UP-REGULATION OF MIRNA-222 PREDICTS POOR PROGNOSIS AND TUMOR PROGRESSION IN PATIENTS WITH ADVANCED STAGE NON-SMALL CELL LUNG CANCER

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Abstract
MicroRNAs (miRNAs) are endogenous small noncoding RNAs that play important roles in regulating cancers. We previously indicated that miR-222 promotes non-small cell lung cancer growth. Here, we aimed to investigate whether the miR-222 expression level was associated with clinic pathological factors and prognostic values of non-small cell lung cancer (NSCLC). The expression level of miR-222 in the pathological tissues and paired normal tissues of NSCLC was evaluated using a quantitative real-time RT-PCR. A Kaplan-Meier survival curve was demonstrated following a log-rank test. Results showed that miR-222 expression level is up-regulated in NSCLC tissues compared with matched normal tissues (mean ± SD: 3.55 ± 1.71 vs. 2.08 ± 1.01, P < 0.001). The high expression of miR-222 in NSCLC cancer tissues was also remarkably related with aggressive clinic pathological features. The expression level of miR-222 was associated with tumor grade (P=0.031) and lymph node metastasis (P = 0.0028). Moreover, the results of Kaplan-Meier analyses indicated that patients with high expression level of microRNA-222 had shorter overall survival and progression free survival (P < 0.001). The multivariate analysis clearly indicated that the high miR-222 expression in biopsy samples might be a useful independent biomarker for NSCLC prognosis (RR 3.23; 95%CI 2.12–5.47). Our data demonstrated that the aberrant expression of microRNA-222 is associated with NSCLC progression and it might be a potential biomarker for NSCLC prognosis.

Keywords: microRNA-222, non-small cell lung cancer, progression and prognosis.

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Introduction
Lung cancer is the leading cause of cancer-related mortality worldwide and annually responsible for more than 500,000 deaths worldwide(1). Approximately 80% of the lung cancer patients have non-small cell lung cancer (NSCLC). Once diagnosed, survival rates are low. Even diagnosed early, grade I lung cancer patients have only a 60-70% five-year survival rate(2-4). Although interventions have been improved in recent years, the prognosis and long-term survival of the NSCLC patients are still poor(5). Thus, there is a need to identify novel, noninvasive prognostic biomarkers for NSCLC in order to improve diagnostic and therapeutic strategies.

MicroRNAs (miRNAs) are an abundant class of 17-25 nucleotides small non-coding RNAs and would post-transcriptionally regulate gene expression through directly binding to the 3’ untranslated region (3’ UTR) of target mRNAs(6). Through the post-transcriptional path, miRNAs have been shown to play fundamental roles in diverse biological and pathological processes, including cell pro-
liferation, differentiation, apoptosis, and carcinogenesis\(^{(7-9)}\). Moreover, many studies have shown that miRNAs exhibit altered expression in various cancers and may serve as important prognostic biomarker of cancers\(^{(10)}\).

MiR-222 belongs to the miR-221/222 family, and has been reported to play a potential oncogenic role in multiple cancer types including hepatocellular carcinoma, breast cancer, prostate cancer, bladder cancer, endometrial carcinoma and glioblastoma\(^{(11-17)}\). We previously reported that miR-221/222 promoted the growth of human NSCLC cell line H460\(^{(18-19)}\). MiR-222 was reported to be a prognostic factor for hepatocellular carcinoma, papillary thyroid carcinoma, glioblastoma, gastric carcinoma, pancreatic cancer et al\(^{(20-25)}\).

Here, we studied the miR-222 expression in NSCLC to evaluate their value in prognosis and progression. We examined the up-regulation of miR-222 in 178 NSCLC cases by means of quantitative real-time reverse-transcription PCR (RT-PCR). We found that miR-222 is overexpressed in NSCLC. More importantly, those patients with increased miR222 expressions were also found to have worse prognosis and this prognostic impact appears to be independent of other factors in multivariate Cox regression analysis Table 2.

Materials and methods

**Ethics Statement**

The Research Ethics Committee of the Second Affiliated Hospital of Nantong University approved the study. All participants were informed about the purpose, procedures, and potential risks and benefits of the study, and their written informed consent were obtained.

**Patient tissue samples and clinical information collection**

178 patients with NSCLC confirmed by pathological and clinical diagnoses in the Second Affiliated Hospital of Nantong University were enrolled in this study from January 2006 to January 2010. None of the patients had received chemotherapy or radiotherapy before surgery. The collected pathological tissues and matched normal tissues of the enrolled patients were put in liquid nitrogen immediately and stored at -80 °C before use.

**RNA extraction**

The total RNA of the pathological tissues and corresponding normal tissues were extracted using TRIZOL reagent (Invitrogen, USA) following the manufacturer’s instructions. Reverse transcription reaction was performed using Bulge-Loop™ miRNA qRT-PCR Primer Set (Ribobio, China) according to the manufacturer’s protocols. Then cDNA was subjected into 40 cycles of quantitative PCR with Takara SYBR Premix Ex TaqTM (Takara, Japan) in CFX96TM Real-Time PCR Detection System (Bio-Rad, USA), using the following cycling conditions: 95°C for 10 min; 95°C for 10 s, 60°C for 20 s, 72°C for 10 s, 40 cycles.

The U6 small nuclear (sn) RNA was used to normalize miR-222.

**Statistical analyses**

To analyze clinic pathologic characteristics, Chi-square tests were used for the categorical data and Mann-Whitney tests for continuous data when comparing patient and control data. Differences in miRNA-222 expression levels between NSCLC and matched normal tissues were compared using an Independent sample Student’s t-test. Associations between miR-222 levels and over survival of the patients with NSCLC were estimated using adjusted relative risks and 95% confidence intervals (95 % CIs) from multivariate logistic regression. Survival time was calculated from the date of NSCLC diagnosis to the date of death or last follow-up. Survival analysis was estimated using the Kaplan-Meier method, log-rank test, and Cox-proportional hazards regression model. P < 0.05 indicated significant difference. The Statistical Product and Service Solutions (SPSS) software version 20.0 for Windows was used for statistical analysis and Figures were constructed using Graph Pad Prism 5.0 (La Jolla, CA, USA).

**Results**

**Relative expression of miR-222 in patients with NSCLC**

178 patients were recruited in this study. The clinical information of the NSCLC patients was summarized in Table 1. The median age of the NSCLC patients at diagnosis was 52.8 year (range, 32-81 year). QRT-PCR was used to detect the relative expression of microRNA-222 in the pathological tissues and corresponding normal tissues of the NSCLC patients. The relative expression level in pathological tissues was 3.55 ± 1.71 while the corresponding in normal tissues was 2.08 ± 1.01.
There was a significant difference between them (P < 0.001, Fig. 1). In addition, there was also a significant difference in miR-21 expression between high-grade (IIb-IIIa) and low-grade (Ia-IIa) NSCLC tissue specimens (P < 0.001, Fig. 1).

The relationship between miR-222 expression and survival of NSCLC patients

Kaplan-Meier method was used for survival analysis. The patients with high expression level of miR-222 had shorter overall survival (P < 0.001, Fig. 2a). However, when the pathological grades were considered, the higher miR-222 expression was a risk of poor prognosis in both the high grade NSCLC (IIb-IIIa, P < 0.001, Fig. 2b) and the low grade NSCLC (Ia-IIa, P < 0.001, Fig. 2c).

To further evaluate whether miR-222 levels can predict NSCLC prognosis, we valued the progression-free survival, which proved shorter in patients with high miR-222 expression (P < 0.001, Fig. 3a). The higher miR-222 expression was a risk of shorter progression-free survival in both the high grade NSCLC (IIb-IIIa, P = 0.0123, Fig. 3b) and the low grade NSCLC (Ia-IIa, P < 0.001, Fig. 3c).

Multivariate Cox proportional Hazard analysis

Multivariate Cox regression analysis was performed to estimate the prognostic value of miR-222 in NSCLC patients and the results were shown in Table 2. From the table, we found that the expression level of miR-222 was associated with the prognosis in biopsy samples (RR, 3.23, 95% CI, 2.12–5.47, P < 0.001), tumor grade (RR 2.19; 95% CI, 1.32-4.31, P = 0.019) and lymph node metastasis (RR, 2.87; 95% CI, 1.23-4.25, P = 0.004) and it could act as an independent biomarker for prognosis of NSCLC patients.

Discussion

Although with the development of diagnosis and surgical techniques, the patients with NSCLC still demonstrated a poor prognosis. Therefore, identification of novel molecular biomarkers associated with the prognosis of patients with NSCLC was urgently needed now. miRNAs have been indicated to be critical regulators of carcinogenesis and tumor progression in NSCLC.
In 2011, Zhang et al. found that miR-222 was up-regulated in NSCLC patients (26). We further indicated that miR-222 promotes human non-small cell lung cancer growth via targeting p27 (19). However, the clinical significance of miR-222 expression in this tumor remains unclear. In this study, we showed that miR-222 expression was up-regulated in NSCLC tissues compared with matched non-cancerous lung tissues. The overexpression of miR-222 in NSCLC tissues was also significantly related with aggressive clinic pathological features. Besides, patients with high miR-222 have higher tumor grade and are in higher risk of lymph node metastasis. Moreover, the results of Kaplan-Meier analyses showed that NSCLC patients with the high miR-222 expression incline to have shorter overall survival and progression free survival. The multivariate analysis indicated that the increased miR-222 expression in biopsy samples might act as an independent prognostic parameter in NSCLC for reduced survival.

miRNAs play important roles in regulating kinds of cancers. The regulation includes activation of oncogenes and inactivation of tumor suppressor genes. Among them miRNAs are reported to locate in the cancer-associated genomic region or in fragile sites (27). So they could act as gene regulators of metastasis by taking part in different signaling pathways (28). Such as miR-126, miR-195 and let-7c were reported to be associated with cancers (29-31). For the up-regulated miR-222, its potential target genes include the tumor suppressor gene p27, p57, PTEN and et al. Zhang et al. found that the expression of high mobility group A1 (HMGA1) was in positive correlation with miR-222 in NSCLC samples (26). In 2013, Wang et al. found that metformin inhibited lung cancer cells proliferation through repressing miR-222 (32).
Up-regulation of miR-222 has been indicated in several types of cancers, suggesting its important role in occurrence and development of cancers. Moreover, previous studies have verified the prognostic value of miR-222 and as non-invasive biomarkers. Zhang et al showed that high level of miR-222 confers increased cell invasion and poor prognosis in glioma\(^ (22)\). Kim et al found that miR-222 is up-regulated in advanced gastric carcinoma\(^ (23)\). Lee et al indicated that miR-222 is associated with poor prognosis in pancreatic cancer. In addition, miR-222 was also reported to be a prognostic factor for hepatocellular carcinoma, papillary thyroid carcinoma et al\(^ (20, 21)\). In line with these studies, we confirmed that the expression levels of miR-222 in NSCLC tissues were significantly higher than those in normal adjacent tissue, and increased miR-222 expression was independently associated with poor survival and tumor progression.

In conclusion, the results have demonstrated that the levels of miR-222 are higher in NSCLC tissues than those in matched normal tissues and correlated with tumor grade and lymph node metastasis. Our findings promote better understanding of the role of miR-222 in NSCLC progression and suggest that miR-222 may function as tumor promoter genes in patient with NSCLC. These results make the potential clinical value of microRNA measurements clear, particularly in evaluating prognosis for NSCLC patients. Large well-designed studies with diverse populations and functional evaluations are warranted to confirm and extend our findings. MiR-222, a potential prognostic biomarker, may be a novel therapy target for patients with NSCLC in future.

References


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