The clinical significance of downregulation of mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p in gastric cancer tumorigenesis

HAILONG LI[1,2], SHOUPIN XIE[3], MIN LIU[1], ZHAOFENG CHEN[1,2], XIAOJUN LIU[1], LI WANG[1], DAYAN LI[1] and YONGNING ZHOU[1,2]

[1]Division of Gastroenterology and Hepatology, First Hospital of Lanzhou University; [2]Key Laboratory for Gastrointestinal Diseases of Gansu, Lanzhou, Gansu 730000; [3]First People's Hospital of Lanzhou, Lanzhou, Gansu 730050, P.R. China

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Abstract. Dysregulated miRNAs in gastric cancer are usually screened by miRNA microarray from clinical samples, however, reports have indicated that results of each miRNA microarray screening are considerably different, and dysregulated miRNAs, especially downregulated miRNAs were contradictory. In view of this, the Human Cancer Pathway Finder miRNA PCR array was applied to compare 7 gastric cancer cell lines AGS, SGC-7901, MKN-45, MKN-28, MGC-803, BCG-823, and HGC-27 with an immortalized normal gastric cell line, GES-1 in cancer pathway-related miRNA expression profile, followed by qPCR verification, the clinical significance of downregulated miRNAs and the Enriched KEGG pathways and GO terms of their target genes were analyzed. Thirty-eight miRNAs were upregulated, and four miRNAs were downregulated in gastric cancer cell lines. Clinical significance of 4 miRNAs including mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p in gastric cancer tissue compared with adjacent non-tumor tissues of 58 patients indicated that the low-expression group of mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p showed more extensive lymph node metastasis, lymphatic invasion, venous invasion, high-stage Borrmann type, lymphatic invasion and poor differentiation than that of the high-expression groups, respectively ($P<0.05$; χ^2 test). Enriched KEGG pathway analyses showed that most of the targeted genes of the 4 miRNAs concentrated on 37 signaling pathways, and were involved in the same pathways related to cancer. Enriched GO terms showed that targeted genes of the 4 miRNAs concentrated on 339 terms, 24 of 339 terms are associated with cancer tumorigenesis. The Human Cancer Pathway Finder miRNA PCR array could be used to screen dysregulated miRNAs effectively, and 4 screened miRNAs, mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p were found to be downregulated in gastric cancer. Clinical significance and bioinformatic analysis on the target genes of these 4 miRNAs indicated that they were deeply involved in tumorigenesis, suggesting roles such as miRNA tumor suppressors in gastric cancer tumorigenesis which could be applied in gastric cancer diagnosis and prognosis.

Correspondence to: Professor Yongning Zhou, Division of Gastroenterology and Hepatology, The First Hospital of Lanzhou University, Lanzhou, Gansu 730000, P.R. China
E-mail: 642101301@qq.com; yongningzhou@sina.com

Key words: miRNA, PCR array, gastric cancer, tumor suppressors

Introduction

miRNAs are 20-to-25 mer non-coding RNAs which incompletely bind to the 3' untranslated regions (UTR) of multiple target mRNAs, enhancing their degradation and inhibiting their translation. MiRNAs participate in the regulation of cell differentiation, cell cycle progression and apoptosis. Dysregulated miRNAs play critical roles during carcinogenesis and cancer progression. The levels of many miRNAs in cancer tissue are lower than those in normal tissue, a state that contributes to cancer progression. Thus, abnormally downregulated miRNAs function as tumor suppressor genes and upregulated miRNAs function as oncogenic genes (1-3) in tumorigenesis as well as in gastric cancer. Therefore, it is necessary to identify new aberrant expression miRNAs in gastric cancers by screening all miRNAs closely related to tumorigenesis and progression. In recent years, miRNA microarray including Agilent, Exiqon, Affymetrix, Phalanx (4-8) or TaqMan miRNA (9,10) assays have been used to screen candidate miRNA biomarkers from fresh samples or frozen samples. Because of the differences in clinical cancer tissue and various methods employed by different miRNA microarray manufacturers, the screening results are considerable different, and some screening results of miRNA microarray are contradictory (11) also in gastric cancer (9,12-18). Aberrantly expressed miRNAs between gastric cell and gastric cancer cells were not fully identified and understood. The profiling results from this array may identify aberrantly expressed miRNAs as biomarkers of gastric cancers.

To resolve the issue, we decided to utilize Human Cancer Pathway Finder miRNA PCR array (MIHS-102Z,

Qiagen, Hilgen, Germany) which profiles the expression of 84 miRNAs differentially expressed in tumors versus normal tissue. This array provides cancer researchers with a convenient way to quickly analyze the miRNAs most relevant to tumorigenesis and screen and identify aberrantly expressed miRNAs between gastric cell gastric cancer cells. This array detected expression between gastric cancer and adjacent non-tumor tissues, and the clinical significance was analyzed.

Materials and methods

Cell lines. Seven gastric cancer cell lines, AGS, SGC-7901, MKN-45, MKN-28, MGC-803, BCG-823, HGC-27 and GES-1 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and Cancer Institute and Hospital, Chinese Academy of Medical Sciences (CAMS) (Beijing, China). All the gastric cancer cell lines were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum in a humidified cell incubator having an atmosphere of 5% CO_2 at 37˚C. Exponentially growing cells were used for experiments. All miRNA PCR primers were purchased from GeneCopoeia™ Inc (Rockville, MD, USA).

Clinical samples. Fifty-eight gastric cancer samples were obtained during surgery and used after obtaining informed consent. All patients underwent curative resection of the primary tumor at WuWei City Tumor Hospital from the year 2010 to 2012 (WuWei, China, a high incidene rate area with gastric cancer) (19,20). All patients had a clear histological diagnosis of gastric cancer, based on the clinicopathologic criteria. All data, including age, gender, histological grade, depth, lymph node metastasis, local invasion, depth of tumor invasion, lymph node metastasis, lymphatic invasion, venous invasion, Borrmann type and clinical stage were obtained from clinical and pathologic records. No patient received neoadjuvant chemotherapy or radiotherapy before surgery and adjuvant radiotherapy after surgery. Resected cancerous tissues (T) and paired non-cancerous tissues (N) were immediately cut and stored in frozen liquid nitrogen, at -80˚C until RNA extraction. Written informed consent was obtained from each patient for his or her participation in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the ethics committee of the First Hospital of Lanzhou University.

Total RNA isolation and quality analysis. Total RNA of gastric cancer cell lines and frozen tissues of gastric cancer were extracted using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Concentrations and purity of the RNA samples were assayed by electrophoresis and spectrophotometric methods.

Human Cancer Pathway Finder miRNA PCR array expression profiling. Human Cancer Pathway Finder miRNA PCR array were used to amplify and quantify the expression levels of 84 miRNAs in GES-1 cell line and AGS, SGC-7901, MKN-45, MKN-28, MGC-803, BCG-823, HGC-27, 7 gastric cancer cell lines, the differentially expressed miRNAs were analyzed using the $2^{-(CTmiRNA\text{-}CTRNU6B\ RNA)}$ method of relative quantification. qRT-PCR was performed in Bio-Rad CFX96 Real-time PCR system. Each reaction was performed in a final volume of 25 μl containing 1 μl of cDNA, 0.5 mM of each primer and 1X SYBR Green PCR Master mix (Qiagen). The amplification program was: denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec, 60˚C for 1 min, in which fluorescence was acquired. Relative changes in gene expression levels were calculated using $^{\Delta\Delta}$Ct (threshold cycle) method. The housekeeping genes, such as SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A and RNU6-2, were used to normalize to the amount of miRNAs. The differentially expressed miRNAs were analyzed using the formula $2^{-(CTmiRNA\text{-}CTRNU6B\ RNA)}$ method of relative quantification. Hierarchical clustering of aberrantly expressed miRNAs with significantly different expression was performed using online data analysis tool of Sabiosciences (Qiagen).

miRNA quantification by real-time qRT-PCR. miRNA quantification by real-time qRT-PCR. SYBR green qRT-PCR assay was used for miRNA quantification. In brief, 40 ng of total RNA containing miRNA was polyadenylated by poly(A) polymerase and was reversely transcripted to cDNA using miScript Reverse Transcription kit according to the manufacturer's instructions (GeneCopoeia). miScript SYBR Green PCR kit was used and miscript Universal primer was provided by the manufacturer (GeneCopoeia), qRT-PCR was performed in Bio-Rad CFX96 Real-time PCR system. Each reaction was performed in a final volume of 10 μl containing 2 μl of cDNA, 0.5 mM of each primer and 1X SYBR Green PCR Master mix (GeneCopoeia). The amplification program was: denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec, 60˚C for 30 sec and 72˚C for 30 sec, in which fluorescence was acquired. At the end of the PCR cycles, melting curve analyses were performed as well as electrophoresis of the products on 2.5% agarose gels in order to validate the specific generation of the expected PCR product. Each sample was run in triplicates for analysis. The expression levels of miRNAs were normalized to RNU6B. Relative gene expression was calculated as $2^{-(CTmiRNA\text{-}CTRNU6B\ RNA)}$. Hierarchical clustering of aberrantly expressed miRNAs with significantly different expression was performed using the MeV (Multiple Experiment Viewer) 4.9.1 software and visualized with Treeview v1.60.

miRNA targeted gene prediction and signal pathway analyses. We utilized a miRNA target gene prediction database mirfocus 2.0 (http://mirfocus.org/index.php) to select validated targets of the differently expressed miRNAs to analyse enriched KEGG pathways and to annotate the molecular function of the miRNA targeted genes. The mirfocus 2.0 integrated 5 bioinformatical Target Prediction Tools: MiRanda, MirTarget2, PicTar, microT and TargetScanS, and the experimental validated Target Tools include miRecords, miR2Disease, TarBase and miRTarBase. Enriched KEGG pathway and Enriched GO term analyses of 4 miRNA-targeted genes also were performed by mirfocus 2.0. Prediction database support number was 3, P-value of Fisher Test was $P<0.05$.

Statistical analysis. Student's unpaired t-test was used to compare values of samples of 7 gastric cancer cell lines

Table I. mir-124-3p level and clinicopathological factors in patients with gastric cancer.

Characteristics	mir-124-3p Low expression (n=45) n (%)	mir-124-3p High expression (n=13) n (%)	Significance χ^2	Significance P-value
Age			0.481	0.488
<59 years	29 (64.4)	7 (53.8)		
≥59 years	16 (35.6)	6 (46.2)		
Gender			0.157	0.692
Male	27 (60.0)	7 (53.8)		
Female	18 (40.0)	6 (46.2)		
Degree of tumor cell differentiation			0.259	0.611
Moderate-to-well differentiated	16 (35.6)	3 (23.1)		
Poorly differentiated	29 (64.4)	10 (76.9)		
TNM stage			0.098	0.755
I + II	11 (24.4)	2 (15.4)		
III + IV	34 (75.6)	11 (84.6)		
Local invasion			0.000	1.000
T1 + T2	10 (22.2)	3 (23.1)		
T3	35 (77.8)	10 (76.9)		
Lymph node metastasis			5.784	0.016[a]
Positive	36 (80.0)	6 (46.2)		
Negative	9 (20.0)	7 (53.8)		
Lymphatic invasion			9.120	0.003[b]
Positive	42 (93.3)	8 (61.5)		
Negative	3 (6.7)	5 (38.5)		
Venous invasion			1.581	0.209
Positive	5 (11.1)	0 (0)		
Negative	40 (88.9)	13 (100)		
Depth of tumor invasion			0.646	0.421
Mucosa, submucosa, muscularis propria, subserosa	22 (48.9)	8 (61.5)		
Penetration of serosa, adjacent structures	23 (51.1)	5 (38.5)		
Borrmann type			0.625	0.429
I + II	27 (60.0)	10 (76.9)		
III + IV	18 (40.0)	3 (23.1)		

The expression of mir-124-3p in all cases showed different levels between gastric cancer tissues and adjacent non-cancerous tissues. [a]P<0.05, lymph node metastasis; [b]P<0.01, lymphatic invasion.

and samples of GES-1 gastric cell line. Differences between groups were estimated using the χ^2 test. A probability level of 0.05 was chosen for statistical significance, and all statistical analyses were performed using the SPSS 11.0 software (SPSS Inc., Chicago, IL, USA).

Results

Screening of aberrantly expressed miRNAs in gastric cancer cell lines. When setting average change >2-fold or <0.5 as a cut-off level, we found that 37 miRNAs were upregulated, and 5 miRNAs were downregulated in at least 4 of 7 gastric cancer cell lines compared to GES-1 cell line screened by Human Cancer Pathway Finder miRNA PCR array among 84 miRNAs related to tumorigenesis (Fig. 1).

Validation of aberrantly expressed miRNAs in gastric cancer cell lines. After completing screening, 42 aberrantly expressed miRNAs were validated by qRT-PCR to verify its expression levels. Results showed that 38 miRNAs were upregulated in 7 gastric cancer cell lines compared with the GES-1 cell line, these were mir-1, mir-7-5p, mir-10a-5p, mir-10b-5p, let-7b-5p,

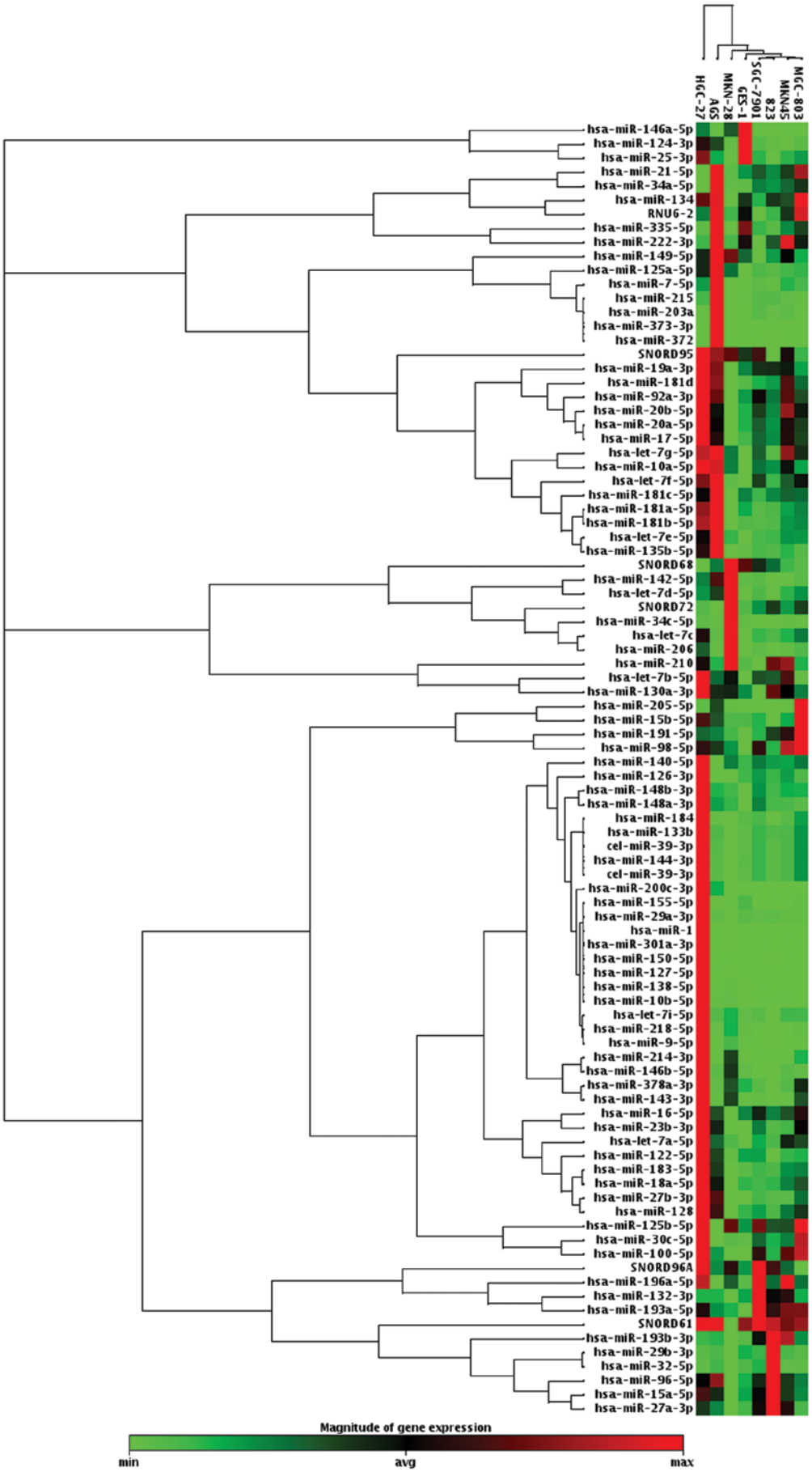

Figure 1. Hierarchical clustering of differently expressed miRNAs in GES-1 cell line and seven gastric cancer cell lines screened by Human Cancer Pathway Finder miRNA PCR array. Eighty-four miRNAs were differentially expressed between GES-1 cell line and the seven gastric cancer cell lines, red denotes high expression levels, whereas green denotes low expression levels.

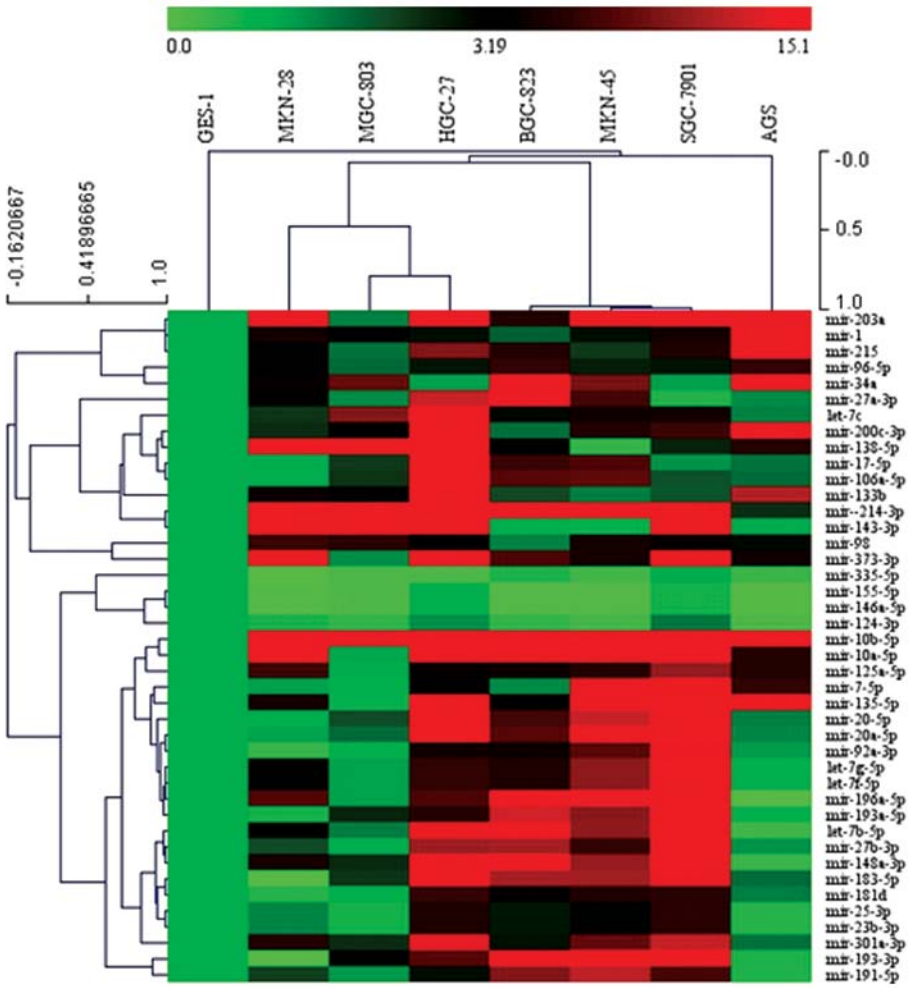

Figure 2. Hierarchical clustering of differently expressed miRNAs in GES-1 cell line and seven gastric cancer cell lines verified by qRT-PCR. Forty-one miRNAs were differentially expressed between GES-1 cell line and the seven gastric cancer cell lines, red denotes high expression levels, whereas green denotes low expression levels.

let-7g-5p, let-7c, let-7f-5p, mir-17-5p, mir-20a-5p, mir-20b-5p, mir-23b-3p, mir-25-3p, mir-27a-3p, mir-27b-3p, mir-34a, mir-92a-3p, mir-96-5p, mir-98, mir-106a-5p, mir-125a-5p, mir-133b, mir-135-5p, mir-136-5p, mir-143-3p, mir-148a-3p, mir-181d, mir-183-5p, mir-191-5p, mir-193-3p, mir-193a-5p, mir-196a-5p, mir-200c-3p, mir-203a, mir-215, mir-301a-3p, mir-214-3p, mir-373-3p and 4 miRNAs were downregulated in 7 gastric cancer cell lines compared with GES-1 cell line, which were mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p. Except mir-25-3p, the expression levels of most miRNAs were consistent with expression of that by Pathway Finder miRNA PCR array expression profiles (Figs. 2 and 3).

Validation of mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p in gastric cancer tissue compared with adjacent non-tumor tissues. In light of confusing studies on the expression of mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p in gastric tissues, the levels of these 4 miRNAs in 58 cancerous and corresponding non-cancerous tissues were detected by qRT-PCR method. Results showed that the number of mir-124-3p in high-expression group (T/N>2) and low-expression group (T/N<0.5) amounts to 13 and 45, respectively, according to the median cancer (T)/non-cancerous (N) tissue ratio of mir-124-3p expression (Table I). The number of mir-146a-5p in high-expression group (T/N>2 and low-expression group (T/N<0.5) was 35 and 20, respectively, according to the median cancer (T)/non-cancerous (N) tissue ratio of mir-146a-5p expression (Table II), the number of mir-155-5p in high-expression group (T/N>2) and low-expression group (T/N<0.5) was 26 and 26, respectively, according to the median cancer (T)/non-cancerous (N) tissue ratio of mir-155-5p expression (Table III), and the number of mir-335-5p in high-expression group (T/N>2) and low-expression group (T/N<0.5) was 30 and 25, respectively, according to the median cancer (T)/non-cancerous (N) tissue ratio of mir-335-5p expression (Table IV).

Clinical significance of mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p in gastric cancer tissue compared with adjacent non-tumor tissues. All clinicopathologic factors were analyzed in relation to these 4 miRNA levels. The mir-124-3p low-expression group showed more extensive lymph node metastasis and lymphatic invasion than the high-expression group ($P<0.05$; χ^2 test). However, no significant differences were observed among age, gender, degree of tumor cell differentiation, local invasion, depth of tumor invasion, venous invasion, Borrmann type, and clinical stage (Table I, Fig. 4A). The mir-146a-5p low-expression

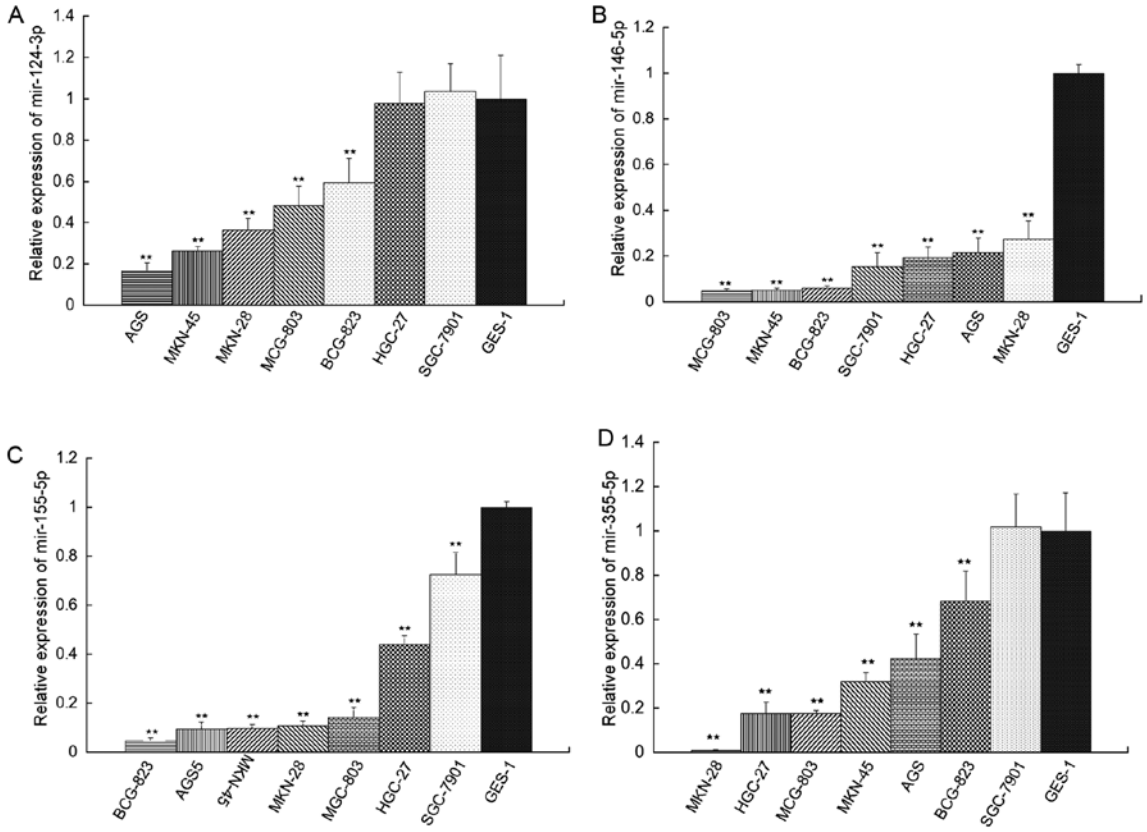

Figure 3. mir-124-3p (A), mir-146a-5p (B), mir-155-5p (C) and mir-335-5p (D) were downregulated in 7 gastric cancer cell lines compared with GES-1 cell line by qRT-PCR experiment validation, results indicating the 4 downregulated miRNAs found by Human Cancer Pathway Finder miRNA PCR array were consistent with qPCR experiment validation. Relative gene expression was calculated as $2^{-(CTmiRNA\text{-}CTRNU6B\ RNA)}$, and each sample was analyzed in triplicate. There was statistically a significant difference between the 7 gastric cancer cell lines and the GES-1 cell line ($^{**}p<0.01$).

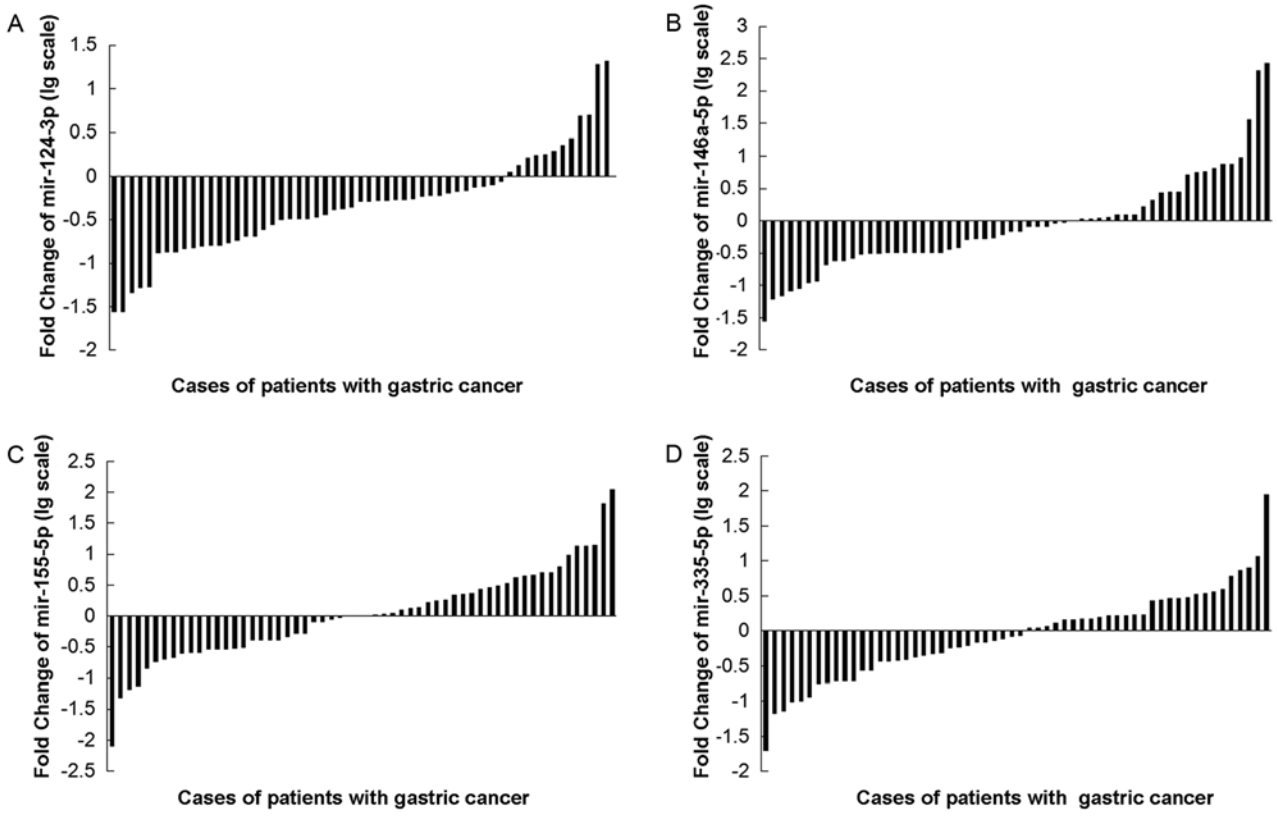

Figure 4. Expression levels of mir-124-3p (A), mir-146a-5p (B), mir-155-5p (C) and mir-335-5p (D) in gastric cancer tissue compared with adjacent non-tumor tissues of 58 gastric cancer samples.

Table II. mir-146a-5p level and clinicopathological factors in patients with gastric cancer.

Characteristics	mir-146a-5p Low expression (n=35) n (%)	mir-146a-5p High expression (n=20) n (%)	Significance χ^2	Significance P-value
Age			0.000	1.000
<59 years	22 (62.9)	12 (60.0)		
≥59 years	13 (37.1)	8 (40.0)		
Gender			3.654	0.056
Male	17 (48.6)	15 (75.0)		
Female	18 (51.4)	5 (25.0)		
Degree of tumor cell differentiation			2.946	0.086
Moderate-to-well differentiated	11 (31.4)	11 (55.0)		
Poorly differentiated	24 (68.6)	9 (45.0		
TNM stage			0.000	1.000
I + II	7 (20.0)	4 (20.0)		
III + IV	28 (80.0)	16 (80.0)		
Local invasion			0.000	1.000
T1 + T2	21 (60.0)	12 (60.0)		
T3	14 (40.0)	8 (40.0)		
Lymph node metastasis			0.074	0.786
Positive	24 (68.6)	13 (65.0)		
Negative	11 (31.4)	7 (35.0)		
Lymphatic invasion			0.000	1.000
Positive	32 (91.4)	18 (90.0)		
Negative	3 (8.6)	2 (10.0)		
Venous invasion			3.956	0.047[a]
Positive	12 (34.3)	2 (10.0)		
Negative	23 (65.7)	18 (90.0)		
Depth of tumor invasion			0.667	0.414
Mucosa, submucosa, muscularis propria, subserosa	18 (51.4)	8 (40.0)		
Penetration of serosa, adjacent strucures	17 (48.6)	12 (60.0)		
Borrmann type			0.000	1.000
I + II	21 (60.0)	12 (60.0)		
III + IV	14 (40.0)	8 (40.0)		

Except in 3 cases, the expression of mir-146a-5p in other cases showed different levels between gastric cancer tissues and adjacent non-cancerous tissues. [a]P<0.05, venous invasion.

group showed more venous invasion than the high-expression group (P<0.05; χ^2 test). However, no significant differences were observed in age, local invasion, depth of tumor invasion, lymph node metastasis, lymphatic invasion, Borrmann type, and clinical stage (Table II, Fig. 4B). The mir-155-5p low-expression group showed higher stage Borrmann type than the high-expression group (P<0.05; χ^2 test). However, no significant differences were observed in age, local invasion, depth of tumor invasion, lymph node metastasis, lymphatic invasion, venous invasion and clinical stage (Table III, Fig. 4C). The mir-335-5p low-expression group showed more lymphatic invasion and high stage Borrmann type than the high-expression group (P<0.05; χ^2 test). However, no significant differences were observed in age, gender, local invasion, depth of tumor invasion, lymph node metastasis, venous invasion and clinical stage (Table IV, Fig. 4D).

Signaling pathway and gene ontology analyses of mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p targeted genes. In order to investigate the possible regulation mechanisms of the mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p in the process of gastric cancer, we utilized a bioinformatics

Table III. mir-155-5p level and clinicopathological factors in patients with gastric cancer.

Characteristics	mir-155-5p Low expression (n=26) n (%)	mir-155-5p High expression (n=26) n (%)	Significance χ^2	P-value
Age			0.000	1.000
<59 years	18 (69.2)	15 (57.7)		
≥59 years	8 (30.8)	11 (42.3)		
Gender			0.000	1.000
Male	15 (57.7)	16 (61.5)		
Female	11 (42.3)	10 (38.5)		
Degree of tumor cell differentiation			0.702	0.402
Moderate-to-well differentiated	10 (38.5)	13 (50.0)		
Poorly differentiated	16 (61.5)	13 (50.0)		
TNM stage			0.115	0.734
I + II	5 (19.2)	6 (23.1)		
III + IV	21 (80.8)	20 (76.9)		
Local invasion			0.495	0.482
T1 + T2	6 (23.1)	4 (15.4)		
T3	20 (76.9)	22 (84.6)		
Lymph node metastasis			0.048	0.827
Positive	19 (73.1)	18 (69.2)		
Negative	7 (26.9)	8 (30.8)		
Lymphatic invasion			0.000	1.000
Positive	24 (92.3)	25 (96.2)		
Negative	2 (7.6)	1 (3.8)		
Venous invasion			0.885	0.347
Positive	22 (84.6)	25 (96.2)		
Negative	4 (15.4)	1 (3.8)		
Depth of tumor invasion			1.238	0.426
Mucosa, submucosa, muscularis propria, subserosa	14 (53.8)	10 (38.5)		
Penetration of serosa, adjacent strucures	12 (46.2)	16 (61.5)		
Borrmann type			3.914	0.048[a]
I + II	12 (46.2)	19 (73.1)		
III + IV	14 (53.8)	7 (26.9)		

Except in 6 cases, the expression of mir-155-5p showed different levels between gastric cancer tissues and adjacent non-cancerous tissues. [a]P<0.05, Borrmann type.

database mirfocus 2.0 to select plausible targets of these 4 miRNAs. A total of 1770, 400, 816, 357 target genes were predicted as the target genes of mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p, respectively. Enriched KEGG pathway analyses and enriched GO terms showed that most of the targeted genes, which were regulated by these 4 miRNAs, were involved in the same pathways.

Results of enrichment KEGG pathway indicated that the target genes of these 4 miRNAs were mainly centralized in cancer associated terms, which were: colorectal cancer, pathways in cancer, acute myeloid leukemia, endometrial cancer, adherens junction, pancreatic cancer, renal cell carcinoma, non-small cell lung cancer, apoptosis, prostate cancer, melanoma, chronic myeloid leukemia, thyroid cancer, small cell lung cancer, bladder cancer, melanogenesis, glioma, and 9 signaling pathways of toll-like receptor, chemokine, erbb signaling pathway, epithelial cell signaling in *helicobacter pylori* infection, cell cycle, NOD-like receptor, mTOR, MAPK and wnt signaling pathways (Fig. 5).

Results of enrichment GO terms indicated that the target genes of these 4 miRNAs were centralized in cancer associated terms, which were: angiogenesis, positive regulation of cell proliferation, cell-matrix adhesion, negative regulation of apoptotic process, positive regulation of focal adhesion

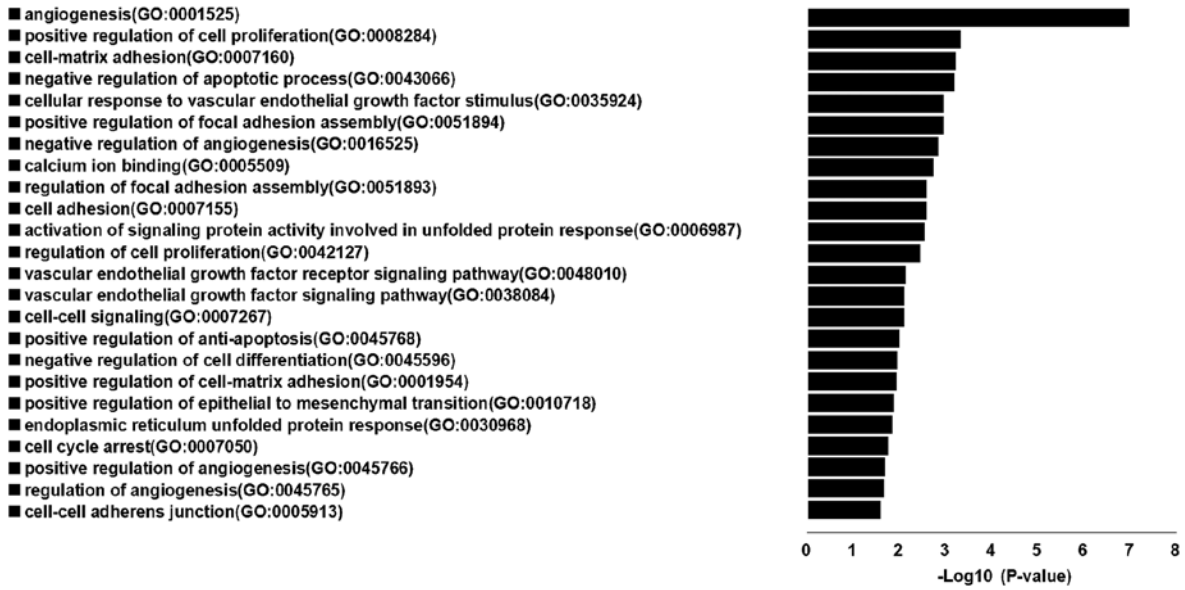

Figure 5. The 24 enriched KEGG pathways from target genes of mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p which were centralized in cancer-associated pathways.

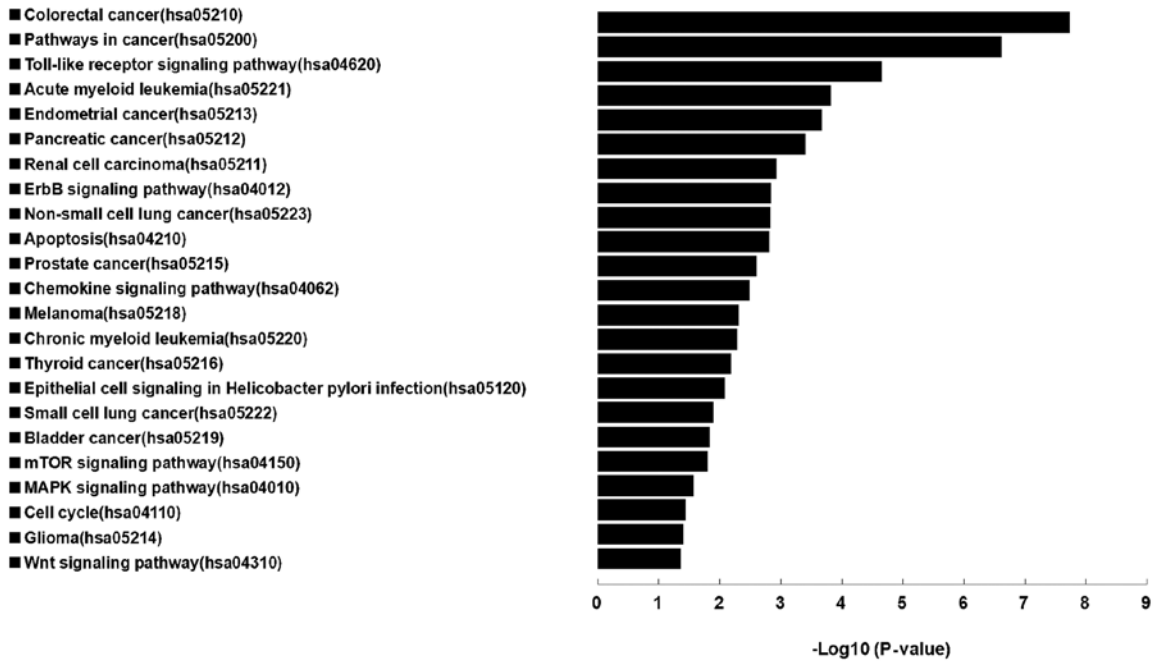

Figure 6. The 24 enriched GO terms from target genes of mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p which were centralized in cancer-associated terms.

assembly, cellular response to vascular endothelial growth factor stimulus, negative regulation of angiogenesis, calcium ion binding, cell adhesion, regulation of focal adhesion assembly, activation of signaling protein activity involved in unfolded protein response, regulation of cell proliferation, vascular endothelial growth factor receptor signaling pathway, cell-cell signaling, vascular endothelial growth factor signaling pathway, positive regulation of anti-apoptosis, negative regulation of cell differentiation, positive regulation of cell-matrix adhesion, positive regulation of epithelial to mesenchymal transition, endoplasmic reticulum unfolded protein response, cell cycle arrest, positive regulation of angiogenesis, regulation of angiogenesis and cell-cell adherens junction. The results showed that genes regulated by these 4 miRNAs participated in most of the important biological process associated with human cancer (Fig. 6).

Discussion

This study showed that 38 miRNAs were upregulated and 4 miRNAs downregulated in 7 gastric cancer cell lines compared to the GES-1 cell line. To our knowledge, this is the first report on the expression levels of 42 miRNAs in gastric cancer cells which could facilitate its discovery

Table IV. mir-335-5p level and clinicopathological factors in patients with gastric cancer.

Characteristics	mir-335-5p Low expression (n=30) n (%)	mir-335-5p High expression (n=25) n (%)	Significance χ^2	Significance P-value
Age			0.603	0.437
<59 years	21 (70.0)	15 (60.0)		
≥59 years	9 (30.0)	10 (40.0)		
Gender			0.09	0.765
Male	18 (60.0)	14 (56.0)		
Female	12 (40.0)	11 (44.0)		
Degree of tumor cell differentiation			6.227	0.013[a]
Moderate-to-well differentiated	8 (26.7)	15 (60.0)		
Poorly differentiated	22 (73.3)	10 (40.0)		
TNM stage			0.128	0.721
I + II	6 (20.0)	6 (24.0)		
III + IV	24 (80.0)	19 (76.0)		
Local invasion			0.484	0.487
T1 + T2	6 (20.0)	7 (28.0)		
T3	24 (80.0)	18 (72.0)		
Lymph node metastasis			1.101	0.294
Positive	22 (73.3)	15 (60.0)		
Negative	8 (26.7)	10 (40.0)		
Lymphatic invasion			4.303	0.038[a]
Positive	28 (93.3)	17 (68.0)		
Negative	2 (6.7)	8 (32.0)		
Venous invasion			0.110	0.740
Positive	3 (10.0)	1 (4.0)		
Negative	27 (90.0)	24 (96.0)		
Depth of tumor invasion			0.022	0.883
Mucosa, submucosa, muscularis propria, subserosa	15 (50.0)	13 (52.0)		
Penetration of serosa, adjacent strucures	15 (50.0)	12 (48.0)		
Borrmann type			0.638	0.425
I + II	16 (53.3)	19 (76.0)		
III + IV	14 (46.7)	6 (24.0)		

Except in 3 cases, the expression of mir-335-5p showed different levels between gastric cancer tissues and adjacent non-cancerous tissues. [a]P<0.05, degree of tumor cell differentiation, lymphatic invasion.

in gastric cancer tissue and find new biomarkers of gastric cancer, the 7 miRNAs mir-1, let-7b-5p, let-7c, let-7g-5p, mir-27b-3p, mir-183-5p, mir-193-3p and mir-203a were first identified upregulated in gastric cancer cell lines, and no studies were found in gastric cancer tissues compared with adjacent normal gastric tissue. In addition, the expression levels of 4 miRNAs of mir-1, mir-7-5p, mir-133b and mir-143 were inconsistent with previous studies screened by miRNA microarray acquired from literature, according to these reports mir-1, mir-7-5p, mir-133b and mir-143 were downregulated in gastric cancer tissues and gastric cancer cells (14,21-26). In previous studies, miRNA microarray has been widely used to screen aberrant miRNAs between cancer tissue and normal tissue, but the results indicated that aberrant expression of miRNAs in each study were different, and the reasons could be associated with different sample collection from different gastric cancer tissues which reflect different representations or associate with the miRNA microarray manufacturing, which employed various methods. Therefore, Human Cancer Pathway

Finder miRNA PCR array was used to screen and analyse aberrant expression of miRNAs which could be used to compensate for the disadvantages of miRNA microarray.

Our data indicated that mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p were downregulated in gastric cancer cell lines and gastric cancer tissues, fully disclosing its expression levels in gastric cancer lines and gastric cancer tissues. The downregulated expression of 4 miRNAs showed clinical significance in gastric cancer tissues comparing with adjacent normal gastric tissues. Therefore, these 4 downregulated miRNAs are markedly changed biomarkers in gastric cancer, and new light should be shed on their target genes and their clinical significance of diagnosis and prognosis.

In previous studies, upregulation of miR-124 dramatically inhibits the proliferation and tumorigenicity of gastric cancer cells both *in vitro* and *in vivo* through downregulation of SPHK1 (27), but its clinical significance in gastric cancer is unknown. Our data found that miR-124-3p was downregulated in gastric cancers, and low-expression group of miR-124-3p indicating more extensive lymph node metastasis and lymphatic invasion than the high-expression group.

Previous studies demonstrate that the level of miR-146a in cancer tissues was significantly lower than that in the corresponding non-cancerous tissue in 90 clinical samples of gastric cancer (28) and associated with lymph node metastasis and venous invasion. Overexpression of miR-146a suppressed the migration and invasion of gastric cancer cells, and the protein level of WASF2 (29). Low expression of miR-146a was correlated with increased tumor size and poor differentiation in gastric cancer. Overall survival time of patients with high miR-146a expression was significantly longer than that of patients with low expression of miR-146a (30). Moreover, overexpression of miR-146a inhibits the invasion and metastasis of MKN-45 cells *in vitro* and *in vivo* in part due to the downregulation of L1CAM (31). In addition, some researchers found that miR-146a expression is upregulated in a majority of gastric cancers in which it targets CARD10 and COPS8 through inhibiting the activation of NF-κB, thus reducing expression of NF-κB-regulated tumor-promoting cytokines and growth factors (32). miR-146a was upregulated in 20 gastric cancer tissues compared to matched non-tumor adjacent tissues by directly target SMAD4 (33). Our data confirmed that mir-146a-5p was downregulated in gastric cancers, and low-expression group of mir-146a-5p showed more venous invasion than the high-expression group.

In previous studies, miR-155 was overexpressed in cancer tissues of 91 patients by formalin-fixed paraffin-embedded (FFPE) (34), and related to tumor penetration through serosa and lymph node metastasis (35). However, there is also a study on miR-155 significantly downregulated in gastric cancer cell lines compared with GES-1, and overexpression of miR-155 significantly reduced the protein levels of SMAD2 (36). Our results showed that mir-155-5p was downregulated in gastric cancers, and low-expression group of mir-155-5p showed higher stage Borrmann type than the high-expression group.

Previous reports demonstrate that miR-335 is downregulated in gastric cancer cell lines SGC-7901, MGC-803, BCG-823 and AGS compared with GES-1, and suppresses gastric cancer invasion *in vitro* and *in vivo*, by targeting SP1 directly and indirectly through a Bcl-w-induced signaling pathway that sequentially involves PI3K, Akt and Sp1 (37). miR-335 has the potential to recognize the recurrence risk and relate to the prognosis of gastric cancer patients (38). In this study, mir-335-5p was also downregulated in gastric cancer lines and gastric cancer tissues, and low-expression group showed more lymphatic invasion and high stage Borrmann type than the high-expression group which was consistent with previous studies (38).

KEGG pathway enrichment analysis showed that targeted genes of mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p concentrated on 37 signaling pathways, 24 of 37 signaling pathways were involved in the same pathways relevant to cancer. Enriched GO terms showed that targeted genes of these 4 miRNAs concentrated on 339 terms, 24 of 339 terms are associated with same terms relevant to cancer.

In conclusion, Human Cancer Pathway Finder miRNA PCR array is a new approach to screen and validate aberrantly expressed miRNAs more effectively and accurately than miRNA microarray. With this array, we found 38 miRNAs upregulated, and 4 miRNAs downregulated in 7 gastric cancer cell lines comparing GES-1 cell line. Among the 38 upregulated miRNAs, 7 miRNAs including mir-1, let-7b-5p, let-7c, let-7g-5p, mir-27b-3p, mir-183-5p, mir-193-3p and mir-203a were first identified in gastric cancer cell lines. In addition, the expression of 4 miRNAs of mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p was also verified in gastric cancer cell lines and gastric cancer tissues which clarify their confusing, even paradoxical reports, as their expression levels in gastric cancer. Clinical significance of these 4 miRNAs in gastric cancer tissues showed downregulation relevant to more extensive lymph node metastasis and lymphatic invasion, more venous invasion, more high stage Borrmann type, more lymphatic invasion and poor differentiation. Moreover, bioinformatics analysis indicated enriched KEGG pathway analysis and gene ontology of mir-124-3p, mir-146a-5p, mir-155-5p and mir-335-5p were mainly centralized in pathways and GO terms related to tumorigenesis suggesting 4 miRNAs could be important biomarkers in gastric cancers.

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