

Expression and significance of miR-24 and miR-101 in patients with advanced gastric cancer

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Abstract. Clinical significance of micro ribonucleic acid (miR)-24 and miR-101 were investigated by evaluating the expression of miR-24 and miR-101 in the tissues of patients with advanced gastric cancer. A total of 247 gastric cancer tissue specimens and 150 cancer-adjacent normal tissues (>5 cm away from the tumor) from patients with advanced gastric cancer who underwent surgical resection in the Surgical Oncology Department of Tianjin Union Medical Centre (Tianjin, China) from April 2013 to May 2016 were collected. The expression of miR-24 and miR-101 in gastric cancer and cancer-adjacent normal tissues were detected via reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and the correlation of the levels of miR-24 and miR-101 in gastric cancer tissues with their clinical and pathological features were explored. The expression level of miR-24 in gastric cancer tissues was significantly higher than that in cancer-adjacent normal tissues ($t=10.26$, $p<0.01$), while the expression level of miR-101 was significantly lower ($t=13.940$, $p<0.01$). The expression of miR-24 and miR-101 in gastric cancer was correlated with the pathological differentiation degree of the tumor, lymph node metastasis and depth of infiltration ($p<0.05$). The multivariate Cox regression analysis revealed that miR-24 and miR-101 were independent prognostic factors affecting the overall survival of patients ($p<0.01$). The results indicated that the expression of miR-24 is upregulated and that of miR-101 is downregulated in gastric cancer tissues. miR-24 and miR-101 may promote the occurrence, development, infiltration and metastasis of gastric cancer, and can be indicators for the prognosis of patients with gastric cancer.

Introduction

Gastric cancer is the most common gastrointestinal cancer in human and originates from malignant tumors of epithelial cells. High-prevalence groups of gastric cancer are the population aged 55-75 years, and its incidence rate in men (ranks fourth among cancers worldwide) is slightly higher than that in women (ranks fifth among cancers worldwide) (1). In recent years, with the development of the society, patients with gastric cancer tend to be increasingly younger, which is closely related to the living environment and habits. According to data provided by the World Health Organization (WHO), there are >1.5 million patients with gastric cancer around the world, and the mortality rate caused by gastric cancer ranks second among the malignant tumors (2). At present, the treatment for patients with gastric cancer and their survival time are not optimistic, and the survival time of patients with early gastric cancer is greatly prolonged compared with that of patients with advanced gastric cancer. The treatment for gastric cancer is mainly based on radical surgery, supplemented by chemotherapy, radiotherapy and other non-surgical treatments (3). Exacerbated gastric cancer affects the survival of patients, and during the exacerbation, tumor cell invasion and metastasis will gradually appear. The metastasis of tumor cells is a multi-factor involved and complex process, during which the cells metastasize from the primary focus to the tissue nearby. After metastasis, tumor cells can invade the vasculature, and proliferate to form secondary tumors, thus leading to the re-development of gastric cancer and resulting in death (4).

The formation and development of gastric cancer are very complicated processes, and no ideal biological prediction indicator for the occurrence, development, invasion, metastasis and prognosis of gastric cancer has been found so far. According to relevant reports, some micro ribonucleic acids (miRNAs) are expressed in gastric cancer and involved in the pathological and physiological development processes of gastric cancer (5). miRNA regulates the post-transcriptional gene level, plays important roles in a series of activities such as growth, senescence and death of organisms, and plays a carcinogenic or tumor suppressive role in tumors (6). Therefore, the role of miRNA in the tumor has been the focus of clinical research, and miRNA plays key roles in the processes of tumorigenesis, development and treatment. miR-24 is widely distributed in various tissues and organs of the human body, participates in

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multi-tissue and systemic cell differentiation of the body and plays an important role in the process of differentiation (7). miR-101, as a tumor suppressor gene, has two precursors, located in human chromosomes 1 and 9, and its mature sequence is highly conserved in various species (8).

At present, there is little research on the expression of miR-24 and miR-101 in gastric cancer, and the combination of both for monitoring the status of gastric cancer has not been reported yet. This study investigated the roles of miR-24 and miR-101 in gastric cancer by observing their expression so as to provide evidence for the treatment of gastric cancer.

Materials and methods

General data. A total of 247 gastric cancer tissue specimens and 150 cancer-adjacent normal tissues (>5 cm away from the tumor) were collected from patients with advanced gastric cancer who underwent surgical resection in the Surgical Oncology Department of Tianjin Union Medical Centre from April 2013 to May 2016. The patients were aged 30-75 years, with an average age of 45.36 ± 7.26 years, including 135 males and 112 females. According to the WHO clinicopathological classification and staging criteria for gastric tumors (2010) (9), patients were divided into high differentiation (n=34), moderate differentiation (n=60) and low differentiation (n=153) in light of the tumor differentiation degree, into stage III (n=114) and stage IV (n=133) on the basis of their clinical stages, and into N0+N1 (n=131) and N2+N3 (n=116) based on the state of lymph node metastasis. Inclusion criteria: patients whose cancer tissues were confirmed by the gold standard of the Pathology Department, those whose cancer tissues were immediately placed in liquid nitrogen after surgical resection and stored in a refrigerator (-80°C) for low temperature storage, and those whose clinical data were complete. Exclusion criteria: patients having undergone chemoradiotherapy before surgery, those with family history of mental illness or psychosis, and those with liver metastasis or lung cancer. This study was approved by the Ethics Committee of Tianjin Union Medicine Centre (Tianjin, China). The study subjects were informed, and signed the informed consent.

Main instruments and reagents. LightCycler real-time quantitative polymerase chain reaction (PCR) instrument was purchased from Roche Diagnostics (Basel, Switzerland), the total RNA extraction kit (TRIzol method) from Invitrogen (Invitrogen: Thermo Fisher Scientific, Carlsbad, CA, USA), Moloney Murine Leukemia Virus (M-MLV) reverse transcription (RT) kit from Vazyme (Nanjing, China), and miR-24 and miR-101 fluorescence quantitative PCR kits from Biomiga (San Diego, CA, USA). miR-24, miR-101 and U6 reference primers for real-time quantitative PCR were synthesized by the Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The required primer sequences are shown in Table I.

Detection methods. Gastric tissue specimens preserved at -80°C were taken out, and 200 mg was cut and placed into liquid nitrogen, and then rapidly and fully grinded for standby application. TRIzol reagent was used for spare tissues, which were vibrated and let stand for 30 min at room temperature for full lysis. The total RNA was extracted in strict accordance

with the manufacturer's instructions. The concentration and purity of the extracted RNA was determined via an ultraviolet spectrophotometer (Bio-Rad, Hercules, CA, USA) and protein electrophoresis. The extracted total RNA was reverse transcribed according to the instructions of the RT kit. The extracted complementary deoxyribonucleic acid (cDNA) specimens were stored at -20°C. RT-q PCR reaction system was prepared according to the instructions, which was made up to 20 μ l using diethyl pyrocarbonate (DEPC). PCR reaction conditions: pre-denaturation at 94°C for 10 min, 94°C for 45 sec, 60°C for 45 sec, 72°C for 45 sec, and a total of 40 cycles. The amplified data were analyzed using the manufacturer's software with U6 as an internal reference gene. The experiment was repeated 3 times to take an average value, and the value was considered as the amplification result, which was processed using $2^{-\Delta\Delta Cq}$ (10).

Statistical analysis. Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) v.19.0 (SPSS, Inc., Shanghai, China). Measurement data were expressed as mean \pm standard deviation (SD), and t-test was used for comparison of measurement data. Comparison of the mean among multiple groups were analyzed via the analysis of variance, and the post hoc test was Dunnett's test. Cox regression equation was used to analyze the independent prognosis. $P < 0.05$ represents that the difference was statistically significant.

Results

Expression of miR-24 in gastric cancer and cancer-adjacent normal tissues. The expression level of miR-24 was 1.906 ± 1.231 in gastric cancer tissues and 0.836 ± 0.437 in cancer-adjacent normal tissues, and the expression level of miR-24 in gastric cancer tissues was significantly higher than that in cancer-adjacent normal tissues ($t=10.26$, $p < 0.01$) (Fig. 1).

Expression of miR-101 in gastric cancer and cancer-adjacent normal tissues. The expression level of miR-101 was 0.316 ± 0.229 in gastric cancer tissues and 1.167 ± 0.914 in cancer-adjacent normal tissues, and the expression level of miR-101 in gastric cancer tissues was significantly lower than that in cancer-adjacent normal tissues ($t=13.940$, $p < 0.01$) (Fig. 2).

Correlation of the expression of miR-24 and miR-101 in gastric cancer tissues with clinicopathological features. The expression of miR-24 and miR-101 in gastric cancer tissues was correlated with the degree of tumor differentiation, lymph node metastasis and depth of infiltration, and the differences were of statistical significance ($p < 0.05$), but they were not associated with age, sex, tumor tissue type, tumor-node-metastasis (TNM) stage, tumor site and tumor size, and the differences were not statistically significant ($p > 0.05$) (Table II).

Survival curves of advanced gastric cancer patients with expression of high and low miR-24 and miR-101. According to the follow-up data, 224 out of 247 patients with advanced gastric cancer died of disease progression. To determine

Table I. miR-24, miR-101 and U6 gene primer sequences.

Genes	Forward primer sequences	Reverse primer sequences
miR-24	5'-GCTCTGGCTCAGTTCAGCAG-3'	5'-CAGTGCAGGGTCCGAGGT-3'
miR-101	5'-TGGGCTACAGTACTGTGATA-3'	5'-TGCGTGTCTGGAGTC-3'
U6	5'-CATTGCACTTGTCTCGGTCT-3'	5'-GGTCCGAGGTATTTCGCACT-3'

miR, micro ribonucleic acid.

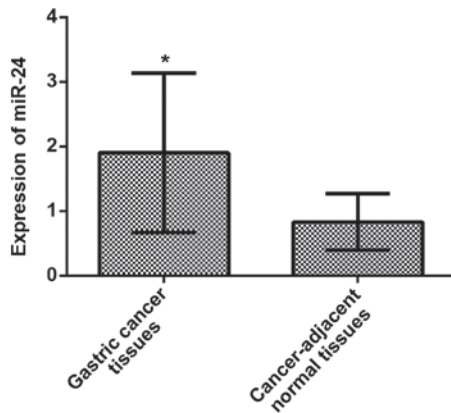


Figure 1. Expression of miR-24 in gastric cancer and cancer-adjacent normal tissues. The expression of miR-24 in gastric cancer and cancer-adjacent normal tissues were detected via RT-qPCR, and the results were statistically analyzed. The expression of miR-24 is 1.906 ± 1.231 in gastric cancer tissues and 0.836 ± 0.437 in cancer-adjacent normal tissues, and the expression of miR-24 in gastric cancer tissues is significantly higher than that in cancer-adjacent normal tissues ($t=10.26$, *compared with cancer-adjacent normal tissues, $p<0.01$). miR, micro ribonucleic acid; RT-qPCR, reverse transcription-polymerase chain reaction.

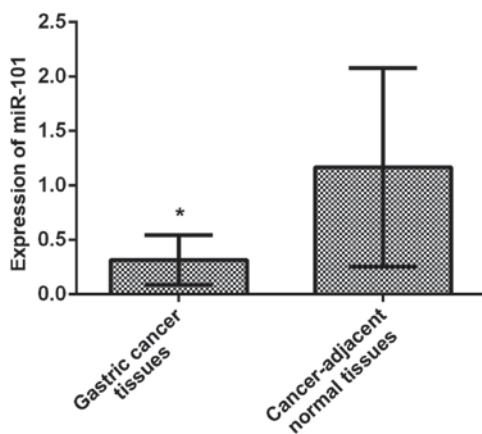


Figure 2. Expression of miR-101 in gastric cancer and cancer-adjacent normal tissues. The expression of miR-101 in gastric cancer and cancer-adjacent normal tissues were detected by RT-qPCR, and the results were statistically analyzed. The expression of miR-101 is 0.316 ± 0.229 in gastric cancer tissues and 1.167 ± 0.914 in cancer-adjacent normal tissues, and the expression of miR-101 in gastric cancer tissues is significantly lower than that in cancer-adjacent normal tissues ($t=13.940$, *compared with cancer-adjacent normal tissues, $p<0.01$). miR, micro ribonucleic acid; RT-qPCR, reverse transcription-polymerase chain reaction.

whether miR-24 and miR-101 could be prognostic biomarkers for patients with advanced gastric cancer, $\Delta\Delta Cq=-2$ was taken

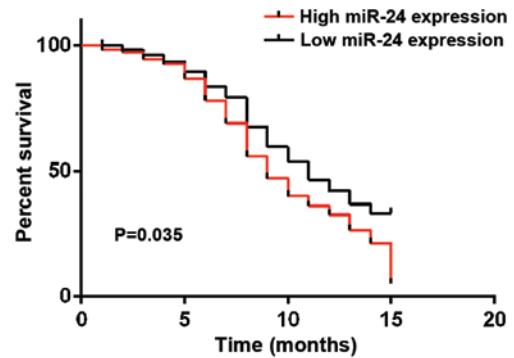


Figure 3. Survival curves of advanced gastric cancer patients with expression of high and low miR-24. The overall survival of patients with advanced gastric cancer is statistically analyzed. The multivariate analysis shows that the overall survival of gastric cancer patients with high miR-24 expression is significantly lower than that of patients with low miR-24 expression ($p<0.05$). miR, micro ribonucleic acid.

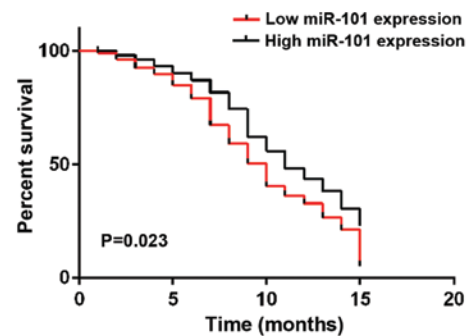


Figure 4. Survival curves of advanced gastric cancer patients with expression of high and low miR-101. The overall survival of patients with advanced gastric cancer was statistically analyzed. The multivariate analysis reveals that the overall survival of gastric cancer patients with low miR-101 expression is significantly lower than that of patients with high miR-101 expression ($p<0.05$). miR, micro ribonucleic acid.

as the critical value in this study to determine the expression levels of miR-24 and miR-101. Multivariate analysis showed that the overall survival of patients with high miR-24 expression was shorter than that of patients with low miR-24 expression ($p<0.05$). The overall survival of patients with low miR-101 expression was shorter than that of patients with high miR-101 expression ($p<0.05$) (Figs. 3 and 4).

Correlation of the expression of miR-24 and miR-101 with the survival time of patients with advanced gastric cancer. Cox regression model analyses of general and clinicopathological

Table II. Correlation of the relative expression levels of miR-24 and miR-101 in gastric cancer tissues with clinicopathological parameters ($\bar{x}\pm SD$).

Types	No.	miR-24	F/t	P-value	miR-101	F/t	P-value
Age			1.074	0.284		1.448	0.149
≤60 years	117	1.943±1.106			0.303±0.351		
>60 years	130	2.107±1.276			0.376±0.432		
Sex			0.453	0.650		0.720	0.471
Male	135	1.837±1.115			0.334±0.386		
Female	112	1.769±1.238			0.371±0.420		
Tissue type			1.326	0.267		1.389	0.251
Adenocarcinoma	168	1.554±1.371			0.341±0.316		
Signet-ring cell carcinoma	45	1.673±1.535			0.298±0.224		
Mucinous carcinoma	34	1.935±1.436			0.256±0.228		
TNM stage			1.880	0.061		1.348	0.179
Stage III	114	1.253±1.011			0.410±0.378		
Stage IV	133	1.493±0.987			0.351±0.310		
Tumor differentiation degree			3.950	0.020		4.508	0.012
High differentiation	34	1.386±1.296			0.376±0.147		
Moderate differentiation	60	1.753±1.224			0.347±0.153		
Low differentiation	153	1.996±1.436			0.264±0.283		
Lymph node metastasis			2.532	0.012		2.165	0.031
N0+N1	131	1.176±0.985			0.228±0.116		
N2+N3	116	1.531±1.192			0.197±0.108		
Tumor site			1.306	0.272		1.296	0.275
Proximal stomach	87	1.731±1.537			0.397±0.263		
Body of the stomach	66	1.934±1.883			0.354±0.273		
Antrum of the stomach	94	2.163±1.982			0.326±0.341		
Tumor diameter			0.756	0.450		1.602	0.110
≤5 cm	139	1.837±0.972			0.374±0.318		
>5 cm	108	1.736±1.124			0.309±0.314		
Depth of infiltration			2.493	0.013		2.332	0.020
T3	37	1.413±1.071			0.273±0.220		
T4	210	1.893±1.129			0.191±0.193		

miR, micro ribonucleic acid; TNM, tumor-node-metastasis.

Table III. Univariate and multivariate Cox regression analyses of the overall survival of patients with advanced gastric cancer.

Variables	Univariate		Multivariate	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age	1.969 (0.483-3.503)	0.376		
Sex	1.890 (0.581-6.161)	0.204		
Pathological differentiation degree	0.421 (0.153-1.177)	0.136		
Lymph node metastasis	3.164 (1.282-7.333)	0.030	2.945 (1.344-4.575)	0.211
Depth of infiltration	2.713 (1.523-3.052)	0.026	2.391 (1.358-3.830)	0.209
miR-24	2.945 (1.344-4.575)	0.016	0.145 (0.055-0.391)	0.021
miR-101	2.391 (1.358-3.830)	0.026	3.162 (1.324-4.544)	0.019

HR, hazard ratio; CI, confidence interval; miR, micro ribonucleic acid.

factors were conducted to investigate the effects of the relative expression levels of miR-24 and miR-101 in patients with advanced gastric cancer and clinicopathological features on the overall survival of patients. The results manifested that miR-24, miR-101, lymph node metastasis and the depth of tumor infiltration may be prognostic factors affecting the overall survival of patients with advanced gastric cancer ($p < 0.05$). Meaningful variables confirmed by the univariate analysis were further analyzed via the multivariate Cox regression model. The results revealed that miR-24 and miR-101 were independent predictors affecting the overall survival of patients ($p < 0.01$) (Table III).

Discussion

Gastric cancer has become a major disease that threatens the health of human beings all over the world. Its morbidity and mortality rates rank first among malignant tumors (11). The survival time of patients with gastric cancer is often closely related to the clinical stage of the tumor. Many patients did not receive examination due to negligence in the early stage, so that they were diagnosed with advanced gastric cancer. With the increase in the cancer stage, tumor cells gradually infiltrate into the serosal or muscular layer, thus the difficulty of treatment is increased and the overall survival of patients with advanced gastric cancer is not satisfactory (12,13). Clinical treatment for gastric cancer is mainly radical surgery, supplemented by chemotherapy or radiotherapy. Although this method has greatly prolonged the patients' survival time, tumor cell metastasis, invasion and local recurrence are still the main causes of death of patients with the deterioration of advanced gastric cancer (14).

miRNA is a newly discovered non-coding small molecular RNA that affects human physiological and pathological changes. The occurrence and development of gastric cancer are multi-step and multi-factor involved processes, and many miRNAs are abnormally expressed in gastric cancer (15). miR-24 plays a key role in the regulation of cell proliferation, apoptosis, invasion and metastasis in biological characteristics. miR-24 has a dual function: it may play a role as a tumor suppressor gene in various tumor development processes on the one hand, and acts as an oncogene on the other hand (16). miR-24 exerts effects on the occurrence and development of the disease by regulating cell differentiation and cell cycle, and it plays inhibitory roles in BRCA1, CDKN1B and CHEK1 at the G2/M restriction sites, thus affecting the cell cycle and indirectly inducing the formation and development of the tumor (17). miR-101 is expressed in a variety of eukaryotic organisms, regulates genes, and has multiple targets associated with oncogenes (18). In this study, the expression of miR-24 was significantly upregulated, and the expression of miR-101 was significantly downregulated in gastric cancer tissues compared with those in cancer-adjacent normal tissues, suggesting that miR-24 and miR-101 may be involved in the occurrence and development of gastric cancer and show certain trends in the progressive process of gastric cancer. miR-24 and miR-101 were correlated with the degree of tumor differentiation, lymph node metastasis and depth of infiltration in gastric cancer, indicating that miR-24 and miR-101 can be used as predictive indicators for detecting progression of

gastric cancer. In addition, the high expression of miR-24 and the low expression of miR-101 may promote the metastasis and invasion of gastric cancer cells, and the combination of the two may help determine the degree of deterioration of advanced gastric cancer. According to the research of Zhang *et al* (19) and Chen *et al* (20), the expression of miR-24 in gastric cancer cell lines and gastric cancer is upregulated in human. miR-24 is related to the process of tumorigenesis of gastric cancer and can induce the occurrence of the tumor by inhibiting the p16 oncogene. The expression of miR-101 is significantly downregulated in gastric cancer tissues, thus losing the inhibitory effect on cyclooxygenase-2 (COX-2) and resulting in the high expression of COX-2 in gastric cancer tissues. Since COX-2 has the ability to promote the generation of tumor-surrounding blood vessels and suppress the apoptosis of tumor cells, the downregulation of miR-101 expression can promote the occurrence and development of gastric cancer. Both miR-24 and miR-101 participate in the metastasis and invasion of gastric cancer. The above is similar to the conclusions of this study.

The overall survival rate of patients with advanced gastric cancer has long been unsatisfactory, and the prognosis of patients has been a hotspot clinically. In this study, the univariate analysis of Cox proportional hazard model for patients with advanced gastric cancer demonstrated that the lymph node metastasis, depth of infiltration, miR-24 and miR-101 may be the prognostic factors affecting the overall survival of patients with advanced gastric cancer. As the impact of confounding factors on the overall survival of patients cannot be controlled in univariate analysis, the intensity of these impacts of indicators observed on the prognosis may be sometimes weakened or enhanced, and false-positive or-negative results may appear; thus, the multivariate Cox model analysis was further performed. The results revealed that the expression of miR-24 and miR-101 was independent prognostic factors for the overall survival of patients, not the lymph node metastasis and depth of infiltration. The study analysis showed that the occurrence of the lymph node metastasis and infiltration in patients represented the further spread of the tumor, and the more serious the disease was, the poorer the prognosis would be, suggesting that miR-24 and miR-101 can be used as indicators for the diagnosis of patients with gastric cancer.

In this study, the selected gastric cancer specimens underwent strict screening to ensure the reliability of the experiment. This study failed to include patients with early gastric cancer, so there were some limitations. The occurrence, development, metastasis and infiltration of the tumor are complex and multi-factor involved processes, and oncogenes may promote the proliferation and apoptosis of tumor cells (21). miR-24 and miR-101 are also abnormally expressed in other tumors, but the mechanisms of the two in gastric cancer remain unclear, so it is expected that the mechanisms of the two in gastric cancer will be elaborated in further studies.

In conclusion, the expression of miR-101 is upregulated and the expression of miR-24 is downregulated in gastric cancer tissues. miR-24 and miR-101 may promote the occurrence, development, infiltration and metastasis of gastric cancer. They may be indicators for the diagnosis of patients with gastric cancer, and have important clinical predictive values.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XD and YL conceived and designed the study, collected, analyzed and interpreted the patient data. XD wrote the manuscript. YL revised the manuscript for important intellectual content. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Tianjin Union Medical Centre (Tianjin, China). Signed informed consents were obtained from the patients or guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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