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Lab Note

Clinical significance of up-regulated miR-181a in prognosis and progression of esophageal cancer

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Human esophageal cancer is one of the most lethal malignancies with a variable regional distribution, which is the sixth leading cause of cancer mortality worldwide [1]. It has two main types, esophageal squamous cell carcinoma (ESCC) and adenocarcinoma, and ESCC is the dominant histological type in China [2]. Tumor metastasis is mostly responsible for ESCC mortality, but the molecular mechanism of metastatic dissemination remains obscure [3]. Therefore, identification of new prognostic biomarkers is urgently needed to understand the molecular mechanisms of metastasis involved in ESCC, and to predict novel targeted therapeutic strategies for this disease.

miRNAs are small non-coding RNAs that act as negative regulators of target gene expression by binding to the 3′-untranslated region (3′-UTR) of target mRNA, resulting in either mRNA degradation or translational suppression [4]. miRNAs are involved in a wide range of important biological processes including cell proliferation, differentiation, apoptosis, and stress resistance [5]. Studies have shown that miRNAs can function as novel oncogenes or tumor suppressors, so miRNAs are emerging as targets for cancer molecular therapy [4].

Human miR-181a is a member of miR-181 family which located at chromosome 1q31.3. This family consists of four miRNAs, including miR-181a, miR-181b, miR-181c, and miR-181d. All of them are highly conserved in the seed-region sequence, suggesting that miR-181 family may have redundancy in targeting genes [5]. Altered expression of miR-181a has been discovered in several kinds of cancers, and it was significantly down-regulated in oral squamous cell carcinoma [6], while significantly up-regulated in thyroid cancer [7]. Studies have shown that aberrantly high miR-181a expression can regulate cell cycle, apoptosis, invasion, proliferation, and metastasis in several kinds of cancer [8]. However, it is unclear whether the expression of miR-181a is altered in ESCC. In this study, we determined the expression level of the miR-181a in ESCC samples and cells, and investigated the clinical significance of miR-181a in human ESCC.

ESCC tumor tissues and paired normal adjacent tissues were retrospectively collected from 105 ESCC patients who underwent routine curative treatments between June 2007 and April 2009 at Hubei Cancer Hospital and Union Hospital (Wuhan, China). Informed consent was obtained from all patients. All samples were handled anonymously according to the ethical and legal standards. None of the patients received chemotherapy or radiotherapy prior to surgery. All patients included in the study were classified according to the classification system of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual (7th Edition). The clinicopathological information of the patients with ESCC was obtained from medical records and summarized in **Supplementary Table S1**.

Five human ESCC cell lines KYSE150, KYSE410, OE19, ECa109, and EC9706, and normal human esophageal epithelial cells (HEECs) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco, Los Angeles, USA). All cells were incubated at 37°C in a 5% CO_2 atmosphere.

The expression of miR-181a in human ESCC samples and cells was detected by real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from frozen tissues or cells using Trizol reagent (Invitrogen, Carlsbad, USA). The concentration and purity of RNA were confirmed and quantified by using the NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Houston, USA). Only the samples with the A_{260}/A_{280} ratio close to 2.0 were used. Subsequently, the cDNAs serving as the templates were amplified for SYBR real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, USA). Primer sequences of *miR-181a* were: forward, 5′-ACACTCC AGCTGGGAACATTCAACGCTGTCGG-3′, reverse, 5′-CT

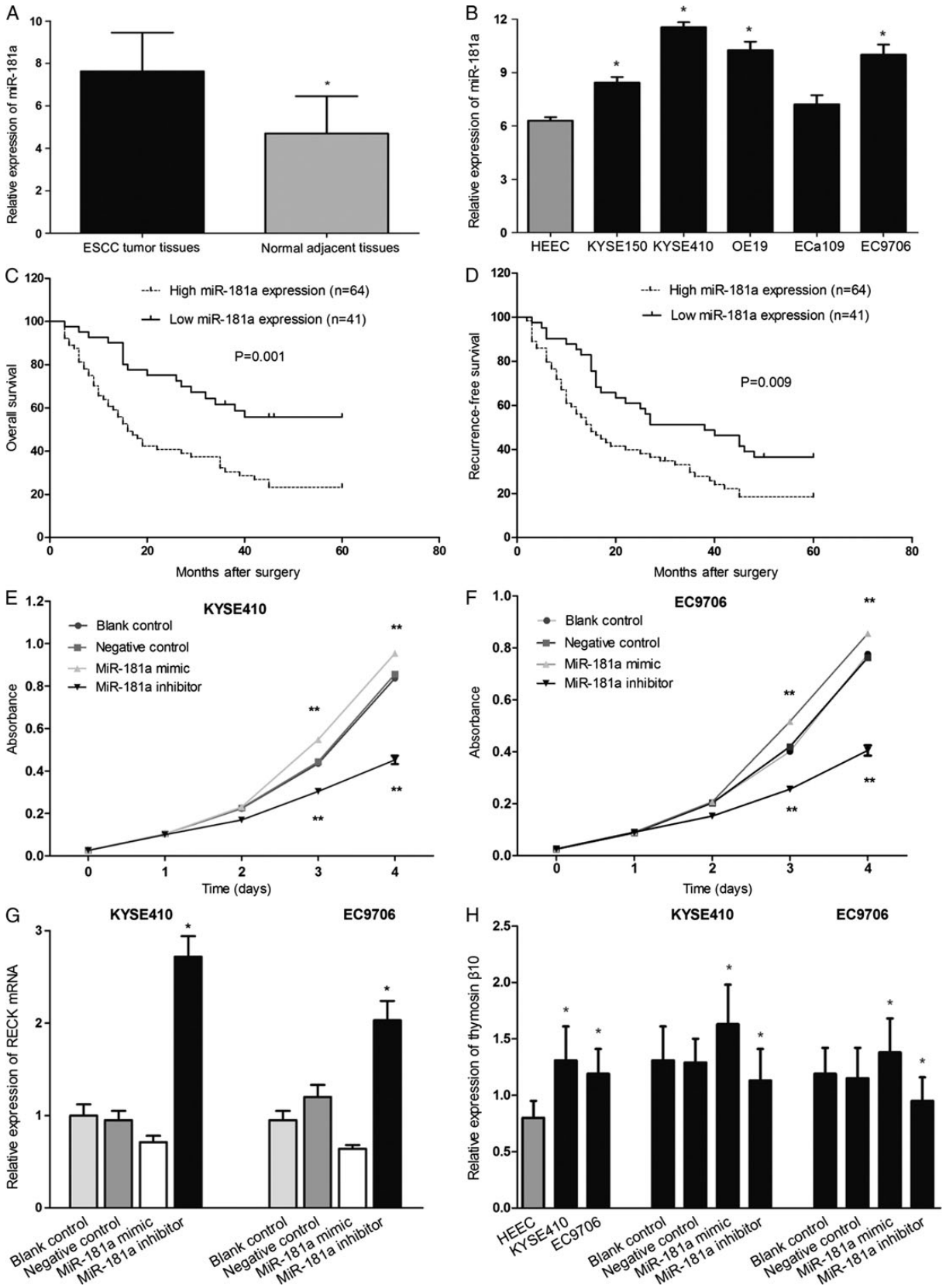

Figure 1. The effect of the up-regulation of miR-181a in ESCC patients and cell lines (A,B) Expression of miR-181a in ESCC samples and human ESCC cell lines as detected by real-time RT-PCR. (A) miR-181a expression in ESCC tissues and paired normal adjacent tissues in 105 patients. (B) miR-181a expression in five human ESCC cell lines and normal HEECs. (C,D) Correlation between miR-181a expression and survival rates in 105 patients with ESCC. (C) Overall survival. (D) Recurrence-free survival. (E,F) Effects of miR-181a on human ESCC cell proliferation. (G,H) Expression of RECK and thymosin β10 in ESCC cell lines before and after transfection with the plasmid. $*P < 0.05$, $**P < 0.01$.

CAACTGGTGTCGTGGA-3′; primer sequences of *RECK*: forward, 5′-AACCAAATGTGCCGTGAT-3′, reverse, 5′-ATGGCTTGACAGTATTCTCG-3′; primer sequences of *thymosin β10*: forward, 5′-AGCTTCGATAAGGCCAAGCTGA-3′; reverse, 5′-CACAGTGCAGCTTGTGGCTC-3′. The small nuclear RNA (RNU6B) served as the internal control for normalization. All PCR reactions were performed in triplicate.

Assessment of cell viability was performed using the colorimetric tetrazolium (MTS) assay (Promega, Beijing, China). Briefly, before transfection, cells were starved in serum-free medium for 12 h. After transfection, cells were harvested at different timepoints (24, 48, 72, and 96 h). And 10 μl of MTS reagent was added into each well, the plates were incubated for 1 h at 37°C in a humidified 5% CO_2 atmosphere, and the absorption was measured at 490 nm with the Model 680 Microplate Reader (Bio-Rad, Philadelphia, USA) for three times.

Results showed that the expression of miR-181a was significantly up-regulated in ESCC tissues compared with normal adjacent tissues (**Fig. 1A**). Moreover, the expression of miR-181a in human ESCC cells was significantly higher than those in HEECs, especially in KYSE410, OE19, and EC9706 cell lines (**Fig. 1B**). For further analysis, all patients were divided into two groups on the basis of the average miR-181a level in 105 ESCC patients. Sixty-four cases were placed in the high expression group and 41 cases in the low expression group. Then, the correlation between the expression of miR-181a and clinicopathological parameters of ESCC patients was analyzed. The results are shown in **Supplementary Table S1**. The expression of miR-181a was significantly up-regulated in patients with lymph node metastasis compared with patients without lymph node metastasis. Moreover, high level of miR-181a expression was also associated with advanced TNM stage in ESCC. But no correlation was observed between miR-181a expression and other clinical parameters such as gender, age, differentiation, location, or depth, suggesting that miR-181a may play an important role in the development or pathogenesis of ESCC. These results were consistent with the previous studies which validated miR-181a as a novel prognostic biomarker for gastric cancer and colorectal cancer [8,9].

Kaplan–Meier survival estimate showed that high miR-181a expression was correlated with shorter overall survival and shorter recurrence-free survival compared with low miR-181a expression (**Fig. 1C,D**). The difference in the prognosis of these two groups was statistically significant, and high miR-181a expression ($P = 0.008$ and 0.013, respectively), advanced TNM stage ($P = 0.045$ and 0.047, respectively), and lymph node metastasis ($P = 0.018$ and 0.041, respectively) were found to be significant prognostic factors for poor overall survival and recurrence-free survival of patients with ESCC as revealed by univariate analysis (**Supplementary Table S2**). These results indicated that miR-181a expression was a statistically significant risk factor which has impact on the overall survival and recurrence-free survival of patients with ESCC, suggesting that the expression of miR-181a could be an independent and significant prognostic factor for predicting the prognosis of this disease, which is also consistent with the previous studies in colorectal cancer and breast cancer [10,11].

Furthermore, to determine whether miR-181a could regulate cell proliferation of ESCC cells. The MTS assay showed that the inhibition of miR-181a markedly inhibited the proliferation activity of two human ESCC cell lines (**Fig. 1E,F**), suggesting that miR-181a may be classified as a 'metastasis promoter gene' in ESCC, which was in line with previous reports in gastric cancer cell lines and HCC cell lines [8,12].

TargetScan, miRBase, and Pictar Targets were exploited to analyze the target genes of miR-181a. It was found that 3′-UTR regions on the RECK gene act as favorably matched sites. To confirm that miR-181a represses RECK expression in human ESCC cells, we performed real-time RT-PCR and found that the transfection of miR-181a inhibitor led to a significant increase in RECK mRNA level compared with the negative control and blank control (**Fig. 1G**). RECK, a tumor suppressor gene, has been reported to play a key role in tumor metastasis and angiogenesis by regulating the activities of matrix metalloproteases [13].

Thymosin β10 has been suggested to play critical roles in tumorigenesis and progression of human cancers [14]. We detected the expression of thymosin β10 by real-time RT-PCR analysis and found that thymosin β10 was highly expressed in human ESCC cells, while significantly low expression of thymosin β10 was found in the cells transfected with miR-181a inhibitor (**Fig. 1H**), which is consistent with what has been observed previously in cholangiocarcinoma [14].

In summary, our results inferred that miR-181a may act as a potent prognostic marker for overall survival and recurrence-free survival of ESCC patients. And we further highlight that miR-181a serves as an oncogene and is involved in the improvement of proliferation of ESCC cells. However, further exploration including the effects of miR-181a inhibitor on the migration and invasion potency in ESCC cells, whether miR-181a directly regulates RECK expression by binding to its 3′-UTR and the *in vivo* study are needed to provide a good understanding of the function and mechanism of miR-181a in ESCC.

Supplementary Data

Supplementary data are available at *ABBS* online.

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