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RESEARCH ARTICLE

THE EXPRESSION OF MIR-126 ASSOCIATED WITH CLINICOPATHOLOGIC FEATURES IN GASTRIC CARCINOMA

[1]Fei Song, [2]Xiu-Lan Su, [2]Ling Yang, [3]Wen-Jian Xu and [1,*]Ming Liu

[1]Department of General Surgery, Affiliated Hospital of Inner Mongolia Medical University, China
[2] Clinical Medical Research Center, Affiliated Hospital of Inner Mongolia Medical University, China
[3]Department of General Surgery, Affiliated Tumor Hospital of Inner Mongolia Medical University, China

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ABSTRACT

Objective: With gene chip technology screening and reference to foreign research reports, we have determined to research the target gene miR-126.We detected the expression of miR-126 in gastric cancer tissues and their non-tumorous control .We also studied the correlation between the expression of miR-126 and clinicopathologicall Preliminary exploration of miR-126 in gastric cancer development, as well as potential tumor markers. **Methods:** 100 cases of fresh gastric carcinoma and adjacent normal tissue specimens were selected from gastric cancer radical resection, using the Trizol method to extract total RNA, and poly-A tail, anti-transcribed into cDNA, and then applied real-time fluorescence quantitative polymerase chain reaction (real-time PCR). Determination we initially selected gastric carcinoma Ct values of miR-126 and miR-126 Ct value, then the corresponding adjacent normal tissue. The 2 -△△Ct calculation method analysis of the measured Ct values were compared between gastric carcinoma cancer and adjacent normal tissues for the mi-R126 relative differences. Finally, we used statistical methods to contact clinicopathological parameters and miR-126 expression level in gastric cancer tissue. **Results:** We found that in the experimental group: miR-126 expression level in the gastric carcinoma group was significantly lower than the control group: namely the adjacent normal tissue group (P <0.001). From medical statistical analysis, miR-126 in gastric cancer tissues was significantly reduced, and the expression level had no correlation with gender, age, histological type, degree of differentiation of gastric cancer cells, lymph node metastasis, depth of invasion and UICC stage (P> 0.05). **Conclusion:** In gastric cancer tissues, the expression of miR-126 was significantly lower than in adjacent normal tissues. MiR-126 may become a potential biological tumor marker. This can provide a reliable basis for the early detection, early diagnosis, individualized treatment and prognosis, monitoring recurrence for patients with gastric cancer.

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INTRODUCTION

World widely, the incidence of stomach cancer ranks the fourth among all malignant tumors, and the second among malignant cancer-related deaths. According to authoritative statistics, the number of stomach cancer-related deaths in China accounts for about half of the total of stomach cancer-related deaths in the world. China is in a high incidence area of gastric cancer, but our level of diagnosis and treatment is far less than that of some developed countries in the world. At present, there are a variety of clinical treatment methods, including surgery, chemotherapy and other comprehensive treatments. But overall, the therapeutic effect of gastric cancer is still not very ideal.

***Corresponding author:** **Ming Liu,**
Department of General Surgery, Affiliated Hospital of Inner Mongolia Medical University, China.

According to relevant reports, the five-year survival rate of gastric cancer patients in China is only about 20-25% (Hartgrink *et al*., 2009). Complete radical resection is still the most effective treatment method at present, which can provide a good chance of survival for patients with early stage gastric cancer. The survival rate of patients with early stage gastric cancer is nearly 90% (Wang *et al*., 2012). However, for advanced gastric cancer, despite partial radical surgery, about 80% of patients still have local recurrence and/or distant metastasis in a very short period of time (30%) or even death (87%). Therefore, early diagnosis and surgical treatment can effectively increase the chance of curing gastric cancer patients or extend the survival time of patients. However, the current situation is that the earliest gastric cancer is difficult to be found through existing detection methods, and the clinical diagnosis rate of early cancer and small cancer is low (Imdahl,

2008). We still lack a thorough understanding of the pathogenesis of gastric cancer, and the existing clinical markers of gastric cancer are not sufficiently sensitive and specific. The high morbidity, low early diagnosis rate and poor prognosis of gastric cancer call for the need of a biological tumor marker which can effectively diagnose gastric cancer and evaluate the prognosis of gastric cancer. This marker can not only be used for early diagnosis of gastric cancer, but can also provide a reliable basis for evaluating prognosis. In recent years, due to the keen desire and continuous exploration of clinical tumor markers, we have found and conducted preliminary studies on micro-RNAs, and achieved gratifying results. Mi-RNA (micro-RNA) is a kind of single-stranded, non-coding small molecule RNA, usually with a length of 21-23nt, which is highly conserved in evolution and widely exists in eukaryotes (7).In terms of human beings, mature mi-RNA, together with other proteins, form the RISC (RNA-induced silencing complex) complex, which binds to the corresponding loci of the 3 'untranslated region (3' -utr) of the target mRNA and participates in genetic regulation(Gregory *et al*., 2005) The binding mode of mi-RNA and mRNA is incomplete complementary pairing, which enables the degradation of target mRNA and negatively regulates the expression of target genes at the level of protein translation (Lund *et al*., 2004).

Since the first mi-RNA was discovered by Lee R *et al* in 1993, a lot of scientific research on mi-RNA had been conducted (Lee *et al*., 1993). Studies have shown abnormal expression of mi-RNA, which is widely present in various diseases, including almost all common cancers (Jay *et al*., 2007). Mi-RNA is involved in the regulation of gastric cancer mainly in two aspects. Firstly, by regulating the activity of proto-oncogenes, mi-RNA can cause excessive cell proliferation, inhibit cell apoptosis and play a role in promoting cancer. Secondly, by regulating the biological activity of tumor cells, mi-RNA inhibits tumor occurrence, promotes apoptosis, reduces invasiveness and metastasis, and plays a role in tumor inhibition. In addition, by analyzing a large number of existing research results, scientists boldly concluded that high affinity nucleotide chemical modification of mi-RNA may become the basic treatment for cancer (Petri *et al*., 2009). Some mi-RNAs with abnormal expression in gastric cancer have been found and played an important role in the occurrence and development of gastric cancer. This study adopts the internationally advanced genetic chip technology to preliminarily screen the mi-RNAs with differential expression in human gastric cancer tissues. The results indicated that the expression level of miR-126 in gastric cancer tissues and normal adjacent tissues might be significantly different. Large clinical sample validation of miR-126 was performed by real-time quantitative PCR. We hope to explore the molecular mechanism of the occurrence of miRNA and gastric cancer through our research work, so as to provide reliable basis for the early detection, early diagnosis, early clinical individualized treatment, prognosis judgment and monitoring of recurrence of gastric cancer.

MATERIALS AND METHODS

Patient selection: In this study, 100 gastric cancer specimens were collected from the Department of Oncology and General Surgery of the Affiliated Hospital of Inner Mongolia Medical University from December 2010 to December 2012. A total of 100 cases of surgical resection of gastric cancer between December 2010 and December 2012 were collected in tumor surgery and general surgery in affiliated hospital of Inner Mongolia medical university. All the specimens were confirmed by pathological diagnosis of the department of pathology, and none of the patients received any treatment including radiotherapy, chemotherapy and biological treatment before surgery. All specimens were approved by the medical ethics committee of Inner Mongolia medical university and received consent from the patient. During the operation, one piece of tumor tissue was collected from each specimen (T, adipose tissue and tumor necrosis area were avoided), together with normal adjacent tissues (N, > 5cm from cancer tissue). Samples to be dissected within 10 minutes away from the body were put in a sterile tube without RNase cryopreserved, cryopreserved in liquid nitrogen, to - 80 □ after cryogenic refrigerator, until the RNA extraction. Patients' names, gender, age, surgical methods, gastroscope data, pathological number, pathological data, preoperative diagnosis and treatment, UICC stage and postoperative follow-up were collected postoperatively.

Study design

Total RNA extraction and concentration determination by Trizol method: Divide Trizol into pieces, and add 1ml Trizol to each EP tube. The tube were then put in 4 □ refrigerator and set aside. Put frozen tissue sizing around 1 cm^3in advance into -80 □ cryogenic refrigerator precooling of stainless steel in the mortar, add the liquid nitrogen trituration, research while, keep frozen tissue, until the compound completely turns into a fine powder, this step also can choose tissue homogenate. Put the tissue powder into EP tube containing 1mlTrizol quickly with the drug spoon, shake it gently with by hand, and place at room temperature for 5min. Note that this step should be done quickly to avoid the tissue melting and RNA degradation. Low temperature centrifuge set to 4 ℃, 10000 g, centrifuge for 10 min without removing the bottom solution. The upper solution was taken to a new EP tube, then 200µl chloroform was added, and the EP tube was covered. Low temperature centrifuge set to 4 ℃, 10000 g, centrifuge for 10 min, the solution is divided into three layers (respectively: the lower organic phase, intermediate phase and water phase of the upper middle). Move the upper water phase to a new EP tube. Add in the new EP tube 500µl isopropyl alcohol, upside down, blending, gently placed 10 minutes at room temperature, low temperature centrifuge set to 4 ℃, 12000 g, centrifuge for 10min. When it becomes clear liquid, add in 1ml 75% ethanol, blend well, and centrifuge at low temperature 4 ℃, 10000 g, centrifuge for 10min. Be careful to refuse to clear liquid, then dry at room temperature for 5-10 minutes, after waiting for ethanol volatilization, with 30µl to 40µl RNase - free H_2O dissolved, move to -80℃ ultra-low temperature freezer and set aside. NANO DROP 2000C was used to determine the purity and concentration of od260/280 by 2µl total RNA sample. Partial shipments, - 80℃. (OD 260/280>1.8, the purity of the sample meets the experimental requirements)

Total RNA integrity was identified by electrophoresis: Prepare 2% agarose gel, agarose buffer = 2g: 100ml; microwave for 2-3minutes, until completely dissolved, and becomes transparent and clear. Cool to 50 to 60□, add GelRed and the solution into good comb in the electrophoresis tank. Cool about 20min at room temperature (visible at room temperature and humidity appropriate adjustments). After the gel is set, pull out the sample comb vertically and upwards. Put the negative pole of the sample hole in the electrophoresis tank

to replenish the electrophoresis buffer. Mix the total RNA of 3-5μl with 2μl loading buffer evenly, add it to the hole of upper sample, connect to the power supply, and set the voltage at 100-120V. When the indicator runs over half of the length of the gel plate, turn off the power and place the electrophoretic gel block under the gel imaging analysis system to observe and shoot the results (see Figure 1).

RNA reverse transcription into cDNA: Poly (A) was applied to the 3 'end of miRNA using the mi-rcute miRNA cDNA first strand synthesis kit. On the ice, precool RNase free reaction tube to join the following reagent to the total volume of 20μl (add e.c. with our fabrication: oli Poly real Polymerase) (A), 0.4μl (including 1 e.c. with our fabrication: oli Poly real Polymerase (A) (5U/(including l), 2 (including 10 × Poly real (A) Polymerase Buffer, 4μl (including 5×rATP solution, including 2-5μl total RNA .Finally, add RNase-free ddH$_2$O to 20μl. Using liquid trace to move after mixing and centrifugal. Set up PCR: reaction temperature of 37 ℃, and the reaction time for 1 hour. The reaction solution obtained can be directly continued for the next experiment. It may also be preserved at - 20 ℃ for short term and long-term at -80℃. The miRNA modified by Poly(A) was then retrotranscriptional and configured with the following table: Poly(A) modified miRNA reaction solution (2μl);10× RTprimer (including 2μl;10× RT Buffer (including 2μl;Superpure dNTP Mixture (2.5mm each) RNasin (u/(including 40 l) 1 (including l;Quant RTase (including 0.5μl; RNase-free ddH$_2$O 11.5μl, total volume 20μl.Use liquid trace to move after mixing and centrifugal, set up PCR at reaction temperature of 37 ℃, reaction time 1hour. Return transcription product to - 20 ℃ refrigerator, or directly use in fluorescent quantitative detection.

Fluorescence quantitative PCR reaction: 4 ℃ low temperature and sample on melt 2×miRcute micrornas Premix and Reveres Primer. When fully melted, mix the 2 - x miRNA Premix gently to avoid bubbling and use in a low speed centrifuge. The following table reaction system (20μl) was configured, including 2× miRNA Premix (SYBR, ROX) and 10μl.Move on to Primer (self-provided). Reverse Primer 0.4μl, 50×ROX Reference Dye 1.6μl. The addition of the first strand of cDNA of miRNAD should not exceed 1/10 of the total volume of RT-PCR.If the concentration of cDNA is too high, non-specific amplification can be diluted appropriately (10 times or 100 times).Add RNase-free ddH$_2$O to 20μl.Reaction system on sample, set the PCR reaction procedure in the following table: starting template degeneration stage 94 ℃ for 2 min 1 cycle; PCR circulation template degeneration in 94 ℃ for 20sec, annealing, 34sec 45cycle extension 60 ℃. The experimental data and Ct values were recorded by adding the Dissociation Stage. Primer sequence Lot No 8400976334 U6F CTCGCTTCGGCA GCACA; Lot No 8400976335 U6R AACGCTTCACGAAT TTGCG. The dissolution and amplification curves of internal parameters U6 and miR-126 were plotted (see Figure 2- Figure 5).

Detection procedure: Ct values of each sample were measured by real-time PCR. Ct value is defined as the number of amplification reaction cycles when the fluorescence signal excited by the amplification product of the desired target gene reaches the set threshold, which is the number of cycles when the target gene starts to show exponential growth. The Ct value was inversely proportional to the copy number of the target gene c-DNA, and the higher the c-DNA content of the target gene, the lower the Ct value. We measured each sample with the method of multihole measurement, and obtained the average value of the data, so as to obtain the final Ct value of each sample. We analyzed the measured Ct values using the calculation method of $2^{-\triangle\triangle Ct}$ to compare the relative differences of miR-126 expression between gastric cancer tissues and adjacent normal tissues. Firstly it was necessary to calculate the Delta Ct (△Ct) to express the relative amount of miR-126. In addition, the proportion of aspirated Ct(the Ct value of miR-126 minus the Ct value) of internal reference U6 in each sample was inversely proportional to the relative amount, and the higher the bound Ct was, the lower the expression of miR-126 was. Then, the value of △ was calculated by using the value of △Ct (the value of △was equal to the value of △Ct of gastric cancer tissue minus the value of △Ct of adjacent normal tissue). Afterwards, a multiple of the relative gene expression difference between gastric cancer tissue samples and adjacent normal tissues in each case was obtained by $2^{-\triangle\triangle Ct}$ .SPSS19.0 was used for statistical analysis of the difference in miR-126 expression between gastric cancer tissues and normal adjacent tissues. Data were described by mean and standard deviation, paired t test and variance analysis was used for inter-group comparison, and a vertical bar chart was drawn. $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics: The results showed that the expression of miR-126 in gastric cancer tissues was 0.3065 and the standard deviation was 0.5618. There was a significant difference in expression multiplier $2^{-\triangle\triangle Ct}$ between adjacent normal tissues, t=12.343, P=0.000. Among patients with gastric cancer, 96 patients (96%) had $2^{-\triangle\triangle Ct} < 1$, of whom 88 were below 0.5. These results suggest that miR-126 may play an important role as an anti-oncogene in the development of gastric cancer. Increasing the expression level of miR-126 may be a potential therapy for gastric cancer.

Association of the expression of mi-RNAs ***with clinical characteristics:*** There was no correlation between the expression level of miR-126 and gender, age, pathological type, degree of differentiation, lymph node metastasis, depth of invasion, and UICC stage. And the expression level was not different (P > 0.05) (see Table 1).

Relationship between miR-126 and pathological types of gastric cancer: MiR-126 expression in adenocarcinoma, mucinous cell carcinoma and signet-ring cell carcinoma was 0.2952 (+ 0.5611), 0.5039 (+ 0.6948) and 0.1872 (+ 0.0567), T or F was 0.513, respectively. There was no significant difference between the groups (P>0.05).

Relationship between miR-126 and differentiation of gastric cancer: The expression levels of microRNAs-126 in the high, middle and low differentiation groups were 0.3356 (+ 0.7507), 0.2922 (+ 0.4282) and 0.2724 (+ 0.3104), respectively, and T or F were 0.090. There was no significant difference between the groups (P>0.05).

Relationship between miR-126 and lymph node metastasis in gastric cancer: The expression levels of miR-126 in lymph node metastasis group and non-lymph node metastasis group were 0.3148+0.5899 and 0.2805+0.4722, T or F 0.259, respectively. There was no significant difference between the two groups (P>0.05).

Table 1. Correlation between the level of mi-r126 expression in gastric cancer tissues and clinical pathological parameters

Characteristic		Number (n=100)	$2^{-\Delta\Delta Ct}$ ($\bar{x}\pm S$)	T or F	P
Gender					
	Male	84	0.3246±0.6049	0.733	0.465
	Female	16	0.2120±0.2169		
Age					
	<65	58	0.3304±0.6877	0.497	0.620
	≥65	42	0.2736±0.3206		
Pathological type					
	Adenocarcinoma	90	0.2952±0.5611	0.513	0.600
	mucinous cell carcinoma	7	0.5039±0.6948		
	Signet ring cell	3	0.1872±0.0567		
Differentiated degree					
	poorly differentiated	39	0.3356±0.7507	0.090	0.914
	moderately differentiated	48	0.2922±0.4282		
	high differentiation	13	0.2724±0.3104		
lymphatic metastasis					
	Yes	76	0.3148±0.5899	0.259	0.796
	No	24	0.2805±0.4722		
The depth of invasion					
	T1T2	34	0.2838±0.4060	0.289	0.773
	T3T4	66	0.3182±0.6298		
UICC Stage					
	1	18	0.3427±0.5319	0.532	0.662
	2	21	0.1971±0.1506		
	3	55	0.3520±0.6863		
	4	6	0.1642±0.0842		

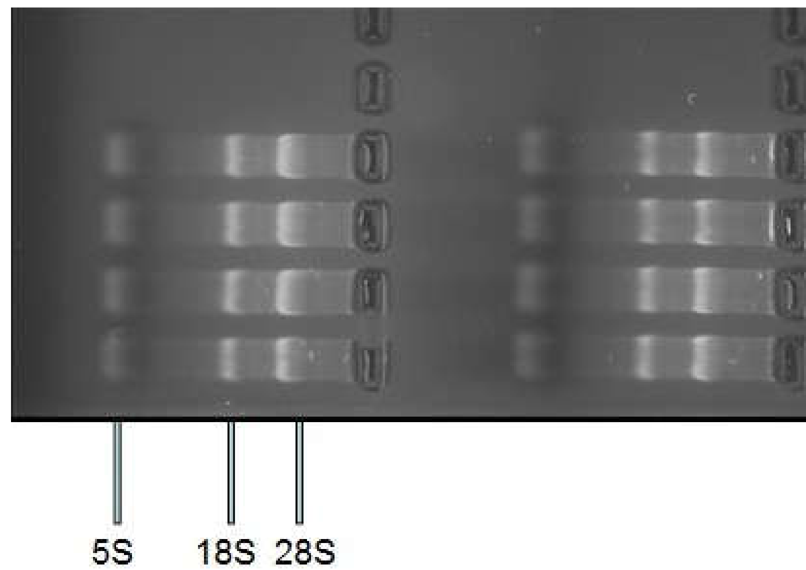

Figure 1. The polymorphisms of the total RNA

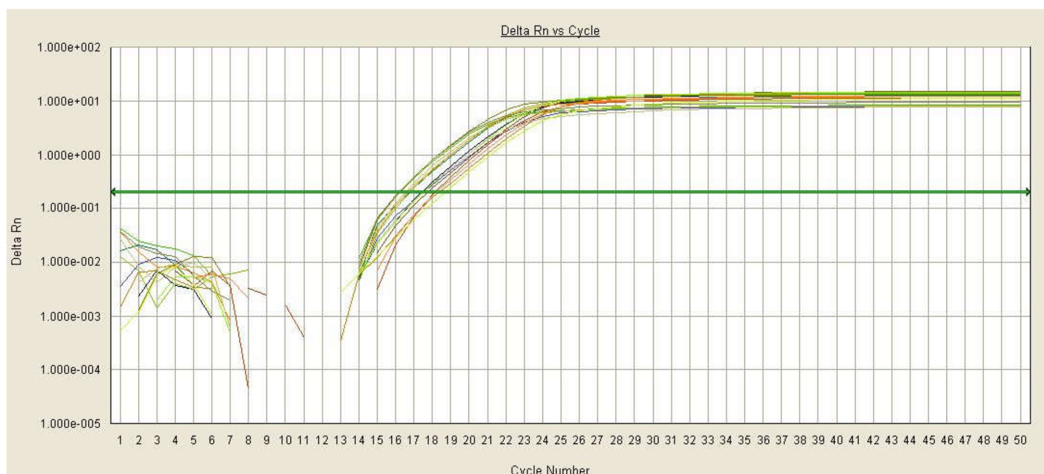

Figure 2. Amplification curve of the internal U6

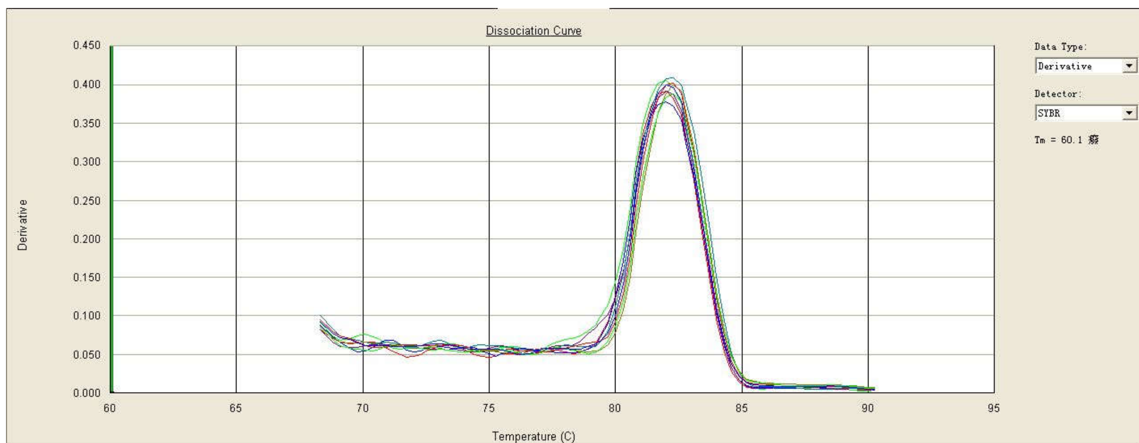

Figure 3. Solubility curve of the internal U6

Figure 4. Amplification curve of miR-126

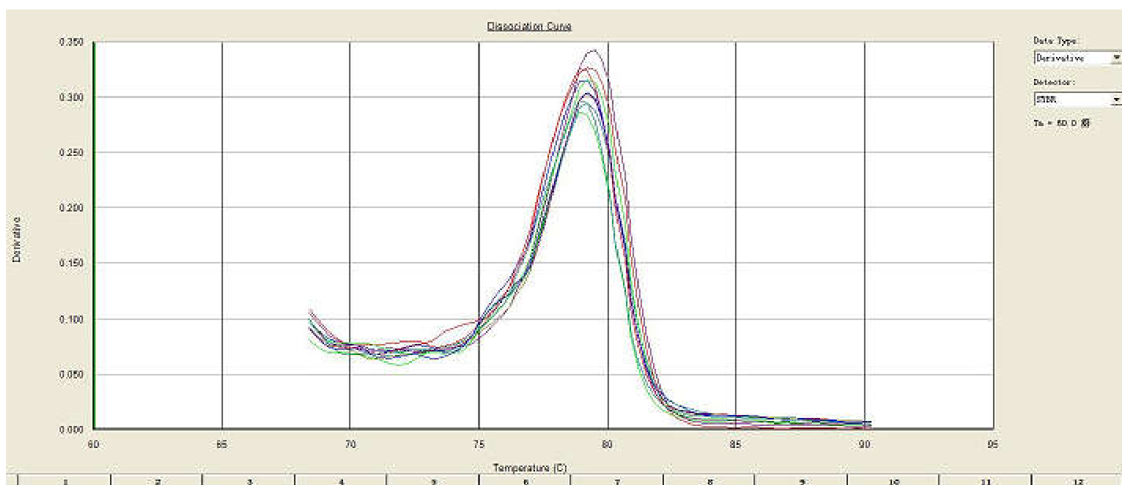

Figure 5. Solubility curve of miR-126

Relationship between miR-126 and invasion depth of gastric cancer: The expression of microRNAs-126 in T1T2 and T3T4 groups was 0.2838 (+ 0.4060) and 0.3182 (+ 0.6298) respectively, and T or F was 0.289. There was no significant difference between the two groups (P>0.05).

Relationship between and UICC staging of gastric cancer: The expression levels of microRNAs-126 in UICC stage I, stage I, stage II and stage IV groups were 0.3427 (+ 0.5319), 0.1971 (+ 0.1506), 0.3520 (+ 0.6863) and 0.1642 (+ 0.0842), t or F 0.532, respectively. There was no significant difference between the groups (P>0.05).

DISCUSSION

Through previous studies, we have known the expression levels of various micro-RNA (mi-RNA) play an important role

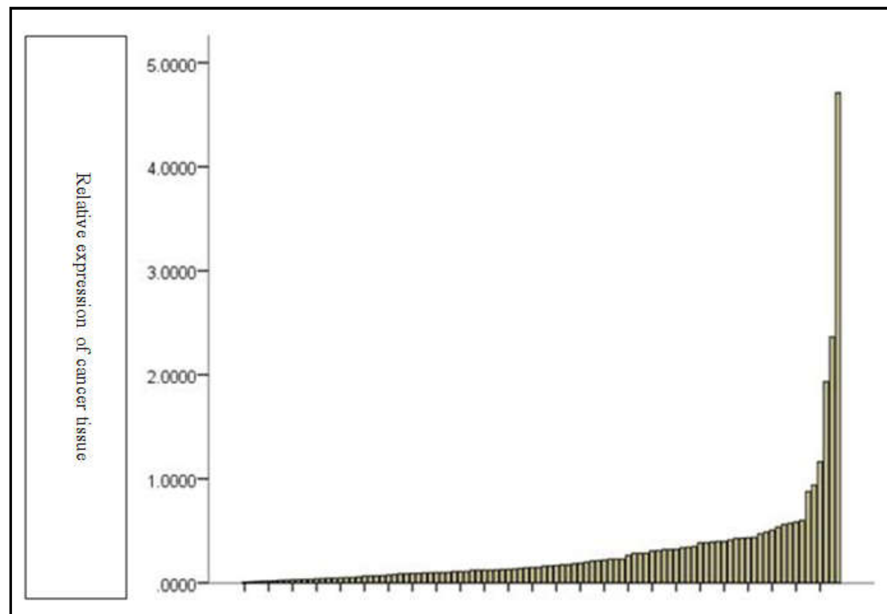

Figure 6. Relative expression of miR-126 in cancer tissue

in diseases, including human malignant tumors. These effects are caused by deletion, amplification or mutation of mi-RNA control site genes, epigenetic silencing or transcription factor maladjustment specific to mi-RNA (Kato, 2008). Due to the research progress of mi-RNA genes in malignant tumor cells, various protein-coding oncogenes or tumor suppressor genes are controlled. These advances in mi-RNA research provided important opportunities for future miRNAs-based treatment (Croce, 2009). Gastric cancer has a highly lethal malignancy, with more than 450,000 new cases per year in China alone (ZOU Xiao-nong, 2012). Therefore, it is urgent to have a better understanding of the etiology and progression of gastric cancer. The role of genetic risk factors of bacteria, environment and host in the occurrence and development of gastric cancer had been studied thoroughly and became a clear cause of gastric cancer, but the molecular study is still very limited (Shah, 2010). Studies have confirmed the role of tumor suppressor genes and cell cycle factors, and the knowledge of post-transcriptional gene expression changes in gastric cancer is still incomplete. We wanted to find a breakthrough here with mi-RNA, so a lot of researchers began to work on mi-RNA related to gastric cancer. The mechanism of mi-RNA production has been basically clarified. Mi-RNAs gene is usually within the nucleus under the function of RNA polymerase II transcribed into Pri-miRNAs, was quickly RNase III Drosha and its cofactor Pasha cut into 70nt hairpin structure of precursor of miRNAs (Pre - miRNAs). Its structure is similar to mRNA, with more than 5 'end cap structure and 3' end poly nuclear acid tail (Fabian *et al*., 2010). These precursors undergo DNA transcription and the cutting of Drosha and Dicer enzymes, and eventually these mature miRNAs combine with other proteins to form the RISC (RNA-induced silencing complex) complex, which binds to the site of the target mRNA and participates in genetic regulation (Lee *et al*., 2003; Filipowicz *et al*., 2008). In recent years, through the continuous efforts of researchers, a considerable number of gastric cancer-related miRNAs have been found, and their roles, mechanism of action and corresponding targeted genes in gastric cancer tissues and cells have been basically defined, such as common oncogenes (miR-21, miR-106a and miR-17) and anti-oncogenes (miR-101, miR-181, miR-499, miR-486 and let-7a).

In addition to the above-mentioned miRNAs that play the role of oncogenes and tumor suppressor genes, there are many other miRNAs that are still controversial and miR-126 plays such a controversial role. MiR-126 is an endothelial cell-specific miRNA, which promotes the generation of blood vessels by targeting SPRED1 and PIK2R2 and regulating VEGF signal of vascular endothelial growth factor (Fish *et al*., 2008; Nicoli *et al*., 2010; Wang *et al*., 2010). In addition, it has also been reported that miR-126 is a tumor cell suppressor that plays a role in tumor inhibition through targeted regulation of p85-courts and irs-1 in colon cancer cells (HEK293 and irs-1) (Guo *et al*., 2008; Zhang *et al*., 2008).

However, in another report by Japanese researchers Otsubo T (Otsubo *et al*., 2011) found that miR-126 through targeted adjustment SOX2 play to the role of the cancer gene. They used the gain and loss of function of experiments, and in and luciferase analysis method, found that miR-126 can inhibit the expression of SOX2, abnormally high expression in gastric cancer cells periodic stagnation and apoptosis had an effect. Gastric cancer cells were obtained by in vitro cell culture and the expression of miR-126 and SOX2 were detected. Abnormal over-expression of miR-126 and consequent down-regulation of SOX2 may contribute to the occurrence of gastric cancer. However, another study reached a different conclusion. The study of Garraway LA *et al* (Garraway, 2006) showed that miR-126 played the role of tumor suppressor genes in gastric cancer, and the low expression level of miR-126 in gastric cancer cells could effectively inhibit the growth, migration and invasion of sgc-7901 gastric cancer cells in vitro cell culture. The tumor forming and metastatic ability tests in animals also showed positive tumor inhibition.

Therefore, in gastric cancer, whether miR-126 is an oncogene or an oncogene is controversial. Some experts have interpreted the presence of these functional differences as lineage - dependent tumors. Considering the different views given by the researchers, further studies are urgently needed, including the molecular mechanism of tumor action, factors and meta-analysis (Feng *et al*., 2016). The expression level of miR-126 in many other malignant tumors was down-regulated, and the tumor inhibition effect and mechanism were confirmed in

leukemia, lung cancer, colon cancer, breast cancer, cervical cancer and other diseases. However, studies on miR-126 and gastric cancer mainly focus on in vitro cell culture and animal experiments, and there is still a lack of histological analysis of human gastric cancer in large sample numbers. In our project, 100 fresh frozen surgical specimens were selected for radical gastrictomy, and qRT-PCR was used for relative quantitative experiments with good sensitivity and specificity. The expression level of miR-126 in gastric cancer was verified by large scale clinical tissue samples, and the correlation between the expression level and gender, age and pathological parameters was statistically analyzed. It has been confirmed that the application of miR-126 as a tumor marker of gastric cancer in clinic has great potential.

CONCLUSION

Real-time fluorescence quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a relative quantitative measurement technique with high sensitivity and specificity. It is an effective method for detecting micro-RNAs. The expression of miR-126 in gastric cancer tissues was significantly lower than that in adjacent normal tissues. MiR-126 expression was low in gastric cancer, and its expression level was not related to sex, age and related pathological parameters. MiR-126 may act as a tumor suppressor gene in the pathogenesis of gastric cancer. The decrease of expression level of miR-126 may weaken the inhibition of some proto-oncogenes in human body and lead to the occurrence and development of gastric cancer. The correlation between miR-126 and gastric cancer opens up new ideas for the pathogenesis and treatment of gastric cancer. Direct detection of its expression may provide a possibility for early diagnosis of gastric cancer.

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