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Original Article

Hypermethylation and low expression of miR-203 in patients with esophageal cancer in Chinese population

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Abstract: The expression of miR-203 has been reported to be significantly down-regulated in esophageal cancer and promoter methylation appeared to mediate it. The purpose of this study was to investigate the relationship between decreased expression of miR-203 and the hypermethylation of promoter gene and evaluate their diagnostic and-prognostic potential inesophageal cancer. Real-time quantitative PCR and methylation-specific PCR were performed to detect the expression levels and methylation status of miR-203 in 50 pairs of esophageal cancer tissues and their corresponding non-tumor adjacent tissues. And we also investigated the association of miR-203 expression and methylation with clinicopathological parameters and survival times. The expression levels of miR-203 were significantly lower in esophageal cancer tissues compared to non-tumor counterparts (P<0.001), lower expression of miR-203 tended to have increased pTNM stage (P=0.0213); MiR-203 is more frequently methylated in esophageal cancer [36/50 (72%)] than in controls [13/50 (26%)] (P<0.01), mir-203 hypermethylation status was found to be closely correlated with pTNM stage (P=0.0218); In addition, Promoter methylation of mir-203 was significantly correlated with the low of mir-203 expression (P<0.001). Furthermore, Multivariate Cox regression models confirmed miR-203 methylation status was an important significant predictor for total survival. Our findings suggest that miR-203 is involved in the etiology ofesophageal cancer and that hypermethylated miR-203 is a potential biomarker for esophageal cancer diagnosis and prognosis. Moreover, targeting miR-203 methylation by demethylating agents may offer a novel strategy for anticancer therapy of esophageal cancer.

Keywords: MicroRNA-203, esophageal squamous cell carcinoma (ESCC), expression, hypermethylation, overall survival (OS)

Introduction

According to the World Health Organization, esophageal cancer is the sixth most common malignancy worldwide [1]. Regarding to different etiologic and pathologic characteristics two main histological types have been identified, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). EAC is common in western countries while ESCC is frequent in east Asia, especially in China whose incidence in the high-risk northern and central China exceeds 100 cases per 100,000 people per year [2]. Although the treatments have made great progress, the prognosis for patients with advanced disease still remains poor and unsatisfactory [3]. Potentially curative treatments are followed by high rates of disease recurrence. All these facts together clearly indicate the need for a molecular biomarker for esophageal cancer enabling earlier detection and prognostic stratification.

MicroRNA (miRNA), a class of small regulatory RNA molecules, acts as tumor suppressors and oncogenes by negatively regulating their mRNA targets in a sequence specific manner through post-transcriptional repression and influencing the proliferation and cell cycle progression, apoptosis, invasion and metastasis of cancer [4]. The altered expression of miRNAs in ESCC has been well documented, supporting their function as tumor suppressors or oncogenes [5]. DNA hypermethylation in the miRNA

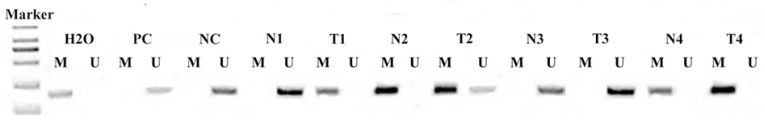

Figure 1. Representative results of the MSP analysis for miR-203 in selected four pairs of samples, respectively. U, unmethylated; M, methylated; NC, negative control (completely unmethylated DNA); PC, positive control (completely methylated DNA); T, tumor tissue; N, adjacent non-tumor tissue.

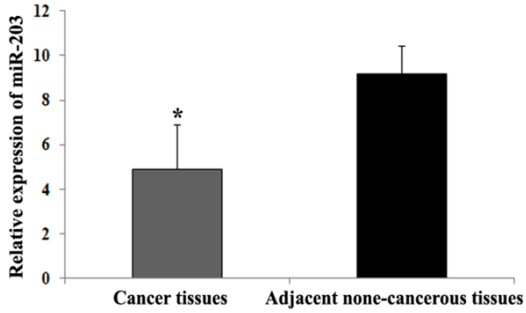

Figure 2. Expression levels of miR-203 in ESCC and adjacent non-cancerous tissues. The expression level of miR-203 was significantly lower in ESCC tissues than in adjacent non-cancerous tissues ($P<0.001$).

5' regulatory region is a crucial mechanism that can account for the downregulation of miRNAs in human tumors, and 10% of miRNAs are likely regulated epigenetically through DNA methylation [6]. Chen et al. have reported that CpG island methylation of miR-34a, miR-34b/c, and miR-129-2 are frequent events, and that this is a crucial mechanism underlying their low expression in ESCC [7]. Epigenetic silencing of miR-375 has also been reported in ESCC [8].

MiR-203 locates at chromosome 14q32-33 and has been identifiedas a stemness-inhibiting miRNA that induces epidermal differentiation by restricting proliferative potential and targeting the stemness-related transcription factor ΔNp63 in esophageal cancer cells [9, 10]. MiR-203 was reported to be downregulated in ESCC and epigenetically silenced in hematopoietic tumor [11, 12], yet the correlation between downregulation/loss of miR-203 expression and promoter methylation in ESCC was not clean. The purpose of this study was to identify the methylation status of miR-203 and to determine whether CpG island methylation is involved in its dysregulation in ESCC. Postoperative esophageal cancer patients enrolled in this study were followed up with regular visits to our hospital, and overall survival rates were recorded.

Materials and methods

Tissue samples

Between October 2010 and May 2011, 50 pairs of esophageal cancer tissues and their corresponding non-tumor adjacent tissues, matched blood samples were obtained from patients who underwent radical resection at the Department of Surgery of Suzhou University Affiliated Changzhou tumor Hospital were enrolled in this study. Fresh samples were snap-frozen, put in liquid nitrogen immediately after surgery, and were stored at -80°C until used. None of the patients received preoperative chemotherapy and/or radiation therapy. Patients whose surgical tissue was studied gave their written informed consent and subsequent analysis. Histological studies were also performed, and all the specimens were characterized as ESCC. Bioethics committee of the institution has approved the study. The histological types and grades of tumor were classified according to WHO criteria. Survival was calculated from the date of operation to the date of the latest follow-up visit or death due to recurrent esophageal cancer. The median follow-up period was 11 months (range, 2-41 months).

Real-time PCR assay

Real-time quantitative RT-PCR for miRNA was performed to detect the expression levels of miR-203 in 50 ESCC tissues and adjacent non-cancerous tissues. Total RNA was extracted from tissue samples with TRIzol (Invitrogen, CA, USA) according to the manufacturer's protocol. and total RNA was reversely-transcribed using the One Step PrimeScript R miRNA cDNA Synthesis Kit (TAKARA, Dalian, China). Quantitative RT-PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems, CA, USA). Each sample was examined in triplicate. The U6 RNA was amplified as an internal control. The primers were as follows: miR-203 F: 5'-ACA CTC CAG CTG GCG TGA AAT GTT TAG GAC CA-3', R: 5'-CTC AAC TGG TGT CGT GGA-3'; U6 F: 5'-CTC GCT TCG GCA GCA CA-3', R: 5'-AAC GCT TCA CGA ATT TGC GT-3'.

Table 1. Association between the expression of miR-203 with clinicopathological features in patients with ESCC

Characteristics	No. of cases tested	miR-203 expression		*p* value
	n=50	Low (n, %)	High (n, %)	
Sex				
Male	30	17	13	
Female	20	10	10	0.2023
Age				
≤64	23	12	11	
>64	27	15	12	0.2295
pTNM stage				
I	14	4	10	
II	10	3	7	
III	26	20	6	0.0213
Tumor size (cm)				
<4	18	8	10	
≥4	32	19	13	0.1418
Tumor grade				
G1	6	3	3	
G2	32	14	18	
G3	12	10	2	0.0723
Invasion into lymphatic vessels				
Negative	31	18	13	
Positive	19	9	10	0.1815

DNA extraction and promoter methylation analysis

Genomic DNA of esophageal cancer and control tissueswas extracted using thetissue DNA kit (TIANGEN, China), and then dissolved in TE bufferand stored at -20°C. Promoter methylation of miR-203 gene was determined by methylation-specific PCR (MS-PCR). Genomic DNA from carcinoma specimens was subjected to bisulfite modifications using EZ DNA Methylation-GoldTM kit (Zymo Research, USA) according to manufacturer's protocol. Then transformed DNA was PCR-amplified using the TaKaRa rTaq Kit (TaKaRa, China). Primers for unmethylated hsa-mir-203 were F: 5'-GGGTTGTGGAGGATTAGTT-3', and R: 5'-AAACAACTAAACTCCAAACA-3', and Primers for methylated hsa-mir-203 were F: 5'-GGGTCGTGGAGGATTAGTC-3' and R 5'-AAACGACTAAACTCCGAACG-3' (same as Bueno) [12]. DNA from GenoMethtm Universal Methylated DNA Standard was used as a positive control for methylated alleles. A water blank and unmodified DNA were used as negative controls with every batch reaction. The PCR for all samples demonstrating methylation for individual genes was repeated to confirm these results (**Figure 1**).

Statistical analysis

The method of $2^{-\Delta\Delta ct}$ was used to analyze the results of RT-PCR in allthe experiments performed in this study. Fisher's exact test, chi-square test, and two-sample t test were used to evaluate the statistical differences among the groups with different clinicopathological data. The methylation ratio of miRNAs between carcinomas and non-tumor tissues were assessed using the χ^2 test or Fisher's exact test. The survival data were analyzed using the Kaplan-Meier method, differences were determined using the log-rank test, and the prognostic significance of clinical characteristics was assessed using the multivariate Cox proportional hazards model. P<0.05 was considered significant. Statistical analysis was performed using Statistical Program for Social Sciences (SPSS) software 18.0 (SPSS Incorporated, Chicago, IL, USA).

Results

Expression of miR-203 in ESCC

For the 50 ESCC tissues, the mean level of miR-203 expression was 4.79 (range 0.26-6.93), while the adjacent non-cancerous tissues was 9.26 (range 4.52-11.98). The statistical analysis showed that the expression level of miR-203 was significantly lower in ESCC tissues than in adjacent non-cancerous tissues (P<0.001) (**Figure 2**). In addition, there was a significant correlation between low expression of miR-203 and clinicopathologic characteristics in ESCC (**Table 1**). Patients with lower expression of miR-203 tended to have increased pTNM stage (P=0.0213). There was no significant difference between low expression of miR-203 and other clinicopathological characteristics such as sex, age, tumor grade.

Table 2. Correlation of promoter methylation of miR-203 with clinicopathological features in patients with ESCC

Characteristics	No. of cases tested	Methylation status of miR-203		*p* value
	n=50	Positive (n=36)	Negative (n=14)	
Sex				
Male	30	22	7	
Female	20	14	4	0.2713
Age				
≤64	23	17	6	
>64	27	19	8	0.2435
pTNM stage				
I	14	7	7	
II	10	7	3	
III	26	22	4	0.0218
Tumor size (cm)				
<4	18	13	5	
≥4	32	23	9	0.2631
Tumor grade				
G1	6	4	2	
G2	32	22	10	
G3	12	10	2	0.3351
Invasion into lymphatic vessels				
Negative	31	22	9	
Positive	19	14	5	0.2559

The CpG island methylation status of miR-203 in carcinoma and non-tumor tissues

MiR-203 promoter methylation was analyzed in tumor from 50 ESCC patients and their normal controls. Among the tested cases, MiR-203 promoter methylation was detected in 36/50 (72%) cases in the tumor DNA and 13/50 (26%) in the normal controls. The difference in miR-203 promoter methylation between tumor tissues and their normal controls was significant statistically (P<0.01). **Table 2** shows the association between miR-203 promoter methylation and clinicopathologic parameters in tumor tissues. Among the patients examined for miR-203 promoter methylation, 50% (7 of 14) cases were found to be methylated in pTNM stage I, 70% (7 of 10) in stages II, and 84% (22/26) in stages III. The correlation between methylation and pTNM stages was found to be statistically significant (P=0.0218).

Association between miR-203 methylation and expression

To further investigate whether DNA methylation contributed to the silenced expression of miR-203 in ESCC, we evaluated the expression levels of miR-203 in both methylated and unmethylated groups. Asshown in **Figure 3**, the expression level of miR-203 was significantly lower in the methylated tumor group than that in the unmethylated tumor group (P=0.026).

Effect of promoter methylation of miR-203 on the prognosis of patients with esophageal cancer

The association of thepromoter methylation of miR-203 with prognosis was analyzed in 50 ESCC patients. The median survival rate for patients with promoter methylation of miR-203 (10.0 months) was lessthan that for patients with unmethylation of miR-203 (29.0 months). In the univariate analysis, miR-203 methylation, miR-203 expression, pTNM stage, tumor grade, lymph node metastasis were correlated with overall survival of ESCC patients (P<0.05) (**Figure 4**). In the multivariate analysis revealedthat mir-203 methylation status and miR-203 expression status are all independent predictors for postoperational survival of esophageal cancer patients (P=0.036 & P=0.0278) (**Table 3**). Univariate andmultivariate analyses suggested that mir-203 methylation status is one ofthe best predictors for the outcome of esophageal cancer in patients.

Discussion

Deregulation of oncogene and tumor-suppressor gene expression has been identified as one of the critical causes of tumorigenesis. The recent discovery of miRNAs provides additional new insights into the molecular mechanism of gene expression control. It has been reported that miRNA could regulate gene expression by inducing target mRNA cleavage or translational repression through binding to its 3'-UTR. Aberrant miRNA expression was recently found in cancer and recognized as a hallmark of cancer [13]. miR-203 is closely related to the dif-

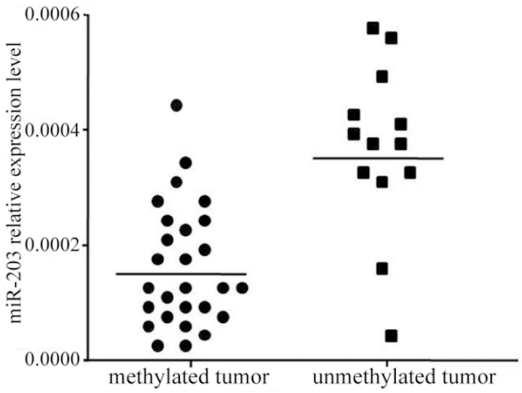

Figure 3. Associations between promoter methylation and mature miR-203 expression among 50 patients. The expression levels of miR-203 was compared between methylated group and unmethylated group in tumor tissues. *P*-values was determined by Mann-Whitney test. Horizontalline represented median value. The result showed that the expression level of miR-203 was significantly lower in methylated tumor group than in unmethylated tumor group (P=0.026).

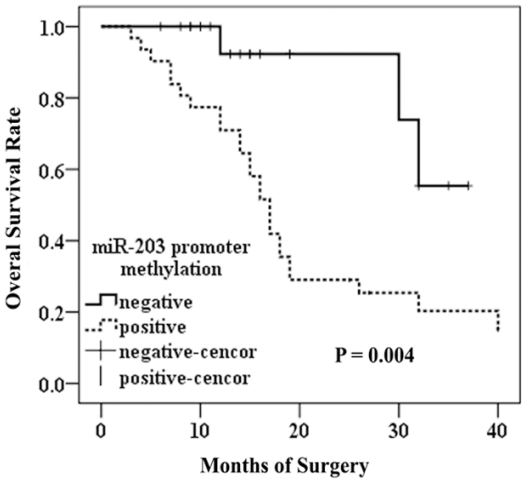

Figure 4. Kaplan-Meier analyses for overall survival rates of patients with ESCC categorized according to mir-203 methylation status. Log-rank test showed that patients with positive miR-203 methylation had significantly poorer overall survival with low miR-203 expression (P=0.004).

ferentiation of stratified squamous epithelia. Because of the function in decreasing cellproliferation, it was thought to be a tumor suppressor gene [9]. Aberrant expression of miR-203 has been found in human cancers: down-regulation of miR-203 was described in oral squamous cell carcinoma, hepatocellular carcinoma (HCC), T cell lymphomas, chronic myelogenous leukemia, B-cell type acute lymphoblastic leukemia, and central nervous system tumor cell lines, whereas its up-regulation was found in lung cancer, pancreatic cancer, bladder cancer, breast cancer and ovarian cancer [14]. In esophageal cancer study, Feber et al. detected the miRNAs exchange in 1 ESCC sample and 7 normal esophageal epithelia samples, and in 7 ESCC samples and 1 normal esophageal epithelia sample with chip, they found that miR-203 is 2-10 times down-regulated in ESCC comparing with normal esophageal epithelial. It indicated that miR-203 might involve in the carcinogenesis of ESCC [11]. The limited specimen couldn't completely reflect the expression level of miR-203 in ESCC, it is essential to identify the expression level of miR-203 in larger specimen. In the present study, we detected the expression of miR-203 in 50 ESCC specimens by real-time quantitative RT-PCR and found that the expression of miR-203 has significantly low expression in esophageal cancer compared to non-tumor counterparts. The role of miR-203 remains unclear in the progression of ESCC. If low expression of miR-203 is causal to the progression of ESCC, it may be correlated with clinicopathologic characteristics of the disease. Our investigation showed that low expression of miR-203 was correlated with increased pTNM stage in ESCC.

Expression of miRNAs could be reduced by manyfactors, including mutations, transcription factors, deletions, and methylation. In previous study, Furuta et al. reported that miR-203 was silenced through CpG-island methylation, and they suggested it was a novel tumorsuppressor in HCC [15]. Furthermore, Bueno et al. also indicated that miR-203 was a tumor suppressor and inactivated in specific hematopoietic malignancies by bothgenetic and epigenetic mechanisms [12]. They found that miR-203 was silenced by the loss of one allele and promoter CpG hypermethylation in the remaining DNA copy. And restoration of miR-203 expression might have therapeutic benefits in specific hematopoietic malignancies. The hypermethylation status of miR-203 has not been extensively reported on in ESCC. Therefore, considering these reasons, the methylation status of miR-203 was examined in our study. We have found hypermethylation of the promoter region of in 72% in ESCC. Interestingly, the 27 low

Table 3. Multivariate analysis of prognostic factors associated with overall survival of esophageal cancer patients with Cox proportional hazards model

Factors	Hazard ratio	95% CI	*P* value
Mir-203 expression status	2.471	1.104-5.533	0.0278
Mir-203 methylation status	2.061	1.050-4.046	0.0360
Age (≤64 vs. >64)	1.052	0.520-2.126	0.8887
Sex (male vs. female)	1.382	0.565-3.388	0.2691
pTNM stage	4.586	2.289-9.189	0.1010
Tumor size	3.703	1.837-7.465	0.4753
Tumor grade	1.930	1.369-2.720	0.4777
Lymph node metastases	1.299	0.754-2.238	0.3457

level of miR-203 expression carcinoma did not show methylation positive miR-203 gene whereas 13 out of 14 methylation-negative carcinoma were the high level for miR-203 expression. Though the number of cases studied was small, our findings suggest that methylation of miR-203 is an early event in the development of ESCC and is effective in silencing miR-203 expression. While this report was different from that of Chen et al. [7]. They results suggested that miR-203 was unmethylated in carcinoma and non-tumor tissues and thus that DNA methylation may not be involved in the regulation of miR-203 in ESCC, so further more study is needed.

We found miR-203 methylation was significantly associated with reduced total survival ($P<0.05$) and suggest tumors without miR-203 function may be more aggressive. This is supported by findings that miR-203 suppress tumor cell invasion in an in vitro study [16]. The association of poor prognosis with miR-203 aberration has also been reported previously in pancreatic adenocarcinoma and ovarian cancer [17, 18].

Most early ESCC cases are asymptomatic, so the patients are usually at intermediate or late stages when they see a doctor. Because of this late diagnosis, the prognosis is very poor. Early detection of ESCC is important for improving cure rates and reducing mortality. The most common current screening technique in high-risk populations, EBC, has only 50% sensitivity for detecting squamous dysplasia. DNA methylation may become a potential indicator in early-stage ESCC by improving the sensitivity. MiR-129-2 methylation was detectable in hepatocellular carcinoma (HCC) tumors [19], as well as inplasma from HCC patients [20], but not in healthy individuals or patients with liver cirrhosis. This selectivity implies its potential utility as an early diagnostic marker for HCC. Renata et al. suggest that altered expression of miR-203 are related to neoplastic transformation and progression of the disease and it could serve as a potential diagnostic and prognostic biomarkers in esophageal cancer [21]. In this study, we found that miR-203 promoter methylation mediate the downregulation and hypermethylation of miR-203 is associated with shorter patient survival time indicating its potential as a methylation biomarker in early diagnosis of ESCC, but it is still not enough to evaluate the early occurrence of carcinoma, more work is needed to confirm the use of DNA methylation of circulating miRNAs as a potential biomarker. We are currently examining whether hypermethylation of miR-203 could serve as a biomarker in plasma from ESCC patients.

In summary, we found that miR-203 was downregulated in ESCC and promoter methylation appeared to mediate the downregulation. Tumors with miR-203 gene methylation may be more aggressive and have worse prognosis. This study suggests miR-203 as a potential biomarker could provide an important diagnostic tool to patient outcomes.

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Disclosure of conflict of interest

None.

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