RESEARCH ARTICLE

Association of miR-193b Down-regulation and miR-196a up-Regulation with Clinicopathological Features and Prognosis in Gastric Cancer

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Abstract

Dysregulated expression of microRNAs (miRNAs) has been shown to be closely associated with tumor development, progression, and carcinogenesis. However, their clinical implications for gastric cancer remain elusive. To investigate the hypothesis that genome-wide alternations of miRNAs differentiate gastric cancer tissues from those matched adjacent non-tumor tissues (ANTTs), miRNA arrays were employed to examine miRNA expression profiles for the 5-pair discovery stage, and the quantitative real-time polymerase chain reaction (qRT-PCR) was applied to validate candidate miRNAs for 48-pair validation stage. Furthermore, the relationship between altered miRNA and clinicopathological features and prognosis of gastric cancer was explored. Among a total of 1,146 miRNAs analyzed, 16 miRNAs were found to be significantly different expressed in tissues from gastric cancer compared to ANTTs ($p<0.05$). qRT-PCR further confirmed the variation in expression of miR-193b and miR-196a in the validation stage. Down-expression of miR-193b was significantly correlated with Lauren type, differentiation, UICC stage, invasion, and metastasis of gastric cancer ($p<0.05$), while over-expression of miR-196a was significantly associated with poor differentiation ($p=0.022$). Moreover, binary logistic regression analysis demonstrated that the UICC stage was a significant risk factor for down-expression of miR-193b (adjusted OR=8.69; 95%CI=1.06-56.91; $p=0.043$). Additionally, Kaplan-Meier survival curves indicated that patients with a high fold-change of down-regulated miR-193b had a significantly shorter survival time (n=19; median survival=29 months) compared to patients with a low fold-change of down-regulated miR-193b (n=29; median survival=54 months) ($p=0.001$). Overall survival time of patients with a low fold-change of up-regulated miR-196a (n=27; median survival=52 months) was significantly longer than that of patients with a high fold-change of up-regulated miR-196a (n=21; median survival=46 months) ($p=0.003$). Hence, miR-193b and miR-196a may be applied as novel and promising prognostic markers in gastric cancer.

Keywords: Gastric cancer - microRNA - miRNA array - gene expression profiling - biomarkers - miR-196a - prognosis

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Introduction

Gastric cancer (GC) is the fourth most common malignant tumor worldwide and the second highest in developing countries (Jemal et al., 2010; Parkin, 2001; Siegel et al., 2012). It also ranks as the second leading cause of cancer death worldwide (Camargo et al., 2012; Yin et al., 2012). Moreover, nearly 47% of worldwide gastric cancer cases occur in China alone (Shen et al., 2013b). Currently radical surgery still offers the best chance of long-term survival; however, the five-year survival rate of gastric cancer patients is only about 20-30% (Crew and Neugut, 2006; Yang, 2006). Therefore, it is necessary to develop novel and more sensitive biomarkers to improve early diagnosis and therapy, which in turn result in better long-term survival for gastric cancer patients.

MicroRNAs (miRNAs) are a small class of nucleic acids (approximately 20-24 bases) that function in transcriptional and post-transcriptional regulation of gene expression (Lee et al., 2003; Tsuchiya et al., 2006). Since the first discovery of two miRNAs (lin-4 and let-7) in the 1990s, an increasing number of miRNAs have been successfully identified in various organisms. Currently almost 2, 000 human miRNAs are listed in miRBase (Kozomara and Griffiths-Jones, 2011), and it is estimated that they control more than 30% of all genes. miRNAs play a vital role in the regulation of most biological and physiological processes, including

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development, cell proliferation, cell cycle, apoptosis, migration, and differentiation (Yin et al., 2012; Chen et al., 2013; Montalban et al., 2014; Wu et al., 2014a; 2014b; Zha et al., 2014; Zhong et al., 2014). They have also gained importance as they carry information about the pathophysiological nature of a disease, and thus can serve as ideal predictive biomarkers and therapeutic targets. More than 50% of miRNA genes are located within cancer-associated genomic regions or in the fragile sites, suggesting that miRNAs are involved in pathogenesis (Calin et al., 2004). High-throughput screening assay studies have recently characterized the identifiable miRNA expression signature in human tumors. Aberrations in the expression level of specific miRNAs and their regulatory targets are shown to be potential tools for diagnosis, classification, and prediction of prognosis in diverse cancer types (Cho, 2010; Yin et al., 2012; Ma et al., 2013).

In human gastric cancer, dysregulation of miRNAs could act as new oncogenes or tumor suppressors in carcinogenesis (Link et al., 2012). For example, over-expression of miR-126 and consequent inhibition of SOX2 promote gastric cancer tumorigenicity (Otsubo et al., 2011). On the contrary, miR-9, miR-16, and miR-21 can target NF-kappaB1 and significantly suppress the growth of cancer cells (Wan et al., 2010; Shin et al., 2011). Moreover, further profiling investigations have displayed a correlation between miRNAs and gastric cancer proliferation, pathology, migration, and invasion through targeting different genes (Yin et al., 2012). For example, the down-regulation of miR-101 expression can be found in gastric cancer tissues and cells, and miR-101 significantly inhibits cellular proliferation, migration, and invasion through targeting EZH2, Cox-2, Mcl-1, and Fos (Wang et al., 2010). Meanwhile, miR-221 and miR-222 could regulate gastric carcinoma cell growth and invasion via modulation of PTEN expression (Chun-Zhi et al., 2010). Over-expression of miRNA let-7f could inhibit invasion and migration through targeting the tumor metastasis-associated gene, MYH9, in human gastric cancer (Liang et al., 2011).

Various miRNAs have been proven to be associated with the clinical outcome of lung adenocarcinoma (Yanaihara et al., 2006; Yang et al., 2013), breast cancer (Guo et al., 2013), endometrioid endometrial cancer (Jia et al., 2013), and bladder cancers (Kozinn et al., 2013; Ratert et al., 2013). Presently, a few studies suggest that unique miRNAs are associated with the progression and prognosis of gastric cancer (Ueda et al., 2010). For instance, low expression levels of miR-451 and miR-125a-5p are significantly correlated with poor prognosis (Bandres et al., 2009; Nishida et al., 2011). miR-107 expression is an independent prognostic factor for overall survival and disease-free survival (Inoue et al., 2012). However, whether miRNA expression can predict the clinical outcomes of gastric cancer remains to be elucidated. In the present study, miRNA expression profiles from snap frozen samples of gastric cancer patients were examined. The expressions of miR-193b and miR-196a were quantified in 48-pair gastric cancer tissues and matched adjacent non-tumorous tissues (ANTTs). Furthermore, we investigated their relationship with clinicopathological features and survival of gastric cancer patients. The findings from our study offer new clinical biomarkers to improve future diagnosis and prognosis of gastric cancer.

Materials and Methods

Patients and tissue specimens

A total of 48 gastric cancer patients were recruited for this study after obtaining informed consent. 96 gastric tissues, including 48 cancer tissues and 48 matched ANTTs, were collected from patients who underwent resection of the primary tumor between July 2009 and March 2010 at the First Affiliated Hospital of Inner Mongolia Medical University, China. These tissue specimens were immediately snap-frozen in liquid nitrogen and stored at -80°C until the preparation of total RNA. Both the cancer tissues and the normal histologically tissues were independently confirmed by two professional pathologists. Pathologic data were collected after histopathological investigation. The depth of tumor invasion was assessed according to the Union for International Cancer Control (UICC) classification criteria (Sobin and Fleming, 1997). Status of lymph node metastasis and differentiation grade was assessed according to the World Health Organization (WHO) classification criteria (Solcia et al.). Tumor location was obtained from histopathology record. Clinical information of all subjects was obtained from medical records at the First Affiliated Hospital of Inner Mongolia Medical University. None of the patients received treatment prior to surgery. All specimens were handled anonymously according to ethical and legal standards. Written informed consent for study participation was obtained from all patients. The present study was approved by the Investigation and Ethics Committee of the First Affiliated Hospital of Inner Mongolia Medical University.

MicroRNA expression profiling assay

Among 48 pairs of freshly frozen gastric tumors and their ANTTs, 5-paired samples were randomly picked initially for genome-wide miRNA microarray screening. (The clinical characteristics of the subjects used for the microarray study are summarized in Supplementary materials). The miRNA microarray was used to examine the expression of 1, 146 human miRNAs (>97% coverage of miRbase release 12). Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion®, Austin, TX, USA) according to the manufacturer's protocol. RNA concentration and integrity were assessed using Agilent 2100 RNA Bioanalyzer (Agilent technologies, Waldbronn, Germany). Each RNA sample had a Bioanalyzer RIN value higher than 7.0, OD 260/280 ratio greater than 1.8, and 260/230 ratio above 1.0. miRNA expression profiling was analyzed using the Illumina® MicroRNA Expression Profiling Assay (Illumina, San Diego, CA, USA). In brief, 200ng RNA was polyadenylated and converted to cDNA, which was then amplified, labeled, and hybridized to a miRNA Bead Chip using the Human v2 MicroRNA Expression Profiling Beadchip (Illumina, San Diego, CA, USA). Hybridization images were collected by iScan System and the signals were captured using the Bead Array Reader software (Illumina, San Diego, CA, USA). Array

raw data processing and analysis were performed with Illumina BeadStudio software v3 (Illumina, San Diego, CA, USA). The array data were filtered with a detection P value of <0.05 in all samples. The selected gene signal values were transformed to log2 ratios and normalized via the average method.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from 96 snap frozen samples (48 gastric cancer tissues and 48 matched ANTTs) to validate the expression levels of miR-193b and miR-196a. All 48 cases were histopathologically diagnosed, among which, 34 were adenocarcinoma and 14 were diffuse adenocarcinoma. The clinical characteristics for these subjects are summarized in Table 1. TaqMan miRNA assays were used to determine the expression levels of miR-193b, miR-196a, and nuclear RNA U6 (RNU6, as an internal control). Briefly, miRNA expression was performed using a two-step TaqMan® MicroRNA kit (Applied Biosystems®, Foster City, CA, USA). First strand cDNA from RNA template was synthesized by priming with gene specific looped primers in a 15μl reaction. Mixtures were incubated for 30min at 16°C, 30min at 42°C, 5min at 85°C, and held at 4°C. After the reverse transcription reaction, RT-PCR was performed by the ABI 7300 Real-Time PCR System (Applied Biosystems®, Foster City, CA, USA). RT-PCR amplification conditions were 94°C for 10 min, followed by 40 cycles of 95°C for 15sec, and 60°C for 60sec in a 20μL reaction volume (Tang et al., 2013a; Tang et al., 2013b). Each sample was performed in triplicate according to the manufacturer's protocol (PN 4364031 TaqMan® MicroRNA Assays Protocol, Applied Biosystems®, Foster City, CA) (Xi et al., 2007). The $2^{-\Delta\Delta Ct}$ method was used to determine the relative miRNA expression ratios of miR-193b and miR-196a, in which data were normalized with RNU6 (Shen et al., 2013a). If the average of Ct_{RNU6} and Ct_{miRNA} were not within 20 and 33 cycles, the assay was repeated.

Table 1. Different Expressions of miRNAs between Gastric Tumor Tissues and Adjacent Non-tumor Tissues

No.	miRNA	Tumor/Normal ratio (fold-change)	*P* value
1	HS_100	8.48	1.30E-02
2	miR-27a	8.18	2.10E-02
3	miR-196a	6.84	3.80E-02
4	solexa-555-1991	5.58	2.50E-02
5	miR-214*	4.32	2.40E-02
6	miR-502-3p, miR-500*	-3.5	1.40E-02
7	miR-551b	-3.87	4.60E-02
8	miR-625*	-4	4.70E-02
9	miR-660	-4.59	2.10E-02
10	miR-582-5p	-5.36	4.70E-02
11	miR-363	-5.55	2.50E-02
12	miR-486-5p	-5.67	2.90E-02
13	miR-642	-6.31	2.40E-03
14	miR-193b	-6.4	2.40E-03
15	miR-29c*	-7.36	3.00E-04
16	miR-30c	-8.36	4.60E-02

MicroRNA target prediction and network pathway analysis

To investigate the biological properties of differentially expressed miRNAs, the validated target genes of miR-193b and miR-196a were retrieved via CyTargetLinker (Kutmon et al., 2013) in Cytoscape v3.1.1 software (San Diego, CA, USA) (Shannon et al., 2003). Pathway enrichment analysis was then performed via ClueGO (ontologies and pathways provided by GO Biological process, GO Molecular function, GO Immune system, Kyoto Encyclopedia of Genes and Genomes (KEGG), and REACTOME) (Bindea et al., 2009). The statistical test was set to a right-sided hypergeometrical test with a Bonferroni (step down) P value correction. The analysis was set with the default settings of the program except that we selected the Global network specificity option. To achieve a visualization of target genes, the function "GO Term fusion" was additionally selected for further reduction of redundancy.

Statistical analysis

All statistical analyses were performed using STATA 10.0 software (StataCorp, College Station, TX, USA). The associations of miR-193b and/or miR-196a expression (s) with clinicopathological characteristics were assessed using one-way analysis of variance (ANOVA). Unconditional logistic regression analysis with odds ratios (OR) and 95% confidence intervals (95% CI) were used to estimate the gastric cancer risk of expression levels of miR-193b and miR-196a; adjusting for depth of invasion, metastasis, tumor differentiation, Lauren type, and UICC stage. To generate survival curves, continuous miRNA fold changes were converted to a dichotomous variable using the respective median fold-change of miR-193b (4.26) and miR-196a (5.69) as a threshold. This procedure enabled the division of samples into classes with high fold-change and low fold-change of miRNA. The effect of each high fold-change or low fold-change of miR-193b and miR-196a on survival was assessed using multivariate Cox proportional hazards regression analysis adjusted for gender, age, Lauren type, tumor differentiation, UICC stage, depth of cancer invasion, distal metastasis, tumor size, and site of tumor. Kaplan-Meier survival curves and log-rank tests were used to evaluate the effect of variants on the time to death (endpoint). The crude OR was computed using the Woolf approximation method. A paired Wilcoxon test was used to compare differences in expression levels of miR-193b and miR-196a between cancer tissues and paired ANTTs. The log-rank test for trend was used to evaluate the relationship between miR-193b and/or miR-196a expression (s) and the prognosis. Unless otherwise noted, $p<0.05$ (2-sided tests) were considered statistically significant.

Results

miRNA expression patterns in gastric cancer tissues and ANTTs

miRNA array data revealed that 16 miRNAs had significantly differential expressions between gastric cancer tissues and ANTTs among the total 1, 146 miRNAs (Figure 1 and Supplementary materials,

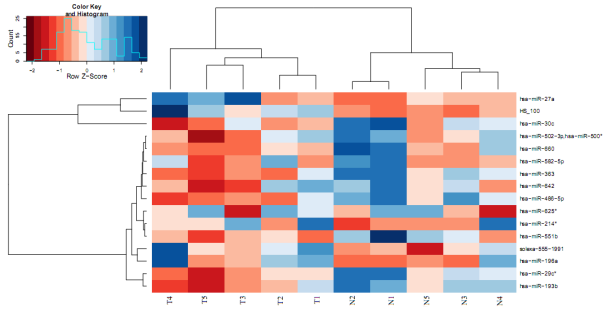

Figure 1. Heat Map of miRNA Differential Expression in Gastric Tumor Tissues and Adjacent Non-tumor Tissues. Data refer to paired samples from 5 Chinese patients with gastric cancer. Both down-regulated (red) and up-regulated (blue) miRNAs were identified in gastric tumor tissues (T1-T5) vs. adjacent non-tumor tissues (N1-N5)

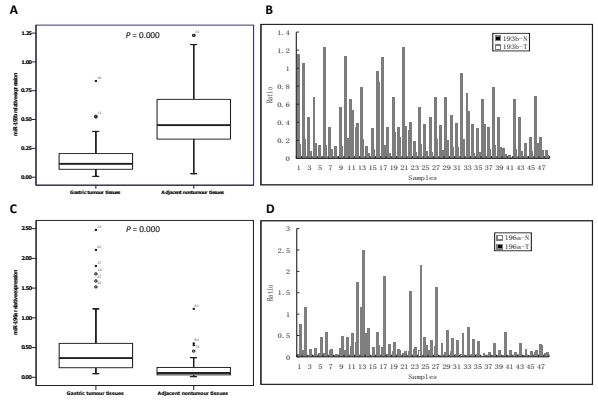

Figure 2. 2 Box Plots Showing miRNA Expressions in Gastric Tumor and Adjacent Non-tumor Specimens. Relative expression levels of miRNA were measured by qRT-PCR with an internal control, RNU6. The box extended from the 25th to the 75th percentile; the line in the box indicated the 50th percentile; and the whisker caps indicated the maximum and the minimum values. (A) miR-193b expression (tumors *vs* normal p=0.000); (B) miR-193b expression of all individual paired samples (48 tumors vs. 48 adjacent non-tumor samples); (C) miR-196a expression (tumors *vs* normal p=0.000); and (D) miR-196a expression of all individual paired samples (48 tumors *vs* 48 adjacent non-tumor samples)

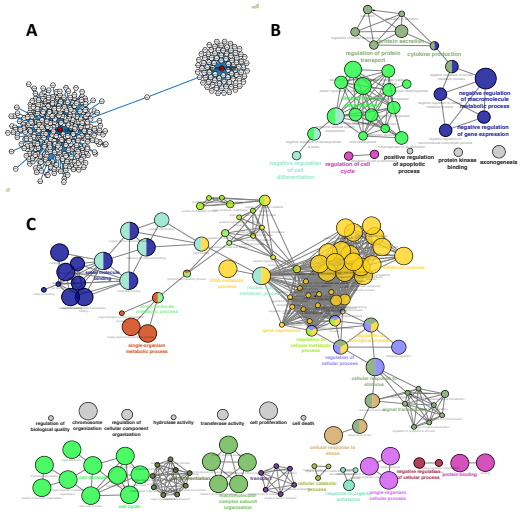

Figure 3. Validated Gene Targets and their Functionally Grouped GO Terms of miR-196a and miR-193b. There were 357 and 87 validated targets for miR-193b and miR-196a, respectively (Figure 3A). Functionally analysis of enriched GO terms with the ClueGO of Cytoscape suggested that the gene targets of miR-193b and miR-196a located into various biological pathways. (Figure 3B and 3C). Each of these cluster groups represents genes with similar biological functions. The size of the nodes corresponds to the statistical significance of the GO terms. Kappa statistics was used to generate the connectivity between the terms (edges)

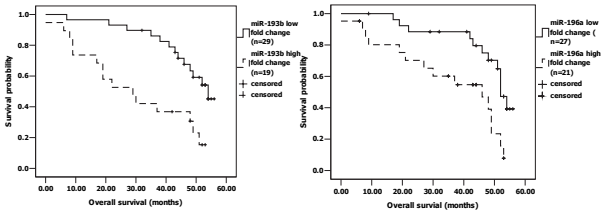

Figure 4. Figure 4 Kaplan-Meier Survival Curves for Overall Survival of Gastric Cancer Patients. Kaplan-Meier survival curves showed that overall survival of gastric cancer patients was associated with (A) low fold-change (n=29) and high fold-change (n=19) of miR-193b expression (p=0.001), and (B) low fold-change (n=27) and high fold-change (n=21) of miR-196a expression (p=0.003). P value was calculated using a log-rank test

p<0.05). More specifically, HS_100, miR-27a, miR-214*, solexa-555-1991, and miR-196 were significantly up-regulated, while miR-502-3p, miR-29c*, miR-642, miR-193b, miR-551b, miR-30c, miR-582-5p, miR-625*, miR-660, miR-363, and miR-486-5p were significantly down-regulated (Table 1 and Supplementary materials). The hierarchical clustering algorithm of Cluster software was employed to generate a cluster tree helping to visualize the correlation of miRNA expression pattern between different tissue types. Based on miRNA expression profiling of each sample, a clear distinction was observed between gastric cancer tissues and paired ANTTs (Figure 1).

miR-193b down-regulation and miR-196a up-regulation in gastric cancer tissues and ANTTs

The expressions of miR-193b and miR-196a were examined in 48 gastric cancer tissues and individually matched ANTTs. As shown in Figure 2, the expression levels of miR-193b and miR-196a were found to be significantly decreased or increased in gastric cancer tissues compared to ANTTs (miR-193b: 0.17±0.16 vs. 0.53±0.32, p=0.000; miR-196a: 0.53±0.57 *vs* 0.15±0.19, p=0.000), respectively. Additionally, the fold changes of down-regulated miR-193b (4.26±2.05) and up-regulated miR-196a (5.69±4.27) in gastric cancer tissues compared to ANTTs were consistent with miRNA array results (6.4-fold down-regulation of miR-193b and 6.84-fold up-regulation of miR-196a).

miR-193b and miR-196a target pathway analysis

Only validated target genes of miR-193b and miR-196a were retrieved using CyTargetLinker from miRTarBase (Hsu et al., 2011). The results showed that there were 357 and 87 validated targets for miR-193b and miR-196a, respectively (Figure 3A). Only one gene ESPL-

Table 2. Association between the Expression Levels of miR-193b or miR-196a and the Clinicolpathological Features of Gastric Cancer Patients were Assessed Using One-way Analysis of Variance (ANOVA)

Items	Clinical characteristics	No. of Patients	%	Expression of miR-193b	*P* value	Expression of miR-196a	*P* value
Gender	Male	37	77.08	4.14±2.12	0.468	5.99±4.37	0.38
	Female	11	22.92	4.66±1.81		4.69±3.89	
Age (years)	>60	23	47.92	4.95±2.16	0.601	6.29±5.03	0.354
	≤60	25	52.08	4.41±1.97		5.14±3.43	
Lauren type	Adenocarcinoma	34	70.83	3.69±1.64	0.002[a]	5.12±3.17	0.15
	Diffusion	14	29.17	5.65±2.32		7.08±5.35	
Tumor differentiation	Moderate-well	29	60.42	3.66±1.84	0.010[a]	4.56±3.39	0.022[a]
	Poor	19	39.58	5.18±2.05		7.41±4.94	
UICC stage	I+II	19	39.58	3.09±1.72	0.001[a]	4.69±3.69	0.19
	III+IV	29	60.42	5.02±1.89		6.35±4.54	
Depth of cancer invasion	$T_{1+}T_2$	27	56.25	3.53±1.61	0.004[a]	5.06±3.89	0.244
	$T_{3+}T_4$	21	43.75	5.20±2.19		6.51±4.67	
Lymphatic metastasis	Negative	11	22.92	3.39±2.14	0.113	4.21±3.37	0.192
	Positive	37	77.08	4.51±1.98		6.13±4.44	
Distal metastasis	M_0	35	72.92	3.82±1.82	0.013[a]	5.51±3.75	0.643
	M_1	13	27.08	5.44±2.21		6.16±5.58	
Site of tumor	Cardia	19	39.58	4.20±2.29	0.878	5.26±4.29	0.577
	Others	29	60.42	4.29±1.92		5.97±4.29	
Tumor size (cm)	≤5cm	22	45.83	4.57±2.13	0.334	5.22±3.66	0.487
	>5cm	26	54.17	3.99±1.97		6.09±4.75	

[a]p<0.05

Table 3. Binary Logistic Regression Analysis for the Association between the Fold Changes of miR-193b or miR-196a and the Clinicopathological Features of Gastric Cancer Patients

Items Variable	miR-193b				miR-196a			
	Crude OR (95% CI)	*p* value	Adjusted OR[a] (95% CI)	*p* valuea	Crude OR (95% CI)	*p* value	Adjusted OR (95% CI)[a]	*p* value[a]
Depth of invasion								
T1+T2	1		1		1		1	
T3+T4	3.81 (1.12-12.90)	0.032[b]	1.02 (0.20-5.12)	0.982	1.68 (0.82-8.65)	0.103	1.43 (0.29-6.95)	0.658
Metastasis								
Negative	1		1		1		1	
Positive	3.49 (0.93-13.14)	0.065	0.81 (0.12-5.32)	0.829	1.14 (0.32-4.11)	0.838	0.29 (0.04-2.17)	0.233
Tumor differentiation								
Moderate-well	1		1		1		1	
Poor	3.61 (1.06-12.25)	0.039[b]	1.59 (0.36-7.05)	0.538	2.61 (0.79-8.59)	0.114	1.73 (0.43-6.99)	0.445
Lauren type								
Adenocarcinoma	1		1		1		1	
Diffusion	4.32 (1.15-16.15)	0.030[b]	1.92 (0.28-13.17)	0.509	2.15 (0.61-7.63)	0.234	2.08 (0.30-14.50)	0.411
UICC stage								
I+II	1		1		1		1	
III+IV	12.04 (2.33-39.83)	0.003[b]	8.69 (1.06-56.91)	0.043[b]	3.45 (0.98-12.10)	0.054	2.91 (0.48-17.74)	0.248

[a]Adjusting for Lauren type, differentiation, UICC stage, invasion, and metastasis; [b]p<0.05.

1 was regulated by both miR-193b and miR-196a (Figure 3A). Further analysis of enriched GO terms via ClueGO suggested that the gene targets of miR-193b and miR-196a located into various biological pathways such as cell cycle, cell differentiation, metabolic process, apoptosis, gene expression, and signal transduction etc. (Figure 3B and 3C). Each of these cluster groups represents genes with similar biological functions.

miR-193b down-regulation and miR-196a up-regulation associates with clinicopathological features of gastric cancer

The associations of miR-193b and miR-196a expression with various clinicopathological parameters of gastric cancer were analyzed. Data in Table 2 and 3 shows that the fold-change of down-regulated miR-193b was significantly associated with Lauren type, tumor differentiation, UICC stage, depth of cancer invasion, and distal metastasis. Whereas the fold-change of up-regulated miR-196a was only significantly correlated with tumor differentiation. Moreover, the fold changes of miR-193b and miR-196a between gastric cancer tissues and ANTTs were dichotomized into low fold-change or high fold-change groups using the median fold-change of miR-193b (4.26) and miR-196a (5.69). This led to 29 patients with low fold-change and 19 patients with high fold-change for miR-193b, and 27 patients with low fold-change and 21 patients with high fold-change for miR-196a. Multivariate binary logistic regression analysis showed that the UICC stage was a significant risk factor for the fold-change of down-regulated miR-193b (adjusted OR=8.69, 95%CI=1.06-56.91, p=0.043) (Table 3). However, there was no significant association between miR-193b expression and other clinicopathological parameters (p>0.05, Table 4). In addition, multivariate binary logistic regression analysis showed miR-196a was

not associated with any clinicopathological parameters.

miR-193b down-regulation and miR-196a up-regulation predicts poor overall survival in gastric cancer patients

To further evaluate the potential clinical relevance of the down-regulated miR-193b and up-regulated miR-196a in gastric cancer prognosis, Kaplan-Meier survival analysis was performed using overall patient survival. Our results indicated that miR-193b was significantly associated with patient survival (Figure 4). Patients with a low fold-change of miR-193b tended to survive longer (n=29; median survival of 54 months) than patients with a high fold-change of miR-193b (n=19; median survival of 29 months) (p=0.001). Similarly, patients with a low fold-change of miR-196a tended to survive longer (n=27; median survival of 52 months) than patients with a high fold-change of miR-196a (n=21; median survival of 46 months) (p=0.003).

Discussion

In the current study, we employed high-throughput microarray technology to screen differential expression of miRNAs in 5-paired gastric tumor and ANTT samples. A total of 16 miRNAs were aberrantly expressed, 11 of which were down-regulated, whereas 5 were up-regulated in carcinoma samples. The number of miRNAs with down-regulation was more than those with up-regulation, which is in line with most previous miRNA profiling studies in cancer (Guo et al., 2009; Presneau et al., 2013). Three differentially expressed miR-196a (up-regulation), miR-27a (up-regulation), and miR-30c (down-regulation) were consistent with previous findings in gastric tumors (Li et al., 2011; Liu et al., 2011; Tsai et al., 2012; Wang et al., 2013). However, we reported here for the first time that miR-193b, miR-214*, solexa-555-1991, miR-502-3p, miR-29c*, miR-64, miR-551b, miR-582-5p, miR-625*, miR-660, miR-363, and miR-486-5p were significantly altered in gastric cancer tissues compared to non-tumorous tissues. Moreover, based on miRNAs profiling of each sample, the hierarchical clustering analysis successfully separated normal samples from carcinoma samples, confirming that gastric cancer tissues have a unique miRNA-profiling pattern compared with normal tissues. Considering our relatively small sample size for microarray analysis, future large sample size investigation is warranted.

The possible functional roles of miRNA have been extensively examined in various cancer types. In this study, we focused on the characteristics of miR-193b and miR-196a in gastric cancer tissues based on the results of miRNA expression in 48-paired samples by qRT-PCR. The results revealed that the miR-193b expression level in gastric cancer tissues was significantly lower, while miR-193b expression is higher in tumor tissues than those in the normal tissues. Many previous studies have demonstrated that miR-193b is involved in apoptosis, metabolism, tumor growth, migration, and invasion (Chen et al., 2010; Xu et al., 2010; Hu et al., 2012; Zhong et al., 2014). More specifically, miR-193b was notably down-regulated in endometrioid adenocarcinoma, melanoma (Chen et al., 2010), hepatocellular carcinoma cells (Xu et al., 2010), and non-small cell lung cancer (NSCLC) (Hu et al., 2012), suggesting that miR-193b may act as a tumor suppressor and play a protective role in the carcinogenesis of these cancers. miR-196a is a known onco-miRNA that plays an important role in tumorigenesis and tumor progression (Peng et al., 2010; Sun et al., 2012). Emerging evidence suggests the aberrant over-expression of miR-196a is a frequent event in various cancers, including head and neck squamous cell carcinomas (Severino et al., 2013), laryngeal cancer (Saito et al., 2013), pancreatic cancer (Liu et al., 2013), and gastric cancer (Tsai et al., 2012). It has been reported that over-expression of miR-196a is associated with apoptosis, invasion, and proliferation in pancreatic cancer (Liu et al., 2013). Also, miR-196a is a putative diagnostic biomarker and therapeutic target for laryngeal cancer (Saito et al., 2013).

To date, little is known about the correlation between clinicopathological factors and miR-193b or miR-196a in gastric cancer. We first exhibited that miR-193b down-regulation was significantly correlated with Lauren type, differentiation, UICC stage, invasion, and metastasis, and that the over-expression of miR-196a is significantly correlated with poor differentiation. Similar findings have been observed in other cancer types. Up-regulation of miR-196a expression is associated with an advanced clinical stage in both NSCLC and cervical cancer (Liu et al., 2012; Hou et al., 2014). Therefore, miR-193b and miR-196a may be applied as a promising marker for gastric tumor aggressiveness.

The potential role of miRNAs as prognostic biomarkers is of interest. miR-196 has shown to play an important role in the malignant progression of gliomas and thus, can be used as a prognostic predictor in glioblastomas (Guan et al., 2010). In breast cancer, the association of the loss of miR-193b with metastasis implies the use of miRNA in prognostic stratification of cancer patients, in addition to conventional clinical and pathological staging markers (Li et al., 2009). Yet, there has been no study that attempts to explore the prognostic value of miR-193b or miR-196a for gastric cancer patients. Our clinical data revealed that down-regulation of miR-193b or up-regulation of miR-196a was significantly correlated with poorer overall survival of patients, indicating these two molecules are suitable to predict poor prognosis in gastric cancer after surgery.

miRNAs exert their biological effects by direct cleavage of target gene mRNAs or by inhibition of protein synthesis (Mattick and Makunin, 2006). In this study, miR-193b and miR-196a were chosen for further elucidation due to their respective pathogenic role and molecular mechanism of action in gastric cancer. The pathway enrichment analysis revealed that these two miRNAs might be involved in several crucial cellular activities and biological processes related to the cancer initiation and progression. The results also indicated that both cell cycle, cell differentiation, metabolic process, apoptosis/cell death, regulation of gene expression, and signal transduction pathways were under the regulation of miR-193b and miR-196a. Further experiments that focus on the identification and validation of these regulatory

targets and functions in gastric cancer are needed.

To the best of our knowledge, this is the first study examining the relationship between altered miR-193b or miR-196a and clinicopathological features and prognosis in patients with gastric cancer. Aberrant expression of miR-193b or miR-196a was demonstrated to be independently associated with pathological features and clinical outcomes, highlighting that these two molecules may be employed as promising diagnosis markers and useful therapeutic targets to improve the survival of gastric cancer patients. This study lays the groundwork for further larger sample-based prospective and experimental studies to explore the biologic functions and underlying molecular mechanism of miRNAs in the development, progression, diagnosis, and prognosis of gastric cancer.

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