Aberrant methylation of microRNA-193b in human Barrett's esophagus and esophageal adenocarcinoma

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Abstract. The present study aimed to investigate the expression and regulation of microRNA-193b (miR-193b) in tissues and cells from esophageal cancer and Barrett's esophagus (BE). Surgical biopsies of esophageal lesions and adjacent normal tissues were obtained, and the miR-193b expression and promoter methylation status were examined. Human BE and esophageal cancer cells were analyzed for miR-193b expression and promoter methylation, with or without treatment with the hypomethylating agent 5-azacytidine. Immunohistochemistry was performed to determine the expression and distribution of Kirsten rat sarcoma viral oncogene homolog (K-Ras), a target of miR-193b. miR-193b expression was significantly downregulated in BE and esophageal cancer tissues compared with corresponding normal tissues. The miR-193b level was significantly reduced in esophageal cancer compared with BE tissue. 5-Azacytidine treatment resulted in a significant upregulation of miR-193b in BE and esophageal cancer cells. Methylation-specific polymerase chain reaction analysis and bisulfite pyrosequencing confirmed hypermethylation of miR-193b promoter regions in esophageal cancer and BE cells, whereas hypermethylation was not observed in normal esophageal squamous epithelial cells. The methylation rate in BE and esophageal cancer tissues was significantly increased compared with the adjacent normal esophageal tissues. BE and esophageal cancer tissues exhibited increased K-Ras protein expression levels compared with the adjacent normal tissues. To the best of our knowledge, this is the first report describing DNA methylation-mediated silencing of miR-193b in esophageal cancer and BE tissues.

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Introduction

Esophageal cancer is the seventh most common type of cancer and sixth leading cause of cancer-associated mortality worldwide (1). There are 2 major histological types of esophageal cancer, squamous cell carcinoma (SCC) and adenocarcinoma. SSC is the most prevalent esophageal cancer worldwide, particularly in developing countries (2). However, the incidence of esophageal adenocarcinoma (EAC) has dramatically increased in the past 40 years (2). From 1975 to 2004, the incidence of EAC among Caucasian American males increased by >460% and over the same time period, the incidence among Caucasian American females increased by 335% (3). Barrett's esophagus (BE) is a metaplastic lesion of the distal esophagus characterized by the replacement of the normal stratified squamous epithelium by a metaplastic, columnar-lined epithelium. Untreated BE can develop dysplasia and progress to adenocarcinoma. Thus, BE is regarded as the precursor to EAC. Patients with BE have a 30- to 60-fold increased risk of developing EAC compared with the general population (4-7).

MicroRNAs (miRNAs) are a class of small, non-coding regulatory RNAs of ~22 nucleotides in length, that are important in various aspects of biology (8). Typically, miRNAs negatively regulate gene expression by binding to the 3'-untranslated region (UTR) of target mRNAs, leading to mRNA degradation and/or translational repression (9). Dysregulation of miRNAs is observed in various types of human cancer, including esophageal cancer (10,11). A large number of miRNAs have been identified to act as oncogenes or tumor suppressor genes, contributing to cancer development and progression (12). Compelling evidence suggests that miRNAs may be novel molecular biomarkers for cancer detection and targeted therapies (13).

DNA methylation is a crucial epigenetic mechanism associated with the dysregulation of miRNAs in cancer (14). Several tumor-suppressing miRNAs have been identified to be epigenetically silenced in esophageal cancer. miR-375 expression is downregulated by hypermethylation of its promoter in esophageal cancer compared with adjacent non-tumorous tissues (15). Additionally, miR-34a methylation is associated with its downregulation in esophageal cancer (16). miR-193b

functions as a tumor suppressor in multiple malignancies, including melanoma (17), prostate (18), and breast (19) cancer. Downregulation of miR-193b via DNA methylation has been previously observed in prostate cancer (18). Despite these findings, the importance of miR-193b expression and regulation in the pathogenesis of esophageal cancer remains unclear.

Therefore, the present study aimed to investigate the expression and epigenetic regulation of miR-193b in esophageal cancer and patients with BE.

Materials and methods

Tissue samples. A total of 22 patients with esophageal cancer (16 males and 6 females) and 14 with BE (4 males and 10 females) were enrolled in the current study. The patients were treated at the Second Affiliated Hospital, Chongqing Medical University (Chongqing, China). The mean age ± standard deviation of patients with esophageal cancer and BE was 65±9 and 45±10 years, respectively. Definitive histological diagnosis of esophageal cancer or BE was performed for each patient. Surgical biopsies of esophageal lesions and adjacent normal tissues (>5 cm from the tumor margin) were obtained from all the patients with informed consent. One part of the tissue samples was immediately snap-frozen in liquid nitrogen and stored at -80˚C prior to DNA or RNA extraction. The remaining samples were fixed in 4% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) overnight, embedded in paraffin, sectioned and then stained with hematoxylin and eosin (Sigma-Aldrich). The study was approved by the Ethics Committee of Chongqing Medical University (approval number, CMU-2012-024).

Cell culture and demethylation treatment. Human BE cell lines (B-T, B-T9 and B-T10), esophageal cancer (EC) cell lines (EC109, TE-10 and SEG-1), and normal esophageal squamous epithelial cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified environment at 37˚C with a 5% CO_2 atmosphere. For demethylation studies, cells in 6-well plates ($5x10^6$ cells/well) were serum-starved for 8 h and subsequently exposed to 5-azacytidine (10 μM; Sigma-Aldrich) in the presence of 10% FBS for a further 72 h. Cellular DNA and RNA were isolated and subjected to gene expression and methylation analysis, as described below.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of miR-193b expression. Total RNA was isolated from the BC and EC cells using the miRNA Isolation kit (Takara Biotechnology Co., Ltd., Dalian, China) and treaed with DNase I (Takara Biotechnology Co., Ltd.). The level of mature human miR-193b (5'-AACUGGCCC UCAAAGUCCCGCU-3') was measured using the TaqMan MicroRNA Assay (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Briefly, RT was performed with an miRNA-specific stem-loop primer (cat. no A25576; Applied Biosystems; Thermo Fisher Scientific, Inc.) and the reaction was performed at 16˚C for 30 min, followed by 42˚C for 30 min and 85˚C for 5 min. qPCR was performed using 0.5 μg of cDNA and a TaqMan MicroRNA Assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a 7900HT Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling conditions were as follows: 95˚C for 2 min, followed by 40 cycles of 95˚C for 10 s and 60˚C for 30 s. The primer sequences were as follows: miR-193b, forward 5'-CTGACTCAGCTCGTTTGTGATG-3' and reverse 5'-AGGTAAACTGGCCCTCAAAGT-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCT TCACGAATTTGCGT-3'. All reactions were performed in triplicate. The relative miR-193b levels were normalized to the level of U6 small nuclear RNA, calculated using the comparative quantification cycle ($\Delta\Delta$Cq) method (20).

DNA methylation analysis. For determination of the methylation status of the promoter region of miR-193b gene, genomic DNA was extracted from the cells using the Genomic DNA Purification kit (Takara Biotechnology Co., Ltd.) and treated with sodium bisulfite using the EZ DNA Methylation-Gold kit (Zymo Research Corporation, Irvine, CA, USA). Methylation-specific PCR (MSP) was performed with the Taq Hot Start Version (Takara Biotechnology Co., Ltd.). The primers specific for methylated and unmethylated sequences are as follows: Methylation-specific sense primer, 5'-TTT TAGGTTTGTTTGTTGGGC-3'; unmethylation-specific sense primer, 5'-GTTTTTAGGTTTGTTTGTTGGGT-3'; and antisense primer, 5'-TCAAAAAATAAATCCCCATTCAC-3'. An enzymatically methylated DNA was included as a positive control, and unmethylated lymphocyte DNA as a negative control. PCR products were separated on 2% agarose gel and stained with ethidium bromide (Takara Biotechnology Co., Ltd.).

miR-193b methylation status was also measured by bisulfite pyrosequencing, as described previously (21). In brief, genomic DNA was extracted and subjected to bisulfite conversion. PCR amplification of the region located ~2,000 bp upstream from the transcription site of miR-193b was performed using the following primers: F 5'-TTTATT TAGCTGGAGATGGGGTG-3'; and R 5'-ACCACAGCC TCCAAAAGCCTC-3'. The region analyzed by bisulfite pyrosequencing included 19 CpG sites from the miR-193b promoter. PCR products were purified and pyrosequencing was performed using a PyroMark Gold reagent kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

Immunohistochemistry. Paraffin sections (4-μm thick) were deparaffinized with xylene (Sigma-Aldrich), rehydrated in a graded ethanol series and heated for 5 min at 100˚C in the presence of 10 mM sodium citrate (pH 6.0; Sigma-Aldrich) to retrieve antigen. Following blocking with normal goat serum for 30 min, sections were incubated with a polyclonal rabbit anti-human Kirsten rat sarcoma viral oncogene homolog (K-ras) antibody (1:1,000; ab84573; Abcam, Cambridge, UK) overnight at 4˚C. Subsequently, section were washed with phosphate-buffered saline (pH 7.4; Thermo Fisher Scientific,

Inc.) and incubated with a biotin-labeled goat anti-mouse antibody (ab6788; Abcam) and horseradish peroxidase-conjugated streptavidin (Sigma-Aldrich) for 1 h at 37˚C. Diaminobenzidine (Sigma-Aldrich) was used as the peroxidase substrate to visualize the positive staining. The nuclei were counterstained using hematoxylin (Sigma-Aldrich). Slides were mounted and observed under a light microscope (Leica DM750; Leica Microsystems GmbH, Wetzlar, Germany). The primary antibody was omitted for negative controls and human colon cancer tissue with strong K-Ras expression was used as a positive control. The slides were examined independently by two pathologists who were blinded to the clinical and pathological data. Immunohistochemical results were quantified based on the extent and intensity of staining. An index value expressed in arbitrary units was calculated to grade the extent and intensity of staining.

Statistical analysis. Statistical significance was evaluated by Student's t-test or one-way analysis of variance followed by the Bonferroni method for multiple comparisons between pairs. Comparison of methylation rates in different tissues were performed using the Fisher's exact test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-193b expression is downregulated in human BE and esophageal cancer tissues. The level of miR-193b was examined in human BE tissues, esophageal cancer tissues, and their adjacent, histologically normal tissues. The results demonstrated that miR-193b expression was significantly downregulated in BE and esophageal cancer tissues compared with the corresponding normal tissues (P=0.002 and P=0.001, respectively; Fig. 1). Additionally, the expression level of miR-193b in esophageal cancer tissues was significantly reduced compared with the BE tissues (P=0.002; Fig. 1).

Upregulation of miR-193b by hypomethylating agent 5-azacytidine. miR-193b expression levels in a number of BE and esophageal cancer cell lines were analyzed by RT-qPCR following demethylation by 5-azacytidine treatment. As demonstrated in Fig. 2, demethylation treatment resulted in a significant upregulation of miR-193b in Barrett's esophagus (B-T and B-T10) and esophageal cancer (EC109, SEG-1 and TE-10) cells, with greater increases observed in the esophageal cancer cell lines. By contrast, the level of miR-193b in B-T9 Barrett's esophagus cells and normal esophageal squamous epithelial cells were not observed to be significantly different following exposure to 5-azacytidine (Fig. 2).

miR-193b promoter hypermethylation in human BE and esophageal cancer cells. miR-193b methylation in human BE and esophageal cancer cells was evaluated by MSP analysis. As presented in Fig. 3A, miR-193b was hypermethylated in the BE and esophageal cancer cell lines. However, normal esophageal squamous epithelial cells exhibited low methylation of the miR-193b promoter region. To confirm the methylation status, cells were treated with 5-azacytidine prior to methylation analysis. The results revealed weak or no methylation of miR-193b in the 5-azacytidine-treated BE and esophageal cancer cells (Fig. 3B).

miR-193b methylation in human BE and esophageal cancer tissues. Table I presents the methylation rates of miR-193b in human BE and esophageal cancer tissues. miR-193b hypermethylation was detected in 37.9±2.3% of BE tissues and in

Table I. Methylation rates of microRNA-193b in human Barrett's esophagus and esophageal cancer tissues.

Sample	n	Rate (%)	P-value
Barrett's esophagus	14	37.9±2.3	
Adjacent tissue[a]	14	28.7±2.9	0.038
Esophageal cancer	22	45.6±14.4	
Adjacent tissue[b]	22	17.5±2.9	0.005

Normal tissue adjacent to [a]Barrett's esophagus and [b]esophageal cancer. Values are presented as the mean ± standard deviation. P-value indicates adjacent tissue vs. tumor tissue.

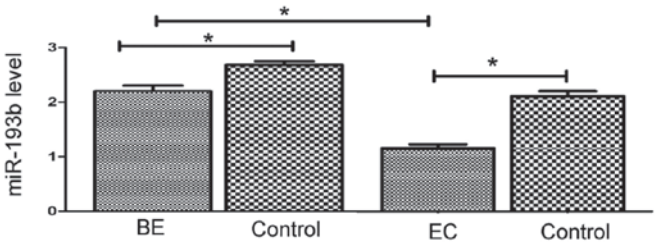

Figure 1. Reverse transcription-quantitative polymerase chain reaction analysis of miR-193b expression in human BE (n=14), EC (n=22) and their adjacent normal tissues. Data are expressed as the mean ± standard deviation of 3 independent experiments. *P<0.05, comparison indicated by brackets. miR, microRNA; BE, Barrett's esophagus; EC, esophageal cancer.

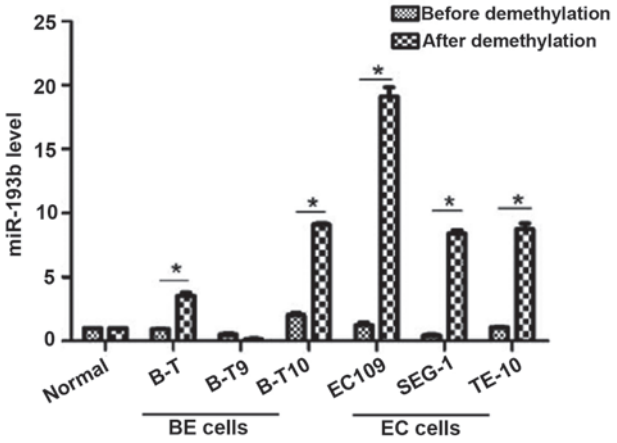

Figure 2. 5-Azacytidine treatment upregulates miR-193b in BE and EC cells. Cells were serum starved for 8 h and exposed to 5-azacytidine (1%) for a further 72 h. miR-193b expression was determined using reverse transcription-quantitative polymerase chain reaction analysis. Data are expressed as the mean ± standard deviation of 3 independent experiments. *P<0.05, comparison indicated by brackets. miR, microRNA; BE, Barrett's esophagus; EC, esophageal cancer.

Table II. Biological roles of microRNA-193b in human cancer.

Author, year	Type of cancer	MicroRNA-193b function	Target	Refs.
Chen *et al*, 2010; Kaukoniemi *et al*, 2015	Prostate cancer, melanoma	Growth suppression	cyclin D1	(17,31)
Li *et al*, 2009; Mitra *et al*, 2015	Breast cancer, ovarian cancer	Inhibition of cell invasion	uPA	(19,32)
Xu *et al*, 2010	Hepatocellular carcinoma	Inhibition of proliferation, migration and invasion	cyclin D1, ETS1	(29)
Mets *et al*, 2015	T-ALL	Tumor suppression	MYB	(33)
Li *et al*, 2014; Jin *et al*, 2015	Pancreatic cancer	Inhibition of growth and metastasis	STMN1, uPA, K-Ras	(28,34)
Lenarduzzi *et al*, 2013	HNSCC	Acceleration of tumor progression	Neurofibromin 1	(30)
Zhong *et al*, 2014	Glioma	Promotion of cell proliferation	Smad3	(35)

T-ALL, T-cell acute lymphoblastic leukemia; HNSCC, head and neck squamous cell carcinoma; uPA, urokinase-type plasminogen activator; ETS1, *v*-ets avian erythroblastosis virus E26 oncogene homolog 1; MYB, *v*-myb avian myeloblastosis viral oncogene homolog; STMN1, stathmin 1; K-Ras, Kirsten rat sarcoma viral oncogene homolog; Smad3, Smad family member 3.

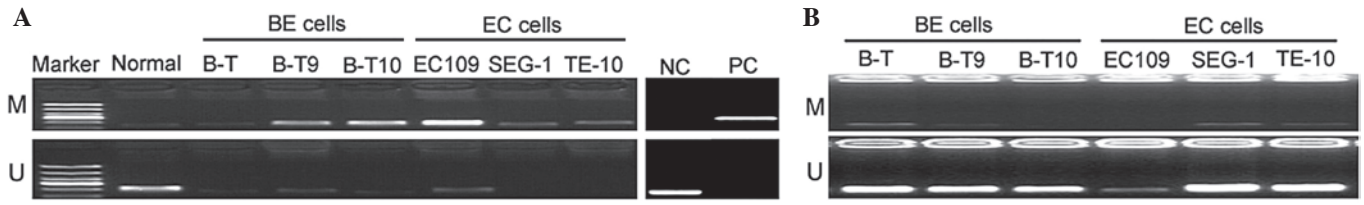

Figure 3. MSP analysis of miR-193 promoter methylation status. BE and EC cells were incubated in the (A) absence or (B) presence of 5-azacytidine for 72 h and subsequently subjected to MSP analysis. Representative gels of MSP products from three independent experiments are presented. MSP, methylation-specific polymerase chain reaction; miR, microRNA; BE, Barrett's esophagus; EC, esophageal cancer; NC, negative control; PC, positive control; M, methylated; U, unmethylated.

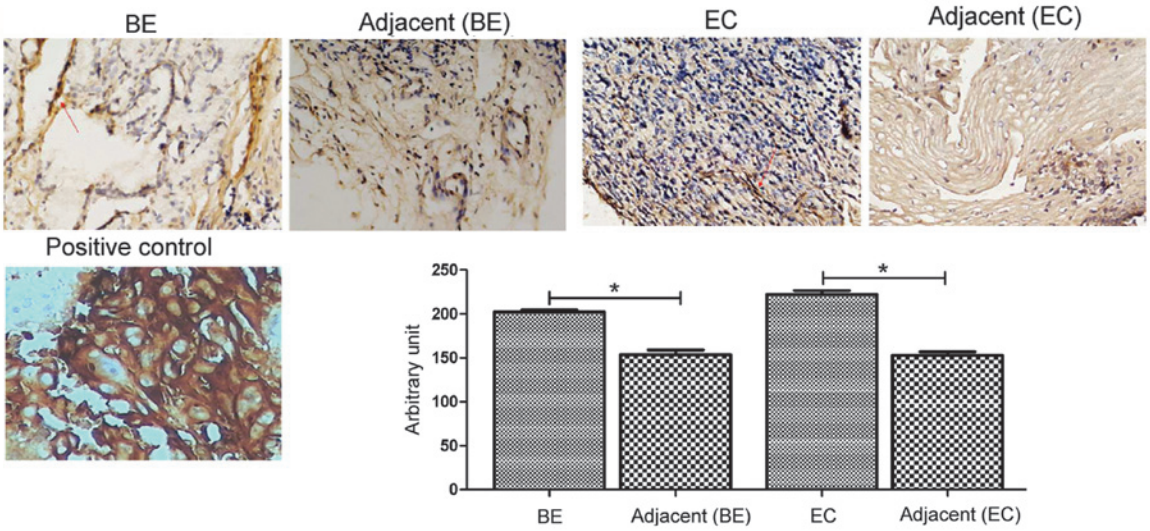

Figure 4. Immunohistochemical analysis of Kirsten rat sarcoma viral oncogene homolog expression in EC and BE tissues and their adjacent normal tissues. Representative images (x100) are presented. Bar graph represents quantification of the immunohistochemical results expressed as arbitrary units. *P<0.05, comparison indicated by brackets. BE, Barrett's esophagus; EC, esophageal cancer.

45.6±14.4% of esophageal cancer tissues. The methylation rates in BE and esophageal cancer tissues were significantly increased compared with the corresponding adjacent normal esophageal tissues (P=0.038 and P=0.005, respectively).

K-Ras protein expression in human BE and esophageal cancer tissues. K-Ras was previously identified as a direct target of miR-193b in epidermal squamous cell carcinoma (22). The current study investigated the expression of K-Ras protein in

esophageal cancer. Immunohistochemistry demonstrated that human BE and esophageal cancer tissues exhibited stronger K-Ras protein levels than the corresponding adjacent normal tissues (P=0.005 and P=0.001, respectively; Fig. 4).

Discussion

Dysregulation of miRNAs is an important mechanism of tumorigenesis (10), and various miRNAs have been identified to be aberrantly expressed in esophageal cancer (23). The data of the present study demonstrated that miR-193b was weakly expressed in esophageal cancer tissues compared with adjacent normal tissues. Additionally, the expression level of miR-193b in esophageal cancer was significantly decreased compared with the level in BE tissues. In agreement with the findings of the current study, previous investigations have reported the downregulation of miR-193b in several other malignancies (19,24,25). For example, Gao *et al* (24) reported that miR-193b acts as a tumor suppressor gene, and is downregulated in acute myeloid leukemia (AML). Using miRNA microarray technology, Wu *et al* (25) profiled the miRNA expression in endometrioid adenocarcinoma and observed that miR-193b was downregulated in adenocarcinoma tissues compared with adjacent non-tumorous endometrium. BE is a premalignant lesion that predisposes patients to EAC (4). The downregulation of miR-193b may contribute to the progression from BE to EAC.

Epigenetic modification of promoter regions, particularly DNA methylation, is frequently associated with the downregulation of genes (26). Epigenetic regulation of miRNA expression commonly occurs in cancer (14). Rauhala *et al* (18) reported that miR-193b DNA is methylated in prostate cancer cells at a CpG island ~1 kb upstream of the miRNA locus. Methylation-mediated silencing of miR-193b has also been described in dedifferentiated liposarcoma cells (27).

The current study demonstrated that miR-193b was hypermethylated in esophageal cancer tissues and cells at its promoter region. Aberrant methylation of the miR-193b promoter occurred less frequently in BE tissues. By contrast, normal esophageal squamous epithelial cells exhibited low methylation of miR-193b compared with the BE and cancer cells. To confirm the methylation status of miR-193b, the present study used the hypomethylating agent 5-azacytidine to treat esophageal cancer and BE cells, and examined the expression levels of miR-193b. Treatment with 5-azacytidine resulted in a significant increase of miR-193b expression compared with untreated cells. To the best of our knowledge, these results provide the first evidence for DNA methylation-mediated downregulation of miR-193b in BE and esophageal cancer tissues.

The reduced expression levels of miR-193b in BE and esophageal cancer tissues suggest that it negatively regulates the pathogenesis of esophageal cancer. Indeed, miR-193b has been previously demonstrated to act as a tumor suppressor gene in several types of human cancer, including melanoma (17), AML (24), pancreatic cancer (28) and hepatocellular carcinoma (29). However, miR-193b has been demonstrated to enhance tumor progression in head and neck squamous cell carcinoma (HNSCC) cells (30). Overexpression of miR-193b occurs in HNSCC cells, and knockdown of its expression has been demonstrated to result in reduced cell proliferation, migration, invasion and tumorigenesis (30). Table II summarizes the biological roles of miR-193b in human cancer. Additional direct evidence is required to confirm the exact biological functions of miR-193b in esophageal cancer.

A single miRNA can modulate hundreds of target genes (36), and several targets of miR-193b have been identified. Zhong *et al* (35) reported that miR-193b is capable of accelerating human glioma cell proliferation via targeting Smad family member 3. Neurofibromin 1 (NF1) is also a target of miR-193b, and downregulation of NF1 promotes the HNSCC aggressiveness induced by miR-193b (30). Gastaldi *et al* (22) identified K-Ras as a direct target of miR-193b. Activation of the K-Ras oncogene has been implicated in tumorigenesis (37). The data of the present study demonstrated that human BE and esophageal cancer tissues exhibited increased expression of K-Ras protein compared with adjacent normal tissues. The upregulation of K-Ras in BE and esophageal cancer tissues may be the result of the epigenetic silencing of miR-193b.

A number of limitations of the present study should be noted. The importance of miR-193b in the development and progression of EC remain unclear. Additionally, it remains to be determined whether the action of miR-193b in esophageal cancer is mediated by targeting K-Ras. No information is available on the action of miR-193b in xenograft *in vivo* models.

In conclusion, to the best of our knowledge, this is the first report investigating epigenetic silencing of miR-193b via DNA methylation in esophageal cancer and BE tissues. Downregulation of miR-193b and upregulation of K-Ras may contribute to the pathogenesis of esophageal cancer. The clinical and biological relevance of miR-193b is unclear and requires further exploration.

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