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Novel miRNA markers and their mechanism of esophageal squamous cell carcinoma (ESCC) based on TCGA

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MicroRNAs(miRNAs) are promising biomarkers for early esophageal squamous cell carcinoma (ESCC) detection and prognostic prediction. This study aimed to explore the potential biomarkers and molecular pathogenesis in the early diagnosis of ESCC. Firstly, 48 differentially expressed miRNAs (DEMs) and 1319 differentially expressed genes (DEGs) were identified between 94 ESCC tissues and 13 normal esophageal tissues in TCGA. From miRNA–mRNA regulatory network, there are 6558 target genes of the 48 DEMs, where 400 target genes are also among 1319 DEGs. Then, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment indicate that the 400 DEGs significantly enriched in cell cycle, proteoglycans in cancer, p53 signaling pathway, protein digestion and absorption, transcriptional dysregulation in cancer, and oocyte meiosis. And there are 66 DEGs among these six biological pathways, which we called GO-DEGs. From miRNA–mRNA regulatory network, 32 DEMs regulated the 66 GO-DEGs, where 22 DEMs were verified by different types of experiments in ESCC tissues, cells, or serum from the literature. For the other novel 10 DEMs, single-factor Cox regression analysis show that only hsa-miR-34b-3p showed no significant correlation with the overall survival of ESCC patients. Finally, we obtained the novel 9 ESCC-related DEMs, where three are down-regulated, and six are up-regulated. We analyzed the expression trends of target genes for five miRNAs and identified three significantly different miRNAs (hsa-miR-205-3p, hsa-miR-452-3p, and hsa-miR-6499-3p) confirmed by qPCR. Moreover, the stage-specific miRNAs were also suggested. These three qPCR validated miRNAs are also specific to the early stages of ESCC: hsa-miR-452-3p is specific to Stage I, II and III; hsa-miR-205-3p is specific in Stage II and III; and hsa-miR-6499-3p is Stage II specific. They might be the potential biomarkers for ESCC stage diagnosis. This study identified three novel miRNA markers potentially related to the diagnosis of ESCC and participated in the occurrence and development of ESCC through cell cycle, proteoglycans in cancer, p53 signaling pathway, protein digestion and absorption, transcriptional dysregulation in cancer, and signaling pathway for oocyte meiosis.

Keywords Esophageal squamous cell carcinoma, miRNA, mRNA, Biomarkers

Abbreviations

ESCC	Esophageal squamous cell carcinoma
DEMs	Differential expression of miRNAs
DEGs	Differentially expressed RNAs
BP	Biological process

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CC Cell component
MF Molecular function

Esophageal cancer is one of the most common gastrointestinal malignant tumors, the eighth incidence rate, the sixth mortality rate of all cancers in the world[1], and the fourth leading cause of cancer-related deaths in China[2]. Esophageal cancer mainly has two pathological subtypes: esophageal adenocarcinoma and esophageal squamous cell carcinoma (ESCC). And in China, about 90% of esophageal cancer are ESCC[3], and most ESCC pathological stage, around 90% were at the severe stage[4]. The prognosis of ESCC in China is very poor, and 5-year survival rate is only 15–25%[5]. The location of ESCC were mainly in the upper and middle esophagus. The cause factors include smoking, alcohol abuse, dietary habits, and genetic factors[6]. However, the specific pathogenesis of ESCC remains unclear.

Studies have identified several miRNAs upregulated in ESCC patients, including miR-10a, miR-18a, miR-19b, miR-21, miR-22, miR-25, miR-31, miR-93, miR-129, miR-1246, miR-1322, miR-451, and miR-365. Conversely, miR-155, miR-203, miR-205, miR-375, miR-377, miR-486, and miR-718 are downregulated in these patients[7]. Recent findings indicate miRNA profiles are crucial for ESCC prognosis. High levels of plasma miR-21 and miR-16 correlate with poor survival, and low miR-375 levels predict an especially poor outcome[8]. A six-miRNA signature outperforms traditional tumour, node and metastasis (TNM) staging in accuracy, while miR-129, miR-103, and miR-107 also relate to worse survival rates[9]. Conversely, miR-377 and miR-15a levels are inversely related to survival, suggesting their potential as new prognostic markers, along with the rising importance of miR-367 serum levels. These results highlight the significant role of miRNA dysregulation in diagnosis and treatment of ESCC[10].

Previous studies show that miRNA may participate in the occurrence, development, invasion, and metastasis of ESCC, thus, miRNAs are promising diagnosis and prognosis biomarkers of ESCC[11,12]. MiRNA is small non-coding RNA regulating gene expression through post-transcriptional regulation, and roles similarly as oncogene or tumor suppressor gene[13]. A meta-analysis[14] of 11 studies revealed that miRNA could be used as a biomarker for early detection of ESCC. Most previous studies on ESCC miRNA markers used The Cancer Genome Atlas (TCGA) database, while few searched miRNACancerMap database and PubMed[15–17].

In this study, we used the transcriptomic data of both miRNA and mRNA, clinical survival information of ESCC from TCGA to explore novel key miRNA markers participating in progression of ESCC, as well as their molecular pathogenic mechanism in ESCC.

Results

Differentially expressed miRNAs (DEM) and genes (DEG) between ESCC and normal

In total, 94 ESCC and 13 normal samples with both miRNA and mRNA transcriptome data from TCGA were included in the study. Compared to the normal esophageal tissue samples, 1319 differentially expressed genes (DEGs) with FDR < 0.05 and 4-FoldChange were obtained, as shown in the volcano plot of Fig. 1A, where 845 were upregulated and 474 were downregulated in ESCC samples. And 48 differentially expressed miRNAs (DEMs) with FDR < 0.05 and 4-FoldChange were identified as shown in the volcano plot of Fig. 1B, where 28 were upregulated and 20 were downregulated in ESCC samples. The heatmaps of these 1319 DEGs and 48 DEMs are shown in Fig. 1C and D, respectively.

The target genes of 48 DEMs

There are 6,559 target genes of 48 DEMs according to miRTarBase database (https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2022/php/index.php).

The comparison of these 6559 target genes with 1319 DEGs was shown in Fig. 2, where Venn diagram in Fig. 2A shows that 400 DEGs are also the target genes regulated by the 48 DEMs, i.e., DEM regulated DEGs. The heatmap of 400 DEM-regulated DEGs in Fig. 2B shows that they well separate the ESCC and normal samples.

GO and KEGG enrichment

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes(KEGG) enrichment analysis of 1319 DEGs, 6559 target genes of the 48 DEMs, and 400 DEM-regulated DEGs were shown in Fig. 3.

From GO enrichment of 6559 DEMs target genes in Fig. 3A, the top enriched biological processes include cell growth, mitotic cell cycle phase transition, gland development, regulation of apoptotic signaling pathway, and regulation of binding; the top cell components include cell-substrate junction, focal adhesion, spindle, chromosomal region, and cell leading edge; and the top molecular function include DNA-binding transcription factor binding, DNA-binding transcription activator activity, RNA polymerase II-specific, RNA polymerase II-specific DNA-binding transcription factor binding cadherin binding.

From GO enrichment of 1319 DEGs in Fig. 3B, the top enriched biological processes include epidermis development, organelle fission, nuclear division, skin development, and mitotic nuclear division. The top enriched cell components include the collagen-containing extracellular matrix, chromosome, chromosomal region, and basal part of the cell. The top enriched molecular functions include glycosaminoglycan binding, DNA-binding transcription activator activity, RNA polymerase II-specific, glycosaminoglycan binding, extracellular matrix structural constituent, and heparin binding.

From GO enrichment of 400 DEM-regulated DEGs in Fig. 3C, the top enriched biological processes include organelle fission, nuclear division, chromosome segregation, mitotic nuclear division, nuclear chromosome segregation, mitotic cell cycle phase transition, sister chromatid segregation, mitotic sister chromatid segregation, microtubule cytoskeleton organization involved in mitosis, and regulation of chromosome segregation; the top cell components include the spindle, chromosomal region, condensed chromosome, chromosome centromeric region, condensed chromosome centromeric region, kinetochore, mitotic spindle, midbody, kinesin complex,

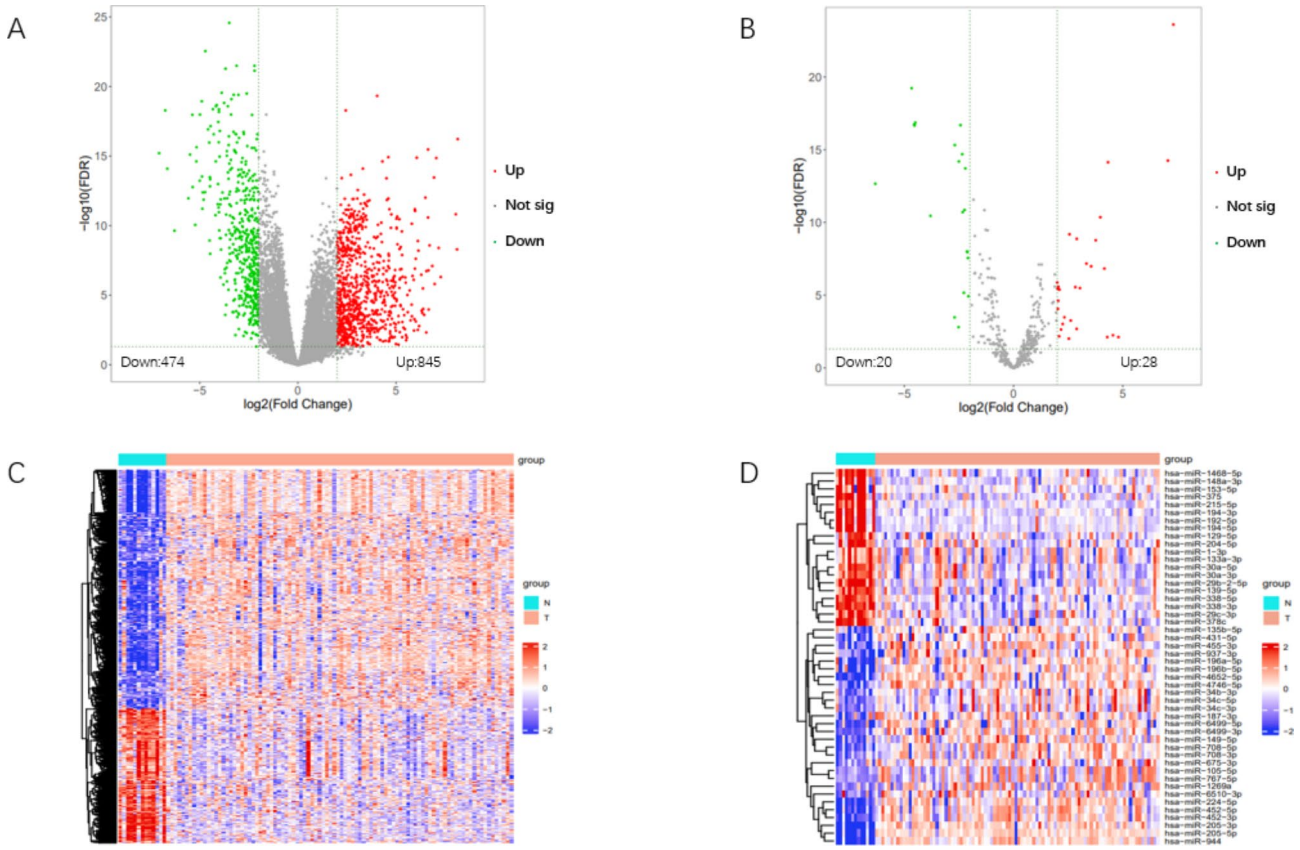

Fig. 1. DEMs and DEGs between ESCC and normal samples. **A** Volcano plot of 1319 DEGs with FDR < 0.05 and 4-FoldChange; **B** Volcano plot of DEMs with FDR < 0.05 and 4-FoldChange; **C** Heatmap of 1319 DEGs; **D** Heatmap of 48 DEMs.

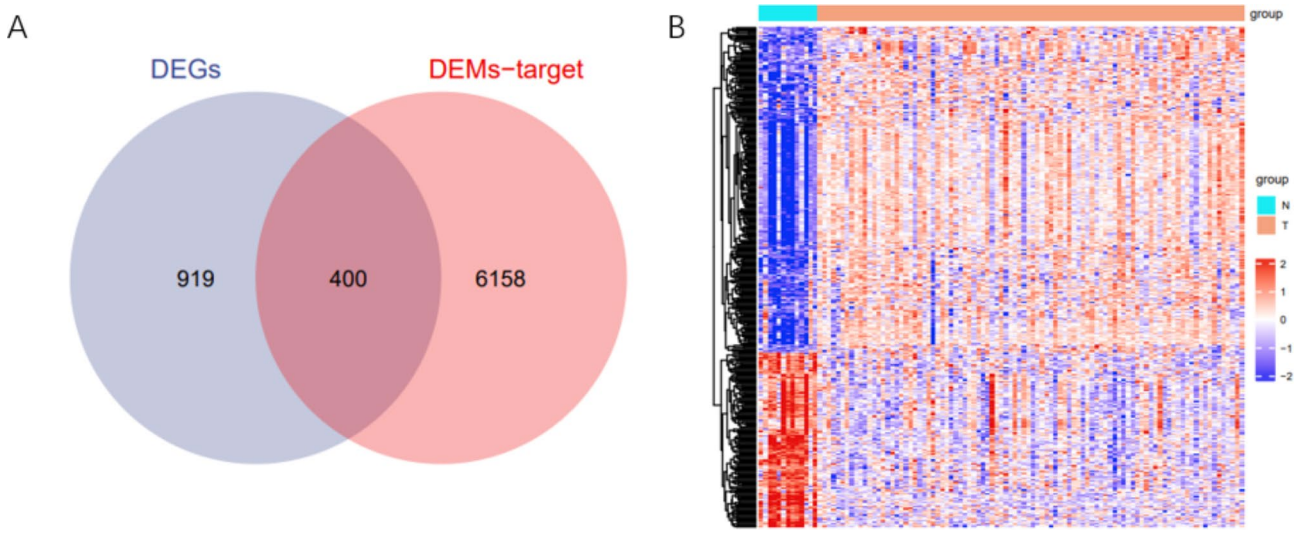

Fig. 2. Common genes between 1319 DEGs and 6,559 target genes of 48 DEMs. **A** Venn diagram of 1319 DEGs and the 6559 target genes of 48 DEMs, where the 400 common genes are DEM regulated DEGs; **B** Heatmap of the 400 DEM regulated DEGs.

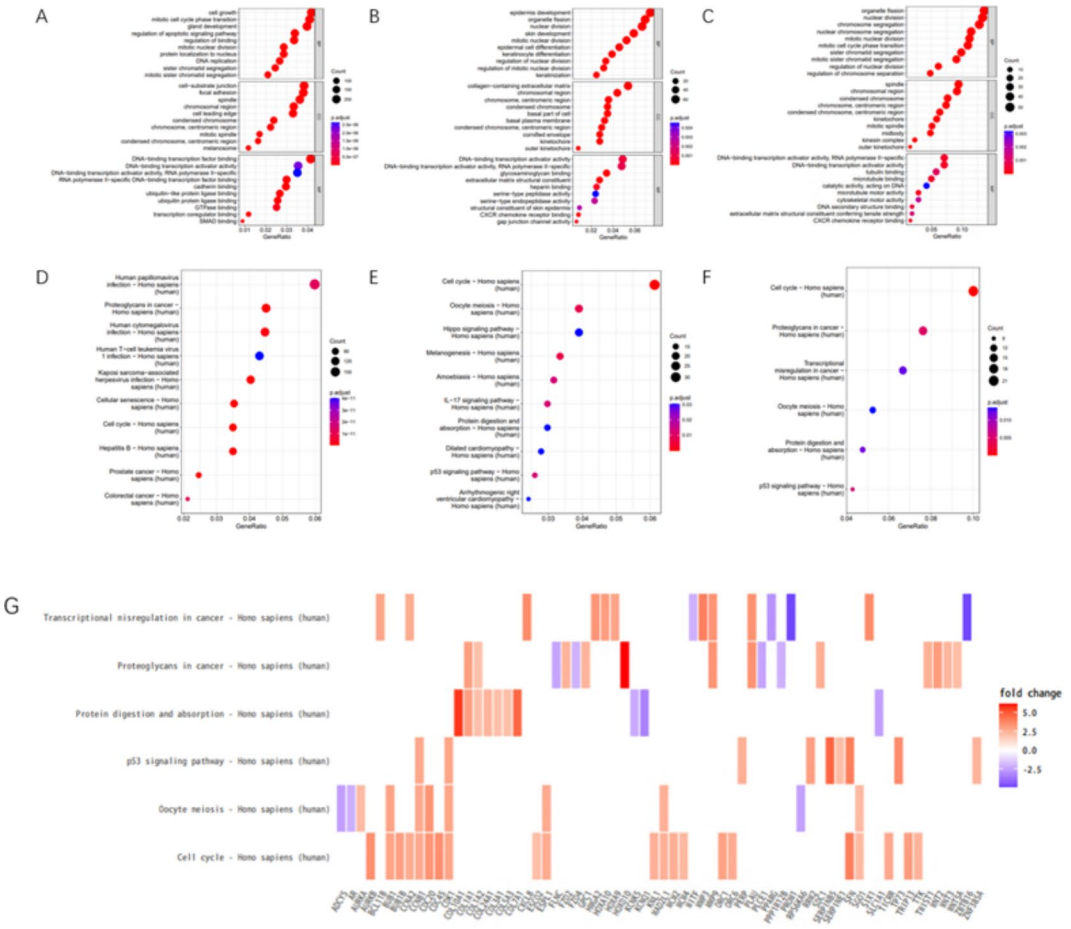

Fig. 3. GO and KEGG enrichment of DEGs and DEMs. **A** Dot plot of top GO terms enriched from 6559 target gene of 48 DEMs; **B** Dot plot of top GO terms enriched from 1319 DEGs; **C** Dot plot of top GO terms enriched from 400 DEM-regulated DEGs; **D** Dot plot of top KEGG pathways enriched from 6559 target gene of 48 DEMs; **E** Dot plot of top KEGG pathways enriched from 1319 DEGs; **F** Dot plot of top KEGG pathways enriched from 400 DEM-regulated DEGs; **G** The occurrence heatmap shows that only 66 DEM-regulated DEGs participate in the top six KEGG pathways.

and outer kinetochore; and the top molecular functions include DNA-binding transcription activator activity RNA polymerase II-specific, DNA-binding transcription activator activity, tubulin binding, microtubule binding, catalytic activity acting on DNA, microtubule motor activity, cytoskeletal motor activity, DNA secondary structure binding, extracellular matrix structural constituent conferring tensile strength, and single-stranded DNA helicase activity.

From KEGG enrichment of 6559 DEMs target genes in Fig. 3D, the top enriched KEGG pathways include Human papillomavirus infection, Proteoglycans in cancer, Cellular senescence, and Cell cycle.

From KEGG enrichment of 1319 DEGs in Fig. 3E, the top enriched KEGG pathways include Cell Cycle, Oocyte meiosis, Hippo signaling pathway, Melanogenesis, IL-17 signaling pathway, and p53 signaling pathway.

Furthermore, KEGG enrichment of 400 DEM-regulated DEGs in Fig. 3F shows that the top enriched KEGG pathways include cell cycle, proteoglycan in cancer, p53 signaling pathway, protein digestion and absorption, transcriptional dysregulation in cancer, and oocyte meiosis.

Both GO and KEGG enrichment indicates that these six pathways were the main mechanisms of both DEM target genes and regulated DEGs, which related to ESCC. We thus filtered the DEM-regulated DEGs in these six ESCC-related pathways, and there are 66 DEM-regulated DEGs among them as shown in their occurrence heatmap of Fig. 3G.

The heatmaps of these 66 DEM-regulated DEGs in the six ESCC-related pathways are shown in Fig. 4A–F, respectively. They show that these 66 DEMs regulated DEGs well separate ESCC and normal samples. We thus looked for the DEMs regulating these 66 DEGs. From the miRNA–mRNA regulatory network in miRTarBase database, there are 32 DEMs in total regulating these 66 DEGs, called as ESCC-related DEMs.

Comparison with ESCC-related DEMs reported in the literature

Among the 32 ESCC-related DEMs identified from our analysis in "GO and KEGG enrichment" section, 22 DEMs (hsa-miR-133a-3p, hsa-miR-196a-5p, hsa-miR-205-5p, hsa-miR-129-5p, hsa-miR-196b-5p, hsa-miR-

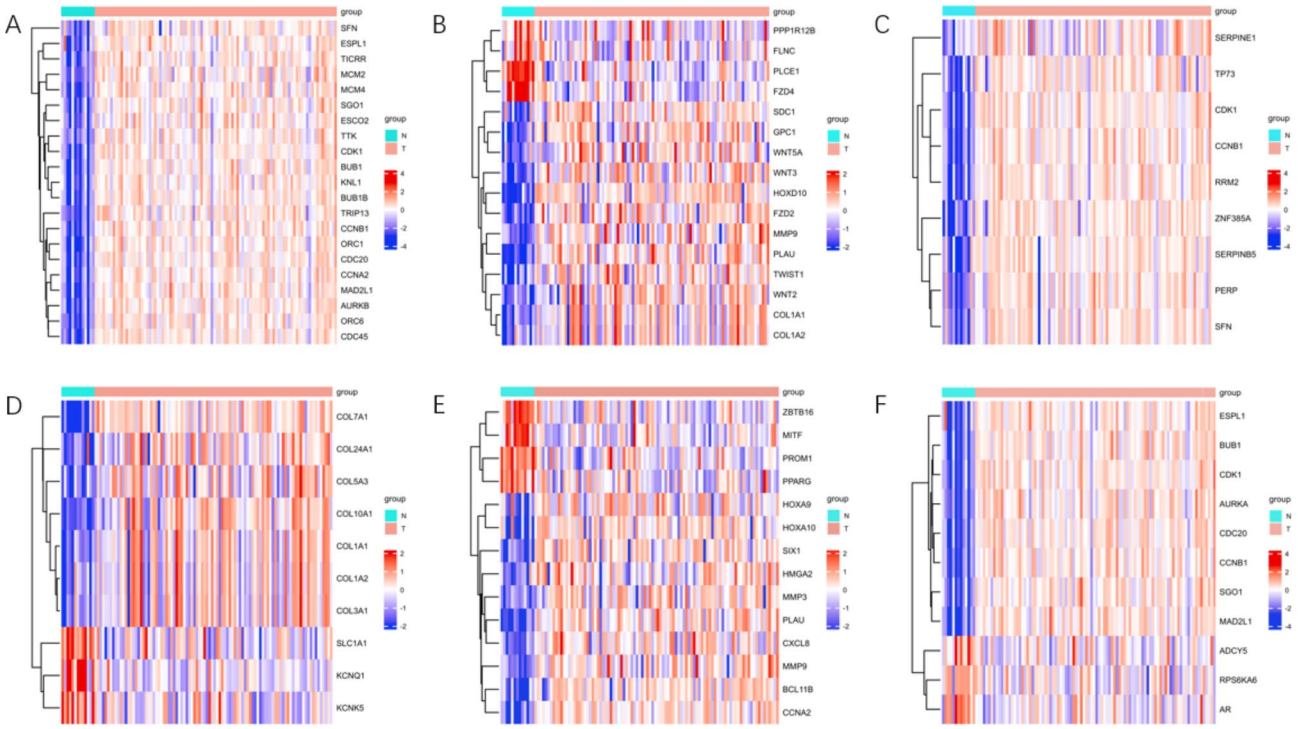

Fig. 4. The heatmaps of the DEM-regulated DEGs in six ESCC-related pathways. **A** Heatmap of DEM-regulated DEGs in cell cycle pathway; **B** Heatmap of DEM-regulated DEGs in proteoglycans in cancer pathway; **C** Heatmap of DEM-regulated DEGs in p53 signaling pathway; **D** Heatmap of DEM-regulated DEGs in protein digestion and absorption pathway; **E** Heatmap of DEM-regulated DEGs in transcriptional misregulation in cancer pathway; **F** Heatmap of DEM-regulated DEGs in oocyte meiosis pathway.

30a-5p, hsa-miR-30a-3p, hsa-miR-148a-3p, hsa-miR-149-5p, hsa-miR-139-5p, hsa-miR-224-5p, hsa-miR-204-5p, hsa-miR-455-3p, hsa-miR-338-3p, hsa-miR-153-5p, hsa-miR-192-5p, hsa-miR-1-3p, hsa-miR-29c-3p, hsa-miR-375, hsa-miR-338-5p, hsa-miR-194-5p, hsa-miR-675-3p) were previously reported in miRCancerMap and PubMed, as verified by different experiments in ESCC tissues, cell lines, or serum, as shown in Table 1.

Univariate Cox regression analysis shows that hsa-miR-34b-3p has no significant correlation with the overall survival of ESCC patients. The other nine ESCC-related DEMs were significantly related to ESCC survival, and have been reported in the literature, thus might be novel miRNA markers related to ESCC. Among them, six upregulated (hsa-miR-944, hsa-miR-205-3p, hsa-miR-4652-5p, hsa-miR-452-3p, hsa-miR-6499-3p, hsa-miR-767-5p) and three downregulated (hsa-miR-215-5p, hsa-miR-194-3p, hsa-miR-29b-2-5p) in ESCC. The differential expression details of these nine miRNA markers, along with their regulated target genes and the ESCC pathways they participate in, were shown in Table 2.

miRNA–mRNA regulatory network

The regulatory network of the 32 DEMs and the 66 DEM-regulated DEGs drawn by Cytoscape 3.7.2 was shown in Fig. 5. The heatmap of the 9 novel ESCC-related DEMs was shown in Fig. 6A. They well separated the ESCC from normal. The regulatory network of the 9 novel and their regulated DEGs was shown in Fig. 6B. The target DEGs of six up-regulated miRNAs and three down-regulated miRNAs were shown in Table 2. The Sankey diagram of the novel 9 DEMs, their regulated DEGs, and the ESCC pathways these DEGs participate in was shown in Fig. 6C.

The 9 ESCC-related DEMs target genes and their GO and KEGG enrichment results

There are 2280 target genes of 9 ESCC-related DEMs according to the miRTarBase database. They were top enriched in biological processes such as organelle fission, chromosome segregation, mitotic cell cycle phase transition, nuclear division, and regulation of cell cycle phase transition; cell components such as spindle, chromosomal region, condensed chromosome, and kinetochore; and molecular functions such as in single-stranded DNA binding, tubulin binding, ubiquitin-like protein ligase binding, and ubiquitin protein ligase binding; and KEGG pathways such as Cell Cycle, p53 signaling pathway pathway, which are consistent with our previous analysis, as shown in Fig. 7.

Identification of novel miRNA markers related to ESCC

To evaluate the expression patterns of novel miRNA biomarkers in ESCC, we employed qRT-PCR to measure the expression levels of nine miRNAs in the ESCC cell line KYSE-30 and in the normal human esophageal epithelial cell line Het-1 A. The qRT-PCR results in Fig. 8 showed that the expression levels of five miRNAs—hsa-miR-944,

miRNA	Expression	Target gene	Detection method	Sample	References
hsa-miR-133a-3p	↓	COL1A1	qRT-PCR	Tissues and cells	[18]
hsa-miR-196a-5p	↑	–	qPCR	Cell line	[19]
hsa-miR-205-5p	↑	–	miRNA microarrays	Cell line	[20]
hsa-miR-129-5p	↓	CCND1	qRT-PCR	Cells	[21]
hsa-miR-196b-5p	↑	PPP1R12B	qRT-PCR	Tissues and cells	[22]
hsa-miR-30a-5p	↓	FOXD1	RT-qPCR	Tissues	[23]
hsa-miR-30a-3p	↓	Wnt2	Microarray	Tissues	[24]
hsa-miR-148a-3p	↓	TUG1	qRT-PCR	Tissues and cells	[25]
hsa-miR-149-5p	↓	IL-6	RT-PCR	Cells	[26]
hsa-miR-139-5p	↓	TBX1	qRT-PCR	Tissues	[27]
hsa-miR-224-5p	↑	CPEB3	qRT-PCR	Tissues and cells	[28]
hsa-miR-204-5p	↓	Nestin	qPCR	Tissues and cell lines	[29]
hsa-miR-455-3p	↑	FAM83F	qRT-PCR	Tissues and cell lines	[30]
hsa-miR-338-3p	↓	–	qRT-PCR	Tissues	[31]
hsa-miR-153-5p	↓	SNAI1	qRT-PCR	Tissues	[32]
hsa-miR-192-5p	↑	–	qRT-PCR	Tissue and serum	[33]
hsa-miR-1-3p	↓	–	qPCR	Cell line	[19,34]
hsa-miR-29c-3p	↓	CCNA2	qRT-PCR	Tissues	[35]
hsa-miR-375	↓	PRDX1、XPR1	RT-qPCR	Tissue, cell, serum	[36,37]
hsa-miR-338-5p	↓	FERMT2	RT-PCR	Cells	[38]
hsa-miR-194-5p	↓	JMJD1C	qRT-PCR	Tissues and cells	[39]
hsa-miR-675-3p	↑	–	RT-qPCR	Tissues	[40]

Table 1. ESCC-related DEMs reported in miRNACancerMAP and PuBMed.

miRNA	logFC	PValue	FDR	Up/down	Target gene	Pathway
hsa-miR-215-5p	−4.553848153	1.68E−19	1.88E−17	Down	KNL1, BUB1B, TRIP13, MAD2L1, CDC20, ORC1, TICRR, TTK, PLAU, FZD4, WNT3, PLCE1, PERP, PPARG, HOXA10	Cell cycle; proteoglycans in cancer; p53 signaling pathway; transcriptional misregulation in cancer; oocyte meiosis
hsa-miR-194-3p	−4.52886534	2.90E−19	2.16E−17	Down	ORC6, MMP9	Cell cycle; proteoglycans in cancer; transcriptional misregulation in cancer
hsa-miR-29b-2-5p	−2.34622638	3.62E−17	2.02E−15	Down	KCNK5	Protein digestion and absorption
hsa-miR-944	7.069254694	1.12E−16	5.58E−15	Up	BCL11B	Transcriptional misregulation in cancer
hsa-miR-205-3p	4.326054552	1.79E−16	7.29E−15	Up	AR	Oocyte meiosis
hsa-miR-4652-5p	3.763658818	9.39E−11	1.68E−09	Up	SFN	Cell cycle; p53 signaling pathway
hsa-miR-452-3p	3.338949135	4.83E−09	6.75E−08	Up	ZBTB16	Transcriptional misregulation in cancer
hsa-miR-6499-3p	2.832887553	3.48E−07	2.78E−06	Up	SGO1, CCNB1, ESCO2, KNL1, ORC6, FZD4, PPP1R12B	Cell cycle; proteoglycans in cancer; p53 signaling pathway; oocyte meiosis
hsa-miR-767-5p	4.554272941	0.00184551	0.0056892	Up	SGO1, COL3A1, COL10A1	Cell cycle; protein digestion and absorption; oocyte meiosis

Table 2. The target genes and pathways involved in the regulation of 9 newly discovered miRNAs.

hsa-miR-205-3p, hsa-miR-452-3p, hsa-miR-6499-3p, and hsa-miR-767-5p—were significantly elevated in the KYSE-30 cell line compared to the Het-1 A cell line. These findings align with the expression trends observed in the TCGA database.

Given that miRNAs act as negative regulators of mRNA expression, we excluded miRNAs whose expression patterns were congruent with those of their target mRNAs. Consequently, through qRT-PCR analysis coupled with the consideration of miRNA–mRNA negative regulatory relationships, we identified three differentially expressed miRNAs with statistical significance that were also validated by PCR: namely, hsa-miR-205-3p, hsa-miR-452-3p, and hsa-miR-6499-3p (Table 3).

To substantiate the robustness of these three differentially expressed miRNAs and to affirm their consistent differential expression in esophageal squamous cell carcinoma, we extended our qRT-PCR validation on another ESCC cell line, KYSE-180, alongside the Het-1 A, the normal human esophageal epithelial cell line. The validation results as shown in Fig. 9 demonstrated that the expression levels of these three miRNAs were consistent with the discovery results from TCGA database and the validation results observed in the ESCC cell line KYSE-30.

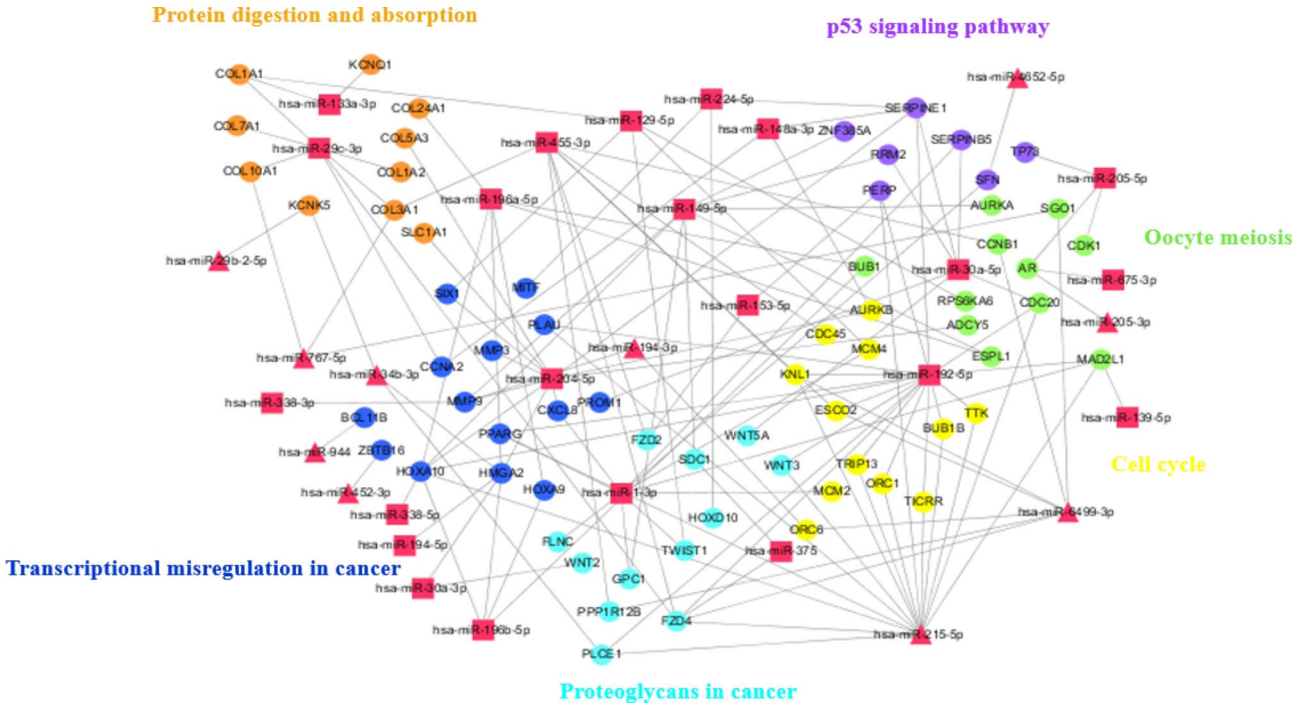

Fig. 5. The regulatory network of the 32 DEMs and the 66 DEM-regulated DEGs was drawn by Cytoscape 3.7.2, where red squares represent DEMs which previously reported related to ESCC in literature; red triangles represent DEMs which were novel reported related to ESCC in our study, and yellow circles represent DEM-regulated DEGs in the cell cycle pathway. Light blue represents DEM-regulated DEGs in proteoglycans in the cancer pathway, and dark blue represents DEM-regulated DEGs in Transcriptional misregulation in the cancer pathway. Orange represents common DEM-regulated DEGs in the protein digestion and absorption pathway, purple represents DEM-regulated DEGs in the p53 signaling pathway, and green represents DEM-regulated DEGs in the Oocyte meiosis pathway.

These validation results confirm the results from our bioinformatics analyses, indicating the potential utilization of these novel miRNA markers in the diagnosis and therapeutics of esophageal squamous cell carcinoma. These three miRNAs are now at the forefront of our ongoing research.

The stage specificity of the novel miRNA markers related to ESCC

We also performed the differential analysis of miRNAs on patients at four ESCC pathologic stages to show the stage specificity of the novel miRNA markers. The results are shown in Supplementary Table 1, among the 9 novel DEMs, hsa-miR-944, hsa-miR-29b-2-5p were common to all of the four stages in ESCC; hsa-miR-452-3p (qPCR validated) and hsa-miR-215-5p is common to Stage I, II and III; hsa-miR-205-3p (qPCR validated), hsa-miR-4652-5p and hsa-miR-194-3p were specific in Stage II and III; hsa-miR-6499-3p (qPCR validated) and hsa-miR-767-5p were Stage II specific. The three qPCR validated miRNAs were also specific to the first three stages of ESCC. They might be the potential biomarkers for ESCC stage diagnosis.

Materials and methods

Data download

The bioinformatics analysis flow chart of this study is shown in Fig. 10. The gene expression profile data, miRNA expression profile data and Clinical data of ESCC were downloaded from the TCGA database website (https://portal.gdc.cancer.gov/) on 11 May 2023. The gene expression profile data include 95 tumor samples and 13 normal samples. The miRNA expression profile data include 96 tumor samples and 13 normal samples. By removing duplicate samples and recurrent samples, 13 normal and 94 ESCC samples have both miRNA and mRNA expression data were included in this study. Among the 94 ESCC samples, 6 patients were in the stage I (Stage IA or Stage IB), 55 patients were in Stage II (Stage II, Stage IIA or Stage IIB), 26 patients were in Stage III (Stage III, Stage IIIA, Stage IIIB, or Stage IIIC), 4 patients in Stage IV (Stage IVA or Stage IV), and the other two have no information about the stage.

Data preprocessing and differential expression analysis

The miRNAs and mRNAs expression data were normalized by using the "GDCRNATools" package in R 4.2.2. The miRNAs and mRNAs with no expression on more than 50% samples were excluded in the following analysis.

The differentially expression analysis of miRNAs and mRNAs between tumor and adjacent normal tissues were conducted using the "limma" package. The screening thresholds are FDR < 0.05 and logFC > 2 (where logFC > 0 indicates upregulated, and logFC < 0 indicates downregulated in tumor samples). The miRNAs and

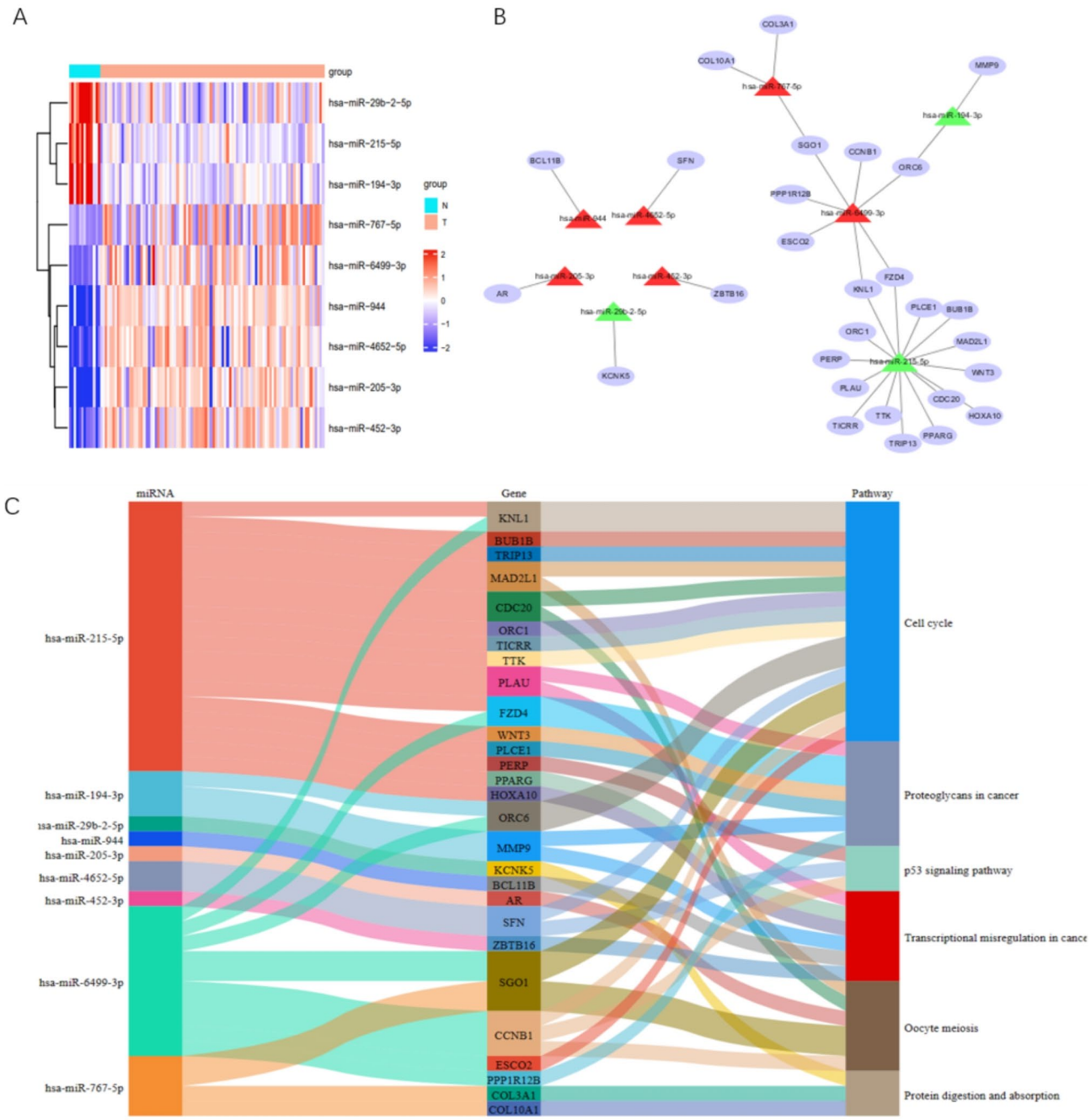

Fig. 6. The 9 novel ESCC-related DEMs. **A** The heatmap of 9 DEMs in ESCC and normal samples; **B** The miRNA–mRNA regulatory networks of the 9 novel DEMs and their regulated DEGs; **C** Sankey diagram of the Sankey diagram of the novel 9 DEMs, their regulated DEGs, and the ESCC pathways these DEGs participate in.

mRNAs satisfying the screening thresholds are the differentially expressed miRNAs (DEMs) and differentially expressed genes (DEGs), respectively. The visualization of differentially expression analysis results was shown in VolcanoPlot using the "gdcVolcanoPlot" package in R 4.2.2. The heatmaps of DEMs and DEGs expression patterns were drawn using the "ComplexHeatmap" package in R 4.2.2, where the clustering distance used Euclidean distance of normalized expression data.

Prediction of DEM target genes

The miRNA–mRNA interactions were collected from miRTarBase database (https://mirtarbase.cuhk.edu.cn). The target genes of the DEMs were then filtered based on these miRNA–mRNA interactions. The intersection of

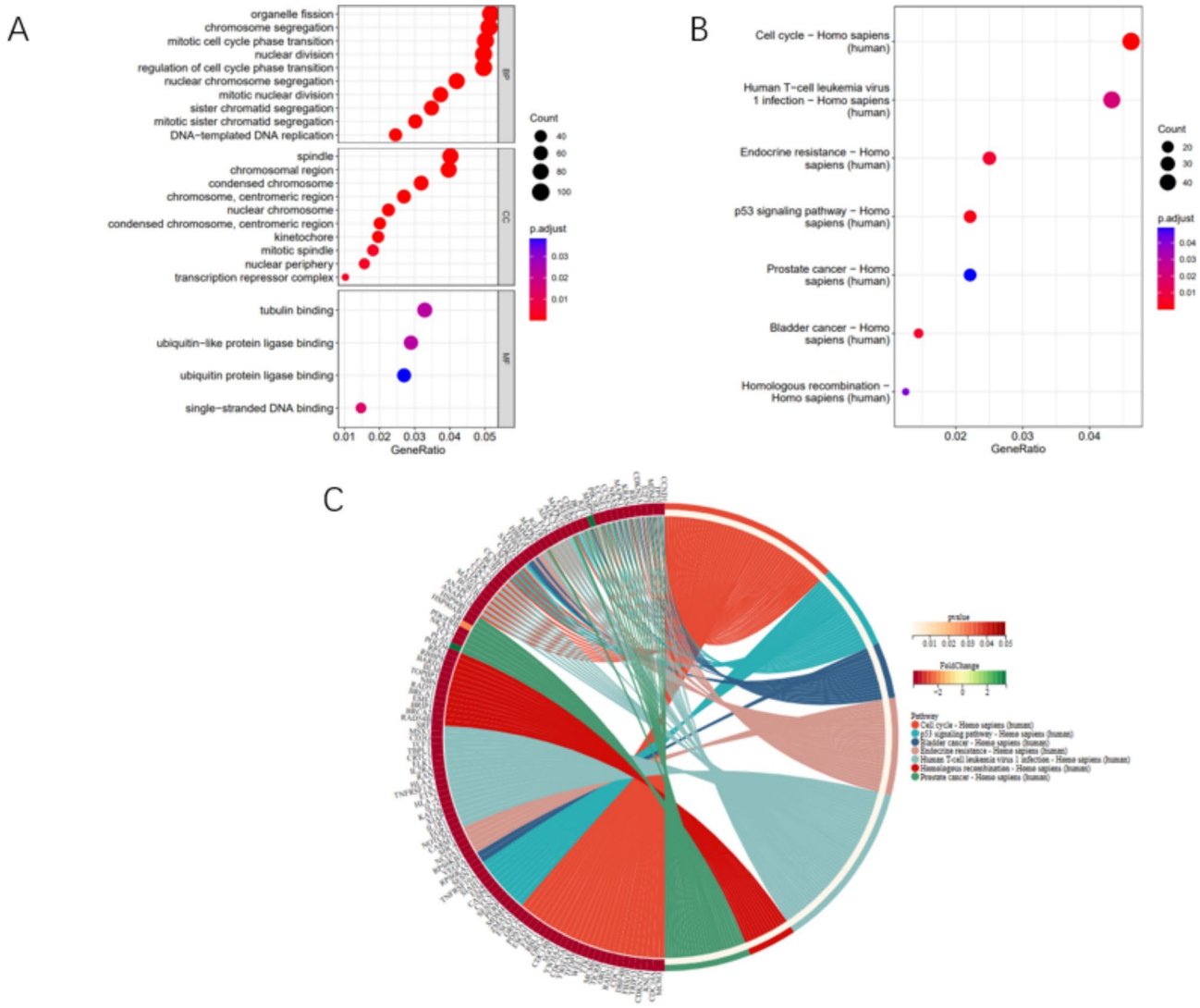

Fig. 7. GO and KEGG enrichment of 2280 target genes of 9 ESCC-related DEMs. **A** Dot plot of top GO terms enriched from 2280 target genes of 9 ESCC-related DEMs; **B** Dot plot of top KEGG pathways enriched from 2280 target genes of 9 ESCC-related DEMs; **C** Chord plot of top KEGG pathways enriched from 2280 target genes of 9 ESCC-related DEMs.

the target genes of DEMs and DEGs, i.e., the DEM-regulated DEGs, were analyzed by "VennDiagram" package in R 4.2.2.

GO and KEGG analysis

The functional annotations of Gene ontology (GO) included biological process (BP), cell component (CC), and molecular function (MF). GO and KEGG[41–43] pathway enrichment analysis of the target genes of DEMs, DEGs and DEM-regulated DEGs, were performed on "clusterProfiler" package in R 4.2.2. The statistically significant enriched functional annotations and pathways were those with enrichment adjusted p-value, i.e., q-value < 0.05. The heatmap of the top enriched KEGG pathway with DEGs expression was drawn by "enrichplot" package in R 4.2.2.

Comparison to miRNACancerMAP and PubMed

The miRNACancerMAP (https://cis.hku.hk/miRNACancerMAP) consists of miRNA expression, related pathways, disease prognosis, and regulatory target genes in various cancers reported in PubMed. Compared the miRNA markers related to ESCC from miRNACancerMAP with our DEMs, the novel ESCC-related miRNA markers were obtained.

Construction of miRNA–mRNA regulatory network

The miRNA–mRNA interactions between DEMs and DEM-regulated DEGs inferred from miRTarBase database was drawn on Cytoscape 3.7.2.

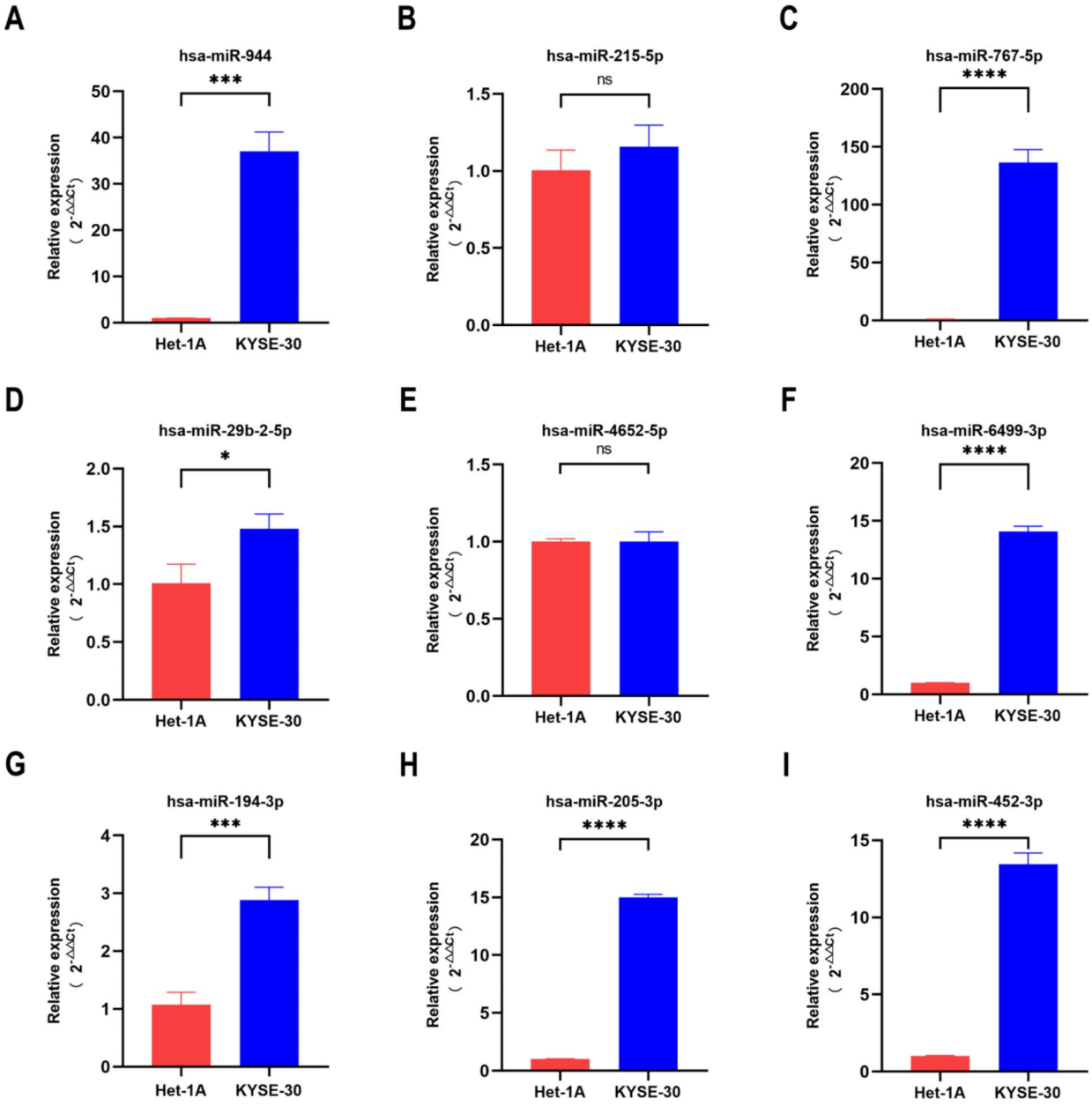

Fig. 8. qRT-PCR for 9 identified candidate novel miRNAs in KYSE-30 and Het-1 A cell lines. ("ns" indicates $p > 0.05$; "*" indicates $p < 0.05$; "***" indicates $p < 0.001$; "****" indicates $p < 0.0001$).

Quantitative real-time polymerase chain reaction (qPCR)

The mRNA levels of newly identified miRNAs were measured using qPCR, with each sample tested in triplicate for accuracy. Primer sequences are provided in Supplementary Table 2, designed for specific primer fragments to ensure reliable gene expression data.

Statistical analysis

Statistical analysis was conducted using R language and GraphPad prism 9. Images were processed with ImageJ, Adobe Photoshop, and Adobe Illustrator CS2 software. Comparisons between two groups were done with t test and multiple groups with one-way analysis of variance (ANOVA). The data are presented as mean ± standard error of the mean (SEM). Normally distributed variables were compared by Student's t test, or one-way analysis of variance (ANOVA) followed by Bonferroni correction for multiple comparisons. Chi-square test was used to analyze the counting data. $P < 0.05$ was considered statistically significant.

miRNA				mRNA			
Name	logFC	PValue	Up/down	Name	logFC	PValue	Up/down
hsa-miR-944	7.069254694	1.12E−16	Up	BCL11B	2.857510637	1.27011E−06	Up
hsa-miR-205-3p	4.326054552	1.79E−16	Up	AR	−2.111226181	1.10152E−06	Down
hsa-miR-452-3p	3.338949135	4.83E−09	Up	ZBTB16	−4.847265048	3.88975E−15	Down
hsa-miR-6499-3p	2.832887553	3.48E−07	Up	SGO1	2.164183845	2.31275E−10	Up
				CCNB1	2.735847075	9.29341E−14	Up
				ESCO2	2.041037953	6.55856E−07	Up
				KNL1	2.501481885	7.09196E−10	Up
				ORC6	2.599817102	4.41595E−14	Up
				FZD4	−2.031767905	7.10455E−14	Down
				PPP1R12B	−2.069975635	2.94068E−05	Down
hsa-miR-767-5p	4.554272941	0.001845505	Up	SGO1	2.164183845	2.31275E−10	Up
				COL3A1	2.044194649	6.73107E−06	Up
				COL10A1	5.604170176	3.01379E−05	Up

Table 3. Differentially regulated target genes of 5 new miRNAs related to ESCC.

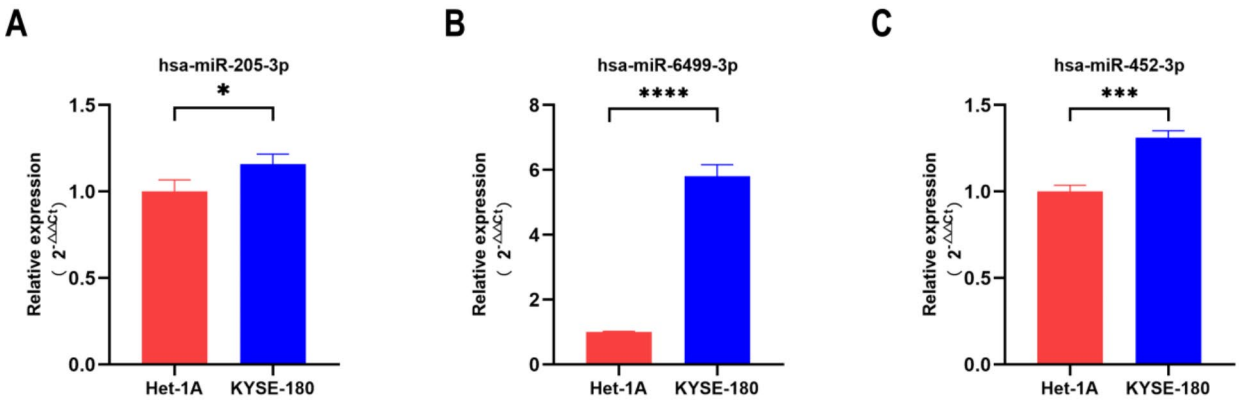

Fig. 9. The qRT-PCR detection results of three novel miRNAs in KYSE-180 and Het-1 A cell lines. ("*" indicates $p < 0.05$; "***" indicates $p < 0.001$; "****" indicates $p < 0.0001$).

Discussion and conclusion

ESCC is a prominent subtype of esophageal cancer in Asia. However, both early diagnosis and effective treatment are quite limited. The pathogenic mechanisms of ESCC remains unclear. The treatment approaches include surgical procedures, chemoradiotherapy, targeted drug therapy, and immunotherapy.

Recent studies demonstrated that miRNA roled as an essential marker for early diagnosis and potential target of cancer. MiRNAs are not very specific as potential drug targets as they negatively regulate many target genes. Thus, the integration analysis of miRNA and mRNA are very important to figure out the specific miRNA–mRNA regulations related to disease progression. TCGA is currently the biggest dataset on various cancers, it included miRNA, mRNA expression data, and the clinical traits. The integration analysis can help to make the target miRNAs more specifically as potential markers for ESCC, and also compensate the information loss from small sample set.

In this study, we jointly analyzed ESCC RNA-seq and miRNA-seq data in the TCGA database. From differential expression analysis, 48 DEMs, 1319 DEGs, and 400 DEM-regulated DEGs were identified. According to KEGG enrichment of 400 DEM-regulated DEGs, the top enriched KEGG pathways include cell cycle, proteoglycan in cancer, p53 signaling pathway, protein digestion and absorption, transcriptional dysregulation in cancer, and oocyte meiosis. We call these six KEGG pathways as ESCC-related pathways. And there are 66 DEM-regulated DEGs in these ESCC-related pathways, and 32 DEMs were their regulators. Among them, 22 DEMs were reported to relate ESCC in PubMed. Survival analysis on the remaining 10 DEMs confirmed their significant relatedness to ESCC except hsa-miR-34b-3p. The other 9 DEMs (hsa-miR-944, hsa-miR-205-3p, hsa-miR-4652-5p, hsa-miR-452-3p, hsa-miR-6499-3p, hsa-miR-767-5p, hsa-miR-215-5p, hsa-miR-194-3p, and hsa-miR-29b-2-5p) might be novel ESCC-related miRNA markers.

Previous studies have reported abnormal expression of hsa-miR-944 in various cancers, such as esophageal adenocarcinoma, gastric cancer, colorectal cancer, breast cancer, oral cancer, lung cancer, cervical cancer, nasopharyngeal cancer, and liver cancer[44]. It plays a vital role in tumor proliferation, invasion, migration,

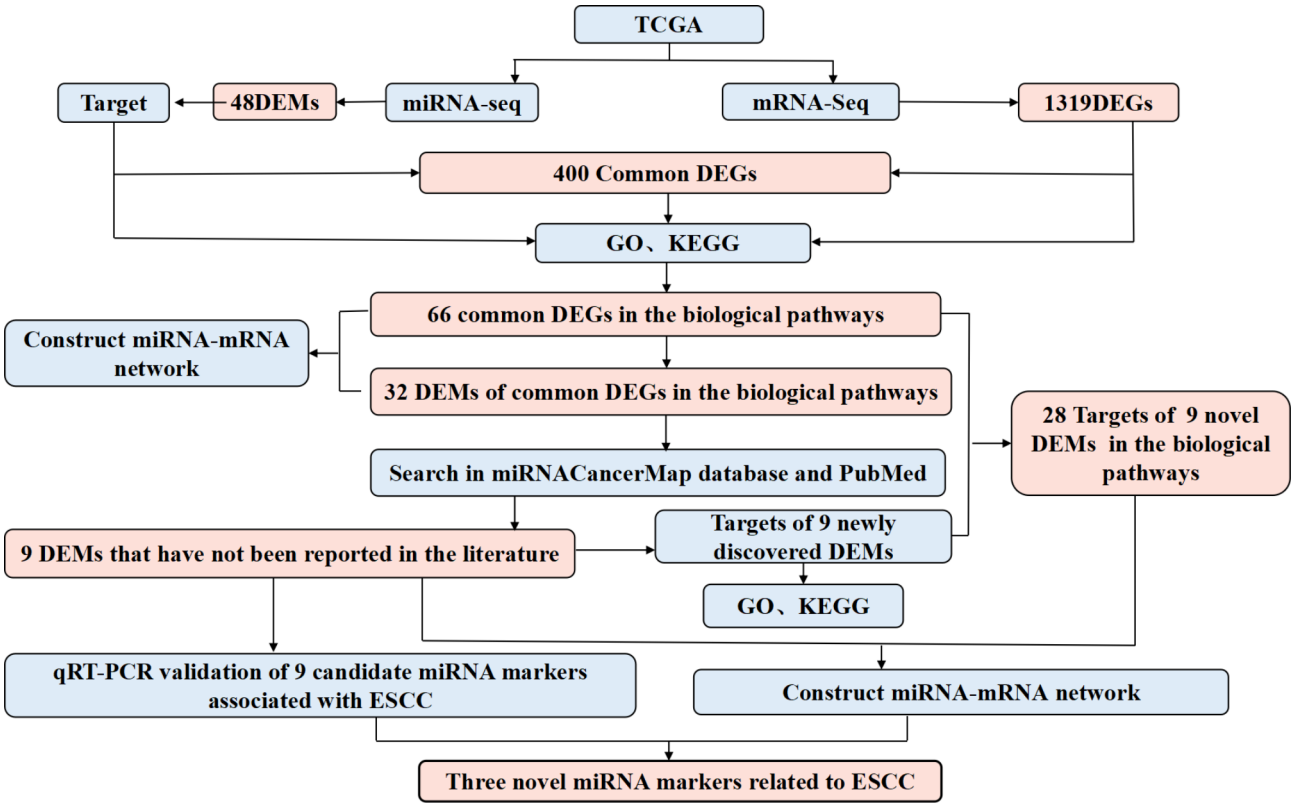

Fig. 10. The bioinformatics analysis flow chart of this study. (Note: TCGA stands for The Cancer Genome Atlas, also known as the Cancer Genome Atlas Project; GO refers to Gene Ontology, the gene ontology; KEGG stands for Kyoto Encyclopedia of Genes and Genomes, the encyclopedia of genes and genomes in Kyoto; DEMs refer to differentially expressed miRNAs, differentially expressed microRNAs; DEG refers to differentially expressed gene; qRT-PCR stands for Quantitative Real-time PCR, quantitative real-time polymerase chain reaction).

epithelial-mesenchymal transition, apoptosis, and drug resistance. Moreover, it has significant clinical application value in cancer diagnosis, treatment, and prognosis judgment. However, its expression in esophageal squamous cell carcinoma (ESCC) remains unexplored. Another novel marker, miR-205-3p is up-regulated in non-small cell lung cancer tissues and cell lines, and it regulates the expression of *APBB2*, promoting the progression of non-small cell lung cancer[45]. In addition, it is significantly associated with poor prognosis in breast cancer patients[46]. MiR-205-3p also exhibits a role in inhibiting cancer in ovarian cancer and gastric cancer[47,48]. Similarly, miR-4652-5p is involved in regulating cell adhesion through down-regulating *RND1* expression, thereby driving the progression of lung squamous cell carcinoma[49]. It could be a potential molecular diagnostic biomarker for laryngeal squamous cell carcinoma and head and neck squamous cell carcinoma[50,51]. Moreover, miR-452-3p is significantly up-regulated in liver cancer tissues and cells, and it promotes the proliferation and migration of liver cancer cells by targeting *CPEB3*[52]. Long-chain noncoding RNA *ZFAS1* promotes the development of oral squamous cell carcinoma through the regulation of the miR-6499-3p/*CCL5* axis[53]. Similarly, miR-767-5p acts as an oncogene, promoting the progression of hepatocellular carcinoma by regulating the expression of the downstream target gene *PMP22*[54]. Studies further highlight its significant relationship with the prognosis of liver cancer patients through weighted gene co-expression network analysis[55]. Additionally, miR-767-5p is one of the ten miRNAs in a prognostic model that accurately predicts tumor recurrence in hepatocellular carcinoma patients after surgical treatment[56]. Conversely, miR-215-5p has been identified as a tumor suppressor gene in several human cancers. For example, it inhibits tumor cell proliferation by targeting *RUNX1* in multiple myeloma[57], inhibits tumor progression in colorectal cancer by regulating the expression of epiregulin and *HOXB9*[58], and inhibits cancer cell invasion by down-regulating the expression of Sox9 in breast cancer[59]. In addition, miR-215-5p down-regulates *RAD54B* expression, promoting cancer cell apoptosis[60]. The molecular panel consisting of miR-215-5p, miR-190b-5p, and miR-527 can serve as a serum biomarker for prostate cancer[61]. Furthermore, miR-194-3p inhibits the proliferation, migration, invasion, and docetaxel resistance of colorectal cancer cells through *KLK10* regulation[62,63]. Moreover, high expression of miR-194-3p is associated with early cancer staging[64]. In pancreatic ductal adenocarcinoma, miR-29b-2-5p inhibits cell proliferation by directly targeting Cbl-b[65]. This miRNA is regarded as one of the most significant hinge genes in digestive system cancers, including esophageal cancer, and may be related to cancer prognosis[66].

In summary, these nine ESCC-related novel miRNAs (hsa-miR-944, hsa-miR-205-3p, hsa-miR-4652-5p, hsa-miR-452-3p, hsa-miR-6499-3p, hsa-miR-767-5p, hsa-miR-215-5p, hsa-miR-194-3p, and hsa-miR-29b-

2-5p) involved in cancer inhibition or carcinogenic effects, and might be promising potential biomarkers for ESCC diagnosis and treatment targets. They participate in the occurrence and development of ESCC through various pathways, including the cell cycle, proteoglycans in cancer, p53 signaling pathway, protein digestion and absorption, transcriptional dysregulation in cancer, and the signaling pathway of oocyte meiosis. These results require further validation in clinical samples. We also performed the qPCR on two ESCC cell lines, and confirmed three novel miRNA markers, hsa-miR-205-3p, hsa-miR-452-3p, hsa-miR-6499-3p, might be the ESCC biomarkers. Interestingly, these three markers were also specific to the first three stages of ESCC, indicating they might be the potential diagnosis biomarkers of ESCC stage.

Data availability

The ESCC RNA-Seq, miRNA-Seq data, clinical data were obtained from The Cancer Genome Atlas(TCGA) (https://portal.gdc.cancer.gov/projects/TCGA-ESCA; dbGaP Study Accession number is phs000178). This study complies with its data use and publication rules.

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Author contributions

WL and JG supervised the study. PY, XG, and MX conduct the bioinformatics analysis and qPCR validation. PY wrote the first draft of the manuscript. WL, XG, MX, LQ, ZQ, JS, ZX, HX, RY, LZ, XL and JG revised the manuscript. All authors approved the final manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical approval and consent to participate
Not applicable.

Consent for publication
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