

# Bioinformatics-based identification of miRNAs, mRNA, and regulatory signaling pathways involved in esophageal squamous cell carcinoma

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## ABSTRACT

**Aim:** The current study analyzed the miRNA microarray dataset (GSE66274) and gene expression microarray dataset (GSE38129) with similar samples to achieve a better understanding of miRNA-mRNA interactions.

**Background:** The most common form of esophageal cancer is esophageal squamous cell carcinoma (ESCC). While, miRNAs are well recognized as having a critical regulatory role in human cancer, their responsibilities and mechanisms of miRNA-mRNA in ESCC are unknown.

**Methods:** Differentially expressed miRNAs (DEmiRNAs) and mRNAs (DEmRNAs) were identified using the LIMMA package in R. In total, 478 DEmRNA (224 upregulated and 254 downregulated) and 39 DEmiRNA (15 upregulated and 24 downregulated) were screened. The RNAInter database analyzed miRNA-mRNA interactions; then, the miRNA-mRNA network was visualized by Cytoscape software. ClusterProfiler packages were used to perform gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses for DEmRNA as targets of DEmiRNAs.

**Results:** KEGG pathway analysis indicated that the p53 signaling pathway, ECM-receptor interaction, and AGE-RAGE signaling pathway were significant. Cellular response to amino acid stimulus, negative regulation of apoptotic signaling pathway, and endoderm formation were most prevalent in the biological process category. Additionally, the collagen-containing extracellular matrix, actomyosin complex collagen trimers, basement membrane, and extracellular matrix structural constituent were more enriched.

**Conclusion:** Overall, the present survey provides evidence that could support the prognosis of esophageal tumors in the future.

**Keywords:** Esophageal squamous cell carcinoma (ESCC), miRNAs, mRNAs, Interaction, Biomarkers

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## Introduction

Esophageal cancer (ESCA) is the world's seventh most frequent cancer and the sixth major cause of cancer-related mortality (1). The most common

histology of ESCA (60–70%) is in esophageal squamous cell carcinoma (ESCC) (2). Despite significant advancements in surgery and radiation treatment, the five-year survival rate of ESCC patients remains less than 25% because of the absence of apparent clinical signs in early-stage ESCA, and many patients are thus already in an advanced disease stage at the time of diagnosis (2, 3). Increased knowledge of the genetics and molecular processes underlying the disease is critical for facilitating early diagnosis, proper

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therapy, and better prognosis in ESCC patients (4). Because ESCC is a multifaceted disease with a complicated genetic etiology, and our understanding of its molecular pathophysiology is lacking, the particular molecular expression patterns will provide novel hints for cancer diagnosis and therapy (5). Molecular markers have been recognized as prognostic indicators of ESCC (3, 6, 7), but these biomarkers are not always helpful in predicting prognosis or early diagnosis. As a result, there is a need to discover novel biomarkers to enhance ESCC diagnosis and prognosis. Genes such as mRNA, miRNA, and other non-coding genes are examples of biomarkers persistently expressed in plasma, serum, and other body parts (8).

MicroRNAs (miRNAs) are small, non-coding RNAs of 18–25 nucleotides that control mRNA translation. Mature miRNAs can identify and bind to the 3' UTR of mRNAs, regulating translation at the post-transcriptional stage by repressing or degrading the targeted mRNAs (9). In cancer research, miRNAs were recently acknowledged as good prognostic or diagnostic biomarkers (10). Many miRNAs, such as miR-21, miR-223 and miR-75 (11), miRNA-1290 (12), miR-455-3p (13), and miR-145 (14), influence their targets, thus playing an important role in the prognosis and survival rates of esophageal cancer. Nonetheless, the regulation of miRNAs and their target mRNAs throughout the incidence and progression of ESCC is crucial. The development of genome-wide technology, such as gene expression microarrays, has allowed for a comprehensive overview of the miRNA and mRNA changes implicated in ESCC. The employment of bioinformatics provides for the study of variations between miRNAs and mRNAs (4). The current study purposed to discover differentially expressed miRNAs (DEmiRNAs) and mRNAs (DEmRNAs) utilizing GEO databases to identify possible disease-associated target gene functional enrichment and protein-protein interaction (PPI) network analysis. Such data provides valuable insights into the molecular progression of esophageal cancer and contributes to the investigation of potential markers of pathogenesis, which will improve diagnosing and predicting the prognosis of esophageal cancer in its early stages. Some people with a family history of cancer, either digestive or other types, face an increased risk for digestive cancers. The significant point in selecting the dataset was the

similarity among the samples in the miRNAs and mRNAs in microarray experimentation.

## Methods

### Data collection and gene expression analysis

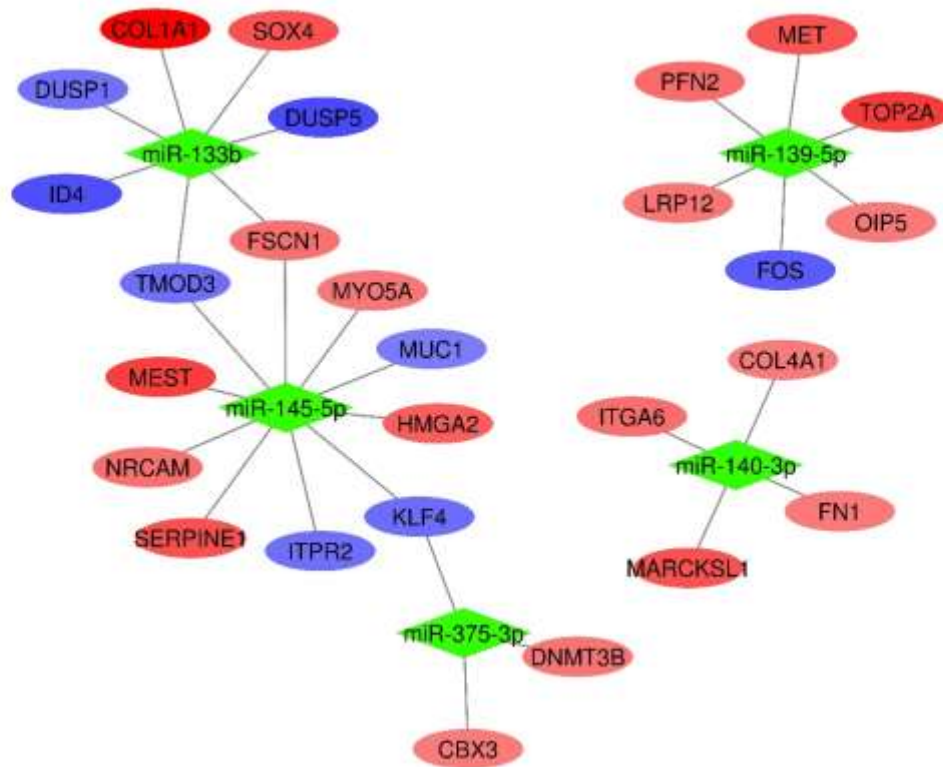
Firstly, miRNA and mRNA expression datasets of esophageal cancer were searched using the keywords: "miRNA," "mRNA," "esophageal squamous cell cancer," and "Homo sapiens" [porgn: txid9606] against the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo>). To better predict the interaction between miRNA-mRNA, the miRNA and mRNA expression datasets were precisely the same (30 tumor and adjacent normal tissues, 60 samples in total for both datasets). Gene expression analysis was performed by limma package in Bioconductor that was utilized to mine statistically significant DEmiRNAs and DEmRNAs based on the differences in their expression values between normal and tumor samples with log2 fold change  $\geq |1.5|$ . An adjusted p-value threshold of 0.01 was considered as significantly differentially expressed (15).

### Interaction assessment between miRNAs-mRNAs

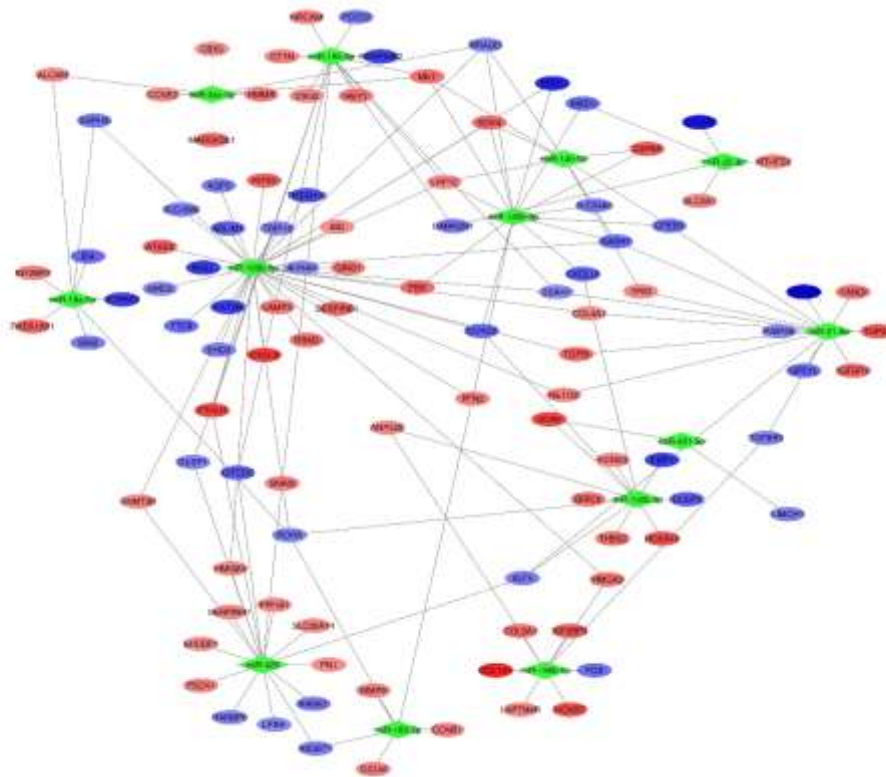
To clearly understand the functions of miRNAs and mRNAs, we developed the network of miRNAs-mRNAs interactions. RNAInter (RNA Interactome Database (<http://www.rna-society.org/raid/home.html>)) is a database that promotes the development of the interactome and increases knowledge of the biological functions and molecular mechanisms of RNAs (16). Through RNAInter, some mRNAs as targets of miRNAs were obtained. Each miRNA was analyzed separately, assigned a category on miRNA, species on homo sapience, interaction type on RNA-RNA, detection method on computational prediction the interval of confidence score between 0.7 to 1, respectively. Some DEmiRNAs had no interactions and were not considered in the continuation of the study.

### Identification of DEmRNA for each DEmiRNA

In this step, we collected all mRNAs as targets of DEmiRNAs from the RNAInter database. The Venny 2.1 tool (<https://bioinfogp.cnb.csic.es/tools/venny/>) was used to compare the obtained mRNA (from RNAInter)



**Figure 1.** Networks were constructed between up-regulated miRNAs and DEMRNAs. Green diamonds represent DEMiRNAs. Red and blue color spectrum circles represent up- and downregulated mRNAs, respectively.



**Figure 2.** Networks were constructed between downregulated miRNAs and DEMRNAs. Green diamonds represent DEMiRNAs. Red and blue color spectrum circles represent up- and downregulated mRNAs, respectively.

targets for each DEmiRNA in this study. Finally, miRNA-mRNA networks were constructed by Cytoscape 3.9.0 software.

### Gene Set Enrichment Analysis (GSEA)

Gene set enrichment analysis (GSEA) is a valuable approach for attaining insight into biological functions, such as pathways and gene ontology (GO) in clouding: biological process (BP), cellular component (CC), and molecular function (MF). GSEA was performed for the DEmRNAs that were targeted by DEmiRNAs. ClusterProfiler packages in R software were used for GO and pathway enrichment analysis of DEmRNAs. GO and Kyoto Gene and Genome Encyclopedia (KEGG) pathways with an adjusted p-value <0.05 are shown by the bar plot (17).

## Results

### Identification of DEmiRNAs and DEmRNAs

Two microarray gene expression datasets (miRNA (GSE66274) and mRNA (GSE38129) datasets) were used in this study. Each dataset contained identical tissue samples, i.e. 30 tumors and 30 adjacent normal tissues (60 samples in total). Each dataset was analyzed separately by limma package. Overall, 478 DEmRNA (224 upregulated and 254 downregulated) and 39 DEmiRNA (15 upregulated and 24 downregulated) were obtained between tumor (n=30) and adjacent normal (n=30) samples.

### Finding mRNAs for each DEmiRNAs

Using RNAInter, the mRNAs targeted by DEmiRNAs were determined. Among 39 DEmiRNAs, only 5 upregulated miRNAs (miR-139-5p, miR-140-3p, miR-375-3p, miR-133b, and miR-145-5p) and 13 downregulated miRNAs (miR-196b-5p, miR-301b-3p, miR-18a-5p, miR-106b-5p, miR-22-3p, miR-130b-3p, miR-21-5p, miR-183-5p, miR-182-5p, miR-34c-5p, miR-135b-5p, miR-431-5p, and miR-429) target some mRNAs based on computational prediction. We

detected 27 and 103 DEmRNAs as targets for up- and downregulated miRNAs, respectively. After conformation between obtained mRNA from RNAInter and DEmRNAs in this study, miRNA-mRNA interactions were constructed by Cytoscape. The networks for up- and downregulated miRNAs are represented separately (Figure 1 and 2, respectively).

### Gene ontology (GO) and pathway enrichment analysis

The clusterProfiler package was utilized to find the enriched pathways and GO with adjusted p <0.05 shown by bar plot. KEGG pathway analysis indicated that the p53 signaling pathway, ECM-receptor interaction, and AGE-RAGE signaling pathway in diabetic complications were significant (Figure 3A). In addition, the genes that were involved in significant pathways are listed in Table 1. According to the results, COL1A1 (collagen type I alpha 1 chain), FN1 (fibronectin 1), and COL4A1 (collagen type IV alpha 1 chain) genes were common between ECM-receptor interaction and AGE-RAGE signaling pathway. However, the p53 signaling pathway and ECM-receptor interaction hadSERPINE1 (serpin family E member 1) as the only overlapping gene.

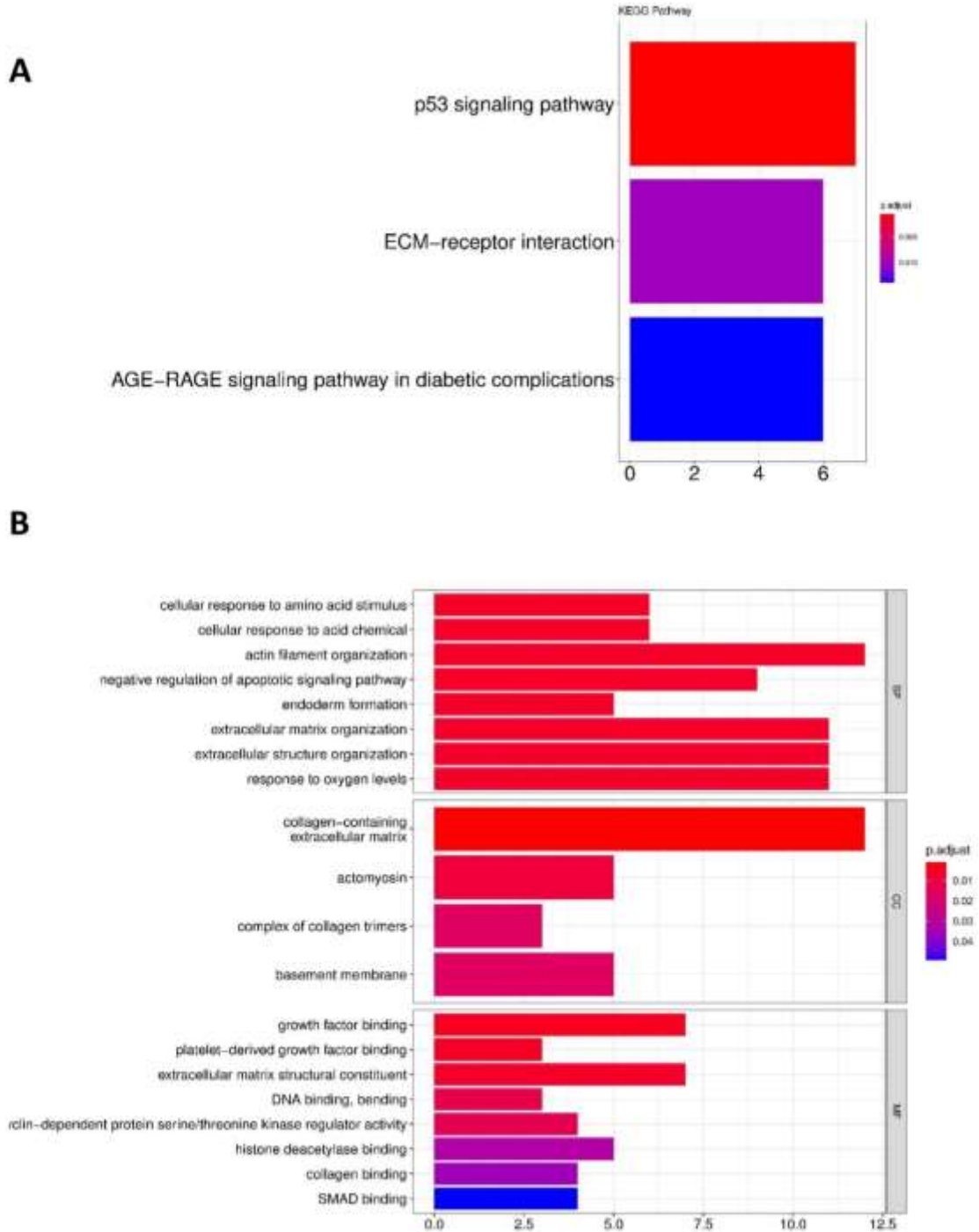
No common genes were found among those three significant pathways. In this study, all overlapping genes were upregulated in ESCC tissue. The Venn diagram in Figure 4 shows the number of overlapping genes. Cellular response to amino acid stimulus, cellular response to acid chemical, actin filament organization, negative regulation of apoptotic signaling pathway, and endoderm formation were most prevalent in the BP category. In CC, the collagen-containing extracellular matrix, actomyosin, complex of collagen trimers, and basement membrane and extracellular matrix structural constituent were more enriched (Figure 3B).

## Discussion

Many studies have investigated microarray data on ESCC. Most datasets were done independently and

**Table 1.** The DEGs that involved in enriched biological pathways

Pathways	Hit	DEGs
p53 signaling pathway	7	RRM2, CCNB1, BID, CCNG2, IGFBP3, CCNE2, SERPINE1
ECM-receptor interaction	6	COL4A1, FN1, ITGA6, HMMR, THBS2, COL1A1
AGE-RAGE signaling pathway in diabetic complications	6	COL3A1, COL4A1, FN1, SERPINE1, CXCL8, COL1A1



**Figure 3.** Functional enrichment analysis of DEMRNAs as targets of DEMiRNAs. A: KEGG pathways, B: GO terms.

with small samples. One of the most critical strengths of the present study is that it systematically integrated two microarray gene expression datasets (miRNA (GSE66274) and mRNA (GSE38129) datasets; 30 tumor and adjacent normal tissues; 60 samples in total for both datasets) to better identify the differentially

expressed miRNAs. This developed the sensitivity to show the up- or downregulated miRNAs and genes in ESCC.

A study of the different pathways in esophageal cancer showed that most pathways are related to cellular metabolism. Some pathways have overlapping



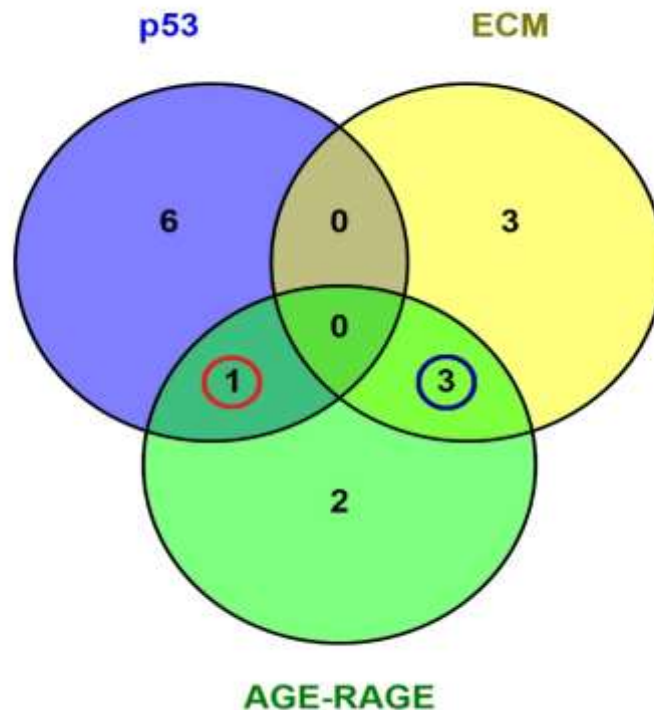
genes targeted by DEmiRNA; for example, COL4A1, FN1, and COL1A1 co-occurred between ECM–receptor interaction and AGE–RAGE signaling pathway. COL1A1 has been linked to the development of various cancers. In one study, the COL1A1 gene was a critical overexpressed gene. By contrast, downregulation of COL1A1 could inhibit the proliferation and migration of human oral squamous cell carcinoma cells. Similar findings were observed in gastric cancer. Thus, COL1A1 expression may serve as an independent biomarker when it comes to ESCC prognosis prediction.

Furthermore, the prognosis of ESCC was associated with COL4A1 (18). Chen et al. reported that the expression of COL4A1 was high in ESCC. According to survival analysis conducted using the GEPIA database of the TCGA, COL4A1 protein expression was associated with a poor prognosis in ESCC. In addition, cancer patients with high COL4A1 expression have worse survival rates and disease-free survival rates. Knocking down COL4A1 reduces cell proliferation and the cell cycle in breast cancer cells (19). A protein-protein interaction network was created

to investigate the interaction between the ESCC-specific genes in system biology studies. This network had FN1 as one of the five most central nodes, suggesting it may be one of the essential genes associated with ESCC carcinogenesis and paclitaxel resistance.

In other studies, overexpression of FN1 was observed in ESCA. A higher pathological stage was observed in patients with elevated levels of FN1 (20, 21). Survival analysis by the GEPIA Association Institute has demonstrated that SERPINE1 is closely related to the worse prognosis of patients with ESCC and that its expression is upregulated in a variety of malignancies such as breast, esophageal, kidney, and liver cancers as well as stomach and colorectal cancers (22).

Cancer cells have a different metabolism compared to normal cells. Recent studies have shown that tumor cells adjust their metabolic pathways according to their needs during proliferation (23). The results of the pathways study showed that in esophageal cancer, the cell cycle, signaling pathways, and cells are changed, which is also effective for metastasis. These changes



**Figure 4.** Number of overlapping genes. Three genes (COL4A1, FN1, and COL1A1) overlapped between ECM–receptor interaction and AGE–RAGE signaling pathway (blue circle). One gene (SERPINE1) was common between the p53 signaling pathway and ECM–receptor interaction (red circle).

can cause epithelial-mesenchymal transition (EMT), which allows cells to migrate from the primary site as well as invasion, resistance to apoptosis, and changes in extracellular matrix (ECM) components (24). Budhu et al. (2008) showed that the expression of miR-219 and miR-20- increased and decreased the expression of miR-124a, respectively, and miR-30c was increased in esophageal cancer, which is associated with metastasis and mortality rate in patients (25). Low ECM1 expression is associated with a high actin ratio; ECM1 plays a significant role in the metastatic process and regulates the skeletal structure of actin in cancer cells through changes in GTPases and Rho A (24). In the analysis of the data obtained from this study, the expression of ECM1 in esophageal cancers decreased.

It was found that miR-139-5p changed in ESCC, making it a potential biomarker for ESCC patient survival. It has been reported that the expression level of miR-139-5P was much higher in 11 cases of esophageal cancer than in cases involving adjacent tissues (26). Yang et al. reported that miR-139-5p was downregulated in ESCC tumor tissue compared to non-tumor tissue samples (27).

The minichromosome maintenance protein 3 (MCM3) is a marker in thyroid, melanoma, liver, and cervical cancers (28). This study also showed an increase in its expression in esophageal cancer, which can be used to diagnose esophageal cancer risk.

Ogawa et al. (2009) examined the association between primary tumor (T), lymph node metastasis (N), vascular invasion (VI), and lymphatic invasion (LI) and reported that 12-microRNA profiles relate to patient survival in esophageal cancer (29). Guo et al. (2008) showed that miRNA profiles are related to age, sex, pathological characteristics, and differentiation of tumor cells in different types of esophageal cancer (30). This study also showed an association between miRNA profiles (miR29-a) and esophageal cancer, suggesting that miRNA profiles could be promising as diagnostic biomarkers in esophageal cancer. The expression of mir-29 is a critical miRNA in many types of cancer. This study showed that the expression of miRNA-21 changed during esophageal cancer. Previous studies have also identified miRNA-21 as a biomarker of esophageal cancer (31). Therefore, the evidence suggests that miRNA-21 may play an essential role in using chemotherapy in patients. The use of

bioinformatics and the development of computational methods can be used in laboratory research. Numerous studies have identified miRNAs as potential biomarkers for human cancer diagnosis and therapeutic agents, but this issue needs further investigation. Biomarkers have potential applications in the screening and diagnosing of cancers in the early stages (32). In gastrointestinal cancers, cell regulation is disrupted. Many studies have used microarray analysis to discover the complex mechanisms of carcinogenesis and abnormal gene expression by cancer cells and find specific biomarkers (33). Gene networks are used to identify genes and gene groups that cause normal tissue to become malignant, in which case transcription network connections can also be examined in cancers (34). In particular, microRNAs are regulators of gene expression, cell proliferation, and survival rate. Moreover, miRNAs are biomarkers as small (18–22nt) non-coding RNA molecules detected in all bodily fluids such as saliva, blood, semen, cerebrospinal fluid (CSF), pleural fluid, amniotic fluid, nasal fluid, and so on.

The results showed that increasing the expression of some genes and decreasing the expression of other genes in esophageal cancer could be a complete study of gastrointestinal cancer pathways, which are associated with increased malignancy in gastrointestinal tumors. These findings could effectively diagnose patients with esophageal tumors and introduce these genes as a suitable prognostic marker for esophageal cancer.

Modified miRNAs lead to tumorigenesis, but little is known about the role of miRNAs in esophageal cancer. The target genes for these miRNAs may be tumor suppressor genes or other oncogene-related genes, such as growth factors, growth factor receptors, apoptosis, transducer signaling transducers, "genes that control cell division, or genes that repair DNA" (30).

In their study, Lane et al. showed that miR-34a expression levels in ESCC were significantly reduced compared to normal esophageal tissue. Lymph node status and advanced clinical stage are related, and studies have shown that miR-34a expression is reduced in human ESCC, indicating a significant decrease in miR-34a expression in ESCC progression and prognosis (35).

Conventional therapies, such as chemotherapy and surgery, do not significantly affect metastatic

esophageal cancer, and all human endeavors aim to inhibit cancer with therapy or gene therapy. Today, much effort is being put into identifying markers that can quickly detect esophageal cancer by increasing or decreasing these markers in the body.

## Conclusion

The current study was based principally on analyses conducted using genetic databases and bioinformatics tools. Essentially, this study aimed to identify miRNA-miRNA networks and associated pathways that might be responsible for ESCC development. Two datasets (mRNA and miRNA) with the same samples were used to demonstrate a valid interaction between miRNA-mRNA. In conclusion, the results indicate that miRNAs are vital regulators of mRNA expressions in ESCC. Comprehending the underlying role of mRNA-miRNA regulation in disease pathology is critical to understanding its occurrence in various biological systems. In addition, the current study analyzed overlapping DEmRNAs in significant pathways that were targeted by DEmiRNAs, finding that all of them played a critical role in ESCC, which has contributed to the identification of therapeutic target sites and biomarkers for ESCC.

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## Conflict of interests

The authors declare no conflicts of interest.

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