

MicroRNA-193b-3p Promotes the Development of Esophageal Squamous Cell Carcinoma by Targeting *CCND1* and *IGF1R*

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Abstract

The abnormal expression of miR-193a-3p was associated mostly with the development and progress of the tumors. In the present study, the expression levels of miR-193b-3p and its associated mRNAs were measured by reverse transcription-quantitative polymerase chain reaction, and the methylation levels of its promoter were detected via the methylation-specific PCR in serum and tissues of patients with esophageal squamous cell carcinoma (ESCC). The results demonstrated that the expression level of miR-193b-3p were significantly decreased in preoperative serum and cancer tissues in patients with ESCC ($p < 0.01$). The expression level of miR-193b-3p significantly was upregulated in serum after surgical removal of the cancerous tissue in ESCC patients ($p < 0.01$). The target gene *CCND1* of miR-193a-3p showed the high expression in the tumor tissues, and its up- and down-stream gene *Rb* and *p16* showed the low expression; and the target gene *IGF1R* and its up- and down-stream gene *IGF1* and *PI3K* showed the high expression. The methylation level of miR-193a-3p in the promoter region was higher significantly in the tumor tissues than that in the adjacent non-cancerous tissues ($p < 0.01$). This showed that the high methylation of genetic promoter region of miR-193b-3p might inhibit the expression of miR-193b-3p and further the regulation of *CCND1* and *IGF1R*, as well as the relevant signaling pathways, facilitating the development and progress of ESCC.

Keywords: miR-193b-3p; *CCND1*; *IGF1R*; Esophageal squamous cell carcinoma; Methylation

Introduction

As one of the common malignant tumors in the world, the esophageal carcinoma can be grouped in two categories based on its pathological features, i.e. esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma. The morbidity and mortality of ESCC in China ranks first in the world [1] while such category accounts for more than 90% of all pathological patterns, featuring the easy metastasis and invasion, as well as recurrence [2]. The major therapies used for the ESCC currently are surgery in combination with neoadjuvant chemoradiation [3]. Thus it may be of great significance if the effective therapeutic targets might be identified based on understanding the mechanism of ESCC at the molecular level.

MicroRNAs (miRNAs) are a group of small non-coding RNAs which may regulate the gene expression at post-transcriptional level. As the important regulator in cell pathway, miRNA may act as the oncogene, anti-oncogene or transfer regulator. In the prior experiment, we had determined that miR-483-5p would be up-regulated in ESCC which might facilitate the occurrence of such cancer [4]. The recent studies had shown that miR-193b-3p as the cancer suppressive factor would be down-regulated in the various tumors [5-7]. Pekow et al. reported that expression of miR-193a-3p decreased in the process in which the ulcerative colitis progressed to tumor, which would cause the up-regulation of target gene *IL17RD* to function in a carcinogenic manner [8]. The *ERBB4* and *S6K2* targeted by miR-193a-3p might suppress the *ERBB* signaling pathway to play a role in inhibiting the lung cancer [9]. The change in its expression had been demonstrated to be related to the tumors such as gastric cancer, colon cancer and endometrial cancer [10-12]. Nevertheless, the potential role and related target gene of which miR-193b-3p functioned in the development and progress of ESCC were unclear yet. The miR-193b-3p may play a part in the inhibition of tumor in many malignant tumors and interact with many target genes, so that it may become the new target of diagnosis and treatment of malignant tumor.

The abnormal expression of *CCND1* would cause the change of cell cycle and then the development of tumor [13]. The recent studies had shown that miR-193b-3p promotes the development of acute myeloid

leukemia by targeting *CCND1* and KIT-RAS-RAF-MEK-ERK (MAPK) [5]. The family of insulin-like growth factors (*IGFs*) was highly correlated with tumor among which *IGF1R* was the focus of many studies for it was closely related to the growth and differentiation of cells [14]. The high expression of *IGF1R* would be seen in liver, breast, prostate and bowel cancer and was associated with the development, progress and metastasis of tumor [15]. The recent studies had shown that miR-193b inhibits the growth and metastasis of renal cell carcinoma by targeting *IGF1R* [16]. Unlike the prior studies, we tested the expression of upstream and downstream gene in signaling pathway *cdk4/Rb/CCND1/p16* and *PI3K/AKT* in which *CCND1* and *IGF1R* located. We predicted that miR-193b-3p might affect the relevant pathways via the target gene, and both pathways, which were associated with the cell survival rate in the ESCC would further influence the development and progress of tumor.

The DNA modification of methylation is the important way in which the expression and regulation of epigenetic gene presents [17-19]. Therefore, this study analyzed further if the abnormal expression of miR-193b-3p in ESCC was associated with the methylation of promoter region. Such efforts will provide the new idea for the miR-193b-3p acting as the new biomarker in the clinical diagnosis of ESCC and further the target therapy.

Materials and Methods

Patients

The study protocol was approved by the Ethics Committee of the

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Xinxiang Central Hospital. A total of 74 ESCC patients from the Xinxiang Central Hospital and 94 healthy subjects were consecutively recruited to this study between September 2016 and June 2017. The diagnosis of ESCC was confirmed histologically in all patients. ESCC tissues and adjacent non-cancerous esophageal tissues (at least 5 cm away from the tumor) from all 23 patients were collected. Three milliliters of peripheral blood was collected from each participant (51 pre-operative and 23 after 7 days of surgery in ESCC patients, 94 healthy persons).

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) according to the instructions of the manufacturer [20].

Peripheral blood was collected in tubes containing separating gel and clot activator, place in water-bath for 20 min at 37°C and centrifuged at 3,500 g for 10 min at room temperature. The supernatants were transferred to Eppendorf tubes. A second centrifugation at 12,000 g for 10 min at 4°C was performed to completely remove all cellular components. The serum was then aliquoted and stored at -80°C until RNA extraction. All blood samples were processed within 3 h after they were obtained. Total serum RNA was isolated from 100 µl serum and eluted in 300 µl of RNase-free water using Trizol (Invitrogen) according to the instructions of the manufacturer for blood samples.

Reverse transcription and quantitative real-time PCR

RT-qPCR individual miRNA tests were performed on independent sets of serum or tissue samples using a two steps procedure. Quantitative real time PCR (qPCR) for miRNA Stem-Loop™ RT primers of miR-193b-3p and miR-16-5p were synthesized by Applied Biosystems (Shanghai, China) (Table 1). PrimeScript™ RT reagent Kit (Perfect Real Time) (TaKaRa) was used to reverse transcribed total RNA. SYBR Green (TaKaRa) qPCR assay was used to detect the expression of miR-193b-3p and miR-16-5p. PCR reaction was performed 45 cycles (95°C, 10 sec; 60°C, 30 sec) after

an initial denaturation step (95°C, 5 min) on the CFX96 system of Bio-Rad CFX Manager 2.0 Software. The expression levels of miRNA were calculated and quantified using the 2-ΔΔCT method [21]. MiR-16-5p was used as the internal control. All reactions were done in triplicate.

Prediction of miR-193b-3p target genes

The prediction of miR-193b-3p target genes was carried out by miRBase (<http://www.microrna.org/microrna/home.do>), TargetScan (<http://www.targetscan.org/>), PicTar (<http://pictar.mdc-berlin.de/>).

Genomic DNA isolation and methylation analysis

The EZ DNA Methylation-Gold™ Kit (Qiagen, Valencia, CA, USA) is used for bisulfate treating DNA for methylation analysis, The primers specific for either unmethylated or methylated alleles are listed in Table 1. As an internal control, all purified genomic DNA samples were successfully tested by polymerase chain reaction (PCR) with TaKaRa EpiTaq™ HS kit (for bisulfite-treated DNA). Normal human peripheral lymphocyte methylated and unmethylated DNA was used as a positive control for the methylated and as a negative control for unmethylated genes, respectively. The samples with H₂O instead of DNA were included for each PCR set. PCR products were analyzed on 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. Each MSP was repeated at least once to confirm the results.

Statistical analysis

Data were reported as mean ± standard deviation (SD) for quantitative variables. The difference in mRNA or miRNA expression levels between paired tissue samples was calculated using the Wilcoxon matched-pairs test. Correlations between independent samplings and RT-qPCR of *CCND1*, *IGF1R* 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 were considered to be statistically significant. The statistical analysis was performed with software SPSS version 17.0.

Primer	Sequence
<i>hsa-miR-16-RT</i>	GTCGTATCCATGGCAGGGTCCGAGGTATTCGCCATGGATACGACCGCCAAT
<i>hsa-miR-193b-3p-RT</i>	GTCGTATCCATGGCAGGGTCCGAGGTATTCGCCATGGATACGACCTCCCT
<i>hsa-miR-16-F</i>	GCGGTAGCAGCACGTAATATT
<i>hsa-miR-193b-3p-F</i>	GCAAGACGGGAGGAAAGAAGGGA
universal reverse	TGGCAGGGTCCGAGGT
<i>GAPDH-F</i>	GCACCGTCAAGGCTGAGAAC
<i>GAPDH-R</i>	TGGTGAAGACGCCAGTGGGA
<i>Rb-F</i>	AAGAGCAAGCATCTGCAGGA
<i>Rb-R</i>	TGGTATTGTGCTTGCTGCTG
<i>CCND1-F</i>	ATTTTGGTTAATACGGTGAATTTTC
<i>CCND1-R</i>	CTATCGCCCAAATAAATACGAT
<i>P16-F</i>	TTTGGTTAATATGGTGAATTTTGT
<i>P16-R</i>	CTCTATCACCCAAATAAATACAAT
<i>IGF1-F</i>	GTGCGGAGACAGGGGCTTT
<i>IGF1-R</i>	ACTTGGCGGGCTTGAGAGG
<i>IGF1R-F</i>	CCAAGGGTGTGGTGAAGAT
<i>IGF1R-R</i>	TCCATGATGACCAGTGTGG
<i>PI3K-F</i>	GGACCCGATGCGGTTAGAG
<i>PI3K-R</i>	ATCAAGTGGATGCCCCACAG

F-Forward; R-Reverse; RT-Reverse Transcription; miR-microRNA; *GAPD*-Glyceraldehyde-3-phosphate dehydrogenase; *Rb*-Retinoblastoma; *CCND1*-Cyclin D1; *P16*-multiple tumor suppressor 1; *IGF1R*-Insulin-like growth factor 1; *IGF1R*-Insulin-like growth factor 1 receptor; *PI3K*-phosphoinositide 3-kinase.

Table 1: The primers used in this work.

Results

Expression levels of miR-193b-3p in ESCC

To investigate the role of miR-193b-3p in ESCC, we first examined the levels of serum miR-193b-3p in ESCC before and after surgical removal, and in normal subjects. It was found that miR-193b-3p level was significantly lower in ESCC patients before surgery than after surgery or in normal subjects ($p < 0.01$) (Figure 1A).

The expression level of miR-193b-3p was significantly decreased in cancer tissues of patients with ESCC compared with those in adjacent non-cancerous tissues ($p < 0.01$), and the difference between cancer tissues was similar to that between the serum samples (Figure 1B).

To evaluate the diagnostic value of serum miR-193b-3p, we further analyzed its ROC curve and AUC value. ROC curve analysis showed that the AUC value of miR-193b-3p was 0.815 (95% CI: 0.679-0.937). And when the cutoff value is set at 0.537, a sensitivity of 81.7% and specificity of 73.7% can be achieved (Figure 1C). AUC can be used to determine the diagnostic value of diagnostic methods, the larger AUC (up to 1), the more accurate and the higher diagnostic value. So, serum miR-193b-3p as a diagnostic marker, showed high accuracy and high diagnostic value.

Analysis of the expression of miR-193b-3p target genes

Two genes (*CCND1* and *IGF1R*) were preliminarily selected as the target genes that may have mediated its effect on ESCC (Figure 2). We

first analyzed the expression level of *cdk4/Rb/CCND1/p16* signaling pathway-related genes by RT-qPCR. They include *CCND1* which is miR193b-3p target gene, its upstream gene *Rb* and its downstream gene *p16*. The results demonstrated that the high expression of *CCND1* and the low expression of *Rb* and *p16* in tumor tissues ($p < 0.05$ or $p < 0.01$) (Figure 3). Pearson correlation coefficient analysis demonstrated that expression levels of miR-193b-3p and *CCND1* were negatively correlated ($r = -0.645$, $p < 0.05$).

We second analyzed the expression level of *PI3K/AKT* signaling pathway-related genes. They include *IGF1R* which is miR193b-3p target gene, its upstream gene *IGF1* and its downstream gene *PI3K*. The results demonstrated that the expression levels of the three genes in cancer tissues was significantly upregulated compared with adjacent non-cancerous tissues ($p < 0.05$ or $p < 0.01$) (Figure 4).

Analysis of miR-373-3p promoter region methylation

It was found that the average methylation level of miR-193b-3p promoter was 61.91% in cancerous tissue, significantly higher than that in pericancerous tissue (38.09%) ($p < 0.01$) (Figure 5).

Discussion

In this study, we tested the expression level of miR-193a-3p in the serum and tissues of the patients with ESCC, and also analyzed pre- and post-operatively the expression in the serum of patient, so that we could determine further if miR-193a-3p was related to the ESCC. The

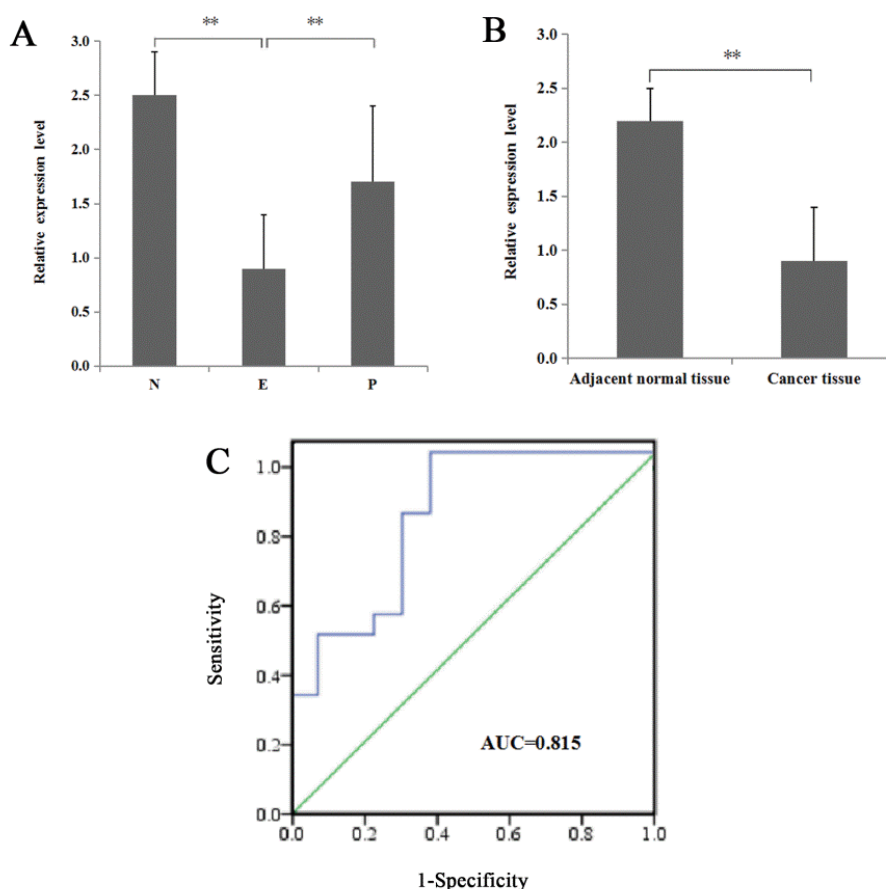
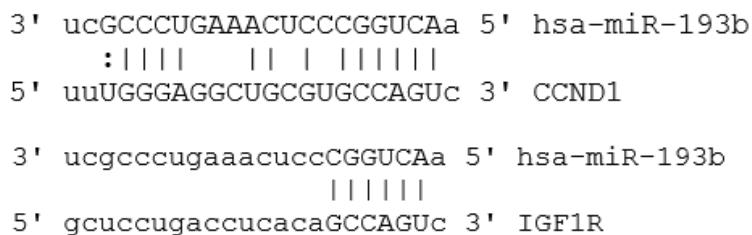
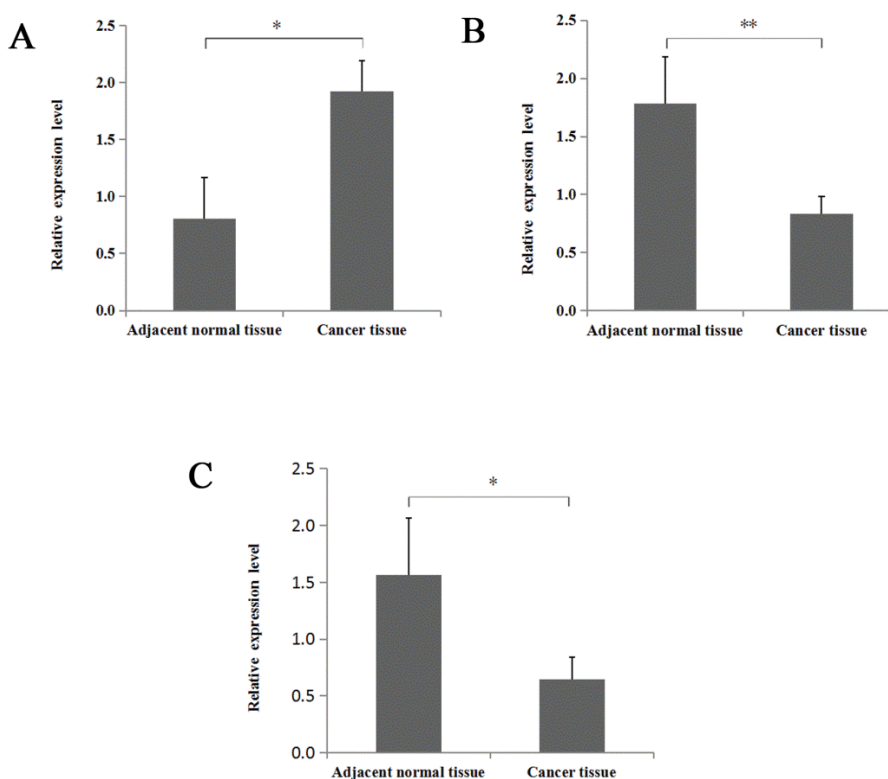


Figure 1: Expression levels of miR-193b-3p in ESCC.



Note: (A) The average expression level of miR-193b-3p in serum from ESCC patients. N, E and P indicated the average expression level of miR-193b-3p in normal serum, esophageal squamous cell carcinoma and postoperative serum, respectively (**p<0.01). (B) The expression level of miR-193b-3p in the serum samples from healthy persons and patients with ESCC prior to and post-surgery (**p<0.01). (C) ROC curve analysis of miR-193b-3p.

Figure 2: The target gene prediction of miR-193b-3p.



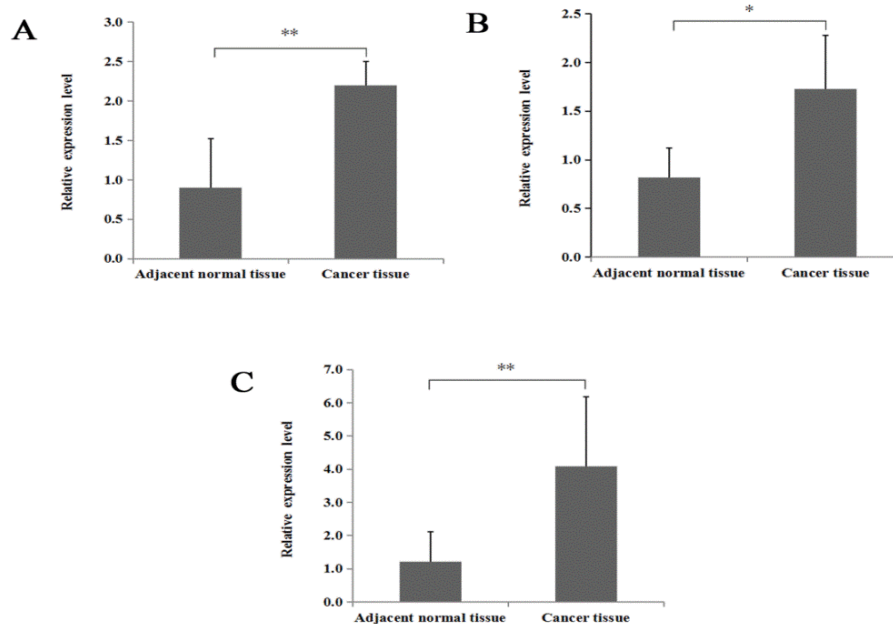
Note: (A) The average expression level of *CCND1* in ESCC patients (**p<0.05). (B) The average expression level of *Rb* in ESCC patients (**p<0.01). (C) The average expression level of *p16* in ESCC patients (**p<0.05).

Figure 3: The expression levels of *cdk4/Rb/CCND1/p16* signaling pathway related genes in ESCC.

results of experiment demonstrated that miR-193b-3p showed the low expression in the serum and tissues of ESCC and the high expression the post-operative and healthy population, which was consistent with the down-regulation of miR-193b-3p expression in other types of tumor as described in the literature [5-7]. We presumed that the miRNA level in serum was affected by the tumor tissues which might recover gradually to the normal level after the tumor tissues were excised in the operation, causing that the expression level of serumal miRNA was separated from the influence of tumor tissues. Moreover, we also tested AUC value of serumal miRNA to evaluate the effect of serumal miRNA on the diagnosis of ESCC. The result suggested that the AUC value of serumal miR-193b-3p was 0.815. For the closer AUC value equaled to 1, the higher value of diagnosis it would have [22], miR-193b-3p as

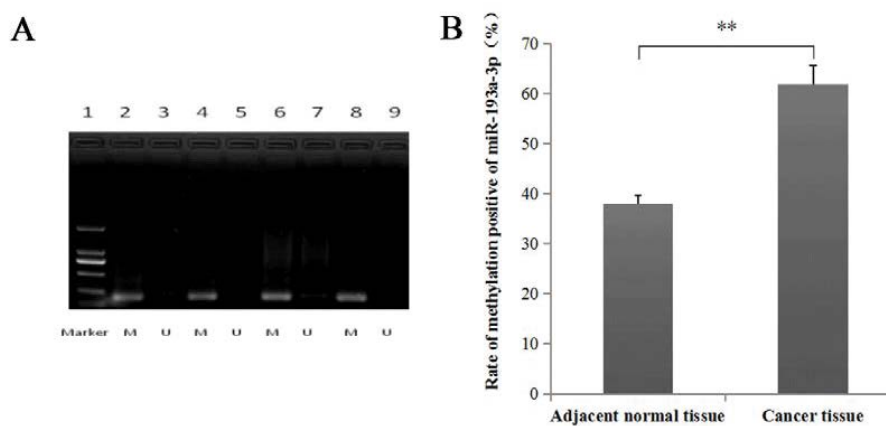
the diagnostic marker of ESCC showed a certain diagnostic value and accuracy.

The miRNA may bind to its complementary mRNA locus *via* base pairs to regulate the expression of gene. There are hundreds of evolutionarily conserved target genes and several times of non-conserved target genes for each of miRNA. Therefore, the identification of target genes of miRNA becomes a big challenge. In this experiment, we tested the mRNA expression level of target gene *CCND1* and *IGF1R* of miR-193a-3p and the expression level of up- and down-stream genes of target gene based on the results from the analysis of report in literatures [5,16] and bioinformatics. The expression level of all these genes was affected by the expression level of miR-193a-3p. The result of experiment supported that miR-193a-3p might act on the target genes



Note: (A) The average expression level of *IGF1R* in ESCC patients (** $p < 0.01$). (B) The average expression level of *IGF1* in ESCC patients (** $p < 0.05$). (C) The average expression level of *PI3K* in ESCC patients (** $p < 0.01$).

Figure 4: The expression levels of *PI3K/AKT* signaling pathway related genes in ESCC.



Note: (A) 1: DL2000 marker; 2,6 Methylation of cancer tissue; 3,7 Nonmethylation of cancer tissue; 4,8 Methylation of adjacent normal tissue; 5,9 Nonmethylation of adjacent normal tissue. (B) The average methylation level of miR-193b-3p promoter in cancer tissue and adjacent normal tissue (** $p < 0.01$).

Figure 5: Detection of methylation status of miR-193b-3p gene promoter region.

and then affect significantly the signaling pathway in which the target gene located, leading to the abnormal expression of such genes in the tumor tissues.

As a member of highly conserved cyclin family, the cyclin D1 protein could be detected at high expression in the various tumors such as head and neck squamous cell carcinoma [23], ESCC [24] and breast cancer [25], etc. The overexpression of miR-193b would inhibit the expression of *CCND1*, and miR-193b, which might act as the tumor suppressor, produced the effect in the gastric cancer when targeted *CCND1* [26]. In this experiment, miR-193b-3p showed the low expression, the *CCND1* showed the high expression. The regulation of *CCND1* targeted by miR-193b-3p would contribute to the occurrence of ESCC. As the important

member of *cdk4/Rb/CCND1/p16* pathway, *CCND1* joined in the regulation of cell cycle as its up- and down-stream gene was *Rb* and *p16* gene, included in anti-oncogenes, respectively [27,28]. *P16* molecule would enter into rivalry with *CCND1* over *cdk4* and *cdk6* binding site, causing *Rb* protein in dephosphorylated active state and to bind closely with E2F molecule, and then to prevent against the cell from G1 phase into S phase [29,30]. The numerous studies showed that the abnormality in *cdk4/Rb/CCND1/p16* pathway might be related to the development and progress of the various tumors as *Rb*, *CCND1* and *p16* would form a feedback regulation loop [31]. We tested if the up- and down-stream gene *Rb* and *p16* in the signaling pathway in which *CCND1* located might be influenced by the abnormality expression of *CCND1*. The result demonstrated that the high expression of *CCND1* and the low

expression of *Rb* and *p16* existed in tumor tissues in which the high expression of *CCND1* was possible in suppressing the expression of *Rb* and *p16*. Some studies suggested that *Rb* gene might play its part in the ESCC via the regulation of *CCND1* [32-34]. Therefore, there was the possibility that miR-193b-3p and *Rb* contributed to the development of ESCC by interaction with *CCND1* together.

As the signaling pathway *cdk4/Rb/CCND1/p16* and *PI3K/AKT* where *CCND1* and *IGF1R* located were associated with the cell viability and affected the development and progress of tumor, we tested the other target gene *IGF1R* and its up- and down-stream gene *IGF1* and *PI3K* of miR-193b-3p, suggesting that the three genes would express highly in the tissues of ESCC. Wang [35] reported that *IGF1R* showed the high expression in ESCC and facilitated the proliferation of cells, so that might induce the development of tumor, which was consistent with the result of present study and illuminated further the effect of *IGF1R* in the ESCC. In addition, the overexpression of down-stream gene *PI3K* of *IGF1R* suggested the activation of *PI3K* pathway in the tissues of ESCC. The study had confirmed that the *PI3K/AKT* signaling pathway showed the abnormal expression in the various types of human tumor [36-39]. The *AKT* activated by *PI3K* might activate or suppress its down-stream target proteins such as Bad, Caspase9, NF-kB, Forkhead, mTOR, Par-4 and P21 via hyperphosphorylation while as the important anti-apoptotic regulatory factor, it might mediate the insulin and the various growth factor to induce the growth of cells and promote the cell viability through the multiple ways [40]. *IGF1R*, *IGF1* and the host gene *IGF2* of miR-483-5p were the members of *IGF* superfamily. Of note, Zhang [41] investigated the regulatory relation among the genes such as *IGF2* and *IGF*, *IGF1R* suggesting that the inconsistency was found in the correlation between the expression of *IGF2* and *IGF1R*. However, the results from our experiment showed the different conclusion compared with that of Zhang, suggesting that the effect of miR-193b-3p might cause the up-regulation of gene expression such as *IGF1R* and then regulate indirectly the *PI3K/AKT* signaling pathway to suppress the apoptosis of tumor and facilitate the malignant progress of ESCC.

As the important part of epigenetic mechanism, DNA methylation plays an important role in the development of human tumor and is one of the new research highlights at present. In the prior studies, we found that DNA in promoter region of miR-483-5p which presented the high expression in ESCC showed the hypomethylation and whether the promoter region of miR-193b-3p with the low expression might show the opposite methylation forms? The result of this experiment suggested that methylation positive rate of in promoter region of miR-193b-3p in the tissues of ESCC reached 61.9%, higher significantly than the methylation positive rate in the peritumoral tissues, i.e. the high methylation in the promoter region of miR-193b-3p might cause the change in the DNA conformation and suppress the process of genetic transcription, leading to the low expression of miR-193b-3p. The transcriptional activity of some miRNA genes in the tumor cell could be suppressed by hypermethylation of DNA. The tumor-suppression effect might be seen in the recovery of the expression activity of such genes, such as miR-212 in gastric cancer [42] and miR-128 in colon cancer [43]. The methylation result of this experiment was consistent with the expression of miR-193b-3p in other malignant tumors on the basis of reports in the literatures [44], supporting further the reliability in the results of DNA methylation. It also shows that methylation in miRNA promoter region regulates miRNA expression, and methylation level is opposite to miRNA expression levels.

Conclusion

In this study, we demonstrated preliminarily that the methylation

in the promoter region of miR-193b-3p might regulate the abnormal expression of miR-193a-3p in the ESCC, the disorder of target gene *CCND1* and *IGF1R*, as well as the related signaling pathways, and finally the development of tumor. The description above explained the effect of epigenetic modification in the etiopathogenesis of ESCC, and provided the new idea for the diagnosis and treatment of advanced ESCC.

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Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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