

Relationship between miR-7 expression and treatment outcomes with gefitinib in non-small cell lung cancer

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Abstract. The aim of the present study was to assess the effects of gefitinib chemotherapy on the serum levels of miR-7 in patients with non-small cell lung cancer (NSCLC). A total of 126 patients were enrolled in the present study (stage I-II, n=54 and stage III-IV, n=72). Patients with stage I-II NSCLC underwent surgery in combination with gefitinib chemotherapy, whereas only gefitinib chemotherapy was administered to patients with stage III-IV disease. Serum levels of miR-7 before and after treatment were measured with quantitative polymerase chain reaction using fluorogenic probes, and miR-7 positivity and scoring in resected specimens were determined by immunohistochemistry. The number of miR-7-positive cases and the number of cases with higher miR-7 scores were significantly lower among patients with stage I-II NSCLC than those with stage III-IV disease. Additionally, serum levels of miR-7 before and after intervention were lower in stage I-II than in stage III-IV NSCLC cases. Serum levels of miR-7 after treatment were significantly lower than those before intervention in the two groups. The treatment success rate was significantly higher in miR-7-negative patients than in miR-7-positive patients in the two patient groups. Adverse event rates in miR-7-negative and -positive patients were comparable between the groups. Among those with stage III-IV NSCLC, the survival rate of miR-7-positive patients was significantly lower than that of miR-7-negative patients. Conversely, among those with I-II NSCLC, the progression-free survival and median survival time of miR-7-positive patients were significantly lower than those of miR-7-negative patients. Our findings suggest that serum and expression levels of miR-7 in the tissue

were closely associated with tumor staging and the therapeutic effects of gefitinib in NSCLC.

Introduction

Lung cancer accounts for 50-65% of all cancer diagnoses. Approximately 60-80% of patients with lung cancer are in advanced stages at diagnosis. For these patients, platinum-based chemotherapy, radiotherapy, and gene-targeted therapy are generally the only treatment options (1). However, the overall efficacy of chemotherapy is only 30-40%, it includes serious side effects, and the overall effective rate over 5 years is only 1-5% (2).

Gefitinib (Iressa[®]), a small molecular-targeted anticancer drug developed by AstraZeneca (New York, NY, USA), is a highly selective oral epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor that was the first of its class to be evaluated in clinical trials. Many large-scale randomized controlled clinical trials demonstrated that gefitinib had better safety and efficacy in controlling renal carcinoma, lung cancer, and moderate and advanced hepatic carcinoma (3-6). Gefitinib, either alone or in combination other chemotherapeutics, is now the first- or second-line treatment option for cancer (7,8).

Recent studies have found that miR-7 was highly expressed in numerous cancers, including breast, lung, liver, and colon cancer (9). Additionally, miR-7 has been shown to play roles in the biological behavior of cancers, including development, migration, and invasion of the tumor, and is a potential target for intervention. Thus, in the present study, we determined the outcomes of gefitinib therapy in patients with non-small cell lung cancer (NSCLC) and its effect on miR-7 expression.

Materials and methods

Subjects. A total of 126 patients with NSCLC who were admitted to People's Hospital of Laiwu city from January, 2013 to January, 2014, were included in the present study. Histopathology was used to diagnose the stage of cancer in each patient; stage I-II and stage III-IV NSCLC was confirmed in 54 and 72 patients, respectively. Exclusion criteria included concomitant primary cancer in other sites; metastasis to the lung from primary cancer at other sites; comorbidities

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including cardiac, hepatic, pulmonary, kidney, and other organ dysfunction; severe post-surgical complications; advanced disease; expected survival time of less than one month; and incomplete clinical data. The present study was approved by the ethics committee of Shandong Cancer Hospital Affiliated to the Shandong University (Shandong, China). Informed consent was obtained from patients or their legal guardians.

Among patients with stage I-II disease, 22 and 32 patients were diagnosed with stage I and II NSCLC, respectively. Twenty-eight patients were male, with an age range of 38-78 (62.4±15.5) years. The maximum diameter of tumor lesions ranged between 0.5 and 4.6 (2.6 ± 1.4) cm, and the number of lesions per patient was between 1 and 3 (1.4±0.6). In this group, 20 patients underwent open radical tumor resection, and minimally invasive surgery under thoracoscopic guidance was performed in 34 patients. Among patients with stage III-IV cancer, stage III and IV NSCLC was diagnosed in 32 and 40 patients, respectively. Thirty-six patients in this group were male, with an age range of 45-79 (65.0±17.2) years. The maximum diameter of lesions was 1.4-6.3 (3.5±1.7) cm, and the number of lesions per patient was 1-3 (1.4±0.6). In this group, liver, bone, lung, and celiac lymph node metastases were found in 16, 15, 3 and 6 patients, respectively.

Clinical approach. Patients with stage I or II NSCLC were treated by surgery in combination with gefitinib chemotherapy, whereas those with stage III or IV NSCLC were treated with gefitinib chemotherapy alone. The same surgical and nursing team attended to all the patients that underwent surgery, according to standard medical procedures. Gefitinib was administered orally at a dose of 250 mg/day, and all the subjects were monitored for any adverse side effects. If necessary, gefitinib treatment was stopped, and patients were observed.

Outcome measures. The effective rate, rate of adverse effects, survival rate, progression-free survival (PFS), and median survival time following treatment were analyzed. Serum miR-7 and tumor positivity for miR-7 before and after surgery in patients with stage I or II NSCLC and before treatment and 3 months after treatment in patients with stage III or IV NSCLC were compared.

According to the response evaluation criteria in solid tumors (RECIST), radiological assessment was conducted once every three months to categorize outcomes as complete remission (CR), partial remission (PR), stable disease (SD), or progressive disease (PD). Specifically, CR was defined as the disappearance of any tumor lesions lasting 4 weeks, and PR was defined as a >30% decrease in lesion size lasting 4 weeks. SD was defined as the reduction in lesion size by <30% or increase by ≤20% lasting at least 4 weeks, whereas PD was defined as >20%-increase in lesion size. Effective rate was defined as follows: CR+PR+SD/total number of cases. Acute and chronic toxic and side effects of gefitinib were evaluated and recorded according to the expression and grading standards of the World Health Organization for anticancer drugs (10).

Serum mRNA levels of miR-7 were measured in peripheral mononuclear cells isolated from subjects using reverse transcription-quantitative polymerase chain reaction (RT-PCR)

and fluorogenic probes. TRIzol® was used to extract total RNA from the cells, followed by 1.2% agarose gel electrophoresis and ultraviolet spectrophotometer. The TaqMan® kit was used to determine the quality and concentration of the total RNA. PCR volume was 20 µl and included 1 µl TaqMan microRNA Assay (x20) probe, 10 µl 2X TaqMan general PCR master mix, 7.67 µl nuclease-free water, and 1.33 µl reverse-transcribed product. The reaction conditions were as follows: 95°C for 10 min, 95°C for 15 sec, 60°C for 1 min; for 40 cycles; 3 replicates were run for each sample. Primers for miR-7 were: forward: 5'-CCAGGACCAGAGGAAACC-3', and reverse: 5'-GCTAGCCTTCTTCTGGATTTGA-3'.

The 2^{-ΔΔC_q} method was used to determine the relative expression of miR-7. Levels of miR-7 expression in resected specimens was determined by immunohistochemistry using previously described steps in the following order: Preparation of paraffin sections, de-waxing, antigen retrieval, endogenous peroxidase activity inhibition, blocking with serum, antibody incubation, 3,3'-diaminobenzidine (DAB) staining, counterstaining, transparency, and mounting.

For the semiquantitative analysis of miR-7 expression, images from five high-power fields were randomly captured, and specimens with ≥10% of tumor cells with positive cytoplasmic miR-7 were determined as positive. Additionally, specimens were scored according to the intensity of cellular miR-7 staining and the number of positive cells. Briefly, specimens with dark (reddish brown) DAB staining and ≥50% immunopositive cells received a score of 3, specimens with brownish yellow DAB staining and 26-50% immunopositive cells received a score of 2, specimens with light yellow DAB staining and 10-25% immunopositive cells received a score of 1, and specimens with no visible DAB staining and <10% immunopositive cells received a score of 0.

Statistical analysis. SPSS version 20.0 (IBM SPSS, Armonk, NY, USA) was used for all statistical analyses. Quantitative data were expressed as means ± standard deviation. Comparisons between groups were determined using the Student's t-test, and within-group comparisons were determined using the paired Student's t-test. Countable data were expressed as numbers with percentages. Groups were compared using Chi-square analysis with Yates' correction, and survival times between groups were determined using the Kaplan-Meier method and log-rank χ^2 test. P<0.05 was considered to indicate a statistically significant difference.

Results

Comparison of tissue and serum miR-7 expression. Immunohistological examination revealed that the number of cases positive for miR-7 and those with higher miR-7 scores were significantly lower in patients with stage I or II NSCLC that were treated with surgery plus gefitinib than those with stage III or IV NSCLC that received only gefitinib chemotherapy (Table I, P<0.05). Serum levels of miR-7 in patients with stage I-II NSCLC before and after intervention were significantly lower than those in patients with stage III-IV NSCLC. Furthermore, within-group serum levels of miR-7 after intervention were significantly lower than those before intervention (Table I, P<0.05).

Table I. Comparison of tissue and serum levels of miR-7 expression.

Group	No. of cases	Tissue			Serum	
		Positive	Negative	Score	Before treatment	After treatment
Stage I-II	54	22 (40.7)	32 (59.3)	1.8±0.6	0.12±0.04	0.05±0.02
Stage III-IV	72	45 (62.5)	27 (37.5)	2.6±0.7	0.46±0.13	0.27±0.10
t (χ^2)		5.868		6.125	6.237	6.529
P-value		0.015		0.020	0.018	0.015

Table II. Comparison of treatment success rates and rates of adverse effects (no., %).

Group	No. of cases	CR	PR	SD	PD	Success rate
Stage I-II						
Positive	22	3 (13.6)	5 (22.7)	6 (27.3)	8 (36.4)	14 (63.6)
Negative	32	9 (28.1)	10 (31.3)	10 (31.3)	3 (9.4)	29 (90.6)
χ^2						4.309
P-value						0.038
Stage III-IV						
Positive	45	0	3 (6.7)	9 (20.0)	33 (73.3)	12 (26.7)
Negative	27	0	6 (22.2)	8 (29.6)	13 (48.1)	14 (51.9)
χ^2						4.639
P-value						0.031

CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease.

Table III. Comparison of survival and progression-free survival (PFS) rates and median survival times between early and advanced clinical stages.

Group	No. of cases	Survival rate, no. (%)	PFS, month	Median survival time, month
Stage I-II				
Positive	22	15 (68.2)	10.3	26.8
Negative	32	27 (84.4)	14.5	31.3
χ^1		1.152	6.238	6.859
P-value		0.283	0.016	0.010
Stage III-IV				
Positive	45	12 (26.7)	5.6	12.6
Negative	27	14 (51.9)	8.4	20.2
χ^0		4.639	6.529	7.234
P-value		0.031	0.013	0.008

Comparison of treatment success rates and rates of adverse effects between early and advanced clinical stages. The treatment success rate was higher in miR-7-negative patients than in miR-7-positive patients in stage I-II and III-IV groups; the difference was statistically significant (Table II, P<0.05). The rate of adverse effects was not significantly different between the miR-7-negative and miR-7-positive patients in either stage I-II or stage III-IV NSCLC cases (Table II, P>0.05).

Comparison of survival and PFS rates and median survival time between early and advanced clinical stages. The follow-up time was 23.0-35.0 (30.5±10.4) and 20.0-32.0 (25.6±7.4) months for patients with stage I-II and III-IV NSCLC, respectively. There were no significant differences in survival rates between miR-7-positive and miR-7-negative patients with stage I or II NSCLC. By contrast, the survival rates were lower in miR-7-positive patients than in miR-7-negative

patients among those with stage III or IV NSCLC; this difference was statistically significant (Table III, $P < 0.05$). The PFS and median survival time were lower in miR-7-positive patients than in miR-7-negative patients among those with stage I or II NSCLC as well as in those with stage III or IV NSCLC. Differences were statistically significant ($P < 0.05$).

Discussion

In two double-blind, parallel, multicenter, large-scale, phase II randomized clinical trials, IDEAL1 and IDEAL2 (3,4), gefitinib was used as second-line therapy for advanced NSCLC. The two studies included two groups that received either 250 or 500 mg/day gefitinib. The results of IDEAL1 demonstrated that the objective response rate, symptom improvement rate, and median survival time were 18.4, 43%, and 7.6 months, respectively, in subjects treated with 250 mg/day gefitinib. Conversely, the objective response rate, symptom improvement rate, and median survival time were 19.0, 37%, and 8.0 months, respectively, in subjects treated with 500 mg/day gefitinib. The findings of IDEAL2 indicated that the patients receiving 250 mg/day gefitinib achieved an objective response rate of 11.8% and that the median survival time was 6.1 months, whereas the objective response rate and the median survival time were 8.8% and 6.0 months, respectively, in patients treated with 500 mg/day gefitinib.

SIGN, a randomized, phase II clinical trial, was conducted to compare gefitinib with established chemotherapy regimens in Asian populations (5). The results showed that, compared to patients treated with docetaxel, those that received gefitinib as second-line treatment for NSCLC had higher rates of symptom and life quality improvements and objective response rates as well as increased median survival times and fewer drug-related adverse effects. A double-blind, placebo-controlled, multicenter, phase III randomized clinical trial, ISEL, concluded that maximal benefits of gefitinib were observed in patients with adenocarcinoma and those with good PE scores, non-smokers, females, and those carrying EGFR mutations (6). Furthermore, the outcomes of V15-32 (7), INTEREST (8), and ISTANA (9) demonstrated that the efficacy of gefitinib in advanced NSCLC was not inferior to that of docetaxel.

As a randomized phase III clinical trial, IPASS (11) evaluated gefitinib as the first-line treatment for advanced NSCLC and concluded that EGFR mutations were strong predictive biomarkers for the superiority of gefitinib to carboplatin/taxol. Two Japanese trials, WJTOG3405 (12) and NEJGSG002 (13), showed that the PFS of patients with NSCLC and EGFR mutations who were treated with gefitinib was significantly longer than that of patients treated with cisplatin/docetaxel chemotherapy. The Korean First-SIGNAL (14) study also concluded that there were significant differences in PFS rates between gefitinib and platinum-based double-agent chemotherapy as first-line treatment regimen; however, this was not reflected in overall survival rates as the outcomes of the study showed that maximum benefit with gefitinib was observed in patients with EGFR mutations.

Genetic analysis in a randomized phase III trial comparing gefitinib with docetaxel as second-line chemotherapy in NSCLC (7) found that EGFR mutations were present in

40-52.5% of advanced NSCLC cases and were detectable in 30-40% of patients with early-stage NSCLC. Moreover, long-term treatment with gefitinib led to the transformation of EGFR mutation-positive tumors to EGFR mutation-negative lesions, which could lead to chemoresistance or weaker chemotherapeutic response and tumor recurrence. Studies have shown that miR-7 expression is closely correlated with the presence and development of lung cancer. Specifically, Xiong *et al* (15) utilized miR-7 mimics to rescue endogenous miR-7 expression and showed that miR-7 had noticeable inhibitory effects on migration, clone formation, and cell cycle regulation of A549 and H1299 human NSCLC cells *in vitro*. Conversely, in a nude mouse model, they demonstrated that inhibition of miR-7 gene expression led to the induction of tumor growth and metastasis. Similarly, upregulation of miR-7 may reduce cyclin D1 expression and inhibit the proliferation of NSCLC cell lines.

miR-7 regulates the growth and the metastatic and invasive capabilities of lung cancer cells through multiple mechanisms. Previous studies showed that miR-7 inhibits the proliferation of 95D lung cancer cells by targeting EGFR (16,17). Xiong *et al* (15) found that miR-7 targeted the complementary sites of the 3' terminal untranslated region of B-cell lymphoma 2 (Bcl-2) to inhibit the expression of Bcl-2, thus inhibiting proliferation and promoting apoptosis in A549 NSCLC cells. They also identified proteasome activator 28 subunit γ (PA28 γ) as a target of miR-7; miR-7 overexpression led to the downregulation of PA28 γ and inhibited the occurrence of NSCLC (17).

In addition to its roles in tumor formation and invasion, miR-7 has been shown to play a role in signal transmission at the plasma membrane in lung cancer cells. Specifically, miR-7 has been shown to negatively regulate the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway through the activation of the regulatory subunit of its target protein, PI3K regulatory subunit 3, which led to the inhibition of growth and migration in lung cancer cells (18). Rai *et al* (19) transfected miR-7-containing expression vectors into nude mice in an *in vivo* model of human lung cancer and found that the growth of RPC-9 and H1975 lung cancer cells with high resistance to TKI was significantly inhibited in nude mice bearing tumors, suggesting miR-7 as potential target for gene-targeted therapy in lung cancer.

In this clinical study, we found that the number of miR-7-positive cases and the number of cases with higher levels of miR-7 expression was lower among patients with stage I or II NSCLC than those with stage III or IV cancer. Furthermore, serum levels of miR-7 before and after intervention were lower in stage I-II than in stage III-IV NSCLC cases. Among the early- and advanced-stage patients, serum levels of miR-7 after intervention were lower than those before intervention; these differences in serum levels of miR-7 were statistically significant. The treatment success rate was significantly higher in miR-7-negative patients than in miR-7-positive patients among the early- and advanced-stage patients. The number of adverse effects in miR-7-negative and in miR-7-positive patients was comparable between the early and advanced clinical stage groups. There were no differences in the survival rates of miR-7-positive and miR-7-negative patients with stage I or II NSCLC; however,

the survival rate of miR-7-positive patients with stage III-IV NSCLC was significantly lower than that of miR-7-negative patients. The PFS and median survival time of miR-7-positive patients with stage I-II NSCLC were significantly lower than those of miR-7-negative patients. In conclusion, serum and tissue miR-7 expression was closely associated with tumor staging and therapeutic effects of gefitinib in NSCLC.

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