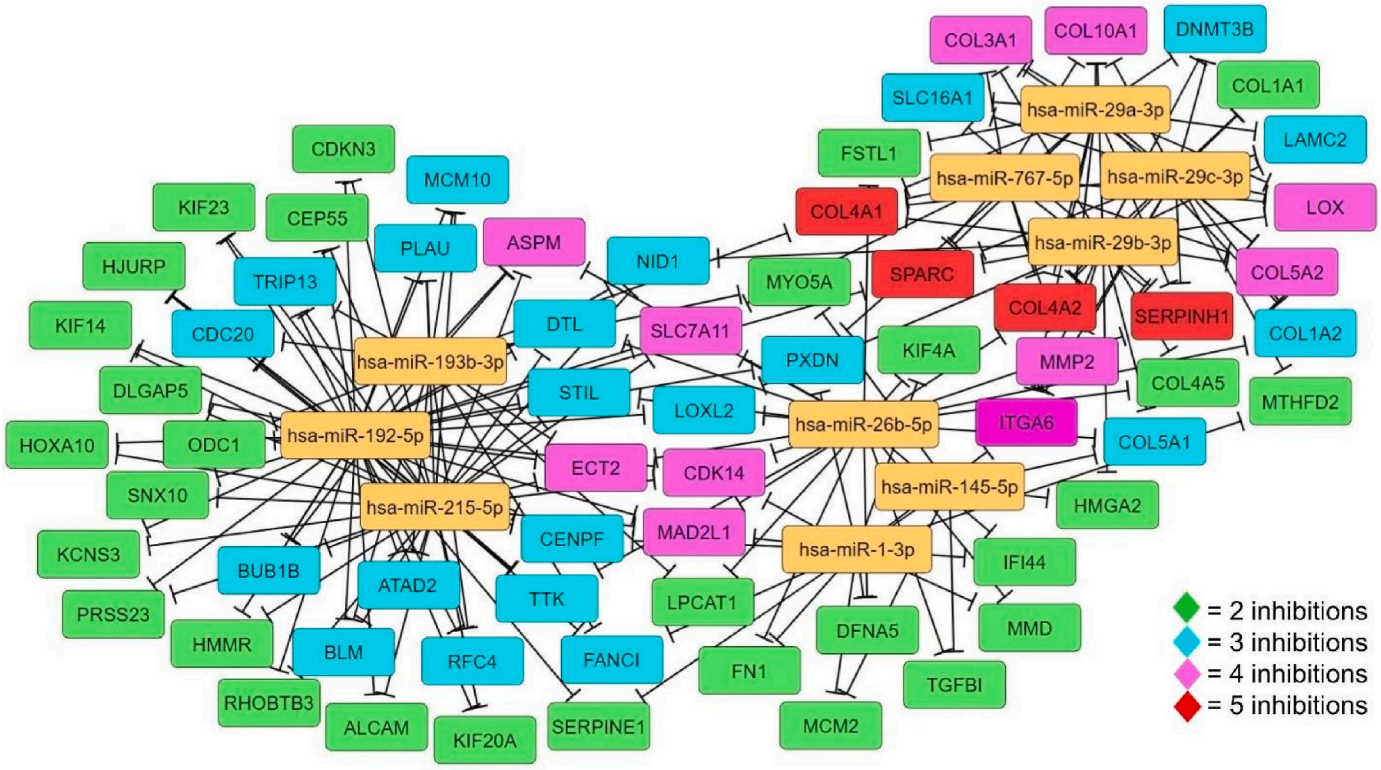


Fig. 5. miRNA-target gene network constructed for the top 10 miRNAs targeting the up-regulated DEGs, and their associated target genes in ESCC (miRNAs were selected based on the number of target genes and p-value <0.05.

Our miRNA-gene target analysis revealed miR-26b-5p, miR-192–5p and miR-215–5p, as the top miRNAs targeting the highest number of the up-regulated genes in ESCC. miR-26b is a tumor suppressor in many cancer types such as colorectal cancer, breast cancer and osteosarcoma [71–73]. In ESCC, it has been shown that down-regulation of miR-26b leads to overexpression of MYC binding protein (MYCB), and subsequently enhanced activity of the c-MYC pathway [74]. The miR-192/215 family is revealed to be down-regulated in various malignancies such as colorectal cancer, multiple myeloma, and renal cell carcinoma [75–77]. Down-regulation of miR-192–5P, and overexpression of its target genes (TYMS and ABCC3) was shown in 5-FU resistant esophageal adenocarcinoma cells [78]. Moreover, tumor suppressive effects of miR-215–5p was observed in the case of liver metastasis of colorectal cancer cells through regulation of ECM-receptor interactions, and focal adhesion [79]. Notably, for miR-215–5p, only one study has shown that it is significantly down-regulated in esophageal adenocarcinoma compared to Barrett's esophagus [80]. Therefore, further research is required to clarify the role of miR-215–5p in ESCC pathogenesis.

Moreover, in the miRNA-target gene network of the up-regulated genes in ESCC, four key miRNAs (miR-29a-3p, miR-29b-3p, miR-29c-3p and miR-767–5p) were shown to have the largest number of common targets. Also, among the up-regulated DEGs, COL4A1, COL4A2, SPARC, and SERPINH1 were targeted by 5 miRNAs, The miR-29 family, comprising miR-29a, miR-29b and miR-29c, are aberrantly expressed in various cancers [81]. Pan et al. demonstrated that knockdown of miR-29b-3p enhances radioresistance in CSCs. It was suggested that miR-29b-3p inhibits the kinetic process of DNA damage repair followed by radiation, by decreasing the expression of DNMT3B, Bcl-2, PI3KR1, AKT2 and RBL1, thereby regulating radiosensitivity [82]. miR-29c is down-regulated in ESCC, leading to increased expression of cyclin E, and consequently uncontrolled cell cycle progression [83]. Down-regulation of miR-29a was reported to stimulate overexpression of LOX2 and SERPINH1; which contribute significantly to collagen biosynthesis, thus promoting proliferation, invasion and metastasis in lung cancer cells [84]. Also a recent study showed that COL4A1 is negatively regulated by XPD-miR-29a-3p axis, further leading to liver cancer progression.

In this study we identified promising candidate biomarkers involved in ESCC progression, using microarray analysis. As a high-throughput technology and powerful research method, microarray has been widely used to identify candidate biomarkers in cancer onset and progression [85]. However, this technology has limitations such as low sensitivity due to profiling predefined genes or transcripts through hybridization [86]. RNA sequencing avoids the limitations of microarray and offers full sequencing of the transcriptome, thus provides a powerful way to determine gene expression profiles with greater accuracy and higher efficiency [87]. Therefore, future work using RNA-Seq analysis can help identify genes with very low or extremely abundant expression and non-coding transcripts (e.g., lncRNAs and miRNAs), and these additional data may facilitate discovery of biomarkers involved in ESCC progression [88]. Moreover, our study is merely based on bioinformatics analysis, therefore, further experimental investigation of the proposed miRNAs and target genes involved in ESCC pathogenesis, in larger number of samples should be undertaken to validate these findings.

5. Conclusion

In conclusion, we have identified potential DEGs and their upstream regulators, hub genes, metabolites, miRNAs and signaling pathways involved in ESCC development and progression. The identified factors and pathways may provide valuable insights into the underlying molecular mechanisms that result in ESCC pathogenesis, and may be considered as potential therapeutic targets or prognostic biomarkers for future research. Although, more experiments and rigorous testing in large case-controls, and cohort studies is required in the future to confirm these data.

Table 2
Top metabolites associated with the up- and down-regulated DEGs (p-value ≤0.05).

	Term	P-value	Genes
Up-regulated DEGs	Ursodeoxycholic acid (HMDB00946)	0.006206	AKR1C1; SLCO1B3
	Chenodeoxycholic acid (HMDB00518)	0.007393	AKR1C1; SLCO1B3
	Lithocholic acid (HMDB00761)	0.007393	AKR1C1; SLCO1B3
	Copper (HMDB00657)	0.01761	SPARC; LOX; LOXL2
	Hyaluronic acid (HMDB02061)	0.018212	TNFAIP6; HMMR
	Simvastatin (HMDB05007)	0.030606	SERPINE1; MMP3
Down-regulated DEGs	NAP (HMDB00217)	1.55E-08	CYP2J2; ADH1B; CYP4F3; CYP4B1; FMO2; ADH7; CYP3A5; TM7SF2; ALDH3A1; ALDH3B2; CYP2C9; GMDS; DHRS9; DPYD; CYP2E1; ALDH9A1
	NADPH (HMDB00221)	2.15E-08	CYP2J2; ADH1B; CYP4F3; CYP4B1; FMO2; ADH7; CYP2C18; CYP3A5; TM7SF2; ALDH3B2; CYP2C9; DUOX1; DHRS9; DPYD; CYP2E1; ALDH9A1
	3,4-Dihydroxymandelaldehyde (HMDB06242)	4.34E-07	ALDH3A1; ALDH3B2; MAOA; ADH1B; ADH7
	5,6-Epoxy-8,11,14-eicosatrienoic acid (HMDB02190)	1.69E-06	CYP2J2; CYP2C9; EPHX2; CYP4B1; CYP2E1; CYP3A5
	8,9-EET (HMDB02232)	1.69E-06	CYP2J2; CYP2C9; EPHX2; CYP4B1; CYP2E1; CYP3A5
	14,15-Epoxy-5,8,11-eicosatrienoic acid (HMDB04264)	1.69E-06	CYP2J2; CYP2C9; EPHX2; CYP4B1; CYP2E1; CYP3A5
	11,12-EET (HMDB04673)	1.69E-06	CYP2J2; CYP2C9; EPHX2; CYP4B1; CYP2E1; CYP3A5
	Citalopram (HMDB05038)	2.81E-06	CYP2J2; CYP2C9; MAOA; CYP4B1; CYP2E1; CYP3A5
	Acetaldehyde (HMDB00990)	3.84E-06	ALDH3A1; ALDH3B2; ADH1B; ADH7; ALDH9A1
	Oxygen (HMDB01377)	4.79E-06	CYP2J2; CH25H; CYP2C9; MAOA; ACOX1; CYP4F3; BBOX1; CYP4B1; ALOX12; FMO2; CYP2E1; CYP3A5

CRediT authorship contribution statement

Amir Mokhlesi: Formal analysis, Data curation, Writing – review & editing, Writing – original draft, Conceptualization, analyzed the data, reviewed the literature and wrote the original draft and conceptualization, All authors provided critical feedback and helped to shape the manuscript. **Zahra Sharifi:** Formal analysis, Data curation, Writing – review & editing, Writing – original draft, Conceptualization, analyzed the data, reviewed the literature and wrote the original draft and conceptualization, All authors provided critical feedback and helped to shape the manuscript. **Ahmad Berimipour:** Formal analysis, Data curation, Writing – review & editing, Writing – original draft, Conceptualization, analyzed the data, reviewed the literature and wrote the original draft and conceptualization, All authors provided critical feedback and helped to shape the manuscript. **Sara Taleahmad:** Writing – original draft, took the lead in writing the manuscript, All authors provided critical feedback and helped to shape the manuscript. **Mahmood Talkhabi:** Writing – original draft, took the lead in writing the manuscript, All authors provided critical feedback and helped to shape the manuscript.

Declaration of competing interest

The authors declared that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2023.05.009.

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