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# miR-26a and miR-26b inhibit esophageal squamous cancer cell proliferation through suppression of c-MYC pathway

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ABSTRACT

Dysregulation of c-Myc is one of the most common abnormalities in human malignancies, including esophageal cancer, one of the world's most lethal cancers. MicroRNA-26 family, including miR-26a and miR-26b, is transcriptionally suppressed by c-MYC. Our previous microarray data indicated a decreased-expression of miR-26 family in esophageal squamous cell carcinoma (ESCC). However, its roles in c-MYC pathway regulation and esophageal cancer tumorigenesis have yet not been elucidated. In this study, we expanded the detection of miR-26 expression in ESCC patients and found that the great majority of ESCC tissues showed an > 50% reduction, even in the early-staged tumor. Furthermore, ectopic expression of miR-26a or miR-26b induced ESCC cell growth inhibition and G1 phase arrest. MYC binding protein (MYCBP) was identified as a direct target of miR-26. MiR-26 could dramatically decrease MYCBP mRNA and protein levels, as well as the expression of luciferase carrying MYCBP 3′-untranslated region. Moreover, knock-down of MYCBP mimicked the effect of miR-26. More importantly, miR-26 overexpression could downregulate a series of c-MYC target genes as MYCBP silence did. Taken together, these results indicate that miR-26 family can suppress esophageal cancer cell proliferation by inhibition of MYCBP, subsequently downregulate c-MYC pathway. Besides, we also found that reduction of miR-26 expression in ESCC was not due to DNA methylation. Hence, our study reveals a novel feedback loop for c-MYC pathway and implicates miR-26 as a potential target for prevention and treatment of esophageal cancer.

## 1. Introduction

Esophageal cancer is one of the frequently diagnosed cancers with high mortality rates in the world, with the highest incidence rate in Eastern Asia. The two most common types are squamous cell carcinoma, which is the major type in high-risk regions, such as China and adenocarcinoma, which is more common in low-risk regions, such as the United States(Torre et al. 2015). Because esophageal cancer is often undetected until major symptoms appear, it tends to be diagnosed at an advanced stage, which leads to a low 5 year survival rate all over the world. According to the report of Chinese National Cancer Institute, esophageal cancer ranks the fourth leading cause of cancer death in men and the fifth in women(Chen et al. 2014). Thus, in order to find the biomarkers for early detection and treatment of esophageal cancer, more intensive investigations on the mechanism of cancer initiation and progression are required.

The oncogenic transcription factor c-MYC regulates the expression of thousands of genes which involve in various cell properties including proliferation, growth, apoptosis and metabolism. Dysregulated expression or function of c-Myc is one of the most common abnormalities in human malignancies (Meyer and Penn 2008; Wasylishen and Penn 2010). According to records of the catalogue of somatic mutation in cancer (COSMIC), about 16.99% of esophageal cancer patients show c-MYC gene amplification(Forbes et al. 2017). Results from different research groups indicate that gain of 8q24.21 containing the MYC gene is a frequent chromosome alteration in esophageal squamous cell

*Abbreviations:* ESCC, esophageal squamous cell carcinoma; miRNA, microRNA; MYCBP, MYC binding protein; UTR, untranslated-region; COSMIC, the catalogue of somatic mutation in cancer; siRNA, small interfering RNA; CCND1, cyclin D1; CDK4, cyclin-dependent kinase 4; MTA1, metastasis-associated protein 1; LDHA, lactate dehydrogenase A; CAD, carbamoyl phosphate synthase-aspartate transcarbamylase-dihydroorotase; ODC, ornithine decarboxylase; EIF2A, eukaryotic translation initiation factor 2A.

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carcinoma species (Lin et al. 2014; Song et al. 2014). These indicate that c-MYC dysregulation plays critical roles in the tumorigenesis of esophageal cancer.

MicroRNAs are a class of small non-coding RNAs which mainly regulate gene expression at the post-transcriptional level. Enormous studies have found that dysregulation of miRNAs play important roles in carcinogenesis though affecting expression of genes involving in cell proliferation, apoptosis and motility(Lujambio and Lowe 2012). miR-26a and miR-26b, which belongs to a miRNA family, are two of the miRNAs downregulated by c-MYC(Chang et al. 2008). Our previous microarray study indicated that expressions of miR-26a and miR-26b were decreased in ESCC tissues(Meng et al. 2015). It was also reported that miR-26 was downregulated in Burkitt lymphoma, as well as hepatocellular carcinoma and possess the ability to attenuate cell proliferation (Sander et al. 2008; Ji et al. 2009; Kota et al. 2009). However, the role of miR-26 family in esophageal cancer had yet not known. Bioinformatics analysis indicated that there is a conserved binding site for both miR-26a and miR-26b in the 3′ untranslated-region of MYC binding protein (MYCBP) mRNA. MYCBP was a c-MYC binding protein which could bind the N-terminal domain of c-MYC and enhance the *E*-box-dependent transcriptional activation (Taira et al. 1998; Sakamuro and Prendergast 1999). Involving in stress signal pathway feedback regulation is also quite common for miRNAs (Mendell and Olson 2012). Hence, we speculated whether miR-26a and miR-26b could downregulate c-MYC pathway activity through MYCBP in ESCC cells. In this study, we designed experiments to investigate the function and mechanism of miR-26 family in ESCC and also explore the potential mechanism of miR-26 reduction in esophageal cancer.

**Table 1**
Correlation of miR-26 expression and ESCC clinic-pathological parameters.

| Clinical parameters | miR-26a expression (Ca/N) | | | miR-26b expression (Ca/N) | | |
|---|---|---|---|---|---|---|
| | ≤ 0.5 fold | > 0.5 fold | P | ≤ 0.5 fold | > 0.5 fold | P |
| Age (years) | | | | | | |
| ≤ 60 years | 27 | 9 | 0.297 | 20 | 16 | 0.907 |
| > 60 years | 13 | 8 | | 12 | 9 | |
| Gender | | | | | | |
| Male | 32 | 15 | 0.706 | 27 | 20 | 0.735 |
| Female | 8 | 2 | | 5 | 5 | |
| Primary tumor | | | | | | |
| T1-T3 | 37 | 14 | 0.349 | 30 | 21 | 0.388 |
| T4 | 3 | 3 | | 2 | 4 | |
| Regional lymph nodes | | | | | | |
| N0 | 25 | 9 | 0.501 | 18 | 16 | 0.554 |
| N1-N2 | 15 | 8 | | 14 | 9 | |
| Stage | | | | | | |
| I-II | 27 | 9 | 0.279 | 20 | 16 | 0.907 |
| III | 13 | 8 | | 12 | 9 | |
| Grade | | | | | | |
| G1 | 15 | 6 | 0.822 | 10 | 11 | 0.367 |
| G2-G3 | 24 | 11 | | 21 | 14 | |
| Missing | 1 | 0 | | 1 | 0 | |
| Tumor location | | | | | | |
| lower | 6 | 3 | 1.000 | 4 | 5 | 0.485 |
| Middle, upper | 34 | 14 | | 28 | 20 | |

## 2. Materials and methods

### 2.1. Tissue specimens and cell lines

Paired ESCC and the adjacent non-tumor tissues were obtained from patients undergoing surgery at the Thoracic and Cardiac Surgery of South west Hospital in Chongqing, P.R. China from October 2006 to March 2012. All patients had definite pathology diagnosis and none received previous treatment before operation. The clinic staging of esophageal cancer patients was referred to the criteria of the American Joint Committee on Cancer (AJCC) TNM Classification of Carcinoma of the Esophagus and Esophagogastric Junction (2010). Samples were immediately divided into several pieces and putted in liquid nitrogen until use. All patients were given informed consent and the study was approved by the Ethics Committee of the Third Military medical University. Clinical characteristics of patients used in this study were shown in Table 1.

Human esophageal cancer cell line, EC109 was purchased from Cell Bank of Chinese Academy of Sciences, Shanghai, China. KYSE450 and KYSE150 cells were obtained from Cancer Institute, Chinese Academy of Medical Sciences, Beijing, China. These cells were cultured in 1640 supplemented with 10% fetal bovine serum (FBS), at 37 °C in a humidified incubator containing 5% CO2. Het-1A, a human normal esophageal epithelial cell was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in BEGM from Lonza/Clonetics Corporation.

### 2.2. RNA extraction and real-time qRT-PCR

Total RNA was extracted from cells or tissues using RNAiso Plus reagent (Takara, Dalian, China) and quantified with a NanoDrop spectrophotometer (Thermo Scientific, USA). MiRNA expressions were measured using All-in-One miRNA qRT-PCR Kit (GeneCopoeia, Guangzhou, China) according to the manufacturer's instructions. Specific miRNA qPCR primers for hsa-miR-26a and -26b were also purchased from GeneCopoeia. PCR conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 10 s. For mRNA expression detection, 500 ng of total RNA was converted to cDNA using PrimeScript RT reagent Kit With gDNA Eraser (Takara, Dalian, China) and then cDNAs were amplified using SYBR Premix Ex Taq (Takara, Dalian, China). PCR conditions were 95 °C for 30s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s. The expression of miRNAs and mRNAs were normalized against the relative expression of U6 and GAPDH, respectively. All reactions were carried out in triplicate on a bio-rad CFX connect real-time PCR system. Relative expression level was calculated using the $2^{-\Delta\Delta CT}$ method. All primers used were provided in Supplementary Table 1.

### 2.3. miRNAs, RNA interference and transfection

hsa-miR-26a and -26b mimics, small interfering RNA against human MYCBP and their negative controls (NC) were commercially synthesized by GenePharma (Shanghai, China). miRNA mimics and siRNA transfection were performed with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Fifty nanomoles per liter of RNA duplex were used for each transfection. The detailed sequence is listed in Supplementary Table 2.

### 2.4. Cell viability assay

KYSE150 cells were transfected in a 12-well plate and replated into a 96-well plate at a density of $5 \times 10^3$ per well 24 h later. Cell viability was determined by the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan) 24 h, 48 h, 72 h and 96 h after transfection. Two hours' incubation was needed before detection by absorbance at 450 nm. Five repeats were performed for each group.

### 2.5. Cell cycle analysis

KYSE150 cells were seeded in 6 well plates at the density of $3.6 \times 10^5$ per well and transfected with RNA duplexes one day later. 24 h after transfection, each group of cells were replated into a new 6-