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***Observational Study***

**Expression of miR-1304 in patients with esophageal carcinoma and risk factors for recurrence**

Luo YG *et al*. Expression of miR-1304 in esophageal carcinoma

Yun-Gang Luo, Li-Wei Duan, Xuan Ji, Wen-Yuan Jia, Yun Liu, Mao-Lei Sun, Guo-Min Liu

**Yun-Gang Luo, Xuan Ji, Wen-Yuan Jia, Yun Liu, Mao-Lei Sun, Guo-Min Liu,** Jilin Provincial Medicine Anti-Tumor Engineering Center, the Second Hospital of Jilin University, Changchun 130041, Jilin Province, China

**Li-Wei Duan,** Department of Gastroenterology, the Second Hospital of Jilin University, Changchun 130041, Jilin Province, China

**Yun-Gang Luo, Xuan Ji, Yun Liu, Mao-Lei Sun,** Department of Stomatology, the Second Hospital of Jilin University, Changchun 130041, Jilin Province, China

**Wen-Yuan Jia, Guo-Min Liu,** Department of Orthopedics, the Second Hospital of Jilin University, Changchun 130041, Jilin Province, China

**Author contributions:** Luo YG and Duan LW designed research;Liu GM and Ji X performed research; Jia WY analyzed data; Liu Y and Sun ML wrote the paper.

**Corresponding author:** **Guo-Min Liu,** **PhD,** **Director,** Jilin Provincial Medicine Anti-Tumor Engineering Center, the Second Hospital of Jilin University, No. 218 Ziqiang Street, Changchun 130041, Jilin Province, China. l168uw@163.com

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Abstract

BACKGROUND

Esophageal carcinoma is a malignant gastrointestinal tumor with very poor prognosis. miR-1304 is a newly discovered non-coding RNA, which shows differential expression in other cancers, and its clinical value in esophageal carcinoma remains unclear.

AIM

To explore the expression of miR-1304 in patients with esophageal carcinoma and its clinical value.

METHODS

The expression of miR-1304 in patients with esophageal carcinoma was analyzed based on the data about miR in esophageal carcinoma downloaded from the Cancer Genome Atlas database. Quantitative real time polymerase chain reaction was adopted to determine the expression of miR-1304 in tissues and serum of patients. Then the clinical diagnostic value of miR-1304 and independent factors for recurrence and prognosis of esophageal carcinoma were analyzed. The potential target genes of miR-1304 were predicted, and they were analyzed based on gene ontology, Kyoto Encyclopedia of Genes, and Genomes, and protein-protein interaction.

RESULTS

The expression of miR-1304 in tissues and serum of the patients increased, and it also showed an increase according to the database. Patients with high expression of miR-1304 suffered increased rates of tumor ≥ 3 cm, low differentiation and II + III stage. miR-1304 had a diagnostic value in identifying esophageal carcinoma, tumor size, differentiation and TNM stage. Tumor size, differentiation, TNM stage, and miR-1304 were independent risk factors for recurrence of esophageal carcinoma, and they had certain predictive and diagnostic value for recurrence of it. Seventy-eight patients showed a 3-year survival rate of 38.46%, and patients with high expression of miR-1304 suffered a relatively lower survival rate. Multivariate analysis revealed that tumor size, differentiation, recurrence and miR-1304 were independent factors for prognosis of patients. Mirtarbase, miRDB, and targetscan predicted 20 target genes in total. Gene ontology enrichment analysis found 18 functions with a*P* < 0.05, and Kyoto Encyclopedia of Genes, and Genomes analysis found 11 signal pathways with a*P* < 0.05. String analysis of protein co-expression found 269 relationship pairs, of which co-expression with epidermal growth factor was the most common.

CONCLUSION

miR-1304 can be used as a potential observation index for diagnosis and recurrence of esophageal carcinoma and for survival of patients with it.

**Key words:** miR-1304; Recurrence; Prognosis; Diagnosis; Bioinformatics analysis; The Cancer Genome Atlas; Esophageal carcinoma

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**Core tip:** In recent years, the morbidity and mortality of esophageal carcinoma have increased significantly. However, there are few clinical tumor markers related to esophageal carcinoma. miRNA is a hot research direction in recent years. This study analyzed the expression of miR-1304 in patients with esophageal carcinoma, and found that miR-1304 was highly expressed in them, and was an independent factor for recurrence and prognosis of esophageal carcinoma. miR-1304 was expected to become a potential index for predicting prognosis and recurrence of esophageal carcinoma.

INTRODUCTION

Esophageal carcinoma is one of the common digestive tract cancers, with morbidity ranking eighth and mortality ranking sixth in all cancers[1]. According to 2015 cancer statistics in China, there were 477900 new cases of esophageal carcinoma and 375000 deaths for esophageal carcinoma in 2015[2]. Such a high mortality and morbidity causes a big problem for medical works. Patients with esophageal carcinoma in the early stage are prone to neglect due to insidious symptoms of esophageal carcinoma in the early stage, and patients in the intermediate stage suffer a typical symptom of dysphagia[3]. At present, although there are patients with early esophageal carcinoma in hospitals, most patients with esophageal carcinoma are already in the intermediate or late stage when admitted to a hospital[4]. The optimal treatment plan for esophageal carcinoma in clinical practice is surgical resection, but its long-term efficacy is unsatisfactory, which is mainly due to the fact as follows: Esophageal carcinoma has the characteristic that lymph nodes around it are prone to skip metastasis, which leads to recurrence of esophageal carcinoma, thus resulting in failure of surgical treatment[5,6]. If the recurrence of esophageal carcinoma can be predicted by detecting relevant indexes of patients before it, it is bound to further prevent metastasis during treatment[7]. However, there are few clinical indexes for detection and observation of esophageal carcinoma, so it is particularly important and urgent to find a potential biomarker.

Non-coding RNA is a transcript substance without any encoded protein[8]. Up to now, several types of non-coding RNA have been identified by RNA sequencing and bioinformatics methods, among which long-chain non-coding RNA and short-chain non-coding RNA (miR) have attracted much attention in various fields[9-11]. miR is a highly conserved short-chain non-coding RNA about 21-25 nt long. Studies have revealed that miR is in disorder in patients with diseases such as cardiovascular diseases and cancer[12,13]. Many studies have confirmed that miR can suppress the translation of target genes after transcription by binding to untranslated regions[14]. A study by Li *et al*[15] found that miR-377 could suppress the occurrence and development of esophageal carcinoma by mediating CD133 and vascular endothelial growth factor. Some studies showed that miR-506 could be used as a biomarker for prognosis of esophageal squamous cell carcinoma[16]. miR-1304 is a newly discovered miR. A previous study has indicated that miR-1304 is in expression imbalance in nasopharyngeal cancer patients treated with paclitaxel[17], but there is no related study on expression of miR-1304 in patients with esophageal carcinoma. This study analyzed the expression of miR in patients with esophageal carcinoma based on GCGA database and found that miR-1304 was highly expressed in them, which indicated that miR-1304 was expected to be a potential observation index for esophageal carcinoma.

Therefore, this study explored the expression of miR-1304 in patients with esophageal carcinoma and its clinical value, so as to provide reference for clinicians.

**MATERIALS AND METHODS**

***Data downloading from the Cancer Genome Atlas***

Manifest, Cart, and Metadata were downloaded by logging to <https://portal.gdc.cancer.gov/>, selecting Repository-Cases-Esophagus-The Cancer Genome Atlas (TCGA)-ESCA-File-Transcriptome Profiling-miRNA Expression Quantification-HTSeq-Counts, and adding all Files to cart. A total of 198 specimens were collected, including 185 cancer tissue specimens and 13 tumor-adjacent tissue specimens. The files were converted into a matrix to extract data about miR-1304 for analysis.

***Specimen collection from patients***

A total of 78 patients with esophageal carcinoma treated in the Second Hospital of Jilin University from March 2015 to March 2018 were enrolled as a patient group, and other 50 healthy people during the same period were enrolled as a normal group. The patient group consisted of 44 males and 34 females, with an average age of 58.4 ± 5.9 years, and the normal group consisted of 30 males and 20 females, with an average age of 59.4 ± 4.8 years. Inclusion criteria of the patients were as follows: Patients diagnosed with esophageal squamous cell carcinoma based on pathology; patients meeting the 8th edition of TNM stage criteria for esophageal carcinoma released by the American Joint Committee on Cancer in 2017[18]; patients who had not undergone cancer therapy; patients who and whose families signed an informed consent form after understanding the study, and patients with detailed clinical data and willing to cooperate for follow-up. Exclusion criteria of the patients were as follows: Patients comorbid with other tumors; patients with infection before admission, severe cardiac or cerebral function injury, or immune deficiency; patients unable to receive the treatment fully, or unwilling to cooperate for follow-up, and those with expected survival time less than 3 mo.

***Treatment plan***

All the 78 patients were treated with resection of esophageal carcinoma and lymph node dissection, and were also treated with auxiliary therapy after surgery. Twenty-six patients were treated with radiotherapy, and fifty-two patients with chemotherapy. Radiotherapy was performed mainly in the way of three-dimensional conformal radiation therapy and intensity modulated radiation therapy[19], and chemotherapy was mainly performed using fluorouracil and cisplatin specifically as follows: 500 mg/m2 fluorouracil (Hainan Choitec Pharmaceuticals Co. Ltd., Hainan, China) were used for the patients through intravenous drip for 1-5 d, and cisplatin (Guizhou Hanfang Pharmaceutical Co., Ltd., Guizhou, China) were also used for them in the same way for 1-5 d. The two drugs were used for the patients at least 2 cycles, 28 d a cycle.

***Specimen collection and detection***

Cancer tissues and tumor-adjacent tissues were sampled from the patients during surgery, and sent to a laboratory for subsequent analysis in liquid nitrogen. Peripheral fasting venous blood (5 mL) was sampled from each patient in the morning of the day before surgery and each person undergoing physical examination in the morning of the day of physical examination, allowed to stand for 30 min, and then centrifuged at 3000 rpm for 10 min to collect supernatant. The total RNA in the collected serum and tissues was extracted with a TRIzol reagent (Carlsbad Invitrogen Company, California, United States, 15596018), and the purity, concentration and integrity of the total RNA were determined using the ultraviolet spectrophotometry and agarose gel electrophoresis. Reverse transcription was performed using the TransScript® miRNA RT Enzyme Mix and 2 × TS miRNA Reaction Mix in TransScript Green miRNA Two-Step Quantitative real time polymerase chain reaction (qRT-PCR) SuperMix kit (TransGen Biotech, Beijing, China, AQ202-01) in strict accordance with the original kit instructions. The amplification system of miR-1304 consisted of 1 μL of cDNA, 0.4 μL of upstream and downstream primers, respectively, 10 μL of 2 × TransScript® Tip Green qPCR SuperMix, 0.4 μL of Passive Reference Dye (50 ×) and ddH2O (added to make up for 20 μL in total). The amplification conditions were as follows: Pre-denaturation at 94 ℃ for 30 s, denaturation at 94 ℃ for 5 s, and annealing and extension at 60 ℃ for 30 s, 40 cycles in total. Three repeated wells were set for each specimen, and the experiment was repeated three times. U6 was used as an internal reference, and 2-△△ct was used to analyze the data[20]. Experiments were carried out using 7500PCR instrument from ABI, United States. The upstream primer and downstream primer of primer sequence miR-1304 were 5'—3' and 5'—3', respectively, and those of U6 were 5'—3' and 5'—3', respectively.

***Criteria for recurrence***

If the lymph node short diameter was diagnosed to be longer than or equal to 10 mm in terms of pathology or cytology based on puncture, and in terms of imaging, it can be considered as regional lymph node recurrence.

***Follow-up***

The patients were followed up mainly through telephone as well as reexamination or review of electronic archive of them at the 3rd, 6th, 9th and 12th mo in the first year, and every 4 mo in the 2nd and 3rd years.

***Bioinformatics analysis***

Target genes were predicted on three online target gene prediction websites for miR, namely, mirtarbase, miRDB, and targetscan, respectively, and a Venn diagram was drawn. Signal pathways of potential mRNAs were analyzed based on gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) on online websites, DIVAD and KOBAS, and a network map for protein co-expression was drawn on the online website, string.

***Statistical analysis***

In this study, the SPSS 20.0 software package was used to analyze the collected data statistically, and the GraphPad 7 software package was used to draw figures required. *K-S* test was used to analyze the distribution of measurement data. The measurement data in normal distribution were expressed by mean ± SD, and those not in normal distribution were expressed by median and interquartile range P50 (P25-P75), analyzed using non-parametric test, and then expressed by Z. Comparison between groups was analyzed using independent-samples *t* test, and comparison within groups was analyzed using paired *t* test, and expressed by *t*. Enumeration data were analyzed using *χ*2 test, and expressed by *χ2*. Comparison among multiple groups was analyzed using one-way anova, and expressed by F. Post hoc pairwise comparison was analyzed using LSD-*t* test. Receiver operating characteristic (ROC) curves were drawn for diagnostic value of miR-1304 in esophageal carcinoma. Sperman was adopted to analyze the correlation between miR-1304 in tissues and serum of patients. A Kaplan-Meier curve for the 5-year survival of the patients was drawn, and the 5-year survival was analyzed using Log-rank test. Multivariate Cox regression was adopted to analyze independent risk factors for prognosis of patients and Logistic regression to analyze independent risk factors for recurrence of esophageal carcinoma in the patients. a*P* < 0.05 indicated a significant difference.

RESULTS

*Clinical baseline data analysis*

The normal group and patient group were compared in clinical baseline data, and it was found that there were no significant differences between them in age, gender, smoking history and history of alcoholism (Table 1).

***Expression of miR-1304 in patients with esophageal carcinoma and its diagnostic value***

Firstly, the analysis about the expression of miR-1304 from TCGA database revealed that cancer tissues showed significantly higher expression of miR-1304 than normal tissues (b*P* < 0.001). Then the analysis about expression of miR-1304 in serum and tissues of the patients showed that the expression of miR-1304 in tissues and serum of the patients was significantly higher than that in tumor-adjacent tissues and normal serum. Correlation analysis revealed that the expression of miR-1304 in tissues of the patients was positively correlated with that in serum of them (*R* = 0.330, b*P* < 0.001). ROC curve analysis revealed that the area under the curve (AUC) of miR-1304 for diagnosing esophageal carcinoma was 0.912, so it had a high diagnostic value (Figure 1).

***Relationship between miR-1304 and pathological data about the patients, and its diagnostic value***

The patients were divided into a high miR-1304 expression group and a low miR-1304 expression group according to the median expression, and they were compared in pathological data. It was found that the two groups had no significant difference in gender, age, and lesion location, and the high miR-1304 expression group suffered significantly higher rates of tumor size ≥ 3cm, low differentiation and II + III stage than the low miR-1304 expression group (a*P* < 0.05). Therefore, we further analyzed the correlation of miR-1304 expression with tumor size, differentiation, and TNM stage, finding that patients with tumor size ≥ 3 cm and low differentiation at II + III stage showed expression of miR-1304 different from other patients in their groups. ROC curves revealed that the AUCs of miR-1304 for identifying tumor size, differentiation and TNM stage were 0.710, 0.773, and 0.788, respectively, so it had a high diagnostic value (Figure 2, Tables 2 and 3).

***Risk factors for recurrence of esophageal carcinoma in patients***

Statistics about recurrence of esophageal carcinoma in the patients after treatment revealed that the 78 patients showed a recurrence rate of 42.31% with recurrence in 33 patients. Univariate analysis was performed to the collected clinical data about the patients, and it was found that there was no significant difference between the two groups in gender, age, smoking history, history of alcoholism, lesion location and adjuvant chemotherapy method (all *P* > 0.05), while there were differences between them in tumor size, differentiation, TNM stage, and expression of miR-1304 (all a*P* < 0.05) (Table 4). Assignment was carried out to indexes with difference (Table 5). Logistic regression analysis was performed and backward LR was selected for statistics. It was found that tumor size, differentiation, TNM stage, and expression of miR-1304 were independent risk factors for recurrence of esophageal carcinoma (Table 6). In addition, ROC curves were drawn for independent risk factors, and it was found that tumor size, differentiation, TNM stage, and miR-1304 had certain clinical value in predicating recurrence (Figure 3).

***Relationship between miR-1304 and prognosis of patients with esophageal carcinoma in survival***

The patients were followed up for 3 years. The 78 patients were all successfully followed up with no one lost to follow-up, 30 of whom survived for 3 years, showing a 3-year survival rate of 38.46%. Survival curves of the patients were drawn according to the expression of miR-1304, which revealed that patients with low expression of miR-1304 showed a significantly higher survival rate than those with high expression (b*P* < 0.001). Then univariate Cox regression for collected pathological data about the patients revealed that tumor size, differentiation, recurrence, and miR-1304 were prognostic factors for patents, and multivariate analysis of them revealed that those factors were independent factors for prognosis of them (Figure 4 and Table 7).

***Bioinformatics analysis of miR-1304***

Three online target gene prediction websites for miR, namely, mirtarbase, miRDB, and targetscan, were used to predict target genes of miR-1304, and they predicted 20 target genes in total. GO enrichment was performed to target genes predicted through pairwise websites based on DAVID, and it found 18 functions with a*P* < 0.05. KEGG analysis was performed to those target genes through KEGG, and it found 11 signal pathways with a*P* < 0.05. String analysis of protein co-expression found 269 relationship pairs, of which co-expression with epidermal growth factor (EGF) was the most common (Figure 5 and Tables 8-10).

**DISCUSSION**

Esophageal carcinoma, a malignant gastrointestinal tumor, is the 8th most common malignant tumor in the world[21]. In 2018, there were more than 500000 new and dead esophageal carcinoma patients worldwide, and the morbidity and mortality of esophageal carcinoma showed an upward trend[22]. The main reasons for this phenomenon are as follows: Firstly, the diet structure of patients is changed. Secondly, esophageal carcinoma has no obvious clinical characteristics in the early stage, so almost all patients are already in middle or late stage when admitted to a hospital, and have already missed the best treatment opportunity. Finally, there is a lack of tumor markers with high specificity for esophageal carcinoma clinically. Those factors eventually lead to the rising mortality and morbidity[23,24]. Therefore, it is urgent for medical workers to find a clinical diagnostic marker with high specificity to solve this problem.

In this study, we found for the first time that miR-1304 was highly expressed in patients with esophageal carcinoma. miR is a hot research topic in various fields in recent years. As a short-chain non-coding RNA, it can inhibit transcription and expression of downstream target genes by regulating them through 3-untranslated regions end[25]. miR-1304 is an important member of miR family. Previous studies have found that miR-1304 shows differential expression in patients with lung cancer, and can inhibit the growth of non-small cell lung cancer by regulating heme oxygenase-1[26]. However, there are few studies on esophageal carcinoma. It was the first time that miR-1304 was found to be in high expression in patients with esophageal carcinoma based on TCGA, which indicated that miR-1304 was expected to become a potential diagnostic index for esophageal carcinoma. Therefore, we conducted a clinical experiment, finding that the determined expression of miR-1304 in serum and tissues of patients was the same as that in the database; the expression of miR-1304 in tissues was positively correlated with that in serum, and AUC of the expression in the ROC curve was larger than 0.9. It further confirmed the role of miR-1304 in esophageal carcinoma, and also indicated that miR-1304 could be a potential diagnostic index for esophageal carcinoma. Moreover, we analyzed the correlation between high and low expressions of miR-1304 and pathological data about the patients, finding that patients with high expression of miR-1304 suffered significantly higher rates of tumor size ≥ 3 cm, low differentiation, and II + III stage, and the expression of miR-1304 had certain diagnostic value.

At present, the best treatment for esophageal carcinoma is radical resection, which can effectively improve the prognosis of patients to a certain extent together with postoperative radiotherapy and chemotherapy[27]. However, treatment for patients with esophageal carcinoma is prone to failure due to micrometastasis of some lesions and limitation of lymph node dissection, thus resulting in local regional recurrence or distal metastasis[28]. At present, recurrence of esophageal carcinoma in patients is mainly judged by identifying the metastasis in them through imaging after treatment. If there are indexes that can be evaluated to predict metastasis in patients before treatment, it will be able to predictably interfere with treatment of patients and avoid the recurrence of esophageal carcinoma[29]. In this study, 33 patients out of the 78 patients suffered recurrence of esophageal carcinoma, showing a recurrence rate of 42.31%, which was consistent with domestic and foreign studies[30,31]. We collected clinical data about the patients and grouped the patients, and then performed Logistic regression analysis, finding that tumor size, differentiation, TNM stage, and miR-1304 were independent risk factors for prognosis of the patients. In addition, we drew ROC curves, finding that the AUC of miR-1304 for predicting recurrence of esophageal carcinoma was larger than 0.7, and larger than that of tumor size, differentiation, and TNM stage. Many studies have confirmed that tumor size, differentiation and TNM stage are independent risk factors for recurrence in patients[32,33], but it is the first report that miR-1304 can be an independent risk factor for recurrence of esophageal carcinoma, which suggests that the expression of miR-1304 has certain predicative value in recurrence in patients.

In addition, this study followed up patients in terms of the 3-year survival of them, and it was found that the 3-year overall survival rate of them was 38.46%, which was consistent with the domestic and foreign studies[34,35]. We analyzed the 3-year survival of the patients according to the high and low expressions of miR-1304 of them, finding that patients with low expression of miR-1304 showed a significantly higher 3-year survival rate. Prognostic analysis revealed that miR-1304 could be an independent prognostic factor for 3-year survival of patients. Based on the above studies, we can confirm the clinical diagnostic and prognostic value of miR-1304 for esophageal carcinoma, but the specific mechanism of miR-1304 remains unclear. Therefore, at the end of this study, we conducted bioinformatics analysis. Bioinformatics analysis revealed that the three predication networks predicted a total of 20 target genes of miR-1304. GO and KEGG enrichment analysis based on DADID and KOBAS found 18 functions with a*P* < 0.05 and 11 signal pathways with a*P* < 0.05, respectively. What is worth paying attention to is that there have been previous reports indicating that HIF-1 and GnRH signaling pathways are involved in the occurrence and development of esophageal carcinoma[36,37], which may become our main research direction in the future. Finally, we plotted a protein-protein interaction co-expression spectrum, finding that relationship pairs with EGF gene was the most common. EGF, a member of epidermal growth factor super family, is a powerful mitogenic factor with important functions in growth, proliferation and differentiation of various cells, and early studies have pointed out that EGF is closely related to prognosis of esophageal carcinoma. Whether miR-1304 can regulate EGF and suppress the occurrence and development of esophageal carcinoma is the main direction of our future research.

This study has preliminarily proved the clinical value of miR-1304 in esophageal carcinoma, but it still has certain limitations. Firstly, it did not carry out basic experiment, and it did not clarify relevant mechanisms of miR-1304 in esophageal carcinoma. Secondly, the specimens in this study were simple, and the study only compared the difference in expression of serum miR-1304 between patients with esophageal carcinoma and normal people. Whether there is any difference in it between patients with esophageal carcinoma and patients with benign esophageal lesion needs further demonstration. Therefore, we hope to add basic experiments in future research, diversify the specimens and compare miR-1304 and tumor markers common in esophageal carcinoma to further confirm the role of miR-1304 in esophageal carcinoma and supplement our study.

In conclusion, miR-1304 can be used as a potential observation index for diagnosis and recurrence of esophageal carcinoma, and for survival of the patients.

**ARTICLE HIGHLIGHTS**

***Research background***

Esophageal carcinoma is a common digestive tract cancer, which is easy to recur after treatment. MiR-1304 is a newly discovered non-coding RNA, which shows differential expression in other cancers, but its clinical value in esophageal carcinoma remains unclear.

***Research motivation***

To find potential diagnostic and prognostic indicators of esophageal cancer recurrence.

***Research objectives***

To explore the diagnostic and prognostic value of miR-1304 in recurrence of esophageal carcinoma.

***Research methods***

Data about miR with potential difference in esophageal carcinoma were screened from the Cancer Genome Atlas. A quantitative real time polymerase chain reaction was employed to determine the expression of miR-1304 in esophageal carcinoma patients, and clinicopathological features of the patients were collected and analyzed. Based on the analysis of screened data and the expression of miR-1304 in esophageal carcinoma patients, the function of miR-1304 was evaluated. Moreover, the patients were followed up for prognosis analysis. Target genes of miR-1304 were predicted, and the function of them was analyzed.

***Research results***

The expression of miR-1304 in tissues and serum of the patients increased, and it also showed an increase according to the database. Patients with high expression of miR-1304 suffered increased rates of tumor ≥ 3 cm, low differentiation and II + III stage. MiR-1304 had diagnostic value in identifying esophageal carcinoma, tumor size, differentiation and TNM staging. Tumor size, differentiation, TNM staging, and miR-1304 were independent risk factors for recurrence of esophageal carcinoma, and they had certain predictive and diagnostic value for recurrence of it. Patients with high expression of miR-1304-3p showed a lower survival rate. Multivariate analysis revealed that tumor size, differentiation, recurrence and miR-1304 were independent factors for prognosis of patients. Furthermore, there were 18 functions with a*P* < 0.05 according to gene ontology enrichment analysis and 11 signal pathways with a*P* < 0.05 according to Kyoto Encyclopedia of Genes and Genomes. In addition, there were 269 relationship pairs according to string analysis of protein co-expression, of which the co-expression pairs with epidermal growth factor were the most.

***Research conclusion***

MiR-1304 can be used as a potential observation indicator for diagnosis and recurrence of esophageal carcinoma, and survival of the patients.

***Research perspectives***

In future research, the molecular mechanism of miR-1304 in esophageal carcinoma can be studied.

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**Footnotes**

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**Informed consent statement:** All patients in our study provided informed consent.

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**Figure Legends**



**Figure 1** **Expression of miR-1304 in the patients with esophageal carcinoma and its diagnostic value.** A: Expression of miR-1304 in cancer tissues and tumor-adjacent tissues in The Cancer Genome Atlas database; B: Expression of miR-1304 in tissues of the patients; C: Expression of miR-1304 in serum of the patients; D, E: Diagnostic value of serum miR-1304 in esophageal carcinoma. When the cutoff value was 1.154, the specificity, sensitivity and Youden index could be 94.00%, 80.77%, and 71.77%, respectively. b*P* < 0.001.



**Figure 2** **Relationship between miR-1304 and pathological data about the patients and its diagnostic value.** A: Patients with tumor size ≥ 3cm showed an increased expression of miR-1304; B: miR-1304 was highly expressed in patients with low differentiation; C: miR-1304 was highly expressed in patients at I + III stage; D: Receiver operating characteristic curves of miR-1304 for identifying tumor size, differentiation, and TNM stage. a*P* < 0.05.



**Figure 3 Diagnostic values of independent risk factors for recurrence.** The red line represented tumor size, and its area under the curve (AUC) was 0.683; the blue line represented differentiation, and its AUC was 0.642; the green line represented TNM stage, and its AUC was 0.642, and the light brown line represented miR-1304 and its AUC was 0.721.



**Figure 4 Three-year survival of the patients.** A: The 3-year overall survival rate of the patients; B: Survival of high and low miR-1304 expression groups.



**Figure 5 Bioinformatics analysis.** A: Venn diagram. The blue represents the potential target genes predicted by mirtarbase; the red represents the potential target genes predicted by miRDB, and the green represents the potential target genes predicted by targetscan; B: Distribution of the first 30 relationship pairs.

**Table 1 Comparison of baseline data**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Factors** | **Normal group (*n* = 50)** | **Patient group (*n* = 78)** | ***t* / *χ*2** | ***P* value** |
| Gender |  |  | 0.161 | 0.688 |
| Male | 30 (60.00) | 44 (56.41) |  |  |
| Female | 20 (40.00) | 34 (43.59) |  |  |
| Age (yr) | 60.4 ± 4.8 | 58.7 ± 5.4 | 0.310 | 0.757 |
| Smoking history |  |  | 0.295 | 0.587 |
| Yes | 30 (60.00) | 43 (55.13) |  |  |
| None | 20 (40.00) | 35 (44.87) |  |  |
| History of alcoholism |  |  | 0.081 | 0.776 |
| Yes | 8 (16.00) | 14 (17.95) |  |  |
| None | 42 (84.00) | 64 (82.05) |  |  |
| Location |  |  |  |  |
| The upper thoracic part |  | 19 (24.36) |  |  |
| The middle thoracic part |  | 34 (43.59) |  |  |
| The lower thoracic part |  | 25 (32.05) |  |  |
| Tumor size |  |  |  |  |
| ≥ 3 cm |  | 45 (57.69) |  |  |
| < 3cm |  | 33 (42.31) |  |  |
| Differentiation |  |  |  |  |
| Low differentiation |  | 25 (32.05) |  |  |
| Moderate + high differentiation |  | 53 (67.95) |  |  |
| TNM stage |  |  |  |  |
| Stage I |  | 27 (34.62) |  |  |
| II + III stage |  | 51 (65.38) |  |  |

**Table 2 Relationship between miR-1304 and pathological data about the patients**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Factors** | **High expression (*n* = 39)** | **Low expression (*n* = 39)** | ***χ*2** | ***P* value** |
| Gender |  |  | 0.834 | 0.361 |
| Male (*n* = 44) | 20 (51.28) | 24 (61.54) |  |  |
| Female (*n* = 34) | 19 (48.72) | 15 (38.46) |  |  |
| Age (yr) |  |  | 0.821 | 0.365 |
| ≥ 60 (*n* = 38) | 17 (43.59) | 21 (53.85) |  |  |
| < 60 (*n* = 40) | 22 (56.41) | 18 (46.15) |  |  |
| Lesion location |  |  | 1.523 | 0.467 |
| The upper thoracic part (*n* = 19) | 10 (25.64) | 9 (23.08) |  |  |
| The middle thoracic part (*n* = 34) | 19 (48.72) | 15 (38.46) |  |  |
| The lower thoracic part (*n* = 25) | 10 (25.64) | 15 (38.46) |  |  |
| Tumor size |  |  | 6.356 | 0.001 |
| ≥ 3 cm (*n* = 45) | 28 (71.79) | 17 (43.59) |  |  |
| < 3 cm (*n* = 33) | 11 (28.21) | 22 (56.41) |  |  |
| Differentiation |  |  | 4.768 | 0.029 |
| Low differentiation (*n* = 25) | 17 (43.59) | 8 (20.51) |  |  |
| Moderate + high differentiation (*n* = 53) | 22 (56.41) | 31 (79.49) |  |  |
| TNM stage |  |  | 6.854 | 0.009 |
| I stage (*n* = 27) | 8 (20.51) | 19 (48.72) |  |  |
| II + III stage (*n* = 51) | 31 (79.49) | 20 (51.28) |  |  |

**Table 3 Receiver operating characteristic parameters**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Parameters** | **AUC** | **95%CI**  | ***P* value** | **Sensitivity (%)** | **Specificity (%)** | **Cut-off** |
| Tumor size | 0.710 | 0.591-0.829 | 0.002 | 42.42 | 95.56 | < 1.158 |
| Differentiation | 0.773 | 0.665-0.881 | 0.001 | 77.36 | 68.00 | < 1.363 |
| TNM staging | 0.788 | 0.687-0.889 | < 0.001 | 47.06 | 96.30 | > 1.393 |

AUC: Area under the curve.

**Table 4 Univariate analysis**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Factors** | **Recurrence group (*n* = 33)** | **Non-recurrence group (*n* = 45)** | ***t* /*χ*2** | ***P* value** |
| Gender |  |  | 2.792 | 0.095 |
| Male (*n* = 44) | 15 (45.45) | 29 (64.44) |  |  |
| Female (*n* = 34) | 18 (54.55) | 16 (35.56) |  |  |
| Age (yr) |  |  | 0.179 | 0.672 |
| ≥ 60 (*n* = 38) | 17 (51.52) | 21 (46.67) |  |  |
| < 60 (*n* = 40) | 16 (48.48) | 24 (53.33) |  |  |
| Smoking history |  |  | 0.694 | 0.405 |
| Yes (*n*=43) | 20 (60.61) | 23 (51.11) |  |  |
| No (*n*=35) | 13 (39.39) | 22 (48.89) |  |  |
| History of alcoholism |  |  | 1.319 | 0.251 |
| Yes (*n*=14) | 4 (12.12) | 10 (22.22) |  |  |
| No (*n*=64) | 29 (87.88) | 35 (77.78) |  |  |
| Lesion location |  |  | 0.602 | 0.740 |
| The upper thoracic part (*n* = 19) | 7 (21.21) | 12 (26.67) |  |  |
| The middle thoracic part (*n* = 34) | 16 (48.49) | 18 (40.00) |  |  |
| The lower thoracic part (*n* = 25) | 10 (30.30) | 15 (33.33) |  |  |
| Tumor size |  |  | 7.648 | 0.006 |
| ≥ 3cm (*n* = 45) | 25 (75.76) | 20 (44.44) |  |  |
| < 3 cm (*n* = 33) | 8 (24.24) | 25 (55.56) |  |  |
| Differentiation |  |  | 7.093 | 0.008 |
| Low differentiation (*n* = 25) | 16 (48.48) | 9 (20.00) |  |  |
| Moderate + high differentiation (*n* = 53) | 17 (51.52) | 36 (80.00) |  |  |
| TNM staging |  |  | 6.825 | 0.009 |
| I stage (*n* = 27) | 6 (18.18) | 21 (46.67) |  |  |
| II + III stage (*n* = 51) | 27 (81.82) | 24 (53.33) |  |  |
| Adjuvant chemotherapy methods |  |  | 0.946 | 0.331 |
| Chemotherapy (*n* = 52) | 24 (72.73) | 28 (62.22) |  |  |
| Radiotherapy (*n* = 26) | 9 (27.27) | 17 (37.78) |  |  |
| Expression of miR-1304 |  |  | 8.877 | 0.003 |
| High expression (*n* = 39) | 23 (69.70) | 16 (35.56) |  |  |
| Low expression (*n* = 39) | 10 (30.30) | 29 (64.44) |  |  |

**Table 5 Assignment**

|  |  |
| --- | --- |
| **Factors** | **Assignment** |
| Tumor size | ≥ 3 cm = 1，< 3 cm = 0 |
| Differentiation | Low differentiation = 1, Moderate + high differentiation = 0 |
| TNM stage | I stage = 1, II + III stage = 0 |
| Expression of miR-1304 | High differentiation = 1, low differentiation = 0 |
| Recurrence | Recurrence = 1, non-recurrence = 0 |

**Table 6 Logistic regression analysis**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Factors** | **β** | **SD** | ***χ*2** | ***P* value** | **Exp (HR)** | **HR 95%CI** |
| **Up** | **Down** |
| Tumor size | 1.861  | 0.646  | 8.297  | 0.004  | 6.429  | 1.812  | 22.803  |
| Differentiation | 1.811  | 0.665  | 7.414  | 0.006  | 6.114  | 1.661  | 22.506  |
| TNM stage | -1.801  | 0.675  | 7.118  | 0.008  | 0.165  | 0.044  | 0.620  |
| Expression of miR-1304 | 1.838  | 0.627  | 8.586  | 0.003  | 6.286  | 1.838  | 21.501  |

CI: Credibility interval.

**Table 7 Cox regression analysis**

|  |  |  |
| --- | --- | --- |
| **Factors** | **3-yr univariate Cox** | **3-Yr multivariate Cox** |
| ***P* value** | **H (95%CI)** | ***P* value** | **HR (95%CI)** |
| Gender (male *vs* female) | 0.803 | 1.075 (0.608-1.903) |  |  |
| Age (≥ 60 yr *vs* < 65 yr) | 0.534 | 0.835 (0.473-1.474) |  |  |
| Lesion location (upper *vs* middle *vs* lower) | 0.427 | 1.166 (0.798-1.705) |  |  |
| Tumor size (≥ 3 cm *vs* < 3 cm) | 0.000 | 3.473 (1.827-6.601) | 0.010 | 2.402 (1.237-4.665) |
| Differentiation (low differentiation *vs* moderate + high differentiation) | 0.000 | 3.09 (1.724-5.539) | 0.015 | 2.153 (1.159-4.002) |
| TNM stage (I stage *vs* II + III stage) | 0.060 | 0.543 (0.287-1.027) |  |  |
| Adjuvant therapy plan (chemotherapy *vs* radiotherapy) | 0.939 | 1.025 (0.549-1.911) |  |  |
| Recurrence (recurred *vs* not recurred) | 0.002 | 2.561 (1.408-4.657) | 0.034 | 1.949 (1.05-3.619) |
| miR-1304 (high expression *vs* low expression) | 0.001 | 2.614 (1.448-4.717) | 0.036 | 1.93 (1.044-3.565) |

**Table 8 Target genes of miR-1304**

|  |  |
| --- | --- |
| **Gene** | **Target gene** |
| *miR-1304* | *LYPD3, MYC, LMNB1, KLHL15, ZNF99, SPRYD4, CCNT2, PLEKHF2, DSEL, ADIPOR2, CBX5, DDX3X, SMAD5, DARS, PCGF3, FAM83H, RRAS, PDE3A, CALM2,* and *TMBIM6* |

**Table 9 Gene ontology enrichment analysis**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **ID** | **Term** | **Gene** | **Count** | ***P* value** |
| GO: 0043392 | Negative regulation of DNA binding | *WFIKKN2, SP100, HAND2, HMOX1, HMGA2* | 5 | 0.001  |
| GO: 0051091 | Positive regulation of sequence-specific DNA binding transcription factor activity | *IL6, SP100, HIPK2, PYCARD, HMGA2, TRIM22, TCF3, ATF2* | 8 | 0.001  |
| GO: 0000122 | Negative regulation of transcription from RNA polymerase II promoter | *ATF7IP, SATB1, BACH2, SP100, GABPA, DICER1, YBX3, KLF16, ZEB2, HMGA2, ZNF345, ATF2, SUV39H2, VDR, OLIG3, MLX, PRMT6, HIPK2, ZNF431, TCF3, SMARCA2, SUDS3* | 22 | 0.002  |
| GO: 0006351 | Transcription, DNA-templated | *XRCC5, ZNF555, IRX5, CCNT1, YBX3, BBX, TTLL5, ZNF250, ZEB2, ZNF652, ZNF184, VDR, OLIG3, PRMT6, ZNF426, KDM3B, BAZ2A, TCF3, MAP2K6, ATF7IP, SATB1, SP100, ZNF620, ZNF92, KLF16, SCAI, TRIM22, ATMIN, ZNF585B, FAM208A, SUV39H2, ZBTB25, EYA4, ZNF439, MLX, PARP14, HIPK2, ZNF431, ZNF432, SMARCA2, FOXD4L6, SUDS3, ZNF573, ZBTB8A* | 44 | 0.002  |
| GO: 0006355 | Regulation of transcription, DNA-templated | *ZNF555, IRX5, YBX3, BBX, ZNF250, ZNF652, ATF2, VDR, ZNF184, ZNF426, KDM3B, BAZ2A, INSR, TCF3, MAP2K6, SATB1, ZNF620, ZNF92, SCAI, AFF1, HMGA2, TRIM22, ZNF585B, FAM208A, ZBTB25, EYA4, TULP4, ZNF439, PARP14, CDKN2AIP, MLX, ZNF431, ZNF432, SMARCA2, ZBTB8A, ZNF573* | 36 | 0.003  |
| GO: 0005138 | Interleukin-6 receptor binding | *IL6, PYCARD, ADAM17* | 3 | 0.004  |
| GO: 0015031 | Protein transport | *SLC7A6OS, RAB9A, RAB3D, KIF17, DUOXA1, RAB39A, VPS52, PKDCC, HOOK3, GOPC, SNX30, SNX20, EXOC6B* | 13 | 0.011  |
| GO: 0050892 | Intestinal absorption | *F11R, VDR, KCNQ1* | 3 | 0.016  |
| GO: 0042147 | Retrograde transport, endosome to Golgi | *SPAG9, RAB9A, STX16, VPS52, VPS26A* | 5 | 0.017  |
| GO: 0032259 | Methylation | *METTL8, TRMT10B, SETD9, CARNMT1, NNMT* | 5 | 0.020  |
| GO: 0032290 | Peripheral nervous system myelin formation | *NCMAP, DICER1* | 2 | 0.028  |
| GO: 0005509 | Calcium ion binding | *PCDH11Y, PCDH11X, RPH3AL, DAG1, STIM1, MMP17, FSTL4, TTN, IQGAP1, CALU, ITPR2, PLSCR1, CALML4, TPT1, SYTL2, EGF, CALM2, CACNA1B* | 18 | 0.029  |
| GO: 0008544 | Epidermis development | *SATB1, STS, ELF3, INSR, SCEL* | 5 | 0.033  |
| GO: 0003677 | DNA binding | *XRCC5, ZNF555, AGFG1, KIAA1958, CCNT1, YBX3, BBX, ZNF250, ZEB2, ZNF345, ZNF652, ZNF184, VDR, OLIG3, HIST1H4C, ZNF426, BAZ2A, TCF3, SATB1, SP100, ZNF620, CHTF8, GABPA, HMGA2, ZBTB25, PLSCR1, ZNF439, RFC2, MLX, HIPK2, RAD18, ZNF432, ZNF573, ZBTB8A* | 34 | 0.035  |
| GO: 0046872 | Metal ion binding | *ZNF555, MGAT5B, AGFG1, DICER1, ZNF250, ZEB2, ZNF345, ZNF652, ATF2, ZFC3H1, ZNF184, CLEC17A, FGG, HMOX1, ZC3H12B, ZNF426, KDM3B, STS, KCND3, NRXN3, ZNF620, ZNF92, KLF16, RPH3AL, ATMIN, ZNF585B, ZBTB25, EYA4, ZNF439, GNAQ, ADAM17, ZNF431, YME1L1, ZNF432, ADAM12, ZNHIT6, CACNA1D, UGP2, ZBTB8A, ZNF573* | 40 | 0.042  |
| GO: 0070644 | Vitamin D response element binding | *VDR, TCF3* | 2 | 0.042  |
| GO: 0005794 | Golgi apparatus | *STS, RAB39A, FGFRL1, VPS52, PKDCC, PRKG1, SART1, CALU, HOOK3, PLSCR1, NRAS, COPB1, STX16, GOPC, CNTNAP2, CAND1, B4GALT7, TRAPPC3, SLC30A7* | 19 | 0.044  |
| GO: 0007173 | Epidermal growth factor receptor signaling pathway | *NRAS, ADAM17, EGF, IQGAP1* | 4 | 0.045  |

**Table 10 Kyoto Encyclopedia of Genes, and Genomes pathway enrichment analysis**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **ID** | **Term** | **Genes** | **Count** | ***P* value** |
| hsa04912 | GnRH signaling pathway | *NRAS, GNAQ, CACNA1D, MAP2K6, CALM2, ITPR2* | 6 | 0.007 |
| hsa04725 | Cholinergic synapse | *NRAS, GNAQ, CACNA1D, KCNQ1, ITPR2, CACNA1B* | 6 | 0.015 |
| hsa04022 | cGMP-PKG signaling pathway | *GNAQ, PRKG1, CACNA1D, INSR, CALM2, ITPR2, ATF2* | 7 | 0.017 |
| hsa04925 | Aldosterone synthesis and secretion | *GNAQ, CACNA1D, CALM2, ITPR2, ATF2* | 5 | 0.021 |
| hsa04728 | Dopaminergic synapse | *GNAQ, CACNA1D, CALM2, ITPR2, ATF2, CACNA1B* | 6 | 0.026 |
| hsa05164 | Influenza A | *IL6, AGFG1, PYCARD, DNAJC3, MAP2K6, HLA-DRA, ATF2* | 7 | 0.026 |
| hsa04540 | Gap junction | *NRAS, GNAQ, PRKG1, EGF, ITPR2* | 5 | 0.028 |
| hsa04066 | HIF-1 signaling pathway | *IL6, PFKFB3, HMOX1, EGF, INSR* | 5 | 0.037 |
| hsa04915 | Estrogen signaling pathway | *NRAS, GNAQ, CALM2, ITPR2, ATF2* | 5 | 0.040 |
| hsa04922 | Glucagon signaling pathway | *GNAQ, CALM2, G6PC2, ITPR2, ATF2* | 5 | 0.040 |
| hsa04730 | Long-term depression | *NRAS, GNAQ, PRKG1, ITPR2* | 4 | 0.043 |