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Original Article

miR-543 functions as a new marker of circulating tumorcells in gastric cancer patients

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Abstract: Currently, circulating tumor cells (CTCs) has received widespread attention in the non-invasive cancer diagnosis and monitoring field. Studies have confirmed the important roles of miR-543 in cancer occurrence including gastric cancer. This study we aimed to investigate whether miR-543 could be used as a marker for CTCs detection in gastric cancer patients. Previous study suggested the high expression of miR-543 in gastric cancer tissue and cells, in the present study; we first confirmed the role of miR-543 in gastric cancer progress by using miR-543 inhibitor. CCk-8 and transwell assays were applied for cell proliferation, cell invasion and migration detection respectively. In addition, we determined the expression level of miR-543 in mononuclear cells (MNCs) from peripheral blood by using quantitative real-timepolymerase chain reaction (qRT-PCR). Moreover, the receiver operator characteristic curves (ROC) were constructed. Our results suggested that inhibition of miR-543 notably suppressed gastric cancer cell growth, invasion and migration. Compared with the healthy controls, the expression level of miR-543 significantly increased in MNCs from gastric cancer. The area under the ROC curve was 0.796 ± 0.0763. In conclusion, our results indicated that miR-543 may be used as a novel bio-marker for CTCs detection in gastric cancer patients.

Keywords: miR-543, gastric cancer, circulating tumor cells, gene diagnosis, cancer growth

Introduction

Gastric cancer, the second leading cause of cancer-relateddeaths in the world, seriously threaten people's health and aggravate people's economic burden, is still a global problem [1-3]. Due to poor diagnosis, many patients are diagnosed as advanced gastric cancer characterized by extensive invasion andlymphatic metastasis [4, 5]. At present, surgery combined with chemotherapy and chemo-radiation is still the only effective therapy for gastric cancer treatment [6]. Recent years, although many progresses havebeen made in gastric cancer treatment, the prognosis ofgastric cancer patients is still very poor [7]. Thus, it is of great importance to find novel diagnostic markers for early stage and effective therapeutic targets for gastric cancer treatment.

MicroRNAs (miRNAs) are a group of non-coding RNAs that play critical roles in regulating gene expression bybinding to the 3' untranslated region of the target genes [8, 9]. Evidence hasstrongly indicated that miRNAs directly or indirectly impacton a variety of biological progresses, including cell proliferation, apoptosis, invasion and migration [10, 11]. Recently, studies have indicated that miRNAs may play important roles in the diagnosis and treatment of diseases [12-14].

miR-543 is currently a hot researched miRNA. Sun et al. suggest that miR-543 act as a tumor promoter in colorectal cancer progress [15]. In hepatoma carcinoma cells, miR-543 modulates cell proliferation, invasion [16]. Li et al. Demonstrated that miR-543 expression was closely related to tumor size, clinical grade, TNM stage and lymph node metastasis in gastric cancer patients [17].

Detection of circulating tumor cells (CTCs) in peripheral blood of solid cancerpatients has a very important clinical significance [18]. Since miR-543 has oncogenic property, we wonderwhether it can be a marker for detecting CTCs for gastriccancer patients.

Materials and methods

Cell line and cell culture

The human gastric cancer cell line MGC-803 was obtainedfrom Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cellswere routinely cultured in Dulbecco's Modified Eagle Medium(Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 1% penicillin and streptomycin combination at 37°C ina humidified atmosphere of 5% CO_2.

Transfection of miR-543 inhibitor

The day before the transfection, MGC-803 cells were seeded in 6-well plates. Whenthe cellsreached 60-70% confluence, the cells were transfected with miR-543 inhibitor or its control with 30 μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in line with the manufacturer's instructions. 48 h after transfection, cell samples were collected for following analysis.

CCK-8

We detected MGC-803 cell proliferation ability by using CCK-8 kit according to the manufacturer's protocol. At 48 h post-transfection with miR-543 inhibitor or its control, MGC-803 cells were re-suspended and then re-seededinto 96-well plate (3×10^3 cells per well), then, 20 μl CCK-8 (5 mg/ml, Sigma, USA) was added to each well and incubated at 37°C for 4 h. We determined the optical densities (OD) (490 nm) by using a spectrophotometer. Experiments were performed in triplicate.

Cell migration and invasion assay

The migration and invasion ability of MGC-803 cells were detected in 24-well transwell plates (Corning Costar, MA). For cell migration assay, 48 h after transfection, MGC-803 cells were trypsinized and re-suspended in serum-free medium and then re-seeded into the upper chamber (1×10^5 cells per well), and the cell culture medium supplemented with 10% FBS was added into the lower chambers. 24 h after incubation at 37°C, cells in the upper chambers were removed and cells in the under chambers were cleaned, fixed and then stained with hematine. For the invasion assay, chamber inserts were paved with matrigel (BD Biosciences, CA). Tests were performed in triple.

Patients and specimens

The present study was approved by the Ethics Committee of Shanghai Tongren Hospital. A total of 30 cases of peripheral blood samples (2 ml per individual) from preoperative gastric cancer patients as well as 30 cases of peripheral blood samples from 30 healthy volunteers were collected at Shanghai Tongren Hospital. The inclusion criteria was executed as previously described [19, 20].

Total RNA preparation

MNCs were collected from the peripheral blood of patients as described previously [21]. Total RNA from MNCs was extracted by using TRIZOL reagent (Takara, Japan) according to the manufacturer's protocol.

The positive control for CTCs detection was gastriccancer SGC-7901 cells added with standard blood samples fromhealthy volunteers. The effectiveness of theexperimental procedure for enriching CTCs from bloodsamples was assessed by immunohistochemistry using cytokeratin 18 (CK18) and CK20 as gastric cancer-associatedepithelial markers [22].

Detection of miR-543 levels by qRT-PCR

For miRNA detection, we performed qRT-PCR. RT reactions were performed by using TaqMan microRNA Reverse Transcription Kit following the manufacturer's instructions. Real-time PCR was performed by using QuantiTect SYBR Green PCR kit (Qiagen, USA) in line with the manufacturer's instructions. Amplification conditions were 95°C for 10 min, 38 cycles at 95°C for 15 s, and 72°C for 30 s. U6 was used an the internal control. Tests were performed in triplicate. $2^{-\Delta Ct}$ method was used for relative gene expression calculation. The primers for real-time PCR were as shown in **Table 1**.

Detection of CEA levels

An Elecsys 2010 machine (Roche Diagnostics, Basel, Switzerland) was performed to determine serum carcinoembryonic antigen (CEA). 5 μg/l was the cut-off concentration for abnormal and normal identification [23].

Table 1. Primer sequences for real-time PCR

Gene Primer	Sequence (5'-3')
miR-543-F	GGGCAGACCTGTTAGATGTCTCC
miR-543-R	GGGTCAATGCAATCAAAGCACAC
U6-F	GCTTCGGCAGCACATATACTAAAAT
U6-R	CGCTTCACGAATTTGCGTGTCAT3

All primers were obtained from the Gen Script (Nanjing) Co., Ltd (Nanjing, China). miR, microRNA; F, forward; R, reverse.

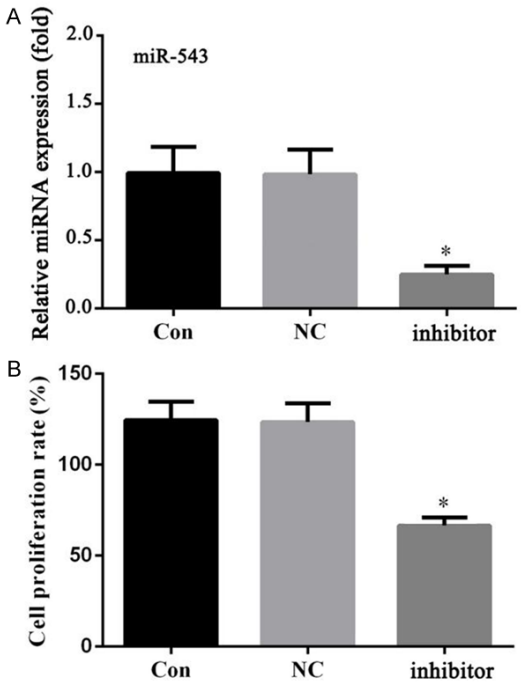

Figure 1. miR-543 inhibition suppresses MGC-803 cell proliferation. A: miR-543 was inhibited by using miR-543 inhibitor, and the inhibition expression of miR-543 was verified by qRT-PCR; B: 48 h after MGC-803 cells were transected with miR-543 inhibitor, cell proliferation was detected by CCK-8 assay. *P<0.05. All data are presented as the mean ± SD of three independent experiments.

Statistical analysis

All statistical analyses were performed by using SPSS 17.0 statistical software (SPSS, Chicago, IL, United States). A level of P<0.05 wasconsidered statistically significant. Receiver operator characteristic (ROC) curves wereconstructed for differentiating gastric cancer cases fromhealthy volunteers [22]. Due to the area under the ROCcurve (AUC) value, the diagnostic value of miR-543 for CTCs detection of gastric cancerpatientswas determined.

Results

Tumor cell growth was significantly suppressed by miR-543 inhibitor

Studies have indicated that miR-543 was upregulated in gastric cancer tissues and cells and acted as a tumor promoter gene. In our present study, to investigate the effect of miR-543 inhibitor on tumor cell growth, we performed CCK-8 assay. Our results suggested that compared with the control, miR-543 inhibitor significantly inhibited tumor cell proliferation (**Figure 1**).

Tumor cell migration and invasion ability was significantly suppressed by miR-543 inhibitor

To investigate the effect of miR-543 inhibitor on tumor cell migration and invasion, transwell assay was performed. 48 h after MGC-803 cells were transfected with miR-543 inhibitor or its control, trans well assay was performed. As shown in **Figure 2**, compared with the control, miR-543 inhibitor significantly inhibited MGC-803 cell migration and invasion ability.

miR-543 expression levels in MNCs from peripheral blood of gastric cancer patients were higher than thosefrom healthy volunteers

To determine the expression level of miR-543 in MNCs from peripheral blood ofgastric cancerpatients, qRT-PCR was performed. As shown in **Figure 3**, the expression level of miR-543 in MNCs from peripheral blood ofgastric cancerpatients was significantly higher than thatfrom the healthy volunteers. Based on the results, the expression of miR-543 was high in MNCs from peripheral blood of gastric cancer patients

Relationship of miR-543 level and clinicopathological factors in gastric cancer patients

Further, we investigate the relationship of miR-543 levels in MNCs with clinicopathological factors of gastric cancer patients. Our results indicated that the expression level of miR-543 was notably associated with tumor size, TNM stage while not significantly associated with other clinicopathological features, including gender, age and differentiation (**Table 2**).

Diagnostic efficiency of miR-543 in the detection of CTCs in patients with gastric cancer

To assess the clinical value of miR-543 as a CTCmarker, the AUC value was detected (**Figure**

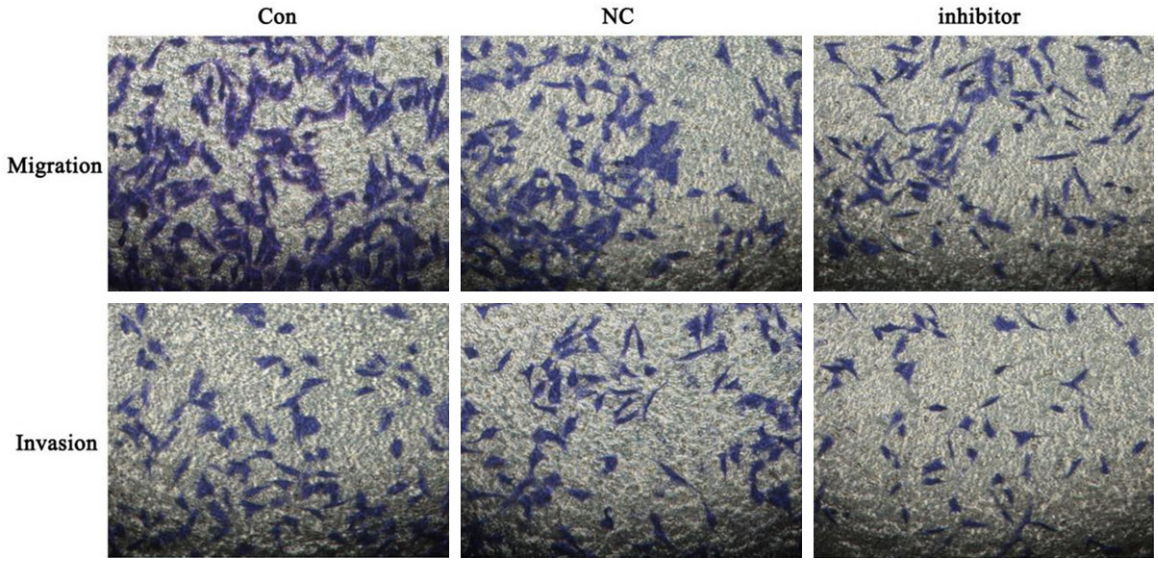

Figure 2. miR-543 inhibition suppresses MGC-803 cell migration and invasion. MGC-803 cells were transected with miR-543 inhibitor or its negative control, respectively. 48 h after the transfection, cell migration and invasion were detected by trans well assay.

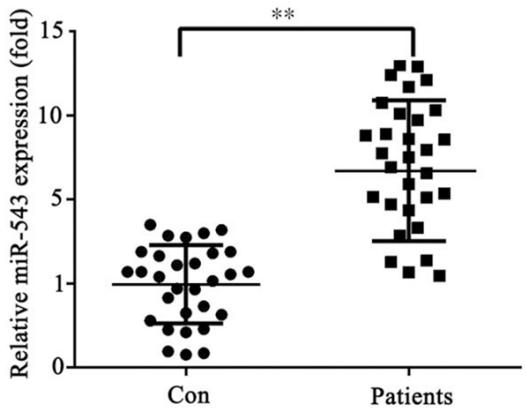

Figure 3. Levels of miR-543 in MNCs from peripheral blood of patients with gastric cancer. miR-543 expression levels in MNCs from peripheral blood of patients with gastric cancer and from the healthy volunteers were detected by qRT-PCR. *P<0.01. All data are presented as the mean ± SD of three independent experiments.

4). The data suggested that the AUC was 0.796 ± 0.0763 (P<0.0001), indicating it might be useful in clinical diagnosis. The 95% confidence interval was 0.767 to 0.939. The criterion value (cutoff value) was 7.14 with a sensitivity of 95.11%, and the specificity was 63.52%. Accordingto this cut-off value, the positive detection rate of patients with gastric cancer was 73.01%. However, when CEA was used as a marker, the positive detection ratewas only 23.81%.

Discussion

To date, the function of miR-543 is still poorly understood. Bing et al. Reported that miR-543 was down-regulated in endometrial cancer and served as a tumor suppressor [24]. Zhang et al. suggested that miR-543 functions as a promoter in non-small cell lung cancer cell proliferation and invasion [25]. Ectopic expression of miR-543 suppressed the proliferation and metastasis of CRC cells in vitro and in vivo, suggesting it may serve as a novel diagnostic and prognostic biomarker for CRC metastasis [26]. Previous studies indicated that miR-543 was up-regulated in gastric cancer tissues and it could promote gastric cancer cell proliferation via the regulation of SIRT1.

In the present study, we investigated the role of miR-543 in the development of gastric cancer by using miR-543 inhibitor, and the results suggested that miR-543 inhibition could suppress gastric cancer cell proliferation, migration and invasion, indicating the tumor promoter role of miR-543 in gastric cancer. We next measured the level of miR-543 in mononuclear cells (MNCs) from peripheral blood of the gastric cancer patients, and our results showed a significantly higher level of miR-543 in MNCs from peripheral blood of the gastric cancer patients compared with the healthy controls. These data indicate that miR-543 detection in MNCs fromperipheral blood may havedi-

Table 2. Association of miR-543 expression with clinicopathological features

Clinicopathological features	N	miR-543 mean (SD)	*p* value
Gender			>0.05
Male	20	105.1 (13.2)	
Femal	10	92.3 (24.6)	
Age (years)			>0.05
<55	12	101.2 (13.9)	
≥55	18	109 (20.7)	
Tumor size (cm)			<0.05
<2	13	70.3 (15.4)	
≥2	17	113.8 (20.7)	
TNM stage			<0.05
I-III	20	81.3 (14.2)	
IV	10	119.1 (15.9)	
Differentiation degree			>0.05
Low	14	80.4 (18.6)	
High	16	103.9 (21.4)	

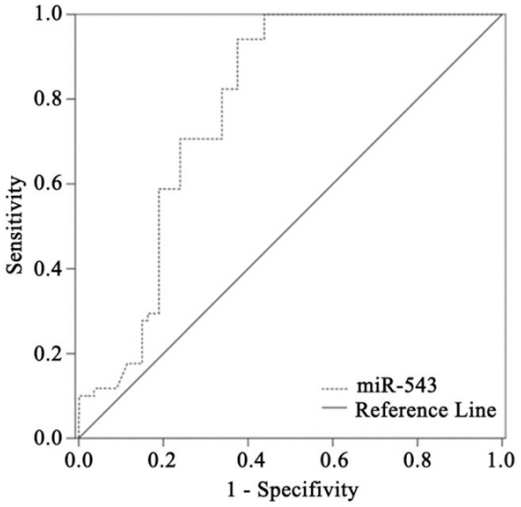

Figure 4. Receiver operation characteristic (ROC) curve. The clinical values were assessed by differentiating 30 preoperative gastric cancer patients from 30 healthy volunteers. The AUC is 0.796 ± 0.0763.

agnostic values forgastric cancerpatients. In order tofurther confirmour view, the relationshipsbetween the expression levels of miR-543 in MNCs from peripheral blood of the gastric cancer patientsandclinicopathological factors were analyzed. And we found that the level of miR-543 was notably associated with tumor size and TNM stage of gastric cancer patients.

Currently, the routine diagnostic methods of gastric cancer such as gastroenterological endoscopy still have their inevitable limitations, for example that tissue biopsy will bring pain and mental torture to patients [27]. Biomarkers which can be extracted without pain aremore acceptable by patients. Detection of peripheral blood seems to be a potential choice. Studies have confirmed the feasibility of miRNAs for the diagnosis of gastric cancer [28]. MiRNAs in CTCs, whose presence reflectthe potential tumor metastasis, arefrom living cells. Hiraiwa et al. [29]. have verified the clinical significanceof CTCs in patients with gastrointestinal cancers. However, the low sensitivity of the CTCs detection is still unsolved. Our results showed thatthe expression levels of miR-543 in MNCs from peripheral blood of the gastric cancer patients were notably higher than thatfrom the healthy control. The positive detection rate of miR-543 in gastric cancer patients was 73.01%, while the positive detection rate of CEA was only 23.81%. These results indicated that miR-543 may be a better choicefor the detection of CTCs.

In summary, miR-543 inhibition ingastric cancer cells significantly inhibitscell proliferation migration and invasion, indicatingthe oncogenic role of miR-543. Detection of miR-543 in MNCs from peripheral blood of gastric cancer patients may be a novel method for detecting CTCs in gastric cancer patients.

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Disclosure of conflict of interest

None.

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