Abstract. The present study aimed to evaluate the correlation between the expression of microRNA-146a (miR-146a) and its target gene, LIN52, in advanced gastric cancer, and determine their potential effects on chemotherapeutic sensitivity and prognosis. Total RNA was extracted from 93 tissue samples of advanced gastric cancer and corresponding adjacent non-tumor tissues to quantify the relative expression levels of miR-146a using reverse transcription-quantitative polymerase chain reaction analysis. The expression of LIN52 was detected in tumors and normal tissues using immunohistochemical analysis. Correlation analysis was performed to assess the correlation between the expression of miR-146a and LIN52 and clinicopathological parameters of gastric cancer, including clinical diagnostic specificity, clinical tumor-necrosis-metastasis staging, lymph node metastasis, differentiation grade, chemotherapeutic sensitivity and prognosis. The expression of miR-146a in advanced gastric cancer tissues was lower, compared with that in the adjacent non-tumor tissues, and was negatively correlated with lymph node metastasis (P<0.05). Gastric cancer tissues with a low expression level of miR146a exhibited an increased expression level of LIN52 (P<0.05). Receiver operating characteristic curve regression analysis showed that miR-146a had 98% sensitivity in distinguishing gastric cancer tissues and adjacent non-tumor tissues. A high expression of miR-146a in gastric cancer was associated with improved treatment efficacy in patients. The chemotherapeutic sensitivity of patients with tumors expressing high levels of miR-146a was significantly higher, compared with that of patients with tumors expressing low levels of miR-146a (P<0.05). The expression of miR-146a was low in advanced gastric cancer tissues. As a tumor suppressor gene in advanced gastric cancer, miR-146a had a significant negative correlation with LIN52. High expression levels of miR-146a in advanced gastric cancer tissue may be associated with improved treatment efficacy of chemotherapy, suggesting that miR-146a may be a molecular marker for the diagnosis, prediction of treatment efficacy and prognosis of advanced gastric cancer.

Introduction

Gastric cancer is a common malignancy affecting the gastrointestinal system, which accounts for 8% of all types of malignant cancer. Although the incidence of gastric cancer has been declining worldwide, it remains high in several countries, particularly in China (1). The development and progression of gastric cancer are associated with multiple genes and numerous transforming steps. Investigating genetic and other risk factors associated with the occurrence and development of gastric cancer, and their correlation with prognosis, is critical to provide theoretical support for improving the diagnosis, prognosis and treatment of gastric cancer (2). MicroRNAs (miRs) are small non-coding RNA molecules of ~22 nucleotides, which regulate gene expression at the post-transcriptional level. The regulatory capacity of miRs includes almost all major cellular activities, including cell proliferation, differentiation and apoptosis. In previous years, a number of studies have shown that microRNAs can have oncogene and tumor suppressor functions, thereby being involved in tumor development and progression (3-5). A previous study showed that miR-146a was associated with gastric cancer metastasis through modulating Wiskott-Aldrich syndrome protein family member 2 (6). Other studies have shown that the expression of miR-146a is associated with tumor cell proliferation and apoptosis in gastric cancer (7,8). miR-146a has direct effects on tumor metastasis and prognosis by affecting epidermal growth factor receptor (EGFR) (8). The expression of LIN52 in gastrointestinal tumors affects drug sensitivity and enhances the effect of imatinib-induced apoptosis in tumor cells (9).
addition, according to the predictions of TargetScan, miR-146a is a target gene regulated by LIN52 (10). The present study further analyzed the effect of miR-146a on the treatment of gastric cancer. The expression of miR-146a was analyzed in 93 clinical cases of advanced gastric cancer using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis, and its expression was correlated with the clinical characteristics of advanced gastric cancer and the prognosis of patients.

Materials and methods

Patients and sampling. A total of 93 patients with advanced gastric cancer (57 men and 36 women; median age, 61 years), who underwent surgical treatment between June 2009 and January 2011 in Henan Provincial People's Hospital (Henan, China) were enrolled in the present study. All patients received an oxaliplatin-based chemotherapeutic regimen following surgical treatment, and the efficacy was evaluated in accordance with the Response Evaluation Criteria in Solid Tumors (RESIST) (11). The histopathological analysis of resected tissues was based on the tumor-node-metastasis (TNM) staging criteria of the World Health Organization (11). From each patient, non-tumor tissues were collected 7 cm adjacent to the tumor lesion as an internal control. The histological sections of each resected specimen were classified by pathologists. All patients signed written informed consent prior to their involvement in the study. The experimental protocol of the present study was approved by the Ethics Committee of Henan Provincial People's Hospital.

Reagents. Mouse anti-human LIN52 monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The streptavidin-peroxidase (SP) immunohistochemical staining kit and 3,3'-diaminobenzidine (DAB) peroxidase substrate kit were purchased from Fuzhou Maxim Biotech, Inc. (Fujian, China). The experimental procedures for the immunohistochemistry were according to the protocol of the SP staining kit manufacturer. Phosphate-buffered saline (PBS) with Tween detergent was used to replace primary antibody in the negative control for the immunohistochemistry assays. TRIzol reagent (1 ml) was added to the tissue powder to lyse the cells for 10 min, followed by transfer of the supernatant to a microcentrifuge tube. Chloroform solution (400 μl) was added to the supernatant, vortexed and subjected to 12,000 g centrifugation at 4˚C for 15 min. The supernatant (200 μl) was then transferred to a new RNase-free microcentrifuge tube and mixed with equal quantities of isopropanol by inverting the tube, followed by incubation for 10 min and 12,000 g centrifugation at 4˚C for 10 min. The supernatant was discarded, followed by the addition of 1 ml 70% ethanol to the pellet and mixing by gentle inversion of the microcentrifuge tube. The mixture was centrifuged at 12,000 g at 4˚C for 10 min, following which the ethanol solution was discarded and the pellet was air-dried. The pellet was then dissolved in diethyl pyrocarbonate-treated distilled water. The total RNA concentration of each sample was determined using an e-Spect ultra-small spectrophotometer (Malcom Co., Ltd., Tokyo, Japan). The RNA quality of each sample was determined based on the optical density (OD) 260/280 ratio, ranging between 1.8 and 2.0. For cDNA synthesis, 20 μl reverse transcriptase solution from the kit was added to 0.1 μg RNA template, miR-146a reverse primer and U6 reverse primer and reverse transcription into cDNA. U6 was the internal reference for analysis of the expression of miR-146a. Details of the primers are listed in Table I. The primers and RNA template were incubated at 65˚C for 5 min, followed by cooling on ice, incubation with the reverse transcription reaction mixture and dNTPs at 42˚C for 60 min, and termination of the reaction at 70˚C for 5 min. The total volume of master mix for the qPCR analysis was 20 μl, containing 1 μl of cDNA (final concentration 5 ng), 12.5 μl of 2X SYBR Green I Master mix, 0.5 μmol/l miR-146a/U6 specific forward primers and 0.5 μmol/l miR-146a/U6 reverse primers. Each sample had three replicates. The qPCR conditions were as follows: 95˚C for 7 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 25 sec and elongation at 72˚C for 25 sec. The quantification cycle (Cq) value of the U6 reaction obtained from the qPCR analysis was used to calculate the Cq value representing the relative expression of miR-146a. qPCR was activated at 95˚C for 5 min, followed by 45 cycles of denaturation at 95˚C for 15 sec, annealing at 62˚C for 30 sec and elongation at 72˚C for 20 sec. The Cq value, standard and melting curves were automatically generated by the RT-qPCR instrument.

RT-qPCR analysis. Following collection of the resected tissue and snap freezing in liquid nitrogen, each tissue specimen was crushed into a powder, followed by total RNA extraction. TRIzol reagent (1 ml) was added to the tissue powder to lyse the cells for 10 min, followed by transfer of the supernatant to a microcentrifuge tube. Chloroform solution (400 μl) was added to the supernatant, vortexed and subjected to 12,000 g centrifugation at 4˚C for 15 min. The supernatant (200 μl) was then transferred to a new RNase-free microcentrifuge tube and mixed with equal quantities of isopropanol by inverting the tube, followed by incubation for 10 min and 12,000 g centrifugation at 4˚C for 10 min. The supernatant was discarded, followed by the addition of 1 ml 70% ethanol to the pellet and mixing by gentle inversion of the microcentrifuge tube. The mixture was centrifuged at 12,000 g at 4˚C for 10 min, following which the ethanol solution was discarded and the pellet was air-dried. The pellet was then dissolved in diethyl pyrocarbonate-treated distilled water. The total RNA concentration of each sample was determined using an e-Spect ultra-small spectrophotometer (Malcom Co., Ltd., Tokyo, Japan). The RNA quality of each sample was determined based on the optical density (OD) 260/280 ratio, ranging between 1.8 and 2.0. For cDNA synthesis, 20 μl reverse transcriptase solution from the kit was added to 0.1 μg RNA template, miR-146a reverse primer and U6 reverse primer and reverse transcription into cDNA. U6 was the internal reference for analysis of the expression of miR-146a. Details of the primers are listed in Table I. The primers and RNA template were incubated at 65˚C for 5 min, followed by cooling on ice, incubation with the reverse transcription reaction mixture and dNTPs at 42˚C for 60 min, and termination of the reaction at 70˚C for 5 min. The total volume of master mix for the qPCR analysis was 20 μl, containing 1 μl of cDNA (final concentration 5 ng), 12.5 μl of 2X SYBR Green I Master mix, 0.5 μmol/l miR-146a/U6 specific forward primers and 0.5 μmol/l miR-146a/U6 reverse primers. Each sample had three replicates. The qPCR conditions were as follows: 95˚C for 7 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 25 sec and elongation at 72˚C for 25 sec. The quantification cycle (Cq) value of the U6 reaction obtained from the qPCR analysis was used to calculate the Cq value representing the relative expression of miR-146a. qPCR was activated at 95˚C for 5 min, followed by 45 cycles of denaturation at 95˚C for 15 sec, annealing at 62˚C for 30 sec and elongation at 72˚C for 20 sec. The Cq value, standard and melting curves were automatically generated by the RT-qPCR instrument.

Statistical analysis. SPSS 13.0 software was used for the non-parametric rank sum test. Relative expression ≥2.0 was
defined as high expression and relative expression <2.0 was defined as low expression following Kaplan-Meier survival analysis. The Cox regression model was based on the forward method of conditional parameter estimates. P<0.05 was considered to indicate a statistically significant difference.

Results

Positive protein expression of LIN52. As shown in Fig. 1A, positive staining of the LIN52 protein, identified as brownish yellow granules, was distributed predominantly in the cytoplasm of the advanced gastric cancer tissues. In the normal tissues adjacent to the tumor, the expression of LIN52 was low (Fig. 1B).

Correlation between the expression of miR-146a in advanced gastric cancer tissue and clinicopathological parameters. The expression of miR-146a in the advanced gastric cancer tissue was significantly correlated with the clinical TNM staging of the patients (P<0.05). The expression of miR-146a in stage III gastric cancer tissues was significantly higher, compared with that in stage IV gastric cancer tissues (P<0.05). Patients with lymph node metastasis had lower expression levels of miR-146a, compared with patients without lymph node metastasis. However, no significant correlation was found between the expression of miR-146a in advanced gastric cancer tissue and other clinicopathological factors, including gender, age and tumor differentiation (P>0.05; Table II).

Correlation between the expression of miR-146a and patient survival rates. The results of the Kaplan-Meier survival analysis showed that the expression of miR-146a had a significant effect on the survival rates of patients with advanced gastric cancer. Patients with high expression levels of miR-146a had significantly higher survival rates, compared with patients with low expression levels of miR-146a (P<0.05; Fig. 2).

Correlation between the expression levels of miR146a and LIN52 in advanced gastric cancer. The present study further analyzed the correlation between the expression levels of miR146a and LIN52. Among the 44 specimens with a high expression of miR146a, only 8 (18.2%) showed LIN52 immunoreactivity (P<0.001; Table III).

Cox regression analysis of prognostic factors in patients with gastric cancer. The clinical prognostic factors of patients with advanced gastric cancer were used as dependent variables and their relevant effects were used as independent variables for Cox regression analysis. As shown in Table IV, TNM staging, lymph node metastasis and the expression of miR-146a were

| Primer | Sequence |
|--------|----------|
| miR-146a | 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAACCCATG-3' |
| RT | 5'-TGGTGTCGTGGAGTCG-3' |
| Forward | 5'-ACACTCCAGCTGGGTGAGAACTGAATTCCATGGGTT-3' |
| Reverse | 5'-AACGCTTCACGAATTTGCGT-3' |
| U6 | 5'-CTCGCTTCGGCAGCACA-3' |
| RT and reverse | 5'-AACGCTTCACGAATTTGCGT-3' |

miR, microRNA; RT, reverse transcription.
Table II. Expression of miR-146a and LIN52 in 93 patients with gastric cancer and their correlation with clinicopathological parameters.

| Parameter                                | miR-146a, n (%) | LIN52, n (%) |
|------------------------------------------|-----------------|--------------|
|                                          | Low  | High  | P-value<sup>a</sup> | Low  | High  | P-value<sup>a</sup> |
| Gender                                   |      |       |                   |      |       |                   |
| Male                                     | 57   | 34 (59.6) 23 (40.4) | 29 (53.7) 25 (46.3) | 0.091 | 0.077 |
| Female                                   | 36   | 15 (41.7) 21 (58.3) | 28 (71.8) 11 (28.2) |       |       |
| Age (years)                              |      |       |                   |      |       |                   |
| <56                                       | 32   | 19 (59.4) 13 (40.6) | 18 (56.3) 14 (43.8) | 0.350 | 0.484 |
| ≥56                                       | 61   | 30 (49.2) 31 (50.8) | 39 (63.9) 22 (36.1) |       |       |
| Grade                                    |      |       |                   |      |       |                   |
| Poorly differentiated                     | 18   | 13 (72.2) 5 (27.8) | 5 (27.8) 13 (72.2) | 0.295 | 0.519 |
| Moderately differentiated                 | 33   | 17 (51.5) 16 (48.5) | 15 (45.5) 18 (54.5) |       |       |
| Well differentiated                       | 22   | 10 (45.5) 12 (54.5) | 11 (50.0) 11 (50.0) |       |       |
| Signet-ring cell carcinoma                | 20   | 9 (45.0) 11 (55.5) | 8 (40.0) 12 (60.0) |       |       |
| Lymph node metastasis                    |      |       |                   |      |       |                   |
| Present                                  | 76   | 44 (57.9) 32 (42.1) | 39 (59.1) 27 (40.9) | 0.033 | 0.754 |
| Not present                              | 17   | 5 (29.4) 12 (70.6) | 15 (55.6) 12 (44.4) |       |       |
| Clinical stage                           |      |       |                   |      |       |                   |
| III                                      | 42   | 16 (38.1) 26 (61.9) | 29 (69.0) 13 (31.0) | 0.011 | 0.051 |
| IV                                       | 51   | 33 (64.7) 18 (35.3) | 25 (49.0) 26 (51.0) |       |       |

<sup>a</sup><sup>χ²</sup> test. miR, microRNA.

Figure 2. Kaplan-Meier analysis of survival rates of patients according to miR-146a and LIN52 expression status. The effects of the expression of (A) miR146a and (B) LIN52 on the prognosis of patients with gastric cancer are shown. miR, microRNA.

Expression of miR-146a and chemotherapeutic sensitivity.

The present study also assessed the association between the expression of miR-146a and chemotherapeutic sensitivity in patients with advanced gastric cancer. The analysis showed that patients with advanced gastric cancer tissues expressing a low level of miR-146a had a poor prognosis (Fig. 3). The present study evaluated the efficacy of chemotherapy in patients with advanced gastric cancer according to the RESIST standard and divided the patients into three groups: Complete remission (CR), partial remission (PR) and disease progression (PD). The characteristics of the expression of miR-146a were analyzed in independent risk factors for the prognosis of patients with advanced gastric cancer.
The expression of miR-146a in the CR group was the highest, with significant differences between the PR and PD groups, and the PR and PD groups (P<0.05), but not between the PR and CR groups (P>0.05).

Evaluation of diagnostic specificity and sensitivity of miR-146a in patients with advanced gastric cancer using ROC curve regression analysis. The area under the ROC curve of miR-146a in the advanced gastric cancer tissues and corresponding adjacent non-tumor tissue was 0.760 [95% confidence interval (CI)=0.589-0.942; P=0.016], suggesting that miR-146a was significant for distinguishing between advanced gastric cancer tissue and normal tissue (P<0.05). Lower expression levels of miR-146a increased the likelihood of advanced gastric cancer. These results indicated the suitability of using miR-146a as an adjuvant diagnostic marker for advanced gastric cancer. In the present study, the relative expression of miR-146a was 19.75, which was used as the evaluation threshold for the diagnosis of advanced gastric cancer, with 94.1% sensitivity and 61.5% specificity (Fig. 4).

Discussion

Gastric cancer is one of the most common types of cancer in China, with a high incidence rate and insidious early symptoms (12). At the time of diagnosis, patients with gastric cancer are often at moderate and/or advanced stages of the disease, with poor prognosis (11). Therefore, the degree of malignancy and the mortality rates of patients with gastric cancer are relatively high. At present, there is no method for the effective early diagnosis of gastric cancer and, if diagnosed with an advanced stage of disease, radical surgery is not an option for patients. Advances in molecular targeted therapy and chemotherapies have not significantly improved the median survival rates of patients with advanced gastric cancer (13). Although α-fetoprotein, carcinoembryonic antigen, CA125 and CA199 enhance the early diagnostic sensitivity of gastric cancer, their diagnostic specificities as markers of gastric cancer are low (14). It is necessary to investigate novel diagnostic markers for gastric cancer to provide guidance for cancer diagnosis and treatment (15). To address this problem, studies are focusing on the use of miRs in gastric cancer as potential biomarkers (16). Evaluation of the expression of miR in tumor tissue and patient serum is important in the early diagnosis and prognosis of gastric cancer (17). In the present study, the expression of miR-146a in gastric cancer tissue was correlated with the early diagnosis of gastric cancer and the prognosis of patients, and predictive analysis on these parameters was performed.

Through binding with the mRNA 3′-untranslated region (3′UTR), a single miR may have regulatory effects against various mRNAs. Typically, there is differential expression of miRs in tumor tissues, compared with normal tissues. miRs usually have specific and stable expression in tissues, therefore, miRs offer potential as prognostic markers (18,19). Binding between miRs and the 3′UTR of mRNA suppresses mRNA transcription and affects the expression of oncogenes and tumor suppressor genes, thereby promoting or inhibiting tumor occurrence, development and metastasis. A previous study showed that miRs can be used as potential targets in anticancer therapy (20). Another prevopis study showed that miRs are associated with tumor metastasis, tolerance of anticancer therapy and tumor progression (21,22). In addition, miRs have...
only ~20 nucleotide bases, which facilitates its stable expression in tissues and its detection (23). Therefore, miRs have received increasing attention in tumor diagnosis, treatment evaluation and determining the prognosis of cancer (24,25).

Although the biological functions of miRs remain to be fully elucidated, the expression of miR in normal tissue, compared with tumor tissue, is often apparent. To date, increasing applications of miRs as tissue-specific markers have become available for the analysis of primary tumor metastasis (26,27). The present study showed that the relative expression of miR-146a in gastric cancer tissue was significantly lower, compared with that in the corresponding adjacent normal gastric mucosa, suggesting that miR-146a acted as a tumor suppressor gene. Early detection of the expression of miR-146a of gastric cancer may assist in the early diagnosis of the disease. In the present study, correlation analysis between the expression of miR-146a and relevant clinicopathological factors in gastric cancer showed that a low expression of miR-146a was significantly associated with lymph node metastasis in the patients. The relative expression of miR-146a in patients with advanced gastric cancer and lymph node metastasis was lower, compared with that in patients with advanced gastric cancer without lymph node metastasis. These results also suggested that miR-146a may act as a tumor suppressor, which increased the degree of malignancy and led to early metastasis of the tumor. As tumors progressed between early stages (stage I and II) and late stages (stage III and IV), the expression of miR-146a decreased, although no statistical significance was found. Previous studies also showed that the expression of miRs in patient tumor tissue had no significant correlation with the clinical staging of advanced gastric cancer (28,29), which was also observed in tissue samples. Further studies are required to investigate the reasons behind this discrepancy. In the present study, the expression of miR-146a was correlated with patient survival rates, which was consistent with the findings of a previous study on gastric cancer (8). Compared with previous findings, the present study showed that the expression of miR-146a was associated with early diagnosis of gastric cancer, and also had specificity in the diagnosis of gastric cancer. The present study also found that the expression of miR-146a was associated with the efficacy of anticancer treatment. Patients with high expression levels of miR-146a in advanced gastric cancer tissues demonstrated a significantly higher treatment efficacy.

According to biological prediction, miR-146a binds to the 3'UTR of LIN52 to regulate the expression of LIN52 and affect LIN52 function. The immunohistochemical staining showed that the protein expression of LIN52 in advanced gastric cancer tissue was negatively correlated with the expression of miR-146a, suggesting that LIN52 was involved as an oncogene, compared with miR-146a. A previous study showed that inhibition of the expression of LIN52 in gastrointestinal stromal tumors promoted imatinib-induced apoptosis and was involved in tumor suppression (9). The present study found that the inhibition of LIN52 by miR-146a resulted in improved survival rates of the patients. Several drugs used for chemotherapy have poor efficacy against tumor cells at the G0 phase of the cell cycle. However, numerous tumor cells are at the G0 phase, which is also a major reason for the resistance to drug chemotherapy (30). The activation of LIN52 directly affects the cell cycle, allowing the majority of cells to remain at the G0 resting phase (31) and preventing the cells from entering the DNA synthesis phase (S phase) (32). miR-146a inhibits the expression of LIN52 and reduces the number of cancer cells remaining in the G0 phase, thereby increasing the ratio of cancer cells at the S phase and improving the efficacy of anticancer therapy. A previous study showed that miRs affect the cell cycle of cancer cells, which may be one of the reasons they can affect the resistance of tumors to drugs (33). Although the present study showed that the expression of miR-146a was significantly correlated with the expression of LIN52 and with chemotherapeutic sensitivity, it is unclear whether miR-146a affects and reverses the effects of chemotherapy through LIN52 to regulate cancer cell cycle. Further investigations are required to verify these mechanisms.

Although miRs are promising markers in the diagnosis and prognosis of cancer, and dozens of miRs can be used for the diagnosis of gastric cancer (34,35), there are several restrictions on methods and technical limitations in the clinical detection of miRs. Currently, several detection methods and software packages are available for miR detection in different types of cancer. However, standardized approaches are different, leading to inconsistent findings among studies (36). For this reason, it is necessary to develop standardized methods of assessment and introduce housekeeping miRs with stable expression, including miR-16 and RUN6B. For the detection of gastric cancer, serum miR-93 is recommended as a marker gene to identify healthy controls (37). Others have also suggested the use of miRs with low expression levels in humans but high expression in lower organisms, including Caenorhabditis elegans, as internal controls (38). With advancements in the future, microRNAs are likely to be of increased clinical use and offer more accurate guidance in cancer investigations.
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