Expression and clinical significance of the microRNA-200 family in gastric cancer

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Abstract. Gastric cancer is one of the most common malignant tumors and one of the leading causes of cancer-related mortality. Recent studies have revealed that there is a difference in microRNA (miR/miRNA) profiles between cancerous and normal tissues. To find a potentially useful prognostic predictor and a promising therapeutic tool for gastric cancer, the present study investigated the expression and clinical significance of the miR-200 family in gastric cancer. The miR-200 family has five members: hsa-miR-200a, hsa-miR-200b, hsa-miR-200c, hsa-miR-141 and hsa-miR-429. In 46 clinical samples of gastric cancer and paired non-cancerous tissues, the present study observed that the expression levels of the miR-200 family in the cancer tissues were significantly lower than those in the non-cancerous tissues ($P<0.001$). Lower levels of the five family members were associated with histological grade and the presence of an intravascular cancer embolus ($P<0.05$). The results revealed that the miR-200 family is downregulated in gastric cancer, and that there are significant differences in the expression of the miR-200 family between normal and cancer tissues. The miR-200 family may therefore become a potentially useful prognostic predictor of the aggressiveness of gastric cancer and a possible therapeutic tool in affected patients.

Introduction

Gastric cancer is one of the most common malignant tumors and one of the primary causes of cancer-related mortality in China. This is mainly a result of patients being diagnosed late and the consequently low surgical resection rate (1-3). An improved understanding of the pathogenesis and pathological characteristics of gastric cancer will provide a novel method to ensure an early diagnosis and treatment.

microRNAs (miR/miRNAs) are a class of small and highly conserved non-coding RNA, which regulate gene expression by binding to the 3' untranslated region (3'UTR) of target mRNAs. Growing evidence indicates that miRNAs play a key role in cancer development, differentiation and progression, acting as tumor oncogenes or suppressors (4-6). Notably, miR-200 family is downregulated in certain types of cancer, such as hepatocellular carcinoma and renal cell carcinoma, while being overexpressed in others, including melanoma, and ovarian and bladder cancer (7-9).

In the present study, in order to identify the changes in expression of miR-200 in normal and cancer tissues, the expression of the miR-200 family was examined in cancerous and paired non-cancerous tissues by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and the distinguishing characteristics of the five members of the miR-200 family were analyzed. Positive results from this study may serve as the foundation for a novel prognostic predictor of grading and a possible therapeutic tool for gastric cancer.

Materials and methods

Human tissue samples. A total of 46 fresh tissue samples, consisting of gastric cancer tissues and paired normal tissues (5 cm away from the tumor), were obtained from surgical resection specimens collected by the Fourth Hospital of Hebei Medical University (Shijiazhuang, Hebei, China) between 2010 and 2011. The tissues were collected and used after obtaining informed consent from the patients. All tissue samples were immediately cut and snap frozen in liquid nitrogen until RNA extraction. Histological typing of the tumor and paired non-cancerous tissues was pathologically confirmed. This study was approved by the ethics committee of the Fourth Hospital of Hebei Medical University.

RT-qPCR. Total RNA, including miRNA, was extracted from the tissues using the miRNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Total RNA was subsequently reverse transcribed to

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Key words: microRNA-200 family, gastric cancer, histological grade, intravascular cancer embolus

Table I. Primer sequences for RT and qPCR.

Gene	Primer	Sequence (5'-3')
miR-200a	RT	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACATCGT-
	qPCR-F	GGCCCGTAACACTGTCTGGTAA
miR-200b	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATCAT
	qPCR-F	GCCGCTTTAATACTGCCTGGTA
miR-200c	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCCATCA
	qPCR-F	GCCGATTTAATACTGCCGGGT
miR-141	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCATCTT
	qPCR-F	GCCGCTAACACTGTCTGGTAAA
miR-429	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACGGTTT
	qPCR-F	GCCGATTAATACTGTCTGGTAA
U6	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATATGGAACTGC
	qPCR-F	GGGTGCTCGCTTCGGCAGC
Common	qPCR-R	CAGTGCAGGGTCCGAGGT

All primers were obtained from the Beijing Genomics Institute (Beijing, China). RT, reverse transcription; qPCR, quantitative polymerase chain reaction; miR, microRNA; F, forward; R, reverse.

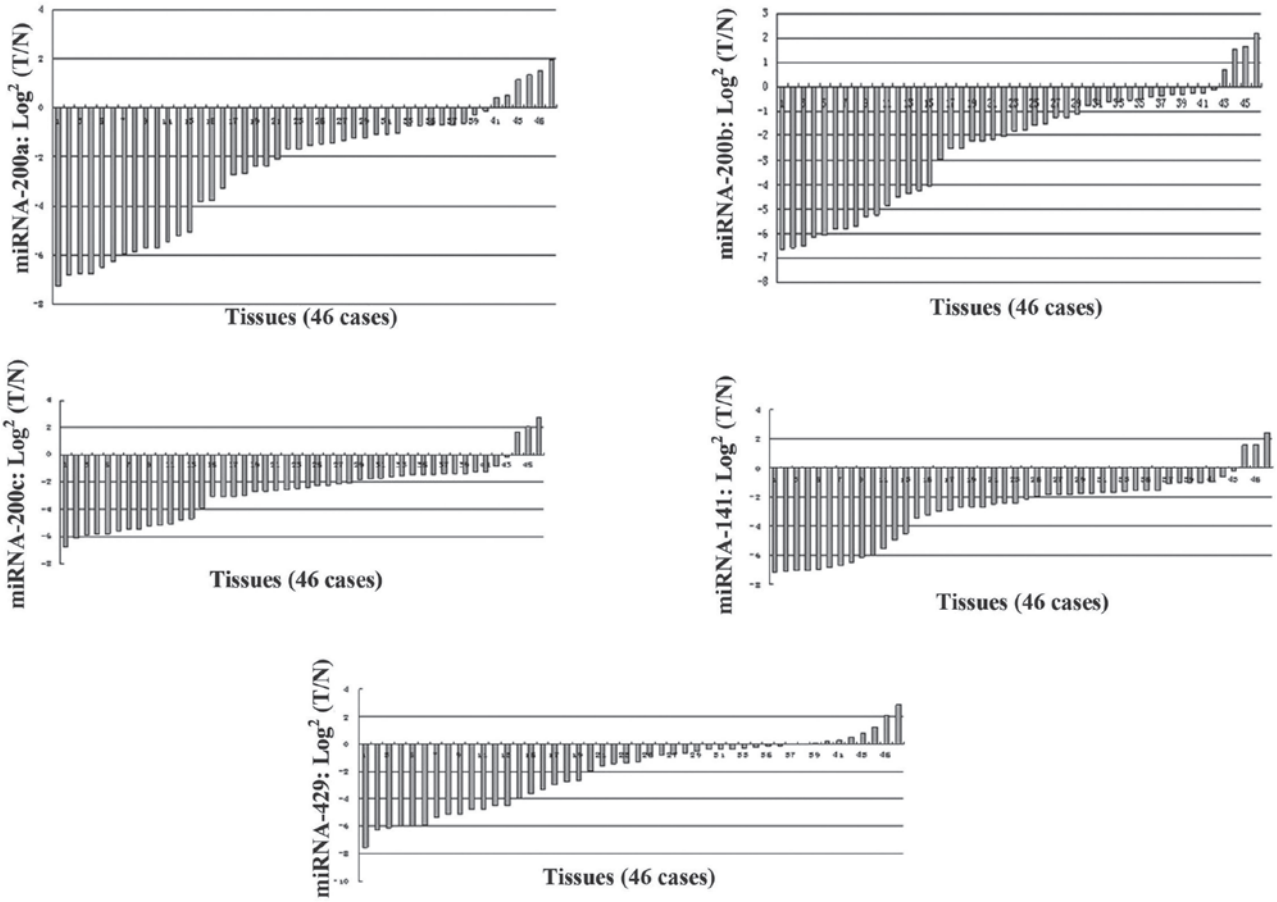

Figure 1. Expression of the miR-200 family in gastric cancer tissues. The expression of the miR-200 family in 46 cancerous (T) and paired non-cancerous (N) tissues were detected by reverse transcription-quantitative polymerase chain reaction. The expression level of the five family members in the cancerous tissues was significantly downregulated compared with the normal tissues ($P<0.05$). miR/miRNA, microRNA.

cDNA with the stem-loop reverse transcription primer (Beijing Genomics Institute, Beijing, China) for miRNA detection. The U6 small nuclear RNA (Beijing Genomics Institute) was used as an internal control for the miRNA. All primer sequences are listed in Table I. RT-qPCR was carried out using SYBR® Premix Ex Taq™ (Takara Biotechnology,

Table II. Expression of miRNA-200a and the correlation with the clinicopathological parameters of gastric cancer.

Clinicopathological parameters	n	miRNA-200a, median (range)	P-value
Gender			0.1631
Male	35	0.071 (0.001-1.635)	
Female	11	0.174 (0.011-1.974)	
Age, years			0.6367
≥60	19	0.157 (0.001-1.635)	
<60	27	0.065 (0.003-1.974)	
Tumor size, cm			0.8354
≤5	22	0.165 (0.003-1.635)	
>5	24	0.033 (0.001-1.974)	
Invasive depth			0.5514
T1+T2+T3	13	0.172 (0.001-0.796)	
T4	33	0.065 (0.003-1.974)	
Lymph node metastasis			0.1678
N0	11	0.157 (0.001-1.635)	
N1	9	0.016 (0.007-0.249)	
N2	10	0.098 (0.016-0.857)	
N3	16	0.367 (0.011-1.974)	
TNM stage			0.9806
I	3	0.486 (0.001-0.796)	
II	11	0.157 (0.003-1.635)	
III	31	0.071 (0.011-1.974)	
IV	1	0.353	
Histological grade			0.0135
G2	24	0.220 (0.013-1.974)	
G3	22	0.054 (0.001-0.796)	
Intravascular cancer embolus			0.0004
Negative	33	0.801 (0.011-1.974)	
Positive	13	0.065 (0.001-0.796)	
Lauren type			0.3632
Intestinal	16	0.020 (0.001-1.635)	
Diffuse	14	0.094 (0.003-1.974)	
Mixed	7	0.481 (0.101-0.965)	
Undetermined	9	0.073 (0.013-0.702)	
Borrmann type			0.9231
I	3	0.101 (0.003-0.796)	
II	12	0.123 (0.007-0.825)	
III	27	0.071 (0.001-1.974)	
IV	4	0.247 (0.016-0.965)	

TNM, tumor-node-metastasis.

Co., Ltd., Dalian, China). The reactions were placed in a 96-well plate (Applied Biosystems Life Technologies, Foster City, CA, USA) using a preheated real-time instrument (ABI 7500HT; Applied Biosystems Life Technologies). The relative levels of expression were quantified and analyzed using Bio-Rad iCycler iQ software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Ct values were used to calculate the RNA expression levels. The amount of target gene expression ($2^{-\Delta\Delta}$Ct) was normalized using the endogenous U6 reference.

Statistical analysis. Data are presented as the median ± standard deviation, unless otherwise stated. All statistical tests were two-sided, and a value of $P<0.05$ was considered to indicate a statistically significant difference. Student's t-test and a one-way analysis of variance (ANOVA) were employed

Table III. Expression of miRNA-200b and the correlation with the clinicopathological parameters of gastric cancer.

Clinicopathological parameters	n	miRNA-200b, median (range)	P-value
Gender			0.4103
Male	35	0.054 (0.001-1.550)	
Female	11	0.161 (0.014-1.300)	
Age, years			0.9843
≥60	19	0.140 (0.001-1.550)	
<60	27	0.061 (0.003-1.471)	
Tumor size, cm			0.4119
≤5	22	0.160 (0.003-1.550)	
>5	24	0.041 (0.001-1.300)	
Invasive depth			0.8398
T1+T2+T3	13	0.166 (0.001-0.799)	
T4	33	0.046 (0.003-1.550)	
Lymph node metastasis			0.1423
N0	11	0.140 (0.001-1.550)	
N1	9	0.022 (0.003-0.210)	
N2	10	0.113 (0.016-0.630)	
N3	16	0.218 (0.013-1.300)	
TNM stage			0.7918
I	3	0.590 (0.001-0.799)	
II	11	0.140 (0.003-1.550)	
III	31	0.054 (0.013-1.300)	
IV	1	0.342	
Histological grade			0.0209
G2	24	0.204 (0.004-1.550)	
G3	22	0.042 (0.001-0.799)	
Intravascular cancer embolus			0.0013
Negative	33	0.504 (0.018-1.550)	
Positive	13	0.046 (0.001-0.799)	
Lauren type			0.7097
Intestinal	16	0.026 (0.001-1.550)	
Diffuse	14	0.133 (0.003-1.300)	
Mixed	7	0.504 (0.054-0.704)	
Undetermined	9	0.061 (0.013-0.590)	
Borrmann type			0.9992
I	3	0.054 (0.003-0.799)	
II	12	0.111 (0.003-1.471)	
III	27	0.099 (0.001-1.550)	
IV	4	0.283 (0.015-0.704)	

TNM, tumor-node-metastasis.

to analyze the differences among groups and comparisons between two groups using Statistical Analysis System V8 software (SAS Institute Inc., Cary, NC, USA).

Results

Expression of the miR-200 family in gastric cancer tissues. To investigate the role of the miR-200 family in gastric cancer, the expression of the miR-200 family in 46 cancerous and paired non-cancerous tissues were detected by RT-qPCR. Through the analysis of data, the results showed that all five members of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) exhibited significantly lower expression levels in the cancerous tissues compared with the corresponding normal tissues (Fig. 1; $P<0.05$). It was observed that the difference in miR-200c and miR-141

Table IV. Expression of miRNA-200b and the correlation with the clinicopathological parameters of gastric cancer.

Clinicopathological parameters	n	miRNA-200c, median (range)	P-value
Gender			0.5526
Male	35	0.059 (0.001-0.739)	
Female	11	0.068 (0.004-0.685)	
Age, years			0.5040
≥60	19	0.071 (0.001-0.560)	
<60	27	0.059 (0.003-0.739)	
Tumor size, cm			0.9137
≤5	22	0.076 (0.003-0.739)	
>5	24	0.040 (0.001-0.685)	
Invasive depth			0.7276
T1+T2+T3	13	0.084 (0.001-0.341)	
T4	33	0.019 (0.003-0.739)	
Lymph node metastasis			0.2434
N0	11	0.059 (0.001-0.739)	
N1	9	0.009 (0.005-0.108)	
N2	10	0.157 (0.005-0.338)	
N3	16	0.102 (0.004-0.685)	
TNM stage			0.9147
I	3	0.068 (0.001-0.310)	
II	11	0.059 (0.003-0.739)	
III	31	0.019 (0.004-0.685)	
IV	1	0.247	
Histological grade			0.0479
G2	24	0.135 (0.006-0.739)	
G3	22	0.020 (0.001-0.354)	
Intravascular cancer embolus			0.0069
Negative	33	0.184 (0.006-0.739)	
Positive	13	0.021 (0.001-0.354)	
Lauren type			0.8673
Intestinal	16	0.009 (0.001-0.739)	
Diffuse	14	0.084 (0.003-0.685)	
Mixed	7	0.184 (0.071-0.354)	
Undetermined	9	0.164 (0.009-0.327)	
Borrmann type			0.7826
I	3	0.068 (0.003-0.071)	
II	12	0.124 (0.005-0.739)	
III	27	0.019 (0.001-0.685)	
IV	4	0.098 (0.009-0.354)	

TNM, tumor-node-metastasis.

expression between the cancerous and normal tissues was similar.

Correlation with histoclinical data. To determine whether the downregulation of the expression of the miR-200 family in gastric cancer tissues was correlated with clinicopathological characteristics, an ANOVA was performed. The median of the relative expression values and the clinicopathological factors are presented in Tables II-VI. The statistical analysis showed that the expression levels of the five family members were associated with histological grade and the presence of an intravascular cancer embolus ($P<0.05$). The expression level of the family was downregulated in G3 grade gastric cancer compared with G2 grade. In the gastric cancer tissues, the expression level of the miR-200 family was decreased when an intravascular cancer embolus was present. However, no

Table V. Expressions of miRNA-141 and the correlation with the clinicopathological parameters of gastric cancer.

Clinicopathological parameters	n	miRNA-141, median (range)	P-value
Gender			0.1709
Male	35	0.038 (0.0004-0.644)	
Female	11	0.101 (0.001-1.050)	
Age, years			0.3072
≥60	19	0.098 (0.0004-0.475)	
<60	27	0.038 (0.001-1.050)	
Tumor size, cm			0.9171
≤5	22	0.101 (0.001-0.644)	
>5	24	0.026 (0.0004-1.050)	
Invasive depth			0.7717
T1+T2+T3	13	0.119 (0.0004-0.415)	
T4	33	0.015 (0.001-1.050)	
Lymph node metastasis			0.2789
N0	11	0.037 (0.0004-0.644)	
N1	9	0.005 (0.003-0.143)	
N2	10	0.143 (0.002-0.371)	
N3	16	0.112 (0.001-1.050)	
TNM stage			0.9970
I	3	0.127 (0.0004-0.242)	
II	11	0.037 (0.002-0.644)	
III	31	0.038 (0.002-1.050)	
IV	1	0.167	
Histological grade			0.0255
G2	24	0.130 (0.003-1.050)	
G3	22	0.014 (0.0004-0.371)	
Intravascular cancer embolus			0.0030
Negative	33	0.181 (0.003-1.050)	
Positive	13	0.019 (0.0004-0.412)	
Lauren type			0.6016
Intestinal	16	0.005 (0.0004-0.644)	
Diffuse	14	0.078 (0.002-1.050)	
Mixed	7	0.171 (0.098-0.244)	
Undetermined	9	0.138 (0.005-0.242)	
Borrmann type			0.8988
I	3	0.098 (0.002-0.127)	
II	12	0.119 (0.003-0.644)	
III	27	0.019 (0.0004-1.050)	
IV	4	0.088 (0.003-0.211)	

TNM, tumor-node-metastasis.

association was observed with regard to gender, age, tumor size, invasive depth, lymph node metastasis, tumor-node-metastasis stage and Borrmann type.

Discussion

Numerous miRNAs have been suggested to play vital roles in gastric cancer development and progression, functioning in a either a cooperative manner or alone (10-12). An independent study by Kogo *et al* reported that miR-146a levels were dramatically decreased in gastric cancer, and more importantly, lower levels of miR-146a were associated with lymph node metastasis and venous invasion (13). The expression of let-7f has also been proven to be decreased in metastatic gastric cancer tissues, while let-7f overexpression in gastric cancer was able to inhibit the invasion and migration of gastric

Table VI. Expressions of miRNA-429 and the correlation with the clinicopathological parameters of gastric cancer.

Clinicopathological parameters	n	miRNA-429, median (range)	P-value
Gender			0.4166
Male	35	0.080 (0.001-1.962)	
Female	11	0.194 (0.009-1.720)	
Age, years			0.5590
≥60	19	0.143 (0.001-1.962)	
<60	27	0.093 (0.002-1.738)	
Tumor size, cm			0.7291
≤5	22	0.173 (0.002-1.962)	
>5	24	0.051 (0.001-1.720)	
Invasive depth			0.5014
T1+T2+T3	13	0.191 (0.001-1.245)	
T4	33	0.072 (0.002-1.962)	
Lymph node metastasis			0.2178
N0	11	0.143 (0.001-1.962)	
N1	9	0.021 (0.008-0.194)	
N2	10	0.123 (0.008-1.377)	
N3	16	0.259 (0.014-1.720)	
TNM stage			0.8561
I	3	0.681 (0.001-1.245)	
II	11	0.143 (0.002-1.962	
III	31	0.080 (0.008-1.720)	
IV	1	0.398	
Histological grade			0.0105
G2	24	0.225 (0.005-1.962)	
G3	22	0.058 (0.001-1.245)	
Intravascular cancer embolus			<0.0001
Negative	33	1.226 (0.015-1.962)	
Positive	13	0.072 (0.001-1.245)	
Lauren type			0.2606
Intestinal	16	0.030 (0.001-1.962)	
Diffuse	14	0.130 (0.002-1.720)	
Mixed	7	0.593 (0.080-1.377)	
Undetermined	9	0.093 (0.010-0.681)	
Borrmann type			0.9877
I	3	0.080 (0.002-1.245)	
II	12	0.144 (0.008-1.738)	
III	27	0.107 (0.001-1.962)	
IV	4	0.150 (0.010-1.337)	

TNM, tumor-node-metastasis.

cancer cells (14). Another study demonstrated the significant downregulation of miR-148b in 106 gastric cancer tissues and the consequent suppression of gastric cancer cell growth, suggesting that miR-148b could become a potential biomarker and therapeutic target against gastric cancer (15).

The five members of the miR-200 family share similar seed sequences, and are located in two distinct genomic clusters. The miR-200a-200b-429 cluster is located on chromosome 1 and the miR-200c-141 cluster is located on chromosome 12. The expression level of the miR-200 family is commonly altered in various biological and pathological processes (16-19). Notably, the expression of the miR-200 family can be downregulated or upregulated in different types of cancers, and the tissue of origin may account for this. One study clearly showed that the expression of miRNA-141 and miRNA-200c was significantly decreased in renal cell carcinoma samples (20). However,

a number of studies have shown that the miR-200 family is overexpressed in bladder, ovarian and cervical cancer. Together, these data suggest that the miR-200 family may play multiple roles in potentially regulating tumor initiation and progression (21,22).

In the present study, the expression and clinical significance of the miR-200 family in gastric cancer was analyzed using a standardized qPCR approach. The results highlight the significance of the miR-200 family as a promising tumor suppressor in gastric cancer. It was shown that the miR-200 family is frequently downregulated in gastric cancer tissues compared with matched non-cancerous tissues. Furthermore, low miR-200 family expression levels were also shown to correlate with histological grade and the presence of an intravascular cancer embolus.

In summary, the present comprehensive analysis showed that the expression of the miR-200 family has a correlation with the development of gastric cancer. Additionally, we found that lower levels of the five family menbers of the miR-200 family were associated with the clinical significance of gastric cancer. These results suggest that the miR-200 family can serve as a new prognostic predictor of the aggressiveness of gastric cancer and as a possible therapeutic tool for affected patients.

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