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Up-regulation of miR-765 predicts a poor prognosis in patients with esophageal squamous cell carcinoma

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Abstract. – OBJECTIVE: Up-regulation of miR-765 in esophageal squamous cell carcinoma (ESCC) has been reported in the previous study. The aim of the present study was to measure the levels of miR-765 expression in ESCC and evaluate its clinical significance in ESCC patients.

PATIENTS AND METHODS: Quantitative Real-time PCR assays were performed to analyze the expression of miR-765 in human ESCC tissues and adjacent esophageal tissues. The relationships between miR-765 expression levels and the clinical factors were investigated by x^2-test. Kaplan-Meier analysis was performed to evaluate the overall survival (OS) and disease-free survival (DFS) of ESCC patients with a different expression level of miR-765. The Cox proportional hazards regression model was used to assess the independent prognostic factors.

RESULTS: The expression level of miR-765 in ESCC tissues was significantly higher than that in their corresponding normal tissues ($p < 0.01$). High miR-765 expression was significantly correlated with tumor stage ($p = 0.001$), lymph nodes metastasis ($p = 0.005$), clinical stage ($p = 0.007$). In addition, Kaplan-Meier analysis showed that patients with higher miR-765 expression had a significantly poorer OS ($p = 0.0010$) and DFS ($p < 0.0001$) than those with lower miR-765 expression. Multivariate analyses revealed that miR-765 expression served as an independent predictor for both OS ($p = 0.001$) and DFS ($p = 0.001$).

CONCLUSIONS: Our findings provided the first evidence that miR-765 may serve as an indicator for prognosis of ESCC.

Key Words:
MiR-765, Esophageal squamous cell carcinoma, Prognosis.

Introduction

Esophageal carcinoma is one of the most frequently fatal malignancies and the sixth common cause of death from cancer[1]. The major histological subtype of esophageal cancer is esophageal squamous cell carcinoma (ESCC), which is more common in men; more than 80% of cases occur in developing countries[2,3]. This disease is usually diagnosed at a locally advanced stage with serious esophageal invasion[4,5]. The prognosis of survival of ESCC patients remains very poor despite rapid advances in surgical techniques and therapies[6]. Therefore, it is necessary to find a useful biomarker of ESCC to predict prognosis and to improve therapeutic efficacy for ESCC patients.

MicroRNAs are small non-coding single-stranded RNAs of about 22 nucleotides in length which can regulate gene levels by binding to the 3'- untranslated regions (3'UTR) of target genes for translational repression or degradation[7]. Increasing findings reveal that miRNAs may be critical regulators of development and cellular homeostasis through their control of various biological processes[8,9]. MiRNAs have emerged as potential therapeutic targets for human cancers, including ESCC[10,11]. For instance, Zhu et al[12] observed that miR-27a attenuated ESCC proliferation and invasion by targeting KRAS. It is reported that miR-889 promoted ESCC cells proliferation through DAB2IP[13]. Li et al[14] showed that miR-140 upregulation inhibited ESCC cells invasion by targeting Slug. Those results suggested miRNAs as important regulators in the progression of ESCC, which may be used for diagnosis and prognostic prediction of ESCC patients. In addition, several miRNAs, such as miR-150[15], miR-375[16] and miR-455-3[17], have been identified to be associated with prognosis of ESCC patients.

MiR-765, located in 1q23.1, has been involved in development and progression of several diseases, including tumors[18,19]. However, research reports about function and molecular mechanism

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of miR-765 in tumors are few. Previously, Zang et al[20], using MicroRNA array, identified dysregulation of miRNAs in ESCC tissues compared to adjacent non-tumor tissues. In their findings, miR-765 was found to be highly expressed in ESCC tissues. However, the effect of miR-765 in ESCC progression has not been reported. In this study, we first determined whether miR-765 was associated with prognosis of ESCC patients.

Patients and Methods

Patients and Specimens

209 pairs of ESCC tissue and the matched normal esophageal epithelial tissue samples were obtained from patients with ESCC who underwent surgical resection between July 2011 and June 2013 at the Affiliated Hospital of Jining University. All patients were pathologically confirmed to have ESCC. The patients were selected for our research only if a follow-up was obtained and clinical data were available. None of the patients received radiotherapy or chemotherapy before surgery. The tumors were classified according to World Health Organization classification. The ESCC patients were aged from 22 to 74 years with a median of 47 years. Clinical information of ESCC patients was collected and summarized in Table I. The study was approved by the Ethical Committee of The Affiliated Hospital of Jining University, and informed consent was obtained from all patients.

Quantitative Real-Time PCR

Total RNA was extracted from ESSC tissues and matched normal tissues using TRIzol® (TaKaRa, Dalian, China), according to the manufacturer's instructions. The RNA samples were then reverse transcribed into cDNA using an All-in-One™ miRNA Q-PCR Detection Kit (Ambion, Austin, TX, USA). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Qiagen, Haidian, Beijing, China) on an ABI 7300HT Real-time PCR system (Applied Qiagen, Haidian, Beijing, China). PCR was performed under the following conditions: 94°C for 4 min followed by 40 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min. GAPDH was used as an internal control for normalization. The primers for miR-765 quantification were purchased from Olivia Technology (Haidian, Beijing, China), and the sequences are shown in Table II. The comparative $2^{-\Delta\Delta Ct}$ method was used for relative quantification and statistical analysis.

Statistical Analysis

All the statistical analyses were performed with GraphPad Prism 6.0 (GraphPad, la Jolla CA, USA). The difference of miR-765 expres-

Table I. MiR-765 expression and clinicopathological features in ESCC patients.

Variable	Number	MiR-765 expression		*p*-value
		High	Low	
Gender				0.106
Male	111	60	51	
Female	96	42	56	
Age (years)				0.186
< 60	102	45	57	
≥ 60	107	57	50	
Tumor size (cm)				0.088
< 4	137	61	76	
≥ 4	72	41	31	
Histological grade				0.158
G1	127	57	70	
G2+G3	82	45	37	
Tumor stage				0.001
T1-T2	135	54	81	
T3-T4	74	48	26	
Lymph nodes metastasis				0.005
Absence	123	50	73	
Presence	86	52	34	
Clinical stage				0.007
I-II	132	55	77	
III-IV	77	47	30	

Table II. The primer sequences included in this study.

Name	primer sequences (5′–3′)
TmiR-765: forward	GCCTGGAGGAGAAGGAA
miR-765: reverse	GTGCAGGGTCCGAGGT
GAPDH: forward	TCAACGACCACTTTGTCAAGCTCAGCT
GAPDH: reverse	GGTGGTCCAGGGGTCTTACT

sion between two groups was compared by independent-samples *t*-test. The x^2-test was applied to the examination of the relationship between miR-765 expression level and clinicopathologic characteristics. Survival curves were constructed with the Kaplan-Meier method and compared by log-rank test. A Cox regression analysis was adopted to assess the prognostic factors. A *p*-value <0.05 was considered statistically significant.

Results

MiR-765 Upregulation in Human ESCC

To examine whether the miR-765 is differentially expressed in human ESCC, we analyzed the expression of miR-765 in 209 pairs of human ESCC tissues and pair-matched adjacent noncancerous tissues. We found that miR-765 expression was significantly upregulated in ESCC tissues compared to adjacent non-cancerous normal tissues ($p < 0.01$) (Figure 1). This result is consistent with Zang et al[20] findings. Our results further confirmed miR-765 upregulation in ESCC patients.

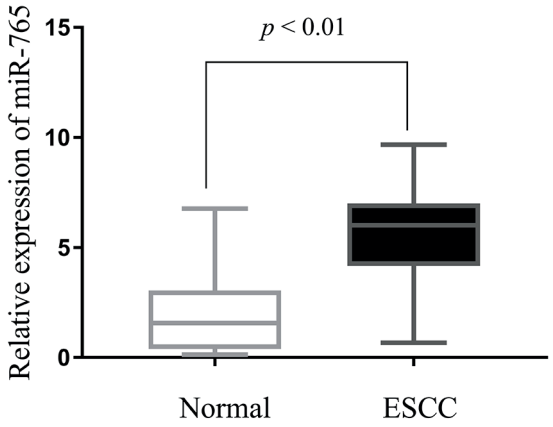

Figure 1. Comparison of miR-765 expression levels between ESCC tissues and adjacent non-tumor tissues.

Correlations Between the Expression of miR-765 and the Clinicopathological Factors in ESCC

To assess the correlation of miR-765 expression with clinicopathological features, ESSC tissue samples were classified into the low-expression group (n = 107) and the high-expression group (n = 102) according to the median expression level of all ESCC samples. As shown in Table I, we observed that ESCC patients with higher miR-765 expression levels tended to have advanced tumor stage ($p = 0.001$), positive lymph nodes metastasis ($p = 0.005$) and advanced clinical stage ($p = 0.007$) for ESCC patients. However, no significant correlations between miR-765 expression and other clinicopathological parameters were observed, such as gender, age, tumor size, histological grade ($p > 0.05$). Taken together, these data revealed that upregulation of miR-765 is associated with an aggressive ESCC phenotype.

High Expression of miR-765 is Correlated With Poor Prognosis of Patients With ESCC

To explore the association between miR-765 expression and the prognosis of ESCC patients, we used a Kaplan-Meier survival analysis and log-rank tests. The results showed that patients in high miR-765 expression group had poorer 5-year overall survival (OS) than those in low miR-765 expression group ($p = 0.0010$) (Figure 2). Similarly, we also found that patients in high miR-765 expression group had poorer 5-year disease-free survival (DFS) than those in low miR-765 expression group ($p = 0.0001$, Figure 3). Subsequently, multivariate analyses were carried out to examine the effect of miR-765 expression on ESCC prognosis using a Cox proportional hazard model. Our results indicated that miR-765 expression was an independent predictor of both OS (HR: 3.672, 95% CI: 1.442-6.692, $p = 0.001$) and DFS (HR: 3.937, 95% CI: 1.633-7.785, $p = 0.001$) (Table III) for ESCC patients.

Table III. Multivariate survival analysis of overall survival and disease-free survival in patients with ESCC.

Variables	Overall survival			Disease-free survival		
	RR	95% CI	*p*-value	RR	95% CI	*p*-value
Gender	1.432	0.783-2.127	0.153	1.642	0.683-1.889	0.114
Age	0.965	0.234-1.674	0.468	0.775	0.324-2.213	0.211
Tumor size	1.778	0.562-3.237	0.156	1.324	0.778-3.673	0.098
Histological grade	2.445	1.213-3.775	0.149	2.892	1.643-4.232	0.085
Tumor stage	4.543	1.734-7.658	0.001	5.239	1.995-8.354	0.001
Lymph nodes metastasis	3.762	1.321-5.231	0.006	4.232	1.667-6.632	0.001
Clinical stage	3.213	1.229-4.438	0.008	3.783	1.556-5.127	0.003
miR-765 expression	3.672	1.442-6.692	0.001	3.937	1.633-7.785	0.001

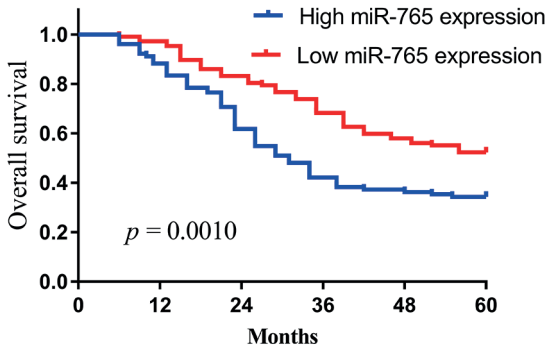

Figure 2. Kaplan-Meier survival curves of patients with ESCC based on miR-765 expression status. The patients with high miR-765 expression had a significantly worse 5-year overall survival rate than those with low miR-765 expression ($p = 0.0010$, log-rank test).

Discussion

ESCC remains the most common type of esophageal cancer in China and an important health problem in high-risk areas[21]. The overall incidence and mortality rates for esophageal cancer are higher in China[22]. Accurate prognostic biomarkers were important for distinguishing high-risk ESCC patients from other patients, so that optimal treatments can be designed[23]. In addition, individualized therapeutic strategies depend on the accurate prognostic prediction. Currently, clinicopathologic features have been used for prognosis prediction for ESCC patients. However, accuracy remains poor. Thus, it is very important to identify novel molecules that take part in ESCC progression. Dysregulation of miRNA expression has been reported to have a prognostic impact in patients with ESCC and other cancer[24]. In the present study, we aimed to determine the prognostic value of miR-765 using our samples.

Growing evidence[25] suggested that dysregulation of certain miRNAs was involved in regulation of various disease. Previous researchers[26,27] found that miR-765 expression was associated with the development of arterial stiffness and human failing hearts. In tumor, miR-765 was reported to exhibit different role according to the types of tumor. For instance, Zheng et al[28] reported that up-regulation of miR-765 suppressed the migration of oral squamous cancer cells via upregulation of E-cadherin. Hong et al[29] found that miR-765 expression was down-regulated in breast cancer and its overexpression could suppress breast cancer cells proliferation, migration and invasion by regulating EMP3. Leung et al[30] also showed that miR-765 was lowly expressed in prostate cancer and its forced expression could inhibit the proliferation and metastasis of prostate cancer cells by targeting HMGA1. However, intriguingly, up-regulation of miR-765 was observed in human hepatocellular carcinoma. *In vitro* assay

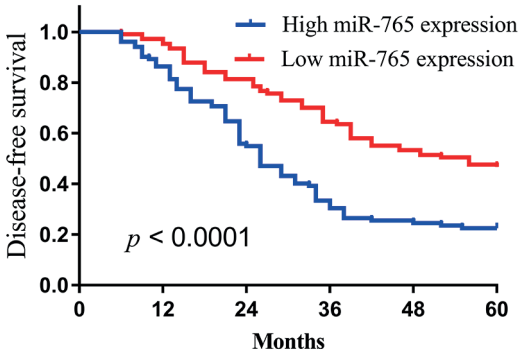

Figure 3. Kaplan-Meier survival curves of patients with ESCC based on miR-765 expression status. The patients with high miR-765 expression had a significantly worse 5-year disease-free survival rate than those with low miR-765 expression ($p < 0.0001$, log-rank test).

revealed that overexpression of miR-765 promoted hepatocellular carcinoma cells proliferation by targeting NPP4B[31]. Those results suggest the diversity of the role of miR-765 in tumor progression. Recently, it was reported that miR-765 expression levels were up-regulated in ESCC tissues by MicroRNA microarrays chip analysis[20]. However, its function and clinical significance in ESCC have not been investigated.

In this study, our results showed that the expression of miR-765 was increased in ESCC tissues compared with non-tumor tissues. Association analysis of miR-765 expression with clinical pathological features of ESCC patients indicated that high miR-765 expression was significantly associated with tumor stage, lymph nodes metastasis and clinical stage, suggesting that miR-765 had the potential to be a therapeutic target for ESCC. In addition, Kaplan-Meier analysis showed that ESCC patients with miR-765 expression tend to have shorter OS and DFS. According to multivariate analysis, miR-765 expression may be used as a novel biomarker to complement traditional biomarkers to predict the outcome of ESCC patients. However, the prognosis prediction reliability of miR-765 is limited by the small sample and large-scale and multi-center studies are needed to confirm our results.

Conclusions

We firstly report the association between high expression of miR-765 and the clinical pathological characteristics and prognosis of ESCC. MiR-765 could be used as an independent prognostic biomarker for patients with ESCC. Further functional study is needed in the future.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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