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Original Article

MiR-153-5p has effects on cell proliferation and invasion and is critical for prognosis of patients with esophageal squamous cell carcinoma

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Abstract: Background: MicroRNAs (miRNAs) modulate a variety of cellular processes by regulating multiple targets, which promote or inhibit the development of malignant behaviors. Accumulating evidence suggests that miR-153-5p plays important roles in human carcinogenesis. This study was designed to explore the role of miR-153-5p inesophageal squamous cell carcinoma (ESCC). Methods: MicroRNA profiles were obtained by miRNA microarray and then validated byquantitative polymerase chain reaction (qPCR) in healthy individuals and ESCC patients. Associations of miR-153-5p with Wilms' tumor suppressor gene 1 (WT1) were assessed by Pearson correlation. In vitro, the biological function of miR-153-5p was examined by Transwell assay, and Western blot in TE-1 cells transfected with miRNA mimics or the empty vector. The miR-153-5p target was validated by a luciferase reporter assay, RT-PCR and Western blot. Results: MiRNA microarray revealed 25 aberrant miRNAs in serum samples from ESCC patients, including miR-153-5p which was significantly decreased (P<0.01); miR-153-5p was expressed at lower levels in patients with aggressive tumors (P<0.01). In addition, WT1 mRNA amounts, which were higher (P<0.01) in ESCC than in healthy individuals, were negatively correlated with miR153-5p levels (r=-0.5983, P<0.001). Moreover, miR-153-5p inhibited the proliferation and invasion abilities of TE-1 cells (P<0.01). Finally, the luciferase activity of the WT1-3'-UTR plasmid was suppressed after miR-153-5p binding (P<0.05). Conclusion: MiR-153-5p is downregulated in patients with ESCC, and plays a critical role in the diagnosis and severity evaluation. In agreement, miR-153-5p inhibits TE-1 cell proliferation and invasion by downregulating WT1.

Keywords: Esophageal squamous cell carcinoma, miR-153-5p, diagnosis, WT1

Introduction

Esophageal cancer incidence has steadily increased in the Western world. In 2015, 16,980 patients were newly diagnosed with esophageal cancer in the United States, and 15,590 individuals died from the disease [1]. Esophageal squamous cell carcinoma (ESCC) is the predominant histologic type, and very common in East Asia, especially Japan and China [2]. Surgery is considered the mainstay of treatment for esophageal cancer [3]. However, many patients develop metastatic disease or locoregional recurrence soon after surgery [4]. It was reported that combining neoadjuvant chemotherapy and radiotherapy reduces tumor size and maximizes local control, improving disease outcome [5, 6]; however, the overall 5-year survival rate for disease localized to the esophagus is 34%, while survival for all stages is much lower with a rate of 16% [7]. Therefore, there is a great need to develop new biomarkers and therapeutic targets for esophageal cancer.

MicroRNAs (miRNAs) are short (approximately 22 nucleotides) non-coding RNAs that regulate target mRNAs predominantly by binding to the 3' untranslated region (UTR) of its target mRNAs, causing degradation or translation inhibition [8, 9]. Increasing evidence indicates microRNAs modulate tumor initiation and progression, with functions in tumor cell invasion and metastasis [10-12]. Indeed, multiple miRNAs have been shown to play vital roles in ESCC. For instance, elevated miR-21 expression was found in esophageal cancer, which could promote cell proliferation by targeting PTEN at the posttranscriptional level. In addi-

Table 1. Differentially expressed miRNAs between healthy individuals and ESCC patients

MiRNA expression profiles					
Upregulated miRNAs			Downregulated miRNAs		
MiRNA name	Log_2 (fold change)	*P*-values	MiRNA name	Log_2 (fold change)	*P*-values
has-miR-155	7.31	0.0136	has-miR-375	9.73	0.0396
has-miR-25	6.20	0.0208	hsa-miR-625	8.37	0.0102
has-miR-92c	5.64	0.0012	hsa-miR-153-5p	8.07	0.0090
hsa-miR-200c	5.39	0.0162	hsa-miR-498	7.21	0.0076
hsa-miR-16	4.74	0.0104	hsa-miR-302b	6.82	0.0281
hsa-miR-214	4.21	0.0108	hsa-miR-518b	6.13	0.0222
hsa-miR-21	4.06	0.0084	hsa-miR-145	5.34	0.0442
hsa-miR-96	3.83	0.0279	hsa-miR-133a	4.16	0.0337
hsa-miR-146a	3.60	0.0411	hsa-miR-203	3.67	0.0076
hsa-miR-296	3.17	0.0032	hsa-miR-91	3.07	0.0168
hsa-miR-20a	2.95	0.0262	hsa-miR-708	2.43	0.0181
hsa-miR-17-5p	2.17	0.0115	hsa-miR-376	2.27	0.0271
hsa-miR-497	2.08	0.0346			

P-values reported are the result of paired class comparison of microRNA expression on serum samples from patients with ESCC and healthy volunteers.

tion, it is able to predict poor overall survival in patients with EC [13, 14]. MiR-625 is associated with tumor depth, stage, and metastasis in ESCC and its down-regulation may constitute a molecular marker to predict progression of aggressive tumors as well as unfavorable prognosis [15, 16]. In addition, Ren *et al*. found miR-183 is upregulated in ESCC tissues, and identified programmed cell death 4 (PDCD4) as a direct target of this miRNA, which promotes ESCC cell proliferation and invasion [17]. Recently, miR-153-5p was described as a tumor suppressor in human cancers, regulating genes or proteins involved in tumor growth, metastasis, and invasion [18-21]. Nevertheless, the exact role of miR-153-5p in ESCC remains unclear. This study, therefore, aimed to assess the role of miR153-5p in ESCC, evaluating clinical data, and exploring its biological functions in esophageal squamous cell carcinoma.

Wilms' tumor suppressor gene 1 (WT1), which maps to chromosomal band 11p13, was identified as a tumor suppressor in a variety of cancers [22-25]. A study demonstrated significant inverse associations of WT1 expression with pathological stage, metastasis, and survival innon-small-cell lung cancer [26]. We searched for direct targets of miR-153-5p in the miRDB and TargetScan, and WT1 was identified as a candidate; this prompted us to assess whether miR-153-5p could modulate WT1 expression in human ESCC cells.

This study identified 25 aberrantly expressed miRNAs in ESCC using miRNA microarray analysis, including miR-153-5p. Further assays demonstrated that miR-153-5p could be used as a biomarker for ESCC diagnosis; indeed, decreased miR-153-5p levels were associated with ESCC severity. Besides, miR-153-5p inhibited TE-1 cell proliferation and invasion *in vitro*. More importantly, *WT1* was confirmed to be a direct target gene for miR-153-5p in ESCC.

Materials and methods

Patients and tissue samples

A total of 47 patients with esophageal carcinoma and 50 healthy individuals were enrolled from 2012 to 2014 at the Huaian Hospital. Inclusion criteria were: ESCC diagnosis; complete clinicopathological data; esophagectomy performed after diagnosis. TNM classification was conducted according to the 7th Edition of AJCC Cancer Staging Manual [27]. Non-tumor tissue specimens were obtained adjacent to tumors, more than 5 cm away from the tumor edge, and validated pathologically. All patients were treated according to the guidelines for standardized diagnosis and treatment of esophageal cancer in China. Tissue samples were snap frozen in liquid nitrogen and stored at -80°C. Serum samples were collected from all participants before any treatment. Clinical characteristics of the patients are shown in **Table 3**. Written informed consent was obtained from each patient. Approval was obtained from the Ethics Committee of the First Affiliated Hospital with of Nanjing Medical University.

MicroRNA microarrays

Serum samples were randomly selected between healthy and ESCC participant groups. Tumor tissues and matched normal tissues were also randomly chosen. A list of miRNAs contained in the array used in this study is available in the Sanger miRB ase V18.0 data-

Table 2. The TaqMan stem-loop primers for reverse transcription PCR and the forward and reverse primers for real-time PCR

Accession	ID		Sequence
MIMAT0000081	Has-miR-25	Sequence	CAUUGCACUUGUCUCGGUCUGA
		TaqMan primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCAGAC
		PCR-F	GCATTGCACTTGTCTCG
MIMAT0002844	Has-mir-518b	Sequence	CAAAGCGCUCCCCUUUAGAGGU
		TaqMan primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACCTCT
		PCR-F	CAAAGCGCTCCCCTTT
MIMAT0000646	Has-mir-155	Sequence	UUAAUGCUAAUCGUGAUAGGGGU
		TaqMan primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACCCCT
		PCR-F	GGGTAATGCTAATCGTGAT
MIMAT0000271	Has-mir-214	Sequence	ACAGCAGGCACAGACAGGCAGU
		TaqMan primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACTGCC
		PCR-F	GGGACAGCAGGCACAGACA
MIMAT0000617	Has-mir-200c	Sequence	UAAUACUGCCGGGUAAUGAUGGA
		TaqMan primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCCATC
		PCR-F	TAATACTGCCGGGTAAT
MIMAT0026480	Has-mir-153-5p	Sequence	UCAUUUUUGUGAUGUUGCAGCU
		TaqMan primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAGCTGC
		PCR-F	TCATGGTTGTGATGTT
	U6	PCR-F	CTCGCTTCGGCAGCACA
		PCR-R	AACGCTTCACGAATTTGCGT
	GAPDH	PCR-F	GCACCGTCAAGGCTGAGAAC
		PCR-R	ATGGTGGTGAAGACGCCAGT
	WT1	PCR-F	GCTTCGGCTTACGGGTCGTT
		PCR-R	GTGAAGGCGCTCAGGCACTG

base. Samples with RNA integrity number (RIN) >8 were processed for hybridization. Total RNA was labeled and hybridized to the miRNA microarray, according to the standard operating procedure provided by CapitalBio Corporation. Scanned images were then imported into a confocal LuxScan scanner (CapitalBio Corp). The SpotData Pro software (CapitalBio Corp) was used for data analysis.

RNA extraction and quantitative real-time PCR

Total RNA was isolated from cultured cells and human tissue samples with TRIzol reagent (Invitrogen) according to the manufacturer's instructions; qRT-PCR was performed to validate microarray data. First strand cDNA was synthesized according to the manufacturer's instructions using TaqMan MicroRNA Reverse Transcription Kit with stem-loop RT primer (Life Technologies, USA). Diluted cDNA was subjected to qRT-PCR using Power SYBR Green PCR Master Mix on a Roche Lightcycler 480 Real Time PCR System, with the following conditions: 2 min of pre-denaturation at 95°C and 40 cycles of denaturation at 95°C (15 s) and annealing at 60°C (60 s). All PCR reactions were assessed in triplicate. Relative miRNA and mRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ method. The primers used for real-time PCR are shown in **Table 2**.

Cell culture

The human esophageal cancer cells (EC190, EC9706, SKGT-5 and TE-1) and human normal esophageal epithelial (HEEC) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were maintained at 37°C in a humidified environment containing 5% CO_2 in DMEM with 10% fetal bovine serum (FBS, Invitrogen) and 1% of 100 U/mL penicillin and streptomycin (Invitrogen, CA).

Western blot

Total protein was extracted from cells with 1% RIPA Lysis Buffer (Beyotime, China). The BCA method was used for protein quantitation. Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes. Then, the membranes were probed with relevant antibodies (anti-WT1, anti-E-cad-

Table 3. Clinicopathological features and the expression of miR-153-5p in patients with esophageal squamous cell carcinoma

Characteristic	Case	MiR-153-5p		*P* value[a]
		Low expression	High expression	
Age (years)				0.365
<60	21	10	11	
≥60	26	9	17	
Gender				0.534
Male	29	16	13	
Female	18	6	12	
Pathology type				0.298
Ulcerative type	18	10	8	
Medullary type	14	5	9	
Fungating type	8	3	5	
Constrictive type	4	0	4	
Plaque type	2	1	1	
Other	1	1	0	
Differentiation				0.135
Well	7	2	5	
Moderate	21	12	9	
Poor	19	12	7	
Tumor size				0.033
≤4 cm	26	10	16	
>4 cm	21	14	7	
TNM Classification				0.009
I	7	4	3	
II	14	10	4	
III	19	10	9	
IV	7	5	2	
Lymph node status				<0.001
Negative	25	13	12	
Positive	22	13	9	

a: t-test was used to analyze the correlation between the expression of miRNAs and clinicopathological features of the patients.

herin, anti-Vimentin and anti-GAPDH) overnight at 4°C, followed by incubation with secondary antibodies (Merck, 1:5000) for 1.5 h at room temperature. Immunoreactive bands were detected using the ECL detection system; intensities of immunoreactive bands were quantified using the Quantity One software.

Cell viability assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay was performed to assess the relative number of viable cells. Cells (1×10^4/well) were seeded into 96-well plates and incubated for 0, 1, 2, 3, and 4 days. Afterwards, 20 μL of 5 mg/mL MTT were added per well for 4 h at 37°C. After careful removal of the supernatant, DMSO was added to dissolve the formazan crystals; absorbance was measured at 490 nm on a microplate reader (BioTEK Instruments, USA) at Growth curves were generated with time and absorbance on the horizontal and vertical axes, respectively.

Invasion assay

Transwell 24-well plates coated with diluted matrigel were used. After transfection, cells were appropriately seeded with serum-free media, with 5% fetal bovine serum (FBS) used as a chemoattractant. After 48 h of incubation at 37°C, the medium was removed, and chambers were washed twice with PBS. Non-invading cells in the upper chamber were gently removed; those migrated to the lower surface were fixed and stained with 0.1% crystal violet. Micrographs were obtained on an Axiovert 200 inverted microscope (Zeiss, Germany) at ×200 magnification.

Luciferase reporter assay

To assess whether miR-153-5p targets the 3'-UTR of WT1, the sequence containing the predicted binding sites was inserted into the pmirGLO plasmid (Promega, USA). The WT1 3'-UTR fragment containing putative binding sites for miR-153-5p was cloned downstream of the firefly luciferase coding region in the pmirGLO plasmid (Promega, USA), to obtain the wild type construct (WT). Mutations in binding sites were introduced by site-directed mutagenesis, and a mutant (Mut) construct was obtained. All constructs were verified by sequencing. For luciferase reporter assay, TE-1 cells were cultured in 96-well plates and co-transfected with 50 nmol/L of miR-153-5p mimic (or negative control) and 50 ng of luciferase reporter, using Lipofectamine 3000. After 48 h of transfection, luciferase activity was assessed in each sample with a luciferase assay kit (Promega).

Statistical analysis

Data are shown as median ± standard deviation. Group comparison was carried out by unpaired two-tailed Student t-test, using the

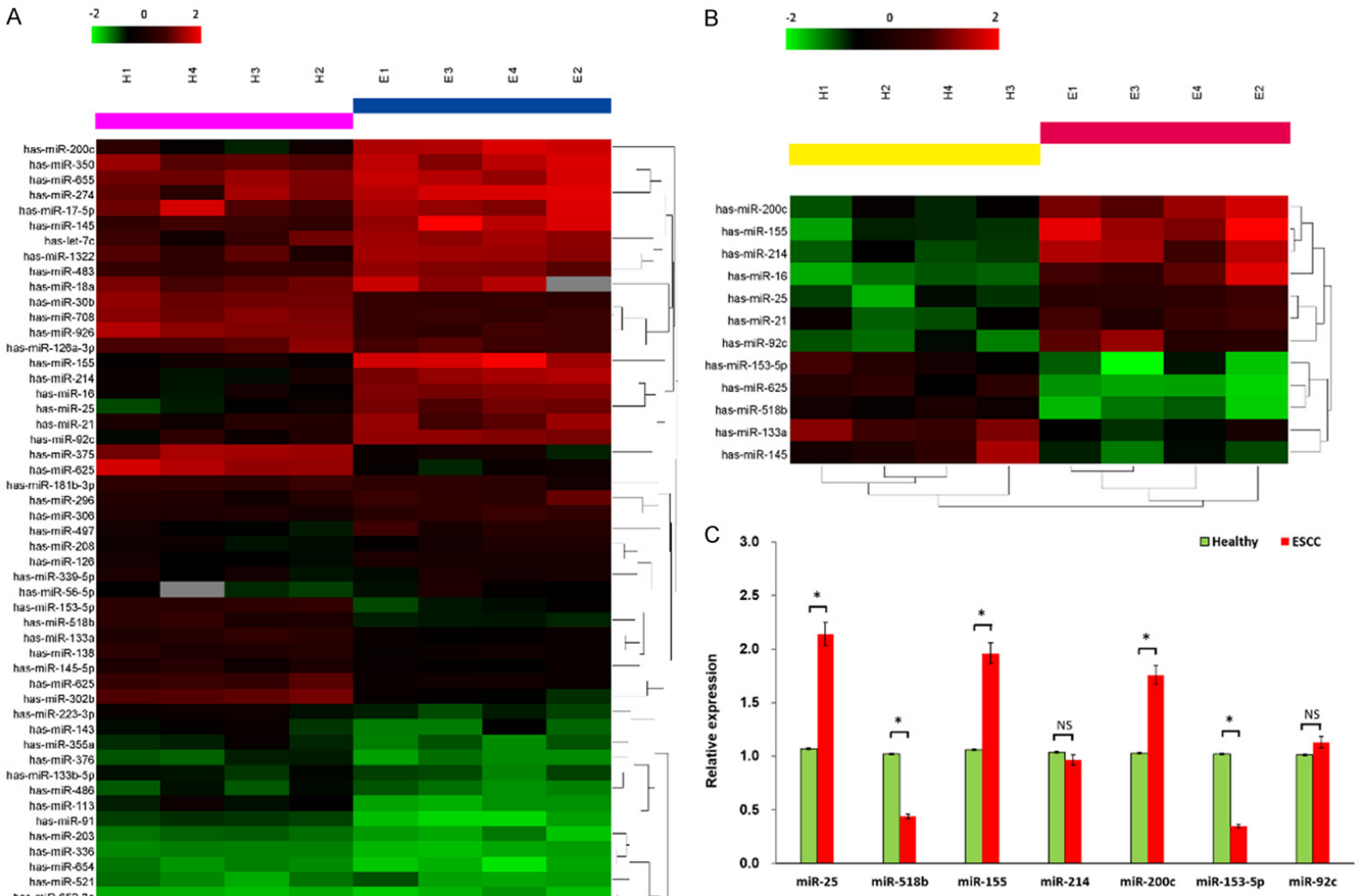

Figure 1. MiRNA profile of ESCC patients' serum samples. Each 4 serum samples were randomly chosen from healthy volunteers (indicates H1-4) and ESCC patients (indicates E1-4). A: Heat map of all miRNA expression differences between H and E serum samples included in the Sanger miRBase V18.0 database. B: Heat map summarizing the patterns of expression for 12 miRNAs whose expression was significantly ($P<0.05$ and foldchange >4) altered in two groups. C: RT-PCR of seven miRNAs expression in serum samples. The green bar means donwregulated, red part means upregulated.

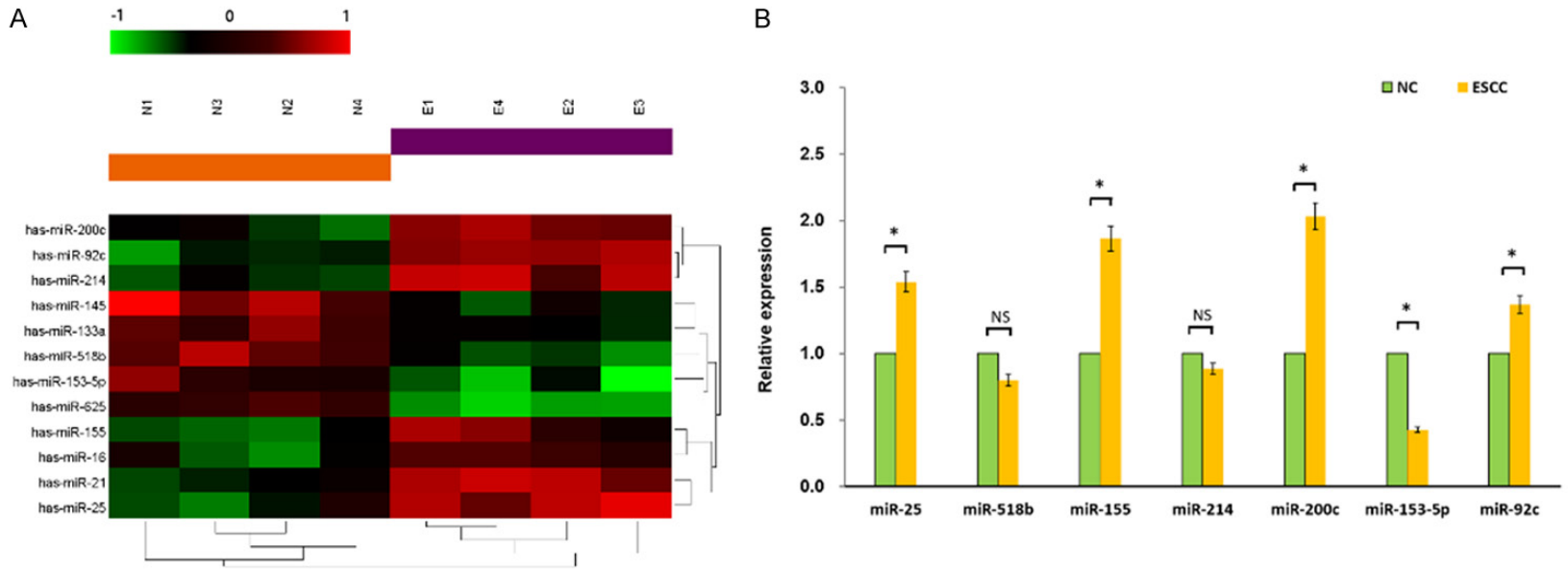

Figure 2. Expression of miRNAs in ESCC tissues. A: Heat map of 12 miRNAs whose expression was significantly ($P<0.05$ and foldchange >4) altered between ESCC tissues (indicates as E1-4) and corresponding normal tissues (indicated as N). B: RT-PCR of seven miRNAs expression in tissues samples.

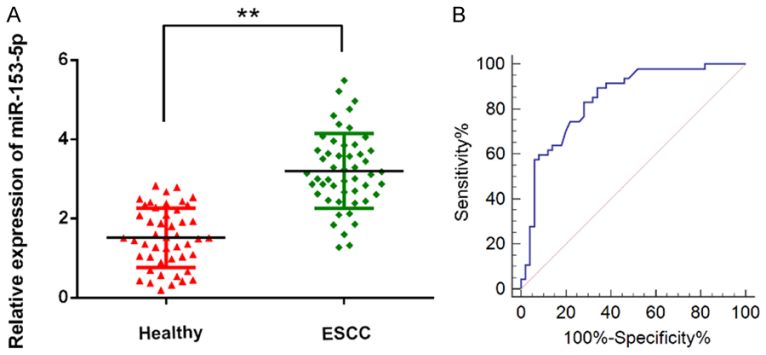

Figure 3. The expression of miR-153-5p and its ROC analysis on the diagnosis. A: Relative expression of miR-153-5p in groups of healthy individuals and ESCC. B: ROC analysis of miR-153-5p on the diagnosis of ESCC; AUC 0.84 (95% CI, 0.75-0.91), P<0.001; Cutoff value is 3.54, sensitivity 89.3% and specificity 66.0%. **P<0.01 vs healthy individuals.

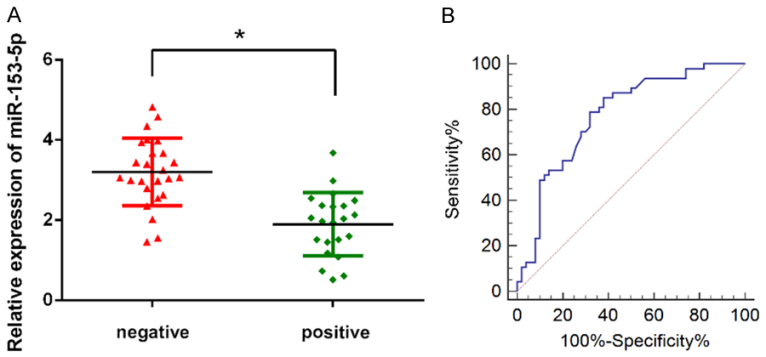

Figure 4. The expression of miR-153-5p and its ROC analysis in predicting lymph node metastasis. A: Relative expression of miR-153-5p in ESCC tissues with or without lymph node metastasis. B: ROC analysis on predicting lymph node metastasis: AUC, 0.77 (95% CI, 0.68-0.85), P<0.001; Cutoff value is 3.70, sensitivity 85.1% and specificity 62.2%.

SPSS20.0 software. P<0.05 was considered statistically significant.

Results

MiR-153-5p is specifically downregulated in human ESCC

To obtain the miRNA expression signature of ESCC, 4 serum samples from patients were randomly selected in the healthy and ESCC subject groups, respectively. Then, miRNA expression levels were evaluated by microRNA microarrays. Interestingly, 25 miRNAs exhibited significantly different expression levels (P<0.05 and fold change ≥2), including 13 upregulated miRNAs and 12 suppressed ones (**Figure 1A** and **Table 1**). MiR-153-5p was one of the most downregulated miRNAs in ESCC serum samples compared with healthy volunteer specimens. The twelve most aberrantly expressed miRNAs are shown in **Figure 1B**. To confirm the microarray findings, seven miRNAs, including miR-25, miR-518b, miR-155, miR-214, miR-200c, miR-92c and miR-153-5p, were assessed by qRT-PCR. Overall, the expression trends were consistent with microarray findings, although fold changes differed somewhat in magnitude between microarray and qRT-PCR data. In addition, the abnormal expression of miR-214 and miR-92c showed no statistical significance (*P* values of 0.574 and 0.601, respectively). Next, the above twelve miRNAs were assessed in ESCC and corresponding normal tissues. As shown in **Figure 2A**, these miRNAs showed levels consistent with serum findings. Meanwhile, qRT-PCR revealed seven miRNAs with different expression results between tissue samples. For example, MiR-92c was upregulated (P=0.037), while there was no sufficient evidence indicating miR-518b downregulation (P=0.542). Such discrepancy may be due to the small sample size. Therefore, we focused on miR-153-5p, which was the only miRNA with consistently significant downregulation.

Decreased miR-153-5p expression in ESCC is correlated with diagnosis and disease severity

To assess the value of serum miR-153-5p in ESCC diagnosis, miR-153-5p levels were compared between 50 healthy individuals and 47 ESCC patients. As showed in **Figure 3A**, miR-153-5p was decreased in the serum of ESCC patients compared with that in the healthy individuals. ROC curve analysis indicated an AUC value for miR-153-5p in diagnosing ESCC of 0.84 (95% CI, 0.75-0.91) (P<0.01); with a cut-off value of 3.54, sensitivity and specificity

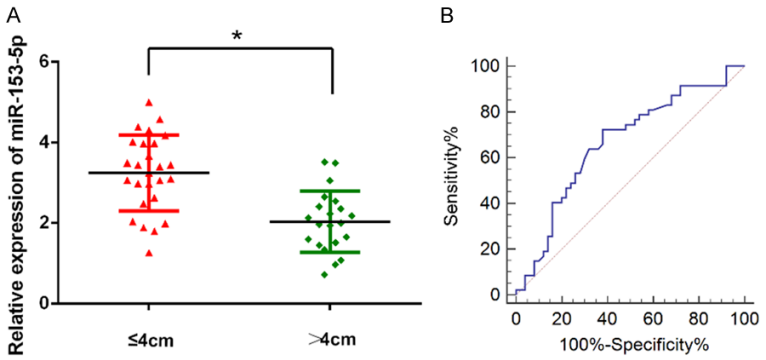

Figure 5. The expression of miR-153-5p and its ROC analysis on the prognosis of tumor size. A: Relative expression of miR-153-5p in ESCC tissues with different size of tumor. B: ROC analysis on predicting tumor size: AUC, 0.66 (95% CI, 0.56-0.75), P=0.037; Cutoff value is 3.93, sensitivity 71.1% and specificity 62.0%.

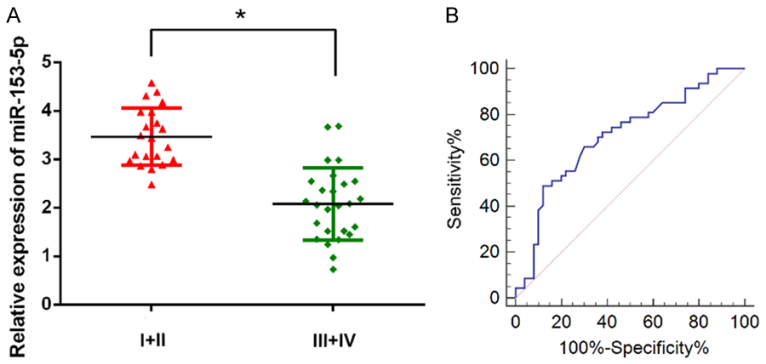

Figure 6. The expression of miR-153-5p and its ROC analysis on the prognosis of tumor staging. A: Relative expression of miR-153-5p in ESCC tissues with different TNM stage. B: ROC analysis on predicting tumor size: AUC, 0.70 (95% CI, 0.61-0.79), P<0.001; Cutoff value is 4.02, sensitivity 48.9% and specificity 88.0%.

were 89.3% and 66.0%, respectively (**Figure 3B**), suggesting that miR-153-5p could be favorably used as a biomarker for ESCC diagnosis. Then, ESCC patients were subdivided into two groups according to clinicopathological features, and ROC curve analysis was used to determine the associations of miR-153-5p with clinical indexes. The results indicated an AUC value for miR-153-5p of 0.77 (95% CI, 0.68-0.85) (P<0.001; **Figure 4**) in predicting lymph node metastasis, with a cut-off value of 3.70 and its sensitivity and specificity was 85.1% and 62.2%, respectively. Furthermore, for predicting tumor size and tumor stage, AUC values were 0.66 (95% CI, 0.56-0.75; P=0.037) (**Figure 5**) and 0.70 (95% CI, 0.61-0.79; P<0.001) (**Figure 6**), respectively. However, miR-153-5p expression showed no significant correlation with tumor differentiation or pathology type in ESCC patients. Detailed clinical characteristics of the patients are shown in **Table 3**. Taken together, miR-153-5p showed a good prospective application in evaluating ESCC severity.

WT1 is negatively correlated with miR-153-5p in ESCC tissues.

Since miR-153-5p plays an important role in ESCC patients, it is essential to elucidate its potential molecular mechanisms in this malignancy. Open access software programs, including TargetScan, miRBase, miRDB and starBase, were used to search for potential miR-153-5p targets. WT1 was identified as a candidate, and is known to be overexpressed in many cancers, with involvement in tumor progression. Therefore, miR-153-5p and WT1 mRNA levels were assessed in ESCC tissue samples using qRT-PCR. As expected, miR-153-5p levels in ESCC tissues were lower than those of the corresponding normal tissues; meanwhile, WT1 mRNA expression was significantly increased and negatively correlated with miR-153-5p levels (P<0.01) (**Figure 7A-C**). In addition, Western blot showed higher WT1 protein levels in cancer tissues compared with paired normal esophageal epithelial tissue specimens (**Figure 7D**). These data supported our hypotheses and required *in vitro* assays to further validate WT1 as a direct target of miR-153-5p.

MiR-153-5p suppresses TE-1 cell growth and invasion in vitro

Based on the above data, *in vitro* experiments were performed to explore the biological function of miR-153-5p. As shown in **Figure 8A**, miR-153-5p expression was significantly higher

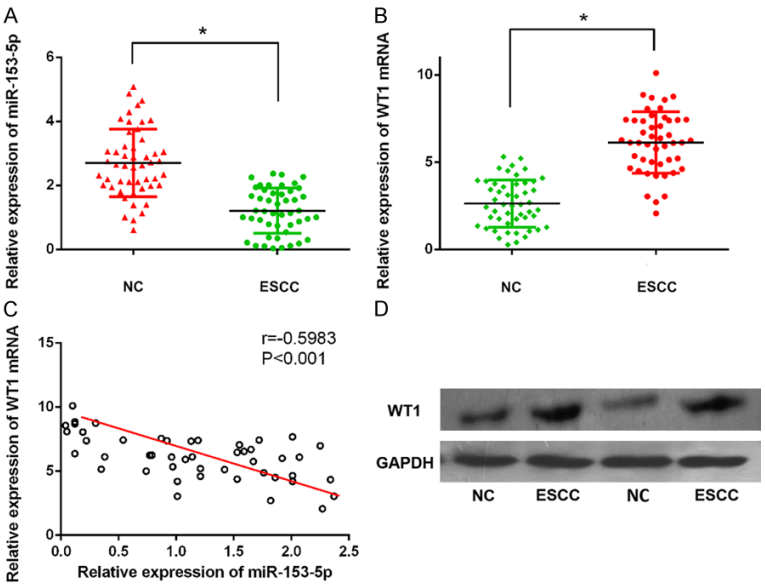

Figure 7. An inverse correlation between WT1 mRNA and miR-153-5p expression in NSCLC tissues. A: The expression of miR-153-5p was significantly decreased in ESCC tissue, compared with normal tissues (*P<0.01). B: The expression of WT mRNA was significantly increased in ESCC tissue (*P<0.01). C: Pearson correlation was used to analyze the relationship between miR-153-5p and WT1 mRNA. D: Western blot showed the expression of WT protein in ESCC tissues and corresponding normal tissues.

in normal esophageal epithelial cells (HEEC) compared with values obtained for the human esophageal cancer cell lines EC190, EC9706, SKGT-5 and TE-1 (P<0.01), as assessed by RT-PCR. Next, miR-153-5p mimics and the corresponding negative control were successfully transfected into TE-1 cells for subsequent studies. MiR-153-5p levels in TE-1 cells were significantly increased after transfection with miR-153-5p mimics (P<0.01) compared with the miR-NC group. Interestingly, TE-1 cell proliferation was suppressed after miR-153-5p overexpression (P<0.01, **Figure 8C**) (MTT assay); meanwhile, Transwell invasion assay indicated that cell invasion was starkly inhibited in the miR-153-5p mimics group (**Figure 8D**, P<0.01). Furthermore, Western blot showed altered expression of proliferation and invasion-related proteins (**Figure 8E**, **8F**). Taken together, these findings suggested that miR-153-5p inhibited TE-1 cell growth and invasion ability *in vitro*.

MiR-153-5p directly downregulates WT1 in ESCC

Because WT1 was negatively correlated with miR-153-5p in ESCC tissues, we explored whether miR-153-5p regulated WT1 through its mRNA's 3'UTR. WT1 expression was assessed in miR-153-5p mimics, miR-NC, and control groups by Western blot. Interestingly, lower WT1 protein levels were found in the miR-153-5p mimics group compared with the two other groups (**Figure 9A**, P<0.01). Then, WT1 mRNA amounts were evaluated by qRT-PCR in TE-1 cells. In agreement with Western blot data, WT1 mRNA levels were lower in the miR-153-5p overexpression group (P<0.01), as shown in **Figure 9B**. Furthermore, the complementary sequence of miR-153-5p was identified in the 3'-UTR of *WT1* mRNA (**Figure 9C**), and a mutant was generated as underlined in the sequence. A luciferase reporter assay was then performed to further validate the interaction of miR-153-5p with WT1. As shown in **Figure 9D**, luciferase activity was suppressed by miR-153-5p mimics in the WT but not MUT vectors (P<0.01). Taken together, these findings suggested that WT1 might be a target of miR-153-5p in TE-1 cells.

Discussion

In Asian countries, ESCC accounts for ~90% of esophageal carcinomas [28] and remains one of the most aggressive malignancies of the gastrointestinal tract. Recently, several meta-analyses showed that chemoradiotherapy plus surgery significantly reduces three-year mortality compared with surgery alone [29, 30]. However, a study found that only patients with esophageal adenocarcinoma (EAC) could benefit from neoadjuvant chemoradiotherapy, suggesting that distinct populations have different responses to neoadjuvant chemoradiotherapy, which may be associated with ethnic differences [31]. Therefore, it is important to identify biological factors involved in the malignant behavior of ESCC.

In recent years, several studies have shown that microRNAs (miRNAs), which are involved in the genesis and development of various can-

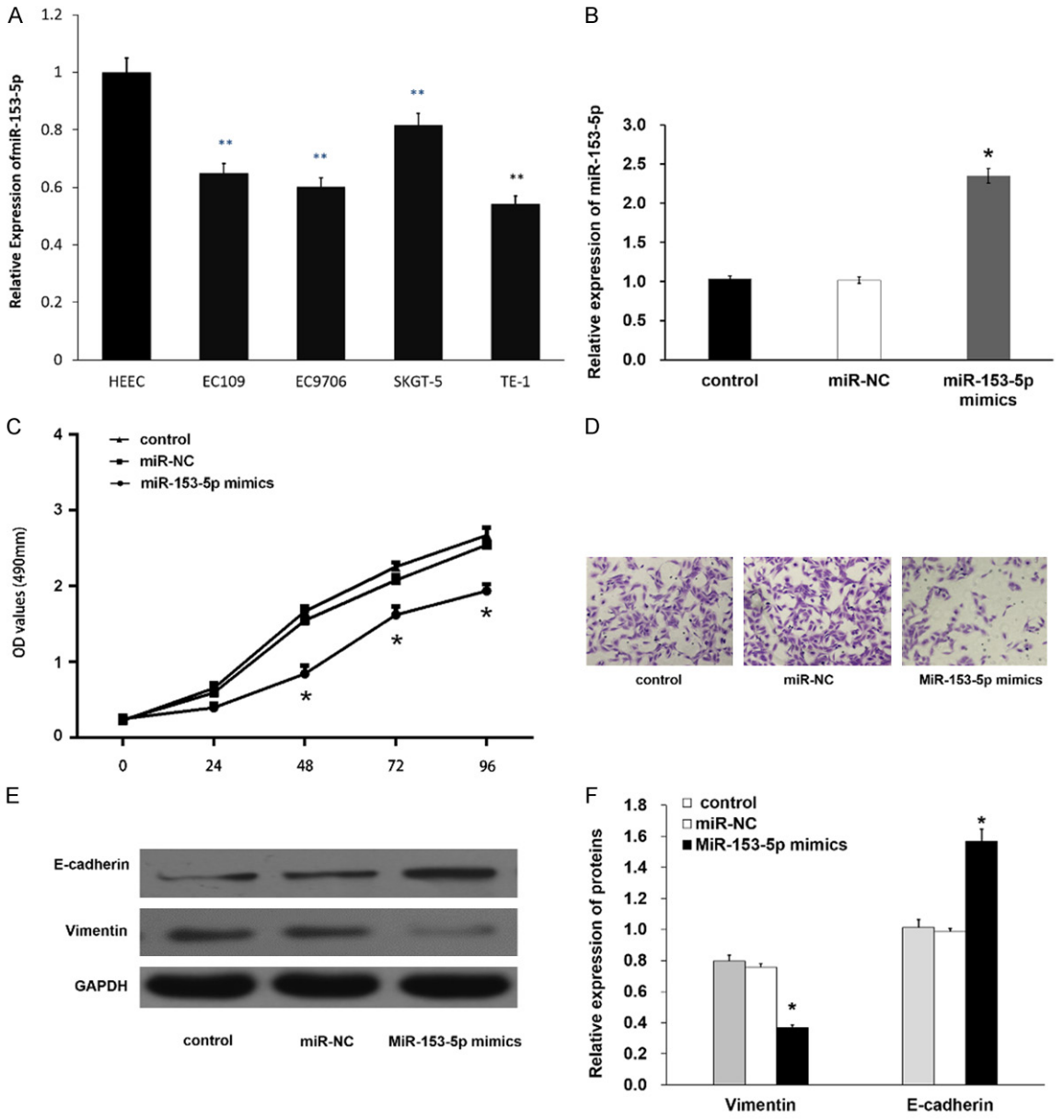

Figure 8. MiR-153-5p inhibited TE-1 cell proliferation and invasion *in vitro*. A: MiR-153-5p was significantly downregulated in different ESCC cell lines compared with normal human esophageal epithelial cells HEEC. B: MiR-153-5p was successfully transfected in TE-1 cells, tested by RT-PCR. C: MTT on TE-1 cells transduced with empty or miR-153-5p vectors. D: Transwell invasion assay was used to determined invasion ability of TE-1 cells in groups. E, F: Western blot showed the different expression of proliferation and invasion-related proteins Vimentin and E-cadherin, between three groups. *P<0.01 vs miR-NC, **P<0.01 vs HEEC.

cers [10], are detectable in plasma or serum [32, 33]. Indeed, growing evidence suggests that miRNAs could be used as novel biomarkers in cancer diagnosis [34], prognosis [35] and treatment [36, 37]. In addition, multiple studies reported that miRNAs dysregulate cell growth, invasion, metastasis, and apoptosis in ESCC [13, 38, 39]. A study indicated that miR-153-5p is noticeably downregulated in hepatocellular carcinoma (HCC) cell lines and tissues, and suppresses cell migration and invasion by binding to the 3'UTR of Snail mRNA [40]. Meanwhile, Xu *et al*. found differential expression of miR-153 in mesenchymal-like cells. After transfecting an miR-153 inhibitor into epithelial-like cells, mesenchymal phenotype

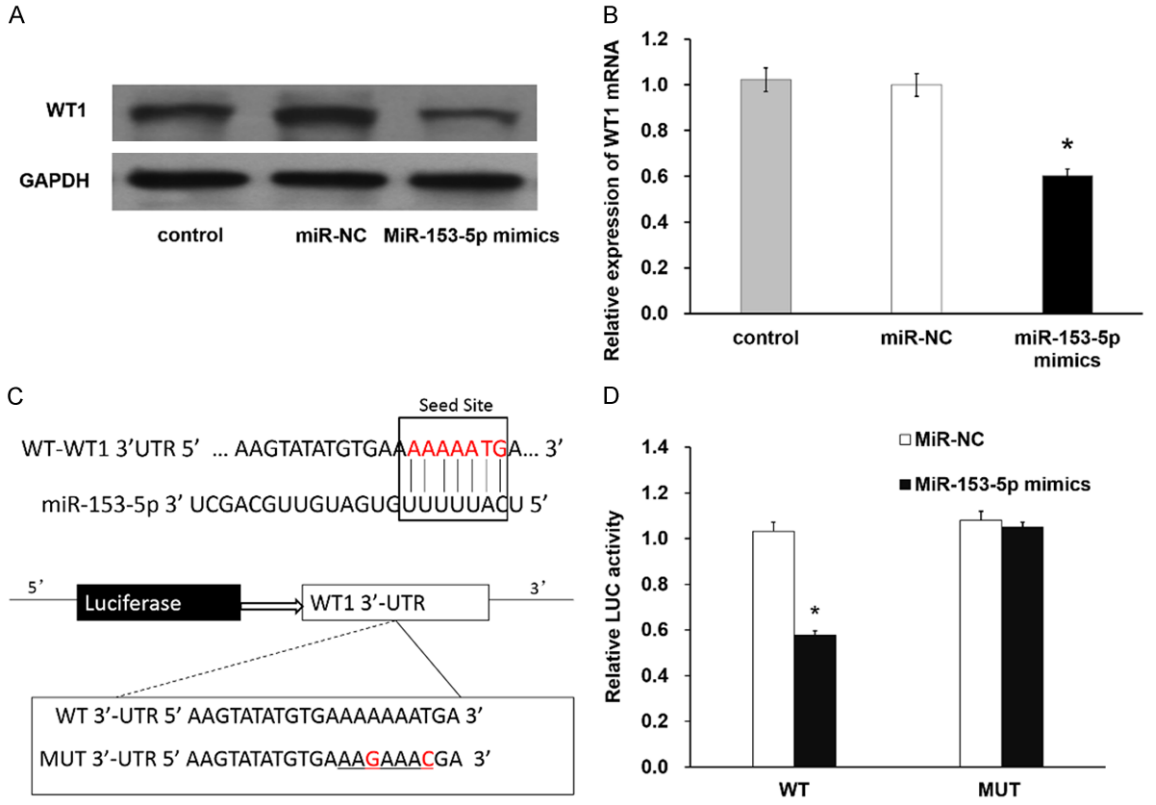

Figure 9. miR-153-5p directly targeted WT1 in TE-1 cells. A: Western blot showed the alteration expression of WT1 protein in groups. B: RT-PCR of WT1 expression in each group. WT1 was significantly suppressed in miR-153-5p mimics group ($P<0.01$). C: Predicted site that miR-153-5p binds the 3'-UTR of WT1 were shown. D: MiR-153-5p inhibited luciferase activity in the WT1 containing 3'UTR of WT binding sites, in contrast to 3'UTR of MUT binding sites in WT1 mRNA.

was acquired and ectopic expression of miR-153 significantly inhibited tumor cell metastasis formation by targeting SNAI1 and ZEB2 in epithelial cancer cells, suggesting its therapeutic value for reducing cancer metastasis [41]; low expression of miR-153 was then found to be significantly correlated with metastasis and poor prognosis in oral cancer patients [41]. Bai *et al.* assessed miR-153 expression levels in human pancreatic ductal adenocarcinoma cell lines and tissues, and found that decreased miR-153 expression inhibits cell migration and invasion by targeting SNAI1, and are closely associated with poor prognostic features and reduced long-term survival time in patients with pancreatic cancer [42]. What's more, Zhang *et al.* evaluated miR-153 expression in gastric cancer tissues, and demonstrated that low expression is prominently correlated with poor prognostic features and short 5-year survival. Meanwhile, *in vitro* experiments revealed miR-153 promotes gastric cancer cell migration and invasion, by inhibiting SNAI1-induced EMT [43]. However, the exact role of miR-153 in esophageal cancer remains undefined. In this study, 25 miRNAs showed differential expression in ESCC, of which 7 were validated by qRT-PCR. Among the latter, miR-153-5p was the only miRNA with consistently significant down-regulation. Therefore, its biological function in ESCC patients was assessed. We examined the clinical significance of miR-153-5p in esophageal cancer progression, and found it may serve as a biomarker for ESCC diagnosis. As shown above, miR-153-5p played a critical role in ESCC severity; besides, *in vitro* experiments demonstrated that miR-153-5p inhibited TE-1 cell proliferation and invasion.

Wilms' tumor suppressor gene 1 (WT1) encodes a protein with four zinc fingers, considered to be involved in transcriptional regulation of several genes, including platelet-derived growth factor A (PDGF-A) chain [44], macrophage colo-

ny stimulating factor-1 (CSF-1) [45], insulin-like growth factor (IGF)-II [46] and retinoic acid receptor (RAR)-α [47], as well as RNA metabolism [48]. In 2013, Xu *et al.* reported high WT1 levels in NSCLC tissues, with WT1 promoting NSCLC cell proliferation [49]. Besides, the therapeutic potential of intravenously infused WT1-specific CTL clones (TAK-1) was revealed; indeed, these clones could efficiently lyse human lung cancer cells in a HLA class I-restricted manner, suggesting WT1-targeted immunotherapy offers a potentially effective treatment option for lung cancer [50]. Importantly, Oji *et al.* found WT1 protein expression is gradually increased through sequential progression from mild to severe dysplasia, carcinoma *in situ* and invasive carcinoma, suggesting that the WT1 gene is involved in the growth of esophageal epithelial cells and plays an important role in ESCC tumorigenesis [51]. In the present study, WT1 mRNA levels were assessed in ESCC tissues, which showed high expression. These findings confirmed that WT1 was a *bona fide* target of miR-153-5p, which was further validated by Western blot, luciferase reporter assay, and RT-PCR, in ESCC cell lines. Our data indicated that WT1 downregulation was mediated by miR-153-5p through its binding to the 3'UTR of WT1 mRNA.

Overall, this study demonstrated that miR-153-5p may serve as a biomarker for ESCC diagnosis, and plays a vital role in ESCC severity. MiR-153-5p inhibits esophageal cancer cell proliferation and invasion by targeting WT1. A direct approach targeting the miR-153-5p/WT1 interaction may constitute a useful therapeutic alternative in ESCC.

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Disclosure of conflict of interest

None.

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References

[1] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015; 65: 5-29.

[2] Lin Y, Totsuka Y, He Y, Kikuchi S, Qiao Y, Ueda J, Wei W, Inoue M and Tanaka H. Epidemiology of esophageal cancer in Japan and China. J Epidemiol 2013; 23: 233-242.

[3] Wu PC and Posner MC. The role of surgery in the management of oesophageal cancer. Lancet Oncol 2003; 4: 481-488.

[4] Lee PC, Port JL, Paul S, Stiles BM and Altorki NK. Predictors of long-term survival after resection of esophageal carcinoma with nonregional nodal metastases. Ann Thorac Surg 2009; 88: 186-192; discussion 192-183.

[5] Cunningham D, Allum WH, Stenning SP, Thompson JN, Van de Velde CJ, Nicolson M, Scarffe JH, Lofts FJ, Falk SJ, Iveson TJ, Smith DB, Langley RE, Verma M, Weeden S, Chua YJ; MAGIC Trial Participants. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. N Engl J Med 2006; 355: 11-20.

[6] Burmeister BH, Smithers BM, Gebski V, Fitzgerald L, Simes RJ, Devitt P, Ackland S, Gotley DC, Joseph D, Millar J, North J, Walpole ET and Denham JW. Surgery alone versus chemoradiotherapy followed by surgery for resectable cancer of the oesophagus: a randomised controlled phase III trial. Lancet Oncol 2005; 6: 659-668.

[7] Meredith KL, Weber JM, Turaga KK, Siegel EM, McLoughlin J, Hoffe S, Marcovalerio M, Shah N, Kelley S and Karl R. Pathologic response after neoadjuvant therapy is the major determinant of survival in patients with esophageal cancer. Ann Surg Oncol 2010; 17: 1159-1167.

[8] Shukla GC, Singh J and Barik S. MicroRNAs: processing, maturation, target recognition and regulatory functions. Mol Cell Pharmacol 2011; 3: 83-92.

[9] Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A and Enright AJ. MiRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res 2006; 34: D140-144.

[10] Berindan-Neagoe I, Monroig Pdel C, Pasculli B and Calin GA. MicroRNAome genome: a treasure for cancer diagnosis and therapy. CA Cancer J Clin 2014; 64: 311-336.

[11] Song YX, Yue ZY, Wang ZN, Xu YY, Luo Y, Xu HM, Zhang X, Jiang L, Xing CZ and Zhang Y. MicroRNA-148b is frequently down-regulated in gastric cancer and acts as a tumor suppressor by inhibiting cell proliferation. Mol Cancer 2011; 10: 1.

[12] Liu N, Chen NY, Cui RX, Li WF, Li Y, Wei RR, Zhang MY, Sun Y, Huang BJ, Chen M, He QM, Jiang N, Chen L, Cho WC, Yun JP, Zeng J, Liu LZ,

Li L, Guo Y, Wang HY and Ma J. Prognostic value of a microRNA signature in nasopharyngeal carcinoma: a microRNA expression analysis. Lancet Oncol 2012; 13: 633-641.
[13] Wen SW, Zhang YF, Li Y, Liu ZX, Lv HL, Li ZH, Xu YZ, Zhu YG and Tian ZQ. Association of miR-21 with esophageal cancer prognosis: a meta-analysis. Genet Mol Res 2015; 14: 6578-6582.
[14] Ma WJ, Lv GD, Tuersun A, Liu Q, Liu H, Zheng ST, Huang CG, Feng JG, Wang X, Lin RY, Sheyhidin I and Lu XM. Role of microRNA-21 and effect on PTEN in Kazakh's esophageal squamous cell carcinoma. Mol Biol Rep 2011; 38: 3253-3260.
[15] Li C, Li DC, Che SS, Ma K, Wang YJ, Xia LH, Dai XM, Zhang GT, Shen Y, Jiao WJ and Tian KH. The decreased expression of miR-625 predicts poor prognosis of esophageal squamous cell carcinoma. Int J Clin Exp Med 2015; 8: 9560-9564.
[16] Wang Z, Qiao Q, Chen M, Li X, Wang Z, Liu C and Xie Z. miR-625 down-regulation promotes proliferation and invasion in esophageal cancer by targeting Sox2. FEBS Lett 2014; 588: 915-921.
[17] Ren LH, Chen WX, Li S, He XY, Zhang ZM, Li M, Cao RS, Hao B, Zhang HJ, Qiu HQ and Shi RH. MicroRNA-183 promotes proliferation and invasion in oesophageal squamous cell carcinoma by targeting programmed cell death 4. Br J Cancer 2014; 111: 2003-2013.
[18] Chen WJ, Zhang EN, Zhong ZK, Jiang MZ, Yang XF, Zhou DM and Wang XW. MicroRNA-153 expression and prognosis in non-small cell lung cancer. Int J Clin Exp Pathol 2015; 8: 8671-8675.
[19] Wang B, Teng Y and Liu Q. MicroRNA-153 regulates NRF2 expression and is associated with breast carcinogenesis. Clin Lab 2016; 62: 39-47.
[20] Ghasemi A, Fallah S and Ansari M. MiR-153 as a tumor suppressor in glioblastoma multiforme is downregulated by DNA methylation. Clin Lab 2016; 62: 573-580.
[21] Sun Y, Jia X, Hou L and Liu X. Screening of differently expressed miRNA and mRNA in prostate cancer by integrated analysis of transcription data. Urology 2016; 94: 313, e311-316.
[22] Bruening W, Gros P, Sato T, Stanimir J, Nakamura Y, Housman D and Pelletier J. Analysis of the 11p13 Wilms' tumor suppressor gene (WT1) in ovarian tumors. Cancer Invest 1993; 11: 393-399.
[23] Kumar-Singh S, Segers K, Rodeck U, Backhovens H, Bogers J, Weyler J, Van Broeckhoven C and Van Marck E. WT1 mutation in malignant mesothelioma and WT1 immunoreactivity in relation to p53 and growth factor receptor expression, cell-type transition, and prognosis. J Pathol 1997; 181: 67-74.
[24] Hayashi S, Oji Y, Kanai Y, Teramoto T, Kitaichi M, Kawaguchi T, Okada M, Sugiyama H and Matsumura A. Low Wilms' tumor gene expression in tumor tissues predicts poor prognosis in patients with non-small-cell lung cancer. Cancer Invest 2012; 30: 165-171.
[25] Oka Y, Tsuboi A, Elisseeva OA, Udaka K and Sugiyama H. WT1 as a novel target antigen for cancer immunotherapy. Curr Cancer Drug Targets 2002; 2: 45-54.
[26] Wu C, Zhu W, Qian J, He S, Wu C, Chen Y and Shu Y. WT1 promotes invasion of NSCLC via suppression of CDH1. J Thorac Oncol 2013; 8: 1163-1169.
[27] Rice TW, Blackstone EH and Rusch VW. 7th edition of the AJCC cancer staging manual: esophagus and esophagogastric junction. Ann Surg Oncol 2010; 17: 1721-1724.
[28] Hiyama T, Yoshihara M, Tanaka S and Chayama K. Genetic polymorphisms and esophageal cancer risk. Int J Cancer 2007; 121: 1643-1658.
[29] Fiorica F, Di Bona D, Schepis F, Licata A, Shahied L, Venturi A, Falchi AM, Craxi A and Camma C. Preoperative chemoradiotherapy for oesophageal cancer: a systematic review and meta-analysis. Gut 2004; 53: 925-930.
[30] Gebski V, Burmeister B, Smithers BM, Foo K, Zalcberg J and Simes J. Survival benefits from neoadjuvant chemoradiotherapy or chemotherapy in oesophageal carcinoma: a meta-analysis. Lancet Oncol 2007; 8: 226-234.
[31] Jin HL, Zhu H, Ling TS, Zhang HJ and Shi RH. Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: a meta-analysis. World J Gastroenterol 2009; 15: 5983-5991.
[32] Calin GA and Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006; 6: 857-866.
[33] Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB and Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008; 105: 10513-10518.
[34] Matikas A, Syrigos KN and Agelaki S. Circulating biomarkers in non-small-cell lung cancer: current status and future challenges. Clin Lung Cancer 2016; 17: 507-516.
[35] Krishnan P, Ghosh S, Wang B, Li D, Narasimhan A, Berendt R, Graham K, Mackey JR, Kovalchuk O and Damaraju S. Next generation sequencing profiling identifies miR-574-3p and miR-660-5p as potential novel prognostic mar-

kers for breast cancer. BMC Genomics 2015; 16: 735.
[36] Huang Q, Gumireddy K, Schrier M, le Sage C, Nagel R, Nair S, Egan DA, Li A, Huang G, Klein-Szanto AJ, Gimotty PA, Katsaros D, Coukos G, Zhang L, Pure E and Agami R. The microRNAs miR-373 and miR-520c promote tumour invasion and metastasis. Nat Cell Biol 2008; 10: 202-210.
[37] Chen X, Jiang Y, Huang Z, Li D, Chen X, Cao M, Meng Q, Pang H, Sun L, Zhao Y and Cai L. MiRNA-378 reverses chemoresistance to cisplatin in lung adenocarcinoma cells by targeting secreted clusterin. Sci Rep 2016; 6: 19455.
[38] Chen BB, Chen XB, Bie LY, Mu Y, Wang HL, Lv HF, Li N, Ma YJ, Ding ZD and Luo SX. Decreased expression of miR-542-3p exerts growth inhibitory functions in esophageal cancer. J Cancer Res Ther 2015; 11 Suppl 1: C24-28.
[39] Li J, Li X, Li Y, Yang H, Wang L, Qin Y, Liu H, Fu L and Guan XY. Cell-specific detection of miR-375 downregulation for predicting the prognosis of esophageal squamous cell carcinoma by miRNA in situ hybridization. PLoS One 2013; 8: e53582.
[40] Xia W, Ma X, Li X, Dong H, Yi J, Zeng W and Yang Z. miR-153 inhibits epithelial-to-mesenchymal transition in hepatocellular carcinoma by targeting snail. Oncol Rep 2015; 34: 655-662.
[41] Xu Q, Sun Q, Zhang J, Yu J, Chen W and Zhang Z. Downregulation of miR-153 contributes to epithelial-mesenchymal transition and tumor metastasis in human epithelial cancer. Carcinogenesis 2013; 34: 539-549.
[42] Bai Z, Sun J, Wang X, Wang H, Pei H and Zhang Z. MicroRNA-153 is a prognostic marker and inhibits cell migration and invasion by targeting SNAI1 in human pancreatic ductal adenocarcinoma. Oncol Rep 2015; 34: 595-602.
[43] Zhang Z, Sun J, Bai Z, Li H, He S, Chen R and Che X. MicroRNA-153 acts as a prognostic marker in gastric cancer and its role in cell migration and invasion. Onco Targets Ther 2015; 8: 357-364.
[44] Gashler AL, Bonthron DT, Madden SL, Rauscher FJ 3rd, Collins T and Sukhatme VP. Human platelet-derived growth factor A chain is transcriptionally repressed by the Wilms tumor suppressor WT1. Proc Natl Acad Sci U S A 1992; 89: 10984-10988.
[45] Harrington MA, Konicek B, Song A, Xia XL, Fredericks WJ and Rauscher FJ 3rd. Inhibition of colony-stimulating factor-1 promoter activity by the product of the Wilms' tumor locus. J Biol Chem 1993; 268: 21271-21275.
[46] Drummond IA, Madden SL, Rohwer-Nutter P, Bell GI, Sukhatme VP and Rauscher FJ 3rd. Repression of the insulin-like growth factor II gene by the Wilms tumor suppressor WT1. Science 1992; 257: 674-678.
[47] Goodyer P, Dehbi M, Torban E, Bruening W and Pelletier J. Repression of the retinoic acid receptor-alpha gene by the Wilms' tumor suppressor gene product, wt1. Oncogene 1995; 10: 1125-1129.
[48] Larsson SH, Charlieu JP, Miyagawa K, Engelkamp D, Rassoulzadegan M, Ross A, Cuzin F, van Heyningen V and Hastie ND. Subnuclear localization of WT1 in splicing or transcription factor domains is regulated by alternative splicing. Cell 1995; 81: 391-401.
[49] Xu C, Wu C, Xia Y, Zhong Z, Liu X, Xu J, Cui F, Chen B, Roe OD, Li A and Chen Y. WT1 promotes cell proliferation in non-small cell lung cancer cell lines through up-regulating cyclin D1 and p-pRb in vitro and in vivo. PLoS One 2013; 8: e68837.
[50] Makita M, Hiraki A, Azuma T, Tsuboi A, Oka Y, Sugiyama H, Fujita S, Tanimoto M, Harada M and Yasukawa M. Antilung cancer effect of WT1-specific cytotoxic T lymphocytes. Clin Cancer Res 2002; 8: 2626-2631.
[51] Oji Y, Yano M, Nakano Y, Abeno S, Nakatsuka S, Ikeba A, Yasuda T, Fujiwara Y, Takiguchi S, Yamamoto H, Fujita S, Kanato K, Ito K, Jomgeow T, Kawakami M, Tsuboi A, Shirakata T, Nishida S, Hosen N, Oka Y, Aozasa K, Monden M and Sugiyama H. Overexpression of the Wilms' tumor gene WT1 in esophageal cancer. Anticancer Res 2004; 24: 3103-3108.