

Epigenetic alterations of the *Igf2* promoter and the effect of miR-483-5p on its target gene expression in esophageal squamous cell carcinoma

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Abstract. Esophageal squamous cell carcinoma (ESCC) is one of the most widespread malignancies in China. MicroRNAs (miRNAs/miRs) are endogenous evolutionarily-conserved small non-coding RNAs that are able to regulate ESCC formation and deterioration by negatively regulating specific target genes. In the present study, the expression levels of miR-483-5p and its associated mRNAs were measured by quantitative polymerase chain reaction (PCR) analysis, and the methylation levels of the insulin-like growth factor 2 (*Igf2*) promoter were detected via the methylation-specific PCR method in serum and tissues from patients with ESCC. The results demonstrated that the expression level of miR-483-5p was significantly upregulated in preoperative serum and cancer tissues from patients with ESCC ($P < 0.01$), and the miR-483-5p expression levels were correlated with the tumor, node, metastasis stage ($P < 0.05$) and lymph node metastasis ($P < 0.05$). In addition, the mRNA levels of miR-483-5p target genes (Rho GDP dissociation inhibitor α , activated leukocyte cell adhesion molecule, and suppressor of cytokine signaling 3) in cancer tissues were significantly decreased compared with adjacent non-cancerous tissues. These results indicated that miR-483-5p and its target genes may be involved in the developmental process of ESCC. The *Igf2* levels in cancer tissues were significantly increased compared with adjacent non-cancerous tissues ($P < 0.01$). Additionally, the methylation levels of the *Igf2* promoter region were 31.82 and 54.55% in cancer tissues and adjacent non-cancerous tissues, respectively, suggesting that low methylation of the *Igf2* gene promoter region may promote

the expression of *Igf2* and miR-483-5p; this, in turn, induces the degradation of miR-483-5p target genes, and leads to the upregulation of oncogenes and the downregulation of tumor suppressors, which promotes the development of ESCC.

Introduction

Esophageal cancer refers to upper gastrointestinal tract tumors of epithelial cellular origin. It is the 6th most common cause of cancer-associated mortality, and the eighth most common malignancy worldwide (1,2). In China, it is the 5th most common cancer and the 4th most common cause of cancer-associated mortality (3,4). Early detection of the disease is essential to improve the survival of patients with esophageal cancer.

MicroRNAs (miRNAs/miRs) are endogenous, single-stranded non-coding RNAs with 19-25 nucleotides, acting as important mediators in the regulation of gene expression, cell differentiation, the cell cycle and apoptosis (5,6). miRNA microarray profiling of human tumors has demonstrated that certain groups of miRNAs may offer opportunities in the identification of novel biomarkers and therapeutic targets (7,8). A number of miRNAs, including miR-21, miR-373 and miR-483, have been reported to be potential biomarkers of esophageal cancer. miR-483 is an intronic miRNA located within the insulin like growth factor 2 (*Igf2*) gene locus in mammalian cells (9), and has been associated with a diverse set of human pathologies, including cancer (10-12). A polymorphism at the miR-483-5p binding site in the 3'-untranslated region of the basigin gene has been demonstrated to be associated with increased susceptibility to esophageal cancer in a Chinese population (13). However, to the best of the authors' knowledge, there has been no study regarding the mechanism underlying the regulatory role of miR-483-5p in the development of esophageal cancer.

Epigenetic alterations have been a subject of research, due to their involvement in malignant transformation and tumor progression. There has been an increase in basic and applied research into the field of the epigenetic regulation of esophageal cancer development (14-16). The aim of the present study was to clarify the association between miR-483-5p expression in serum and tissues from patient with esophageal cancer, with epigenetic alterations in the *Igf2* promoter, in addition to the effect of miR-483-5p on target gene expression.

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Materials and methods

Patients. The study protocol was approved by the Ethics Committee of the Xinxiang Central Hospital (Xinxiang, China). A total of 23 patients (the age range, 25-60 years; median age, 46; 17 males and 6 females.) with esophageal squamous cell carcinoma (ESCC) from the Xinxiang Central Hospital and 50 healthy subjects were recruited to the present study between January 2014 and February 2015. All participants were genetically unrelated ethnic Han Chinese from the same geographic region (Henan, China). The diagnosis of ESCC was confirmed by histopathology in all patients. Written informed consent was obtained from all participants prior to the study. ESCC tissues and adjacent non-cancerous esophageal tissues (at least 5 cm away from the tumor) from all 23 patients were collected. A total of 3 ml peripheral blood was collected from each participant (including 50 healthy persons and 23 patients with ESCC; patient blood samples were collected prior to surgery and at 7 days post-surgery).

Tissue and serum sample processing and RNA isolation. All tissue samples were collected during surgery, immediately snap-frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Total RNA was isolated using TRIzol™ (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's instructions.

Peripheral blood was collected in tubes containing separating gel and clot activator, placed in a water bath for 20 min at 37°C, and centrifuged at 3,500 x g for 10 min at room temperature. The supernatants were transferred to Eppendorf tubes. A second centrifugation at 12,000 x g for 10 min at 4°C was performed to completely remove all cellular components. The serum was subsequently aliquoted and stored at -80°C until RNA extraction. All blood samples were processed within 3 h following collection. Total serum RNA was isolated from 100 µl serum and eluted in 300 µl RNase-free water using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. qPCR for individual miRNAs was performed on independent sets of serum or tissue using a two-step procedure. qPCR for miRNA Stem-Loop™ RT primers for miR-483-5p and miR-16-5p were synthesized by Applied Biosystems (Thermo Fisher Scientific, Inc.) (Table I). A PrimeScript™ RT reagent kit (Perfect Real Time; Takara Biotechnology Co., Ltd., Dalian, China) was used to reverse transcribe the total RNA. A SYBR Green (Takara Biotechnology Co., Ltd.) qPCR assay kit was used to detect the expression of miR-483-5p and miR-16-5p. The qPCR reaction was performed over 45 cycles (95°C, 10 sec; 60°C, 30 sec) following an initial denaturation step (95°C, 5 min) on the CFX96 system using Bio-Rad CFX Manager 2.0 Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The expression levels of miRNA were calculated and quantified using the 2^{-ΔΔCq} method (17). miR-16-5p was used as the internal control. All reactions were performed in triplicate.

Prediction of miR-483-5p target genes. miR-483-5p target genes were predicted using miRBase (www.mirbase.org),

Table I. Primer sequences.

Primer	Sequence
miR-483-5p-RT	GTCGTATCCATGGCAGGGTCCGAG GTATTCCGATGGATACGACCTCCCT
miR-483-5p-F	GCAAGACGGGAGGAAAGAAGGA
universal reverse	TGGCAGGGTCCGAGGT
GAPDH-F	GCACCGTCAAGGCTGAGAAC
GAPDH-R	TGGTGAAGACGCCAGTGA
Socs3-F	CAGGAATGTAGCAGCGATGGAA
Socs3-R	CCTGTCCAGCCCAATACCTGA
ALCAM-F	CCTTGTTGCTGGTGTCTGTCTACT
ALCAM-R	ATTACCGAGGTCTTGTTTACATGT
ARHGDI-A-F	AACCGAGAGATAGTGTCCGGC
ARHGDI-A-R	TCTTGACGCCTTCTCTGTACG
Igf2-F	CCGTGCTTCCGGACAACCTT
Igf2-R	CTGCTTCCAGGTGTCATATTGG
miR-16-RT	GTCGTATCCATGGCAGGGTCCGAGGT ATTCGCCATGGATACGACCGCCAAT
miR-16-F	GCGGTAGCAGCACGTAAATATT
Igf2-MF3	AGCGGTTTCGGTGTCGTTATC
Igf2-MR3	CGAACGCCCAACTCGATT
Igf2-UF3	GGATTGTGGGTGTTTAGTTTGGTT
Igf2-UR3	CCTTTCACACTACATCCCAAAA

F, forward; R, reverse; RT, reverse transcription, ALCAM, activated leukocyte cell adhesion molecule; ARHGDI-A, Rho GDP dissociation inhibitor α; Socs3, suppressor of cytokine signaling 3; Igf2, insulin-like growth factor 2; miR, microRNA; M, methylated; U, unmethylated.

Target Scan (www.targetscan.org), and PicTar (pictar.mdc-berlin.de).

Genomic DNA isolation and methylation analysis. Genomic DNA was extracted using an EZ DNA Methylation-Gold™ kit (Qiagen GmbH, Hilden, Germany). The methylation status of the Igf2 gene was determined using the methylation-specific PCR (MSP) method on bisulfate-treated genomic DNA. The primers specific for either unmethylated or methylated alleles are listed in Table I. As an internal control, all purified genomic DNA samples were successfully tested by PCR with a Takara EpiTaq™ HS kit (for bisulfite-treated DNA; Takara Biotechnology Co., Ltd.). Methylated and unmethylated DNAs of normal human peripheral lymphocytes were used as a positive control for the methylated and as a negative control for unmethylated genes, respectively. Samples with H₂O₂ instead of DNA were included for each PCR set. PCR products were analyzed on a 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light (DL 2000 Marker; Genstar, Beijing, China). Each MSP was repeated at least once to confirm the results.

Statistical analysis. Data were reported as mean ± standard deviation for quantitative variables. The difference in mRNA or miRNA expression levels between paired tissue samples was

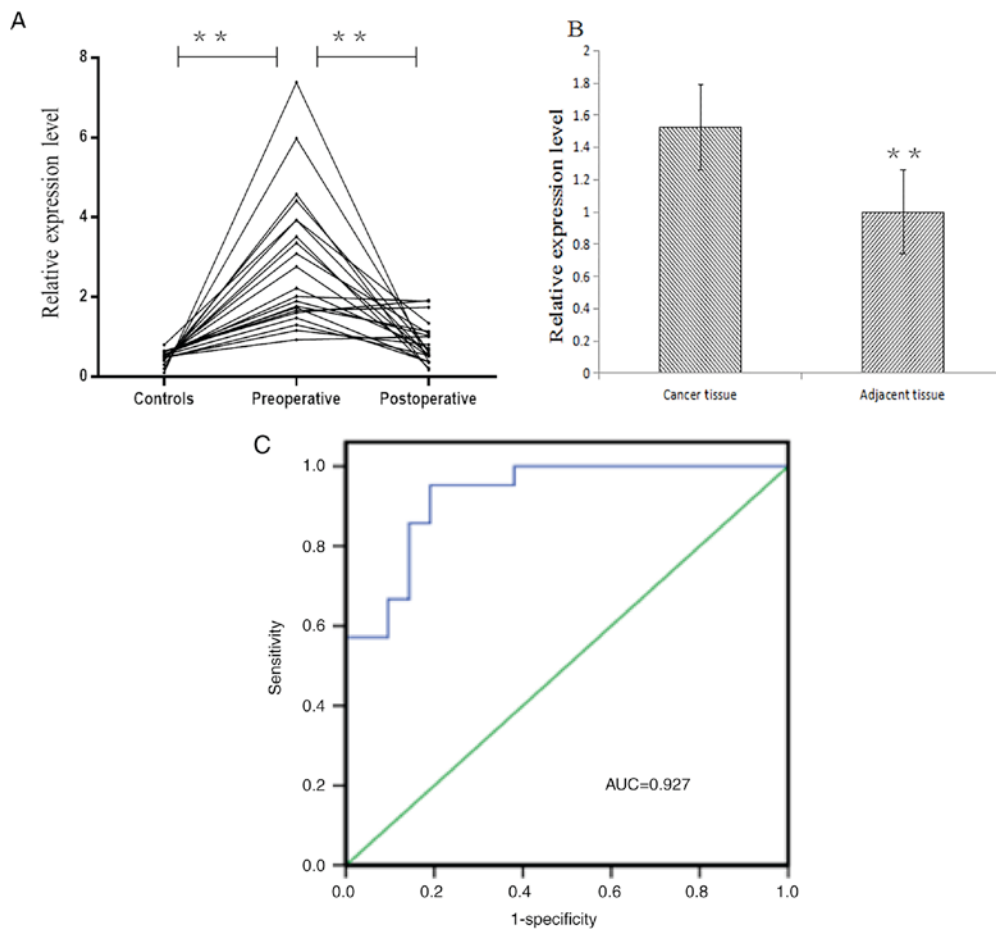


Figure 1. Expression levels of miR-483-5p in ESCC. The data are presented as the mean \pm standard deviation. The asterisks denote a response that was significantly different from the control (** $P<0.01$). (A) The expression level of miR-483-5p in the serum samples from healthy persons and patients with ESCC prior to and post-surgery. (B) The average expression level of miR-483-5p in ESCC tissues and adjacent normal tissues. ** $P<0.01$ vs. cancer tissue. (C) Receiver operating characteristic curve analysis of serum miR-483-5p. ESCC, esophageal squamous cell carcinoma; miR, microRNA; AUC, area under the curve.

calculated using the Wilcoxon matched-pairs test. Correlations between independent samplings and qPCR analysis of Igf2 and miRNA were determined by the Spearman correlation test. The Mann-Whitney test was performed to determine the significance of serum miRNA levels. The area under the curve (AUC) for tissue and serum miRNAs was determined using receiver operator characteristic (ROC) analysis. $P<0.05$ was considered to indicate a statistically significant difference. The statistical analysis was performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Analysis of the expression levels of miR-483-5p in ESCC. To investigate the role of miR-483-5p in ESCC, the miR-483-5p expression levels were evaluated in the serum from patients with ESCC patients prior to and following surgery, and from ESCC-free subjects. The results demonstrated that miR-483-5p was highly expressed in the serum prior to surgery in patients with ESCC, which was significantly increased compared with those following surgery in patients with ESCC and normal subjects ($P<0.05$; Fig. 1A).

The expression level of miR-483-5p in cancer tissues of patients with ESCC was significantly increased compared with those in adjacent non-cancerous tissues ($P<0.01$), and the

Table II. Correlation of miR-483-5p with clinical features in patients with esophageal squamous cell carcinoma.

Characteristic	No. cases	miR-483-5p	
		%	P-value
TNM stage			0.033
I	3	11.5	
II	8	30.8	
III	13	50.0	
IV	2	7.7	
Lymph node metastasis			0.048
No	11	42.3	
Yes	15	57.7	

TNM, tumor, node, metastasis; miR, microRNA.

difference between cancer tissues was similar to that between the serum samples (Fig. 1B). The expression level of miR-483-5p was positively correlated with the clinical tumor, node, metastasis (TNM) staging of patients with ESCC ($P<0.05$), and with the degree of lymph node metastasis ($P<0.05$; Table II).

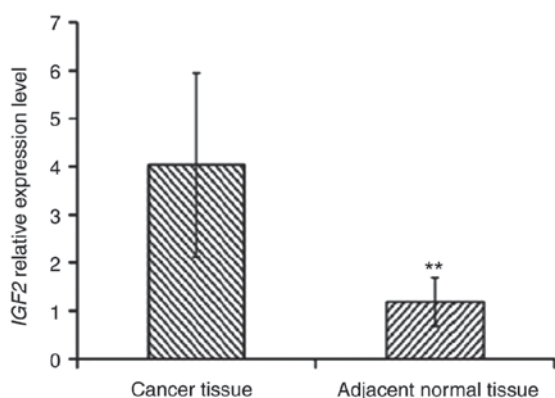


Figure 2. Expression levels of IGF2 in esophageal squamous cell carcinoma. The data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. cancer tissue. IGF2, insulin-like growth factor 2.

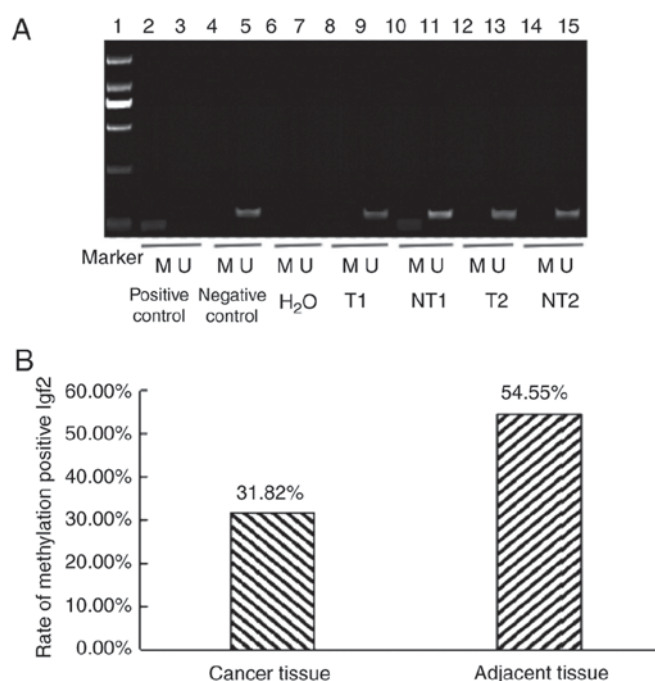


Figure 3. DNA promoter methylation analysis. (A) Detection of methylation status of the IGF2 gene promoter region (Marker DL 2,000). Lane 1, marker (DL 2,000); lane 2, positive control of DNA methylation in peripheral blood lymphocytes from healthy people; lane 3, negative control of DNA methylation in peripheral blood lymphocytes from healthy people; lane 4, positive control of DNA nonmethylation in peripheral blood lymphocytes from healthy people; lane 5, negative control of DNA nonmethylation in peripheral blood lymphocytes from healthy people; lane 6, distilled water as the negative control of the methylated template; lane 7, distilled water as a template for the negative control of nonmethylation; lane 8, methylation of cancer tissue; lane 9, nonmethylation of cancer tissue; lane 10, methylation of adjacent normal tissue; lane 11, nonmethylation of adjacent normal tissue; lane 12, methylation of cancer tissue; lane 13, nonmethylation of cancer tissue; lane 14, methylation of adjacent normal tissue; and lane 15, nonmethylation of adjacent normal tissue. (B) Methylation positive rate of the IGF2 gene in cancer tissue and adjacent normal tissue from patients with esophageal squamous cell carcinoma. IGF2, insulin-like growth factor 2; M, methylated; U, unmethylated; T, tumor tissues; NT, normal tissues.

In order to analyze the diagnostic potential of serum miRNA in ESCC, the ROC curve and AUC value were analyzed to further assess the reliability of serum miR-483-5p expression levels examined by the qPCR. ROC curve analysis



Figure 4. Prediction of miR-483-5p target genes. Target genes were predicted using miRBase, TargetScan and PicTar. The sequences of putative miR-483-5p binding sites in the 3'-UTRs of *ALCAM*, *ARHGDI* and *Socs3* are presented. UTR, untranslated region; *ALCAM*, activated leukocyte cell adhesion molecule; *ARHGDI*, Rho GDP dissociation inhibitor α ; *Socs3*, suppressor of cytokine signaling 3; miR, microRNA.

demonstrated that the AUC value of miR-483-5p was 0.927 (95% confidence interval, 0.85-1.00). When the threshold value was 0.762, the sensitivity was 95.2% and the specificity was 81% (Fig. 1C). AUC may be used as an indicator for the accurate evaluation of certain diagnostic methods, and used for clinical diagnostic tests. A larger AUC value indicates a greater diagnostic value. The closer the AUC is to 1, the higher its accuracy is, suggesting that serum miR-483-5p may be considered to be a diagnostic marker in ESCC.

Analysis of *Igf2* gene expression and promoter region methylation. It has been reported in the literature that miR-483 is located in the second intron of *Igf2*, and that the expression levels of *Igf2* directly affect the expression of miR-483. Therefore, the expression of the *Igf2* gene and the methylation levels in its promoter region were examined. The experimental results demonstrated that the expression levels of *Igf2* in cancer tissues of patients with ESCC were significantly increased compared with those in paracancerous tissues ($P < 0.01$; Fig. 2). The methylation level of the *Igf2* promoter region was decreased in tumor tissues (31.82%) compared with adjacent non-cancerous tissues (54.55%; Fig. 3).

Analysis of the expression of miR-483-5p target genes. miRNAs exert their functions primarily by affecting the expression of their target genes. A bioinformatical analysis was performed for the target genes of miR-483-5p (Fig. 4). A total of three miR-483-5p target genes including Rho GDP dissociation inhibitor α (*ARHGDI*), activated leukocyte cell adhesion molecule (*ALCAM*) and suppressor of cytokine signaling 3 (*Socs3*) were selected for further analysis. The results demonstrated that the expression levels of the three target genes in cancer tissues was significantly decreased compared with adjacent non-cancerous tissues, implying that miR-483-5p may influence the expression of these genes (Fig. 5).

Discussion

The study of miRNAs has been extended into numerous types of tumor. The expression of miR-483 has been demonstrated to be upregulated in approximately one-half of human tumors (18), including adrenocortical carcinoma and hepatocellular carcinoma (11,19), and its oncogenic targets, including

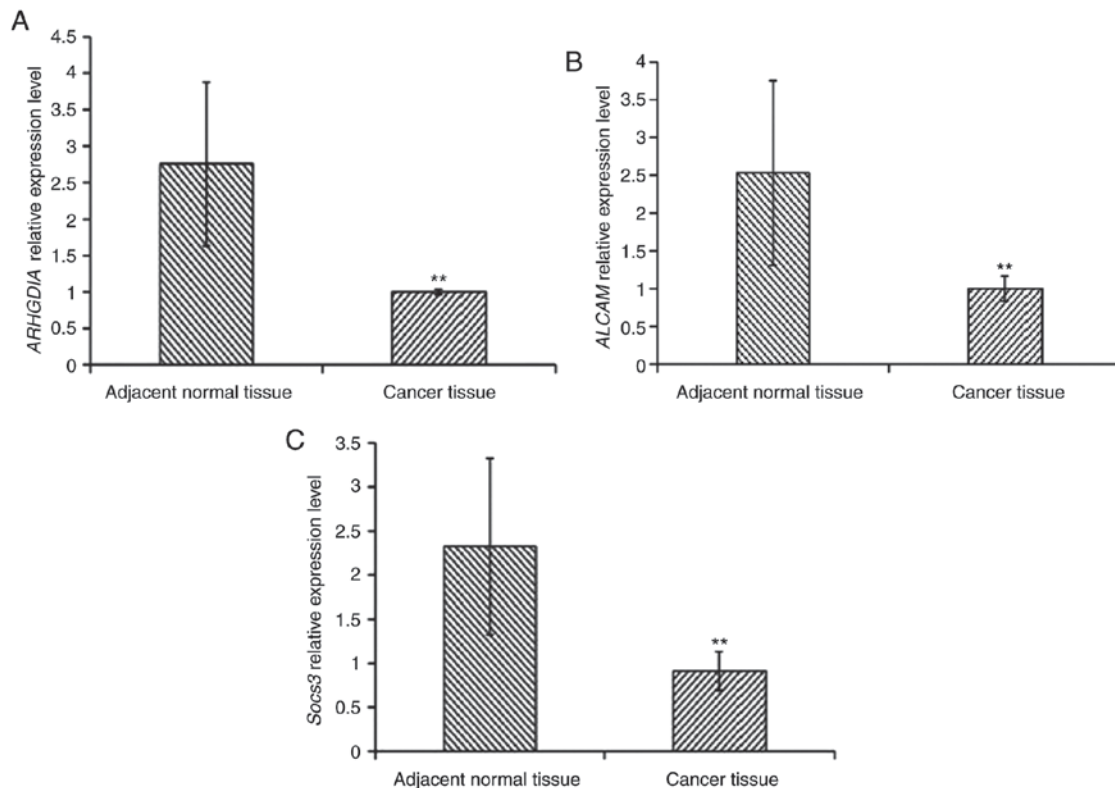


Figure 5. Expression levels of ARHGDI A, ALCAM and Socs3 in ESCC. The data are presented as the mean \pm standard deviation. (A) The expression levels of ARHGDI A in tissues from patients with ESCC. (B) The expression levels of ALCAM in tissues from patients with ESCC. (C) The expression levels of Socs3 in tissues from patients with ESCC. ** $P < 0.01$ vs. adjacent normal tissue. ESCC, esophageal squamous cell carcinoma; ALCAM, activated leukocyte cell adhesion molecule; ARHGDI A, Rho GDP dissociation inhibitor α ; Socs3, suppressor of cytokine signaling 3.

cellular tumor antigen p53, BCL2 binding component 3, catenin β 1, and insulin-like growth factor 1 receptor have been identified (20).

The degree of methylation of the promoter region affects the regional DNA structure and influences gene transcription. The results of the present study demonstrated that the differences in Igf2 promoter methylation resulted in the differential expression of Igf2 between cancer tissues and paracancerous tissues in patients with ESCC. The methylation level of the Igf2 promoter region in cancer tissues was low, although Igf2 gene expression was increased. The methylation of the promoter region of Igf2 in adjacent non-cancerous tissues was high, while the Igf2 expression level was decreased. miR-483-5p is coexpressed with Igf2 (21); thus, the expression of miR-483-5p is enhanced when the expression of Igf2 is increased. Therefore, the extent of methylation in the host gene promoter region influences miRNA expression, indicating that epigenetic modification serves an important role in the regulation of miRNA expression.

It has been demonstrated that miRNAs are able to bind to their complementary mRNA sites through base-pairing to regulate gene expression (22). Each miRNA has hundreds of evolutionarily conserved or non-conservative target genes. Therefore, appraisal of the miRNA target genes has become a challenge. In the present study, the mRNA levels of miR-483-5p target genes, including ARHGDI A, ALCAM and Socs3, were detected, which demonstrated that the levels of these genes were negatively-associated with the expression of miR-483-5p. However, the expression of these genes was

low in cancer tissues, and high in adjacent non-cancerous tissues, suggesting that miR-483-5p may mediate its potential the expression of the target genes ARHGDI A, ALCAM and Socs3, resulting in decreased expression in ESCC.

A recent study demonstrated that ARHGDI A may be a candidate tumor suppressor, and that it was downregulated in hepatoma and mammary cancer (23). Downregulation of ARHGDI A may reverse the activity of Rac family small GTPase 1 and cell division cycle 42, and increase cell migration and invasion to promote tumor metastasis (24). In the present study, the expression levels of miR-483-5p correlated with TNM stage and lymph node metastasis, suggesting that miR-483-5p may promote the development of ESCC by down-regulating the target gene ARHGDI A.

ALCAM is involved in homotypic or heterotypic cellular adsorption. The expression levels of ALCAM vary in distinct cancer tissues or at distinct stages of tumor progression (25-27). Olson *et al* (18) reported a negative correlation between ALCAM levels and the degree of tumor malignancy, and ALCAM expression is elevated in early ESCC (25). Therefore, the reduction of ALCAM expression may be due to the fact that the majority of samples in the present study were advanced ESCC.

The Socs3 gene belongs to the cytokine signaling inhibitor protein family. The Socs3 protein is able to negatively regulate the signaling processes of insulin and a number of cytokines to regulate immune reactions, inflammation and lymphocyte differentiation (28). Similarly, miR-483 negatively regulates the target gene Socs3 to regulate liver cancer cell proliferation

and development (29). The results of the present study demonstrated that miR-483-5p exhibited high expression, although Socs3 exhibited low expression, in ESCC cancer tissues, indicating that Socs3 may serve a role in ESCC pathogenesis.

In conclusion, the results of the present study suggested that miR-483-5p may be involved in ESCC pathogenesis, and that low methylation of the Igf2 gene promoter region led to increased expression of Igf2 and miR-483-5p in ESCC. As a result, the decrease in the ARHGDI1, ALCAM and Socs3 expression levels may cause the upregulation of oncogenes and downregulation of tumor suppressors, thereby inducing ESCC. Further studies are required to investigate the detailed mechanism and function of miR-483-5p in ESCC.

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