

Analysis of salivary microRNA expression profiles and identification of novel biomarkers in esophageal cancer

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Abstract. MicroRNAs (miRNAs/miRs) regulate the expression of target genes and are considered to be associated with human cancer. The aim of the present study was to screen novel miRNA biomarkers in esophageal cancer (EC). The miRNA expression profile GSE41268 was extracted from Gene Expression Omnibus database, and differentially expressed miRNAs between whole saliva samples from patients with EC and healthy controls were identified using the Linear Models for Microarray Data package. Then, the targets of these miRNAs were screened using the miRecords database and used to construct the regulatory network. Gene ontology and pathway enrichment analyses were performed for the target genes of differentially expressed miRNAs to predict their potential functions. A total of 18 differentially expressed miRNAs were identified in saliva samples from patients with EC, and 43 validated target genes corresponding to 7 upregulated miRNAs were identified. Then, 6 miRNAs (miR-144, miR-451, miR-98, miR-10b, miR-486-5p and miR-363) and their target genes were used to construct a regulatory network. Within the network, miR-144 may target Notch homolog 1, fibrinogen α chain and fibrinogen β chain; miR-451 may regulate murine thymoma viral oncogene homolog 1, matrix metalloproteinase (*MMP*)*9* and *MMP**2*; miR-98 may directly target E2F transcription factor (E2F) 1, *E2F2* and v-myc avian myelocytomatosis viral oncogene homolog (*MYC*); miR-10b may modulate peroxisome proliferator-activated receptor α and Kruppel-like factor 4; miR-485-5p and miR-363 may regulate TNF receptor superfamily member 5 and cyclin-dependent kinase inhibitor 1A. In addition, *E2F1*, *E2F2* and *MYC* were associated with the cell cycle, which was the most significantly enriched function and pathway in EC. The results of the present study suggested that miR-144, miR-451, miR-98,

miR-10b and miR-363 may be involved in EC by regulating their target genes, and may be used as biomarkers for EC.

Introduction

Esophageal cancer (EC), also known as oesophageal cancer, is one of the most aggressive tumors of the gastrointestinal tract, and ranks the sixth most common cause of cancer mortality (1). According to the statistical data of National Central Cancer Registry of China, an estimated 287,632 patients were diagnosed with and 208,473 patients succumbed to EC in 2010 in China (2). Although the EC-associated mortality rate has decreased over the past 30 years, it remains a major cancer burden in high risk areas (3). Thus, the early diagnosis and treatment of EC are vital.

Previous studies have indicated that tumor-specific microRNAs (miRNAs/miRs) are considered to be potential biomarkers for the early diagnosis and treatment of EC (4-6). miRNAs, a class of endogenous short non-protein-coding RNA molecules, bind to their target sites within the mRNA molecules via base-pairing with complementary sequences at a post-transcriptional level, and thus certain miRNAs are considered to be oncogenes or tumor suppressor genes (7-9). Previous studies have suggested that miRNAs are primarily expressed in EC tissue and plasma. For example, miR-145, miR-133a and miR-133b were demonstrated to function as tumor suppressors by targeting *FSCN1* in esophageal squamous cell carcinoma (ESCC) (10). miR-375 may inhibit tumor growth and metastasis in ESCC by repressing insulin-like growth factor 1 receptor (11). miR-92a has been revealed to promote tumor metastasis of ESCC via epithelial cadherin (12). In addition, miR-21, miR-184 and miR-221 in the plasma were demonstrated to be correlated with the recurrence of ESCC, and serve as oncogenes in ESCC (13). However, miRNA expression in the saliva of patients with EC remains largely unknown.

Due to the extensive blood supply to the salivary glands, saliva is a promising bodily fluid for use in the early detection of diseases including cancer, infectious and cardiovascular diseases (14,15). Previous studies have also suggested that saliva molecules may be used to detect human organic and systemic diseases (16,17). In addition, Xie *et al* (18) analyzed the expression levels of miRNAs in saliva samples and revealed that miR-10b, miR-144 and miR-451 in whole saliva were significantly upregulated in patients with EC. These

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miRNAs demonstrate promise as biomarkers for the detection of EC due to the convenience and noninvasive nature of saliva collection (18). However, the target genes of these miRNAs were not mentioned and the precise functions of these miRNAs remain to be fully understood. Using the same data as Xie *et al* (18), the present study aimed to identify the validated target genes of the miRNAs that were differentially expressed between whole saliva samples from patients with EC and healthy controls, and to analyze the potential functions of the targets by gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses.

Materials and methods

Microarray data and data preprocessing. The miRNA expression profile (GSE41268) was extracted from the Gene Expression Omnibus (GEO) database (19) (<http://www.ncbi.nlm.nih.gov/geo/>) based on the Agilent-021827 Human miRNA Microarray platform (V3; miRBase release 12.0 miRNA ID version; Agilent Technologies, Inc., Santa Clara, CA, USA). This dataset was deposited by Xie *et al* (18). The microarray contained 10 chips of whole saliva samples from 7 patients with EC and 3 healthy controls. The pathology of the patients with EC was squamous cell carcinoma: 1 patient was stage I, 1 patient was stage II, 3 patients were stage III and 2 patients were stage IV. The probe-level data in CEL files were converted into the miRNA expression profiles. For miRNAs corresponding to multiple probes that exhibited a plurality of expression values, the miRNA expression values of those probes were averaged. Log₂ transformation was performed on probes that mapped with the miRNA names labeled in the annotation platform to normalize the data from a skewed distribution to the approximate normal distribution (20).

Screening of differentially expressed miRNAs. The Linear Models for Microarray Data package (21) was used to normalize the data and identify the differentially expressed miRNAs between patients with EC and healthy controls. miRNAs with the cutoff criteria of log₂fold change (FC) >1 and P<0.05 were considered to be significantly differentially expressed. In order to explore whether the miRNAs were sample-specific, the Pheatmap package (22) in R was used to perform hierarchical clustering by comparing the value of each miRNA in 10 samples.

Target genes for differentially expressed miRNAs. miRecords (<http://cl.accuascience.com/miRecords/download.php>) is an integrated resource for animal miRNA-target interactions (23). The miRecords database currently contains 1,135 records of interactions between 644 miRNAs and 1,901 target genes in 9 animal species. In the present study, the validated target genes were extracted according to the miRecords database.

Interaction network analysis. The Search Tool for the Retrieval of Interacting Genes (STRING) database is a useful tool that provides multiple experimental and predicted data (24,25). In order to study the associations between miRNAs and their targets, the target genes were scanned by the STRING database, and protein-protein interaction (PPI) pairs with the cutoff criterion of a combined score >0.4 were selected.

Then, the regulatory network that contained these miRNAs and the PPI pairs were constructed using Cytoscape software (version 3.2.1; <http://cytoscape.org/>) (26).

Function enrichment analysis. The Database for Annotation, Visualization and Integrated Discovery (DAVID) (27) was used to identify the enriched functions in DEGs. KEGG Orthology-Based Annotation System (KOBAS) is a software to identify significantly enriched pathways using hypergeometric tests (28). To explore the functions and pathways of target genes in the development of EC, the DAVID and KOBAS were used to identify the significantly enriched GO and KEGG categories, respectively. P<0.05 was used as a cutoff criterion.

Results

Screening of differentially expressed miRNAs. With the cutoff criteria of log₂FC >1 and P<0.05, 18 differentially expressed miRNAs were identified in EC samples when compared with the healthy controls (Table I). The top 3 upregulated miRNAs were miR-144, miR-451 and miR-509-3p, while the top 3 downregulated miRNAs were miR-125b-1, miR-129 and miR-636. In addition, to investigate the differences among the 10 samples, a heat map was generated to compare their expression values. The hierarchical clustering analysis revealed a clearly distinct expression of all differentially expressed miRNAs between patients with EC and healthy controls (Fig. 1).

Screening of validated target mRNAs. According to the miRecords database, 43 validated target mRNAs corresponding to 7 upregulated miRNAs (including miR-144, miR-451 and miR-98) were acquired (Table II). No validated target gene for downregulated miRNAs was obtained.

Interaction network analysis. A total of 6 miRNAs and 27 validated target genes were mapped into the network using STRING and Cytoscape. In the network, 5 genes including Notch homolog 1 (*NOTCH1*), fibrinogen γ chain (*FGG*), fibrinogen α chain (*FGA*), and fibrinogen β chain (*FGB*) were modulated by miR-144; while 6 genes including AKT serine/threonine kinase 1 (*AKT1*), matrix metalloproteinase (*MMP*)9, and *MMP2* were modulated by miR-451. A total of 11 genes, including v-myc avian myelocytomatosis viral oncogene homolog (*c-MYC*; *MYC*), E2F transcription factor (*E2F*)1, and *E2F2* were modulated by miR-98. A total of 3 genes, including peroxisome proliferator-activated receptor α (*PPARA*) and Kruppel-like factor 4 (*KLF4*) were modulated by miR-10b. TNF receptor superfamily member 5 (*CD40*) and cyclin-dependent kinase inhibitor 1A (*CDKN1A*) were modulated by miR-485-5p and miR-363, respectively (Fig. 2).

Function and pathway enrichment analysis. To gain additional insight into the underlying functions of all target genes and the target genes in the network, GO and KEGG enrichment analyses were performed. The results demonstrated that the top 3 enriched GO terms were cell cycle (*E2F1*, *E2F2* and *MYC*), blood vessel development (*AKT1*, *NOTCH1* and *MMP2*) and vasculature (*AKT1*, *NOTCH1* and *MMP2*; Table III); and the top 3 enriched KEGG pathways were cell cycle (*E2F1*, *E2F2*

Table I. Differently expressed miRNAs in esophageal cancer tissue compared with normal tissue.

| miRNA ID | P-value | log ₂ FC | miRNA ID | P-value | log ₂ FC |
|----------------|---------|---------------------|----------------|---------|---------------------|
| hsa-miR-125b-1 | 0.0245 | -4.5437 | hsa-miR-144 | 0.0060 | 6.8525 |
| hsa-miR-129 | 0.0024 | -3.7921 | hsa-miR-451 | 0.0107 | 6.8225 |
| hsa-miR-636 | 0.0422 | -2.1583 | hsa-miR-509-3p | 0.0408 | 5.0251 |
| hsa-miR-1909 | 0.0487 | -2.1353 | hsa-miR-486-5p | 0.0105 | 4.6848 |
| hsa-miR-1274b | 0.0008 | -1.8183 | hsa-miR-10b | 0.0497 | 4.5171 |
| hsa-miR-1274a | 0.0119 | -1.5339 | hsa-miR-363 | 0.0396 | 4.2118 |
| hsa-miR-720 | 0.0453 | -1.4932 | hsa-miR-139-3p | 0.0206 | 3.7638 |
| hsa-miR-371-5p | 0.0206 | -1.3268 | hsa-miR-648 | 0.0454 | 3.7265 |
| hsa-miR-933 | 0.0048 | -1.3112 | hsa-miR-98 | 0.0434 | 3.3743 |

FC, fold change; miRNA/miR, microRNAs.

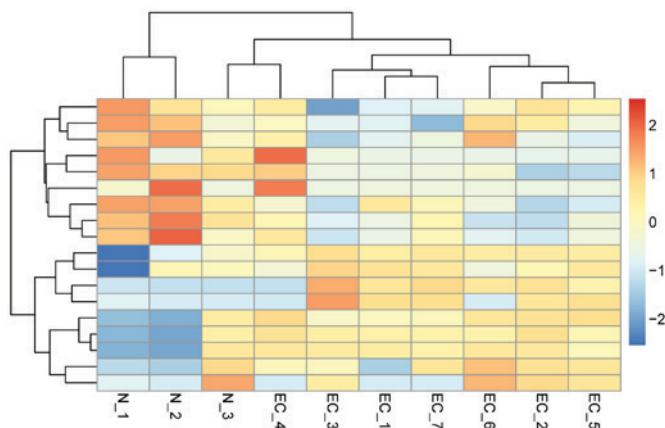


Figure 1. Hierarchical clustering heat map of 18 differentially expressed miRNAs. Each column corresponds to a single microarray, whereas each row indicates the expression profile of a single gene. Red and blue represent high and low miRNA expression, respectively. N-1, 2 and 3 indicate salivary samples in healthy controls, while EC-1, 2, 3, 4, 5, 6 and 7 indicate salivary samples in patients with esophageal cancer. miRNA, microRNA.

and *MYC*), complement and coagulation cascades (*FGG*, *FGA* and *FGB*) and epidermal growth factor receptor signaling pathway (*AKT1*, *CDKN1A* and *MYC*; Table IV).

Discussion

miRNA expression is known to be associated with various types of human cancer (29). In the present study, the salivary miRNA expression profile of EC deposited by Xie *et al* (18) in GEO database was analyzed, to screen for biomarkers for therapeutic intervention. In concordance with the study of Xie *et al* (18), the present study also demonstrated that miR-144, miR-451 and miR-10b were significantly upregulated in saliva samples from patients with EC and healthy controls. In addition, miR-144 was revealed to potentially be involved in EC progression by directly targeting 5 mRNAs, including *NOTCH1*, *FGG*, *FGA* and *FGB*. NOTCH 1 is a member of the NOTCH family. NOTCH signaling serves an important function in a variety of developmental processes by regulating cell differentiation, proliferation and apoptosis (30). NOTCH

signaling has been demonstrated to be upregulated in EC, and may be used as a therapeutic target for EC (31,32). *FGG*, *FGA* and *FGB* belong to the fibrinogen family. Fibrinogen is a glycoprotein in vertebrates that assists with the formation of blood clots and functions as a common component of hemostasis (33). Takeuchi *et al* (34) identified that plasma fibrinogen concentration was significantly associated with chemoradiotherapy responsiveness in patients with ESCC. Matsuda *et al* (35) suggested that the plasma fibrinogen level may be used as a biomarker for postoperative recurrence in advanced EC. The fibrinogen level is correlated with tumor metastasis and recurrence in patients with ESCC (34,35). Thus, by targeting *NOTCH1*, *FGG*, *FGA* and *FGB*, miR-144 may be involved in cell invasion and metastasis of EC.

miR-451 was revealed to target 6 mRNAs in the present study, including *AKT1*, *MMP9* and *MMP2*. The protein kinase B (AKT) family has been demonstrated to integrate extracellular signals with cell processes including proliferation, differentiation, migration and survival (36). Previous data have demonstrated that the phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin pathway is frequently activated in numerous types of human cancer, including colorectal cancer (37). Cancer cells are well known to exhibit a higher proliferation rate compared with normal cells, and cancer cells frequently fail to undergo apoptosis (38). Activated AKT may regulate its downstream targets to increase cell proliferation and decrease apoptosis (39). AKT activation was described as an early cellular response to carcinogen exposure, and may be a key step in environmental carcinogenesis (40). In addition, *MMP2* and *MMP9* were demonstrated to be overexpressed in ESCC and to be associated with esophageal tumorigenesis (41). Therefore, the increased expression of MMPs may promote tumor proliferation and inhibit apoptosis in the development of EC (42).

The present study revealed that miR-10b may regulate 3 mRNAs (including *PPARA* and *KLF4*). *PPARA*, also termed *NR1C1*, is a transcription factor that is a member of the peroxisome proliferator-activated receptor (PPAR) group. PPARs have been demonstrated to stimulate tumor cell proliferation and induce neoangiogenesis in different types of gastrointestinal cancer, including EC, liver and pancreatic cancer (43). *KLF4*, as a known tumor suppressor, may regulate cell proliferation,

Table II. Target genes of differentially expressed miRNAs.

| miRNA | Target | Test methods |
|----------------|-----------------|---|
| hsa-miR-144 | <i>NOTCH1</i> | Luciferase reporter assay |
| hsa-miR-144 | <i>FGA</i> | Luciferase reporter assay |
| hsa-miR-144 | <i>FGB</i> | Luciferase reporter assay |
| hsa-miR-144 | <i>FGG</i> | ELISA; flow; luciferase reporter assay |
| hsa-miR-144 | <i>PLAG1</i> | Luciferase reporter assay |
| hsa-miR-451 | <i>AKT1</i> | RT-qPCR; western blotting |
| hsa-miR-451 | <i>MMP9</i> | RT-qPCR; western blotting |
| hsa-miR-451 | <i>MMP2</i> | RT-qPCR; western blotting |
| hsa-miR-451 | <i>ABCB1</i> | RT-qPCR; luciferase reporter assay; western blotting |
| hsa-miR-451 | <i>BCL2</i> | RT-qPCR; western blotting |
| hsa-miR-451 | <i>CAB39</i> | RT-qPCR; luciferase reporter assay; western blotting; microarray |
| hsa-miR-451 | <i>MIF</i> | ELISA; luciferase reporter assay; microarray; RT-qPCR; western blotting |
| hsa-miR-451 | <i>SSSCA1</i> | RT-qPCR; western blotting |
| hsa-miR-509-3p | <i>NTRK3</i> | Luciferase reporter assay |
| hsa-miR-98 | <i>E2F1</i> | Luciferase reporter assay |
| hsa-miR-98 | <i>E2F2</i> | Northern blot |
| hsa-miR-98 | <i>MYC</i> | Luciferase reporter assay |
| hsa-miR-98 | <i>ACADM</i> | RT-qPCR |
| hsa-miR-98 | <i>AMMECR1</i> | RT-qPCR |
| hsa-miR-98 | <i>CBX5</i> | RT-qPCR |
| hsa-miR-98 | <i>CCL5</i> | ELISA |
| hsa-miR-98 | <i>CHRNB1</i> | RT-qPCR |
| hsa-miR-98 | <i>CNOT4</i> | RT-qPCR |
| hsa-miR-98 | <i>EZH2</i> | Northern blot |
| hsa-miR-98 | <i>HMGA2</i> | Northern blot; RT-qPCR |
| hsa-miR-98 | <i>HNRPDL</i> | RT-qPCR |
| hsa-miR-98 | <i>KLF13</i> | RT-qPCR |
| hsa-miR-98 | <i>MEIS1</i> | RT-qPCR |
| hsa-miR-98 | <i>NCOA3</i> | Luciferase reporter assay |
| hsa-miR-98 | <i>SERP1</i> | RT-qPCR |
| hsa-miR-98 | <i>SLC20A1</i> | RT-qPCR |
| hsa-miR-98 | <i>SOCS4</i> | Luciferase reporter assay; northern blot; RT-qPCR; western blotting |
| hsa-miR-98 | <i>THBS1</i> | RT-qPCR |
| hsa-miR-98 | <i>TUSC2</i> | Luciferase reporter assay; western blotting |
| hsa-miR-98 | <i>ZFP36L1</i> | RT-qPCR |
| hsa-miR-98 | <i>ZMPSTE24</i> | RT-qPCR |
| hsa-miR-10b | <i>PPARA</i> | Luciferase reporter assay; RT-qPCR; western blotting |
| hsa-miR-10b | <i>KLF4</i> | Luciferase reporter assay; western blotting |
| hsa-miR-10b | <i>NCOR2</i> | RT-qPCR; western blotting |
| hsa-miR-10b | <i>HOXD10</i> | Luciferase reporter assay |
| hsa-miR-10b | <i>NF1</i> | GFP reporter assay; luciferase reporter assay; microarray; RT-qPCR |
| hsa-miR-486-5p | <i>CD40</i> | Microarray; RT-qPCR |
| hsa-miR-363 | <i>CDKN1A</i> | RT-qPCR; luciferase reporter assay; western blotting |

miRNA/miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GFP, green fluorescent protein.

differentiation, development and apoptosis (44). KLF4 has been suggested to suppress EC cell migration and invasion, as a direct target of miR-10b (45). Therefore, the associated miRNAs, including miR-144 and miR-451 and miR-10b, may serve important functions in cellular processes including cell differentiation,

proliferation, metastasis and apoptosis in EC by regulating *NOTCH*, *FGG*, *FGA*, *FGB*, *AKT1*, *MMP9*, *MMP2*, *PPARA* and *KLF4*.

Compared with the aforementioned previous study, among the upregulated miRNAs, miR-98 and miR-363 were

Table III. Enriched GO terms of target genes in the microRNA-target regulatory network.

| Term | Count | P-value | Genes |
|---|-------|-----------------------|---|
| GO: 0007049~cell cycle | 10 | 4.27x10 ⁻⁴ | <i>E2F1, E2F2, MYC, AKT1, SSSCA1, TUSC2, CDKN1A, BCL2, HMGA2, THBS1</i> |
| GO: 0001568~blood vessel development | 6 | 8.23x10 ⁻⁴ | <i>AKT1, NOTCH1, MMP2, ZFP36L1, NF1, THBS1</i> |
| GO: 0001944~vasculature development | 6 | 9.18x10 ⁻⁴ | <i>AKT1, NOTCH1, MMP2, ZFP36L1, NF1, THBS1</i> |
| GO: 0022402~cell cycle process | 8 | 1.39x10 ⁻³ | <i>E2F1, MYC, AKT1, SSSCA1, CDKN1A, BCL2, HMGA2, THBS1,</i> |
| GO: 0043067~regulation of programmed cell death | 9 | 2.65x10 ⁻³ | <i>MYC, AKT1, NOTCH1, MMP9, CDKN1A, BCL2, NF1, THBS1, MIF</i> |
| GO: 0010941~regulation of cell death | 9 | 2.71x10 ⁻³ | <i>MYC, AKT1, NOTCH1, MMP9, CDKN1A, BCL2, NF1, THBS1, MIF</i> |
| GO: 0051726~regulation of cell cycle | 6 | 3.09x10 ⁻³ | <i>E2F1, E2F2, MYC, AKT1, CDKN1A, BCL2</i> |
| GO: 0022403~cell cycle phase | 6 | 7.93x10 ⁻³ | <i>E2F1, AKT1, SSSCA1, CDKN1A, BCL2, HMGA2</i> |
| GO: 0008283~cell proliferation | 6 | 9.80x10 ⁻³ | <i>E2F1, MYC, TUSC2, BCL2, CD40, MIF</i> |

GO, gene ontology.

Table IV. Enriched Kyoto Encyclopedia of Genes and Genomes pathways of target genes in the microRNA-target regulatory network.

| Term | Pathway | Count | P-value | Genes |
|----------|--------------------------------------|-------|-----------------------|--------------------------|
| hsa04110 | Cell cycle | 3 | 5.49x10 ⁻⁹ | <i>E2F1, E2F2, MYC</i> |
| hsa04610 | Complement and coagulation cascades | 3 | 1.43x10 ⁻⁴ | <i>FGG, FGA, FGB</i> |
| hsa04012 | ErbB signaling pathway | 3 | 1.52x10 ⁻³ | <i>AKT1, CDKN1A, MYC</i> |
| hsa04620 | Toll-like receptor signaling pathway | 3 | 2.15x10 ⁻³ | <i>AKT1, CD40, CCL5</i> |

ErbB, epidermal growth factor receptor.

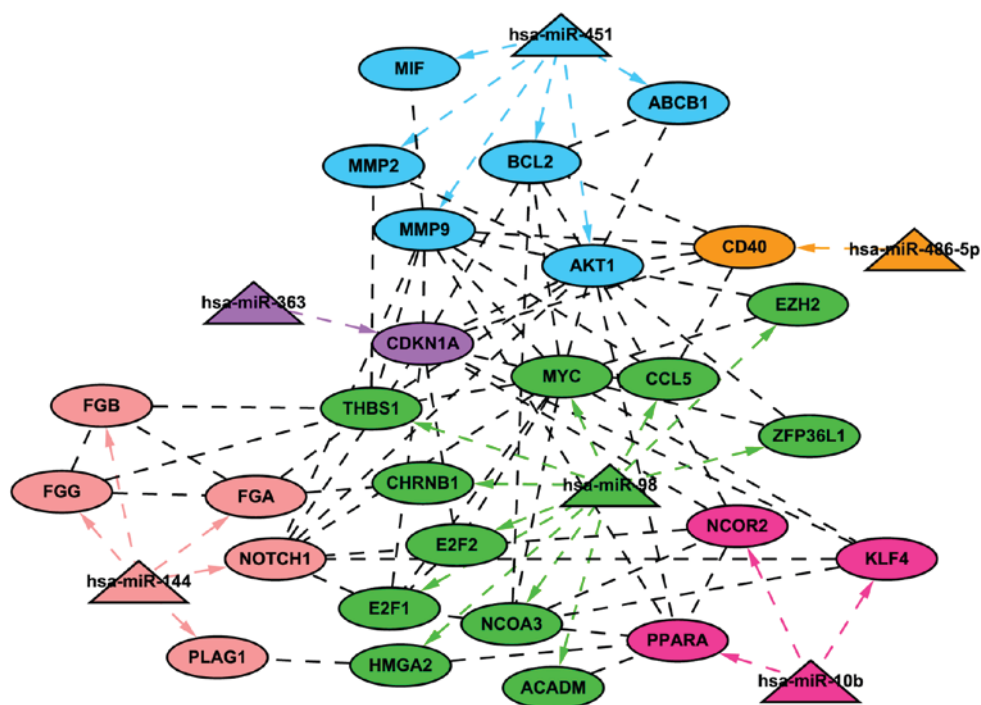


Figure 2. miRNA-target regulatory network. The triangle nodes represent miRNAs, while oval nodes represent target genes. The different miRNAs and their target genes are marked with different colors. miRNA/miR, microRNA.

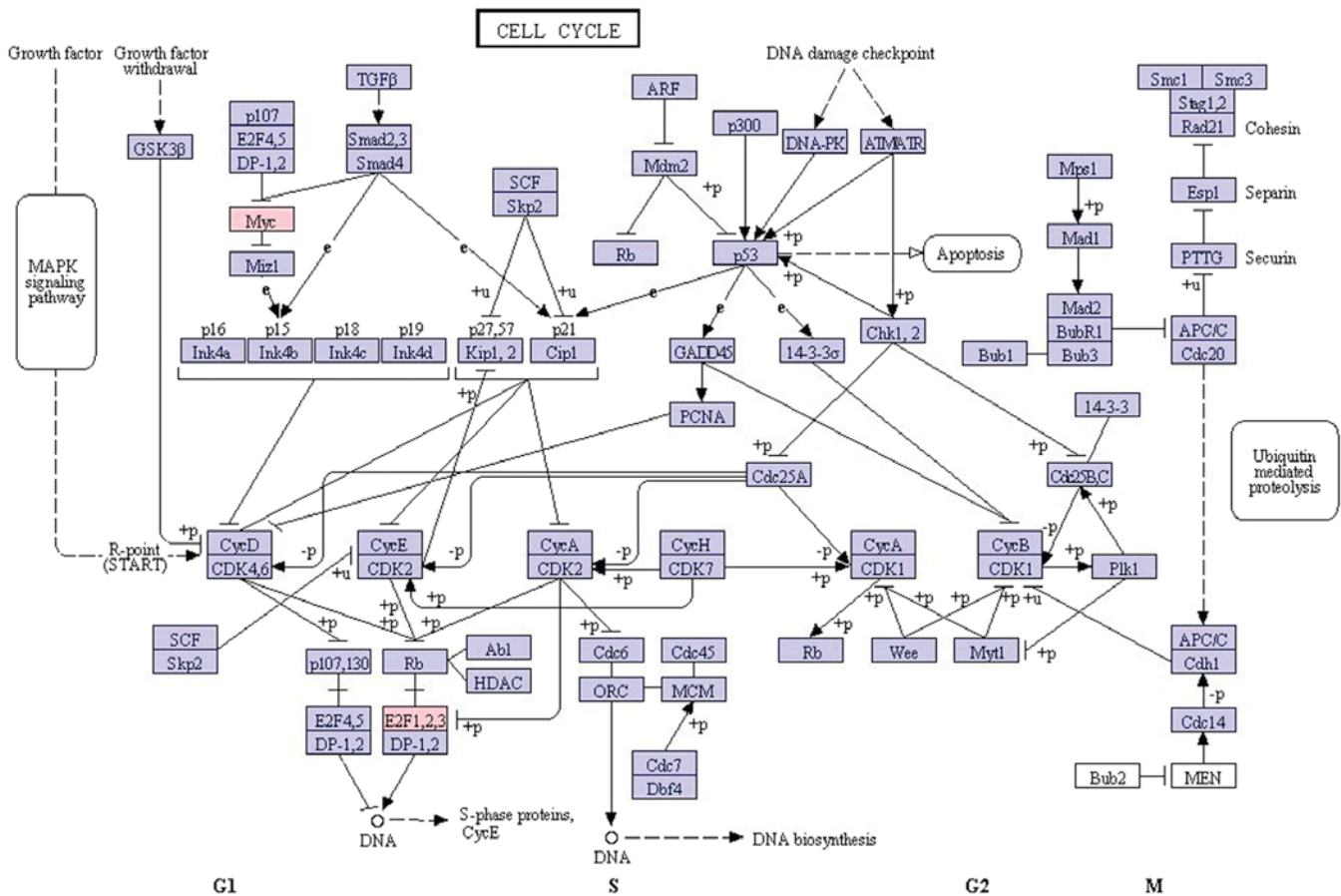


Figure 3. Detailed molecular network of pathways of the cell cycle (Kyoto Encyclopedia of Genes and Genomes ID, has04110). Red genes are the significant genes involved in the pathway. G1, G1 phase; S, S phase; G2, G2 phase; M, mitotic phase.

novel (18). miR-98 belongs to the lethal-7 (let-7) family of miRNAs (let-7f, let-7d, miR-98 and let-7g). The miRNA let-7 may regulate key differentiation processes during development (46). The expression of let-7 has been suggested to predict the response to chemotherapy in EC (47). miR-98 may target almost half of the validated target genes in the network, including MYC, E2F1, and E2F2. MYC, a well-known transcription factor, exhibited a high degree of connectivity in the regulatory network of the present study. E2F1 and E2F2 are transcription factors and members of the E2F family. MYC and E2Fs have been identified as associated with the cell cycle (48). MYC overexpression enhances and MYC downregulation inhibits cell cycle progression (48). E2F1 expression is positively associated with cell proliferation in EC (49). E2F2 has been demonstrated to negatively regulate a subset of genes involved in the processes of DNA metabolism and cell cycle control (50). In addition, E2F1 and E2F2 are expressed in a cell cycle-regulated manner, exhibiting their highest levels in the late G₁ and S phase (51). Cell cycle deregulation is a common feature of human tumors (52). The results of the present study also demonstrated that MYC, E2F1 and E2F2 were enriched in the cell cycle, which was the most significantly enriched GO function and KEGG pathway, suggesting that MYC, E2F1 and E2F2 serve critical functions in the cell cycle (Fig. 3). Therefore, by targeting MYC, E2F1 and E2F2, miR-98 may be involved in the cell cycle of EC cells, and act as novel biomarker for EC.

miR-363 was identified to be downregulated in head and neck squamous cell carcinoma (HNSCC) tissues, and to affect HNSCC cell invasion and metastasis (53). Hsu *et al* (54) indicated that miR-363 served a key function in the increment of gastric carcinogenesis via targeting c-Myc promoter binding protein 1, a negative regulator of MYC. In the present study, miR-363 was suggested to directly target CDKN1A, also termed p21, WAF1 or CIP1. The cyclin-dependent kinase inhibitor CDKN1A is a regulator of cell cycle progression at the G₁ and S phases (55). Therefore, miR-363 may be involved in EC by regulating its target gene, and may be used as a novel biomarker for EC.

In addition, in contrast to the study of Xie *et al* (18), the present study identified that miR-486-5p was significantly upregulated in EC samples (P=0.01052). This may be caused by the different preprocessing methods used in these studies. The present study also identified that miR-486-5p may modulate EC by targeting CD40. CD40, a member of the TNF-receptor superfamily, was originally identified on the surface of B cells and was suggested to regulate B cell growth and differentiation (56). The interaction of CD40 with the CD40 ligand was revealed to modulate immune cells in the lymph nodes and to promote growth of local squamous cell cancer of the head and neck by protecting tumor cells (57,58). miR-486-5p was dysregulated in EC saliva samples and was demonstrated to regulate CD40 in the present study. However, additional studies are needed to verify the involvement of miR-486-5p in EC.

In conclusion, the present study demonstrated that the biomarkers (miR-144, and miR-451 and miR-10b) screened by Xie *et al* (18) may serve an important function in the regulation of EC by targeting *NOTCH*, fibrinogen, *AKT1*, *MMPs*, *PPARA* and *KLF4*. In addition, 2 novel biomarkers, miR-98 and miR-363, were identified. These are associated with the EC and modulate a serial of cell cycle-associated genes, including *MYC*, *E2F1*, *E2F2* and *CDKN1A*. Additional experiments are needed to confirm the expression of miR-486-5p and its involvement in EC, due to the discordant conclusions between the present study and a previous study (18).

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