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ORIGINAL ARTICLE

miR-638 is a new biomarker for outcome prediction of non-small cell lung cancer patients receiving chemotherapy

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MicroRNAs (miRNAs), a class of small non-coding RNAs, mediate gene expression by either cleaving target mRNAs or inhibiting their translation. They have key roles in the tumorigenesis of several cancers, including non-small cell lung cancer (NSCLC). The aim of this study was to investigate the clinical significance of miR-638 in the evaluation of NSCLC patient prognosis in response to chemotherapy. First, we detected miR-638 expression levels *in vitro* in the culture supernatants of the NSCLC cell line SPC-A1 treated with cisplatin, as well as the apoptosis rates of SPC-A1. Second, serum miR-638 expression levels were detected *in vivo* by using nude mice xenograft models bearing SPC-A1 with and without cisplatin treatment. In the clinic, the serum miR-638 levels of 200 cases of NSCLC patients before and after chemotherapy were determined by quantitative real-time PCR, and the associations of clinicopathological features with miR-638 expression patterns after chemotherapy were analyzed. Our data helped in demonstrating that cisplatin induced apoptosis of the SPC-A1 cells in a dose- and time-dependent manner accompanied by increased miR-638 expression levels in the culture supernatants. *In vivo* data further revealed that cisplatin induced miR-638 upregulation in the serum derived from mice xenograft models, and in NSCLC patient sera, miR-638 expression patterns after chemotherapy significantly correlated with lymph node metastasis. Moreover, survival analyses revealed that patients who had increased miR-638 levels after chemotherapy showed significantly longer survival time than those who had decreased miR-638 levels. Our findings suggest that serum miR-638 levels are associated with the survival of NSCLC patients and may be considered a potential independent predictor for NSCLC prognosis.

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INTRODUCTION

Lung cancer is characterized with a high incidence and poor prognosis, and it is the leading cause of cancer-related death worldwide, with a dismal 5-year survival rate of less than 15%.[1–3] The most common lung cancer variant is non-small cell lung cancer (NSCLC), which accounts for ~85% of lung cancer cases. Chemotherapy is the most frequent treatment for NSCLC and helps to improve the life quality and prolong the survival of patients.[4] However, further work is required to monitor NSCLC patient responses to chemotherapy and thereby improving the patient outcomes.

Mature, biologically active microRNAs (miRNAs) are endogenous short non-coding RNAs and are known to have essential roles in the regulation of numerous biological processes, such as development, differentiation, proliferation and apoptosis.[5–7] They achieve this by interfering with the translation or stability of genes by binding the 3′ UTR of target mRNAs at the posttranscriptional level.[8] Over the past few decades, the number of identified miRNAs has continued to grow, and our knowledge of miRNA biology has increased accordingly.[9] On the basis of their role in cancer pathology and the types of gene that they target, miRNAs can be divided into oncogenes and tumor suppressor genes. miR-638 has been

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reported to be downregulated in several types of cancer, such as gastric cancer,[10] leukemia[11] and basal cell carcinoma,[12] and may therefore function as a tumor suppressor gene.

Previously, we successfully prepared a monoclonal antibody named NJ001 that is specific to NSCLC.[13] In this earlier study, the lung cancer cell line SPC-A1 was treated with NJ001 *in vitro* for different periods of time and expression microarray analyses were then performed with the extracted RNA. We found that miR-638 exhibited the most significant time-dependent differential expression in response to NJ001 (GEO accession number: GSE51947). Furthermore, recent studies have revealed that miR-638 is aberrantly expressed in several cancers,[10,14,15] suggesting that it may be involved in cancer development and progression. Thus, we next focused on exploring the potential clinical application of miR-638. Specifically, the aim of this study was to explore the usefulness of serum miR-638 detection for predicting the NSCLC outcome.

MATERIALS AND METHODS

Cell culture and *in vitro* experiments

The SPC-A1 cell line was purchased from the type culture collection of the Chinese Academy of Sciences, Shanghai, China, and was proven to be uncontaminated by bacterium or mycoplasma by the European Collection of Cell Cultures using mass spectrometry. The cells were grown in RPMI1640 medium supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin at 37 °C and 5% CO_2. For *in vitro* experiments, SPC-A1 cells in the exponential growth phase were seeded in six-well plates (2×10^6 per well) and treated with different concentrations (0.5, 2.5 and 5 μg ml^{-1}) of cisplatin or complete RPMI1640 medium alone (as the control group) and incubated at 37 °C and 5% CO_2 for 24, 48 or 72 h. Cultured supernatants were then harvested for miR-638 detection. Each time point was set in triplicates, and the whole experiment was repeated three times.

Detection of apoptosis

The apoptosis rates of the SPC-A1 cells were determined by flow cytometry. Approximately 1×10^5 cells were collected for apoptosis rate detection by flow cytometry. Cells were re-suspended and stained with Annexin V-FITC and propidium iodide (Annexin V-FITC Apoptosis Detection Kit, BD Biosciences, Franklin, NJ, USA) according to the manufacturer's instruction. FACS analysis was then carried out on a FACScan cytometer (BD Biosciences).

In vivo tumor establishment, cisplatin treatment and serum isolation

Experimental nude mice were purchased from Slack Experimental Animal Co. in Shanghai with Certification No.: SCXK (Shanghai, China) 2009-0002 and SPF level status. The mice were housed in an SPF animal facility in the Experimental Animal Center of Jiangsu Province. *In vivo* experiments were carried out using nude mice aged 6 weeks. The xenograft models were established by subcutaneous injection of 2×10^6 SPC-A1 cells, and non-injected mice ($n = 5$) served as a control group. After 2 weeks, when tumor tubercles were significantly observed, the mice were divided into three groups. The first group was injected with cisplatin at a dose of 0.5 μg per 100 μl via the caudal vein ($n = 6$); the second group received an equal volume of normal saline (NS) ($n = 5$) and served as a negative control group; and

the remaining xenograft models were not treated with either cisplatin or NS ($n = 5$). The treatments were performed three times every other day. Blood was collected the day after the last administration and centrifuged at a speed of 16 000 *g* for 10 min at room temperature to isolate serum after coagulation.

Patients and serum specimens

Clinical samples were collected from a cohort of 200 cases of NSCLC patients during their hospitalization from July 2009 to March 2013 at the First Affiliated Hospital of Nanjing Medical University, China. Blood was collected before and after chemotherapy with patients' informed consent. Serum was then separated from whole blood by centrifugation at 2500 *g* for 10 min, followed by 16 000 *g* for 10 min to completely remove cell debris, and then stored at − 70 °C until needed for use.

RNA extraction and quantitative RT–PCR

Total RNA was extracted using the miRNeasy mini kit (QIAgen, Valencia, CA, USA) according to the manufacturer's protocol, with cel-miR-39 (59-ucaccggguguaaaucagcuug-39; final concentration: 10^{-5} pmol ml^{-1}) (Applied Biosystems, Foster City, CA, USA) added as an internal control. RNA concentration and quality were validated by using a UV spectrophotometer at 260 and 280 nm. Quantitative PCR with reverse transcription (RT–PCR) was used to detect miR-638 expression levels. For reverse transcription, 9.16 μl RNA was used in each reaction and mixed with 5.84 μl reverse transcription reagents (Applied Biosystems), including stem-loop reverse transcription primers, according to the manufacturer's instructions. The reverse transcription reactions were performed at 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min and then maintained at 4 °C. Following this, real-time PCR was conducted at 95 °C for 10 min, followed by 95 °C for 15 s with 45 cycles and 60 °C for 1 min in an ABI 7500 Real-Time PCR system. miR-638 expression levels are presented in terms of fold change normalized to cel-miR-39 expression using the formula $2^{-\Delta\Delta Ct}$ in which $\Delta\Delta Ct = (Ct_{miR-638} - Ct_{miR-39})_{treated} - (Ct_{miR-638} - Ct_{miR-39})_{untreated}$.

Statistical analysis

Statistical analyses were performed using Graphpad Prism 5.0 (San Diego, CA, USA) and SPSS 16.0 software (Chicago, IL, USA). A survival analysis was performed with the Kaplan–Meier method, and the log-rank test was used to compare survival times between groups. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Cisplatin induces apoptosis of SPC-A1 cells

To evaluate the effect of cisplatin on SPC-A1 apoptosis, SPC-A1 cells were treated with different doses of cisplatin for different periods of time. The apoptosis rates for 24, 48 and 72 h were then determined after treatment with/without cisplatin using flow cytometry. The apoptosis rates of the SPC-A1 cells cultured for 24, 48 and 72 h with cisplatin at a concentration of either 2.5 or 5 μg ml^{-1} were significantly greater than those of paired untreated control groups (Figures 1a and b), and these apoptosis rates increased more markedly with prolonged exposure to higher concentrations of cisplatin. These data suggest that cisplatin induces apoptosis of SPC-A1 cells in a dose- and time-dependent manner.
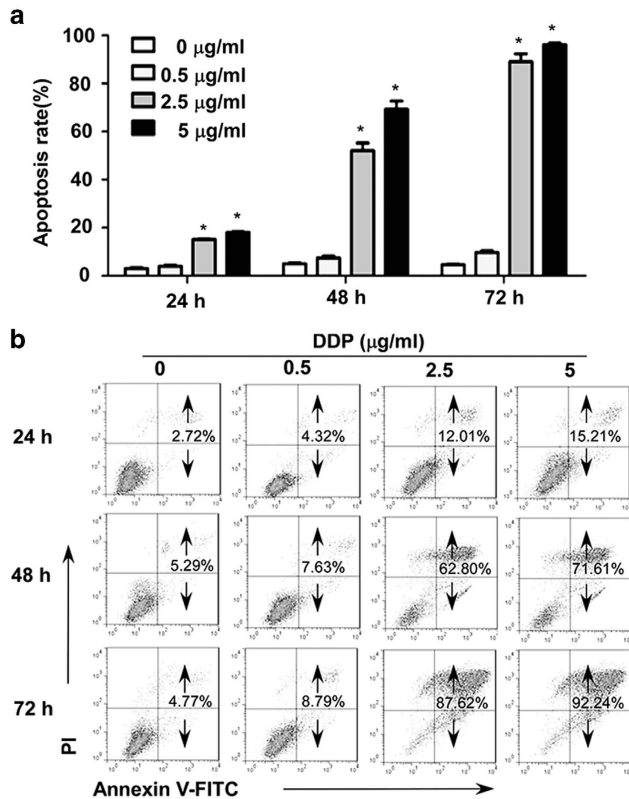
Figure 1 Apoptosis rates of SPC-A1 cells following cisplatin treatment. (a) Apoptosis rates of SPC-A1 cells at different time points post treatment with different doses of cisplatin, as indicated, were measured by flow cytometry. (b) The original flow cytometry plot (*$P < 0.05$).

Figure 2 Relative expression levels of miR-638 in SPC-A1 cultured supernatants treated with cisplatin of $2.5 \mu g \, ml^{-1}$ (a) and $5 \mu g \, ml^{-1}$ (b). SPC-A1 cells were treated with different concentrations (0.5, 2.5 and $5 \mu g \, ml^{-1}$) of cisplatin for 24, 48 and 72 h, and miR-638 expression levels in SPC-A1 cultured supernatants were determined by qRT–PCR. Data represent the mean fold-change in miR-638 expression in three independent groups relative to the untreated control group (*$P < 0.05$).

Cisplatin treatment induces miR-638 expression in a time- and dose-dependent manner in SPC-A1 cultured supernatants

Quantitative RT–PCR (qRT–PCR) was used to detect the expression levels of miR-638 in the culture supernatants of SPC-A1 cells treated with cisplatin. The results showed that the miR-638 expression levels in the SPC-A1-cultured supernatants were more than four-fold higher after being treated with cisplatin for 72 h at a concentration of $2.5 \mu g \, ml^{-1}$ when compared with expression levels in the untreated control group (Figure 2a). Furthermore, treatment of SPC-A1 cells with either 2.5 or $5.0 \mu g \, ml^{-1}$ cisplatin both resulted in a significant increase in miR-638 expression levels in the supernatants compared with that of the control group. Moreover, it was observed that the miR-638 levels in supernatants also increased in a time-dependent manner when $5.0 \mu g \, ml^{-1}$ cisplatin was used for treatment for different periods of time (24–72 h) (Figure 2b).

Cisplatin treatment in vivo up-regulates miR-638 expression levels

To address whether cisplatin treatment affects miR-638 expression in vivo, we analyzed miR-638 expression levels in sera of nude mice SPC-A1 xenograft models with and without
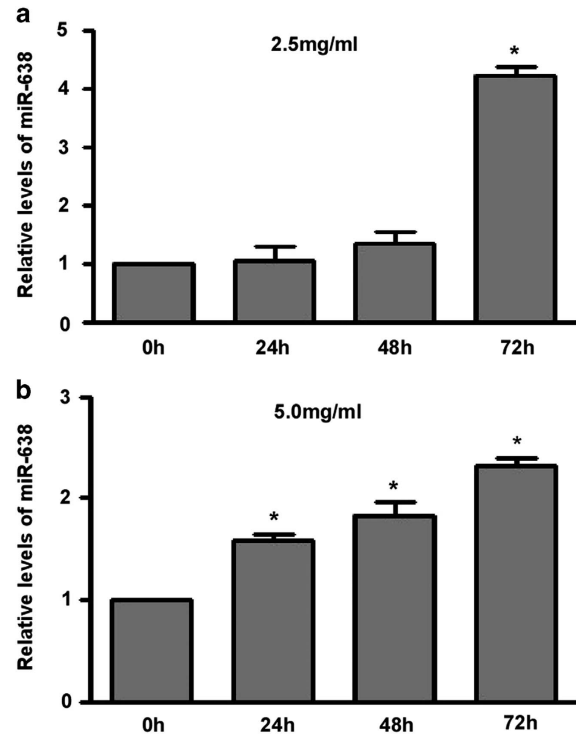
cisplatin treatment by using qRT–PCR. A marked increase in miR-638 expression was observed in the sera of the SPC-A1 lung carcinoma group with cisplatin administration compared with the lung carcinoma group without any treatment, the lung carcinoma group with NS treatment or the blank control group. However, no significant difference in serum miR-638 expression was observed in either of the two lung carcinoma groups without any treatment, the lung carcinoma group with NS treatment or the blank control group. An increase in serum miR-638 expression levels was observed as early as 24 h post-treatment with cisplatin. These data suggest that cisplatin administration in SPC-A1 xenograft models induces upregulation of miR-638 expression as early as 24 h post-treatment (Figure 3).

Associations between clinicopathological features and serum miR-638 expression patterns

To further explore the relationship between serum miR-638 expression and the clinicopathological features of NSCLC, the correlation between serum miR-638 expression patterns after chemotherapy and clinicopathological characteristics was evaluated. The data presented in Table 1 show that the upregulation of miR-638 in serum was significantly correlated with a lower rate of lymph node metastasis ($P = 0.017$);
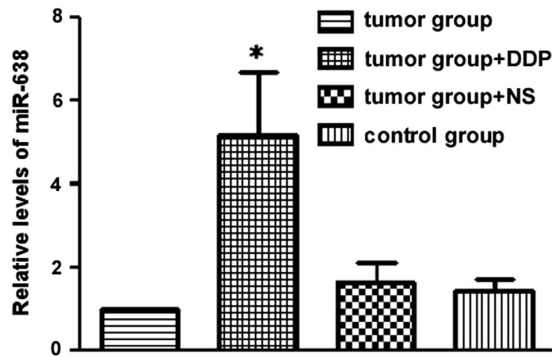
Figure 3 MiR-638 expression levels in serum of nude mice bearing SPC-A1 xenografts. Mice bearing SPC-A1 xenografts were treated with $5 \mu g\, ml^{-1}$ of cisplatin (200 µl, $n = 6$), NS (200 µl, $n = 5$) or received no treatment ($n = 5$). The next day after the last drug administration, serum was collected and miR-638 expression levels were determined. The graph represents the mean serum miR-638 expression levels relative to the control group without SPC-A1 cells ($*P < 0.05$).

however, there were no correlations between serum miR-638 expression and age, gender, smoking history or tumor-lymph node-metastasis (TNM) stage (all $P > 0.05$). These results suggest that elevated miR-638 expression may be inversely correlated with NSCLC progression.

Correlation between miR-638 levels and NSCLC patients' survival

As demonstrated, increased miR-638 expression levels were observed in SPC-A1 culture supernatants in a time-dependant manner after cisplatin treatment. To assess the prognostic value of miR-638 in NSCLC patients receiving chemotherapy, we determined its expression in serum before and after chemotherapy derived from 200 cases of NSCLC patients in which 189 cases displayed miR-638 expression changes. We found that serum miR-638 expression levels increased in 112 cases (~60%) of the NSCLC patients after chemotherapy. After a 1-year follow-up, we observed that the cumulative survival rate of patients with increased miR-638 expression was 59.2% compared with 47.5% of those with decreased miR-638 expression. The survival data for the 183 cases of NSCLC with complete records were analyzed by Kaplan–Meier survival curve and compared with a log-rank test. This analysis revealed a significant association between serum miR-638 levels and the survival of NSCLC patients. Indeed, survival for patients with increased miR-638 expression after chemotherapy was significantly greater than that for patients with a decreased miR-638 expression ($P = 0.0061$, Figures 4 and 5). Moreover, Cox proportional hazards regression analysis performed at the univariate level indicated a significant correlation between overall survival and serum miR-638 expression patterns (HR = 2.142; 95% CI, 1.130–4.060; $P = 0.02$) and regional lymph node metastasis (HR = 6.035; 95% CI, 2.041–17.845; $P = 0.001$) (Table 2). Clinicopathological features that were significantly correlated with overall survival at the univariate level were then

Table 1 Associations between serum miR-638 expression patterns after chemotherapy and clinicopathological characteristics in NSCLC patients ($n = 189$)

	miR-638 Upregulation	miR-638 Downregulation	P-value
Age			
<60	55	37	0.887
>60	57	40	
Gender			
Male	62	42	0.912
Female	50	35	
Smoking history			
Yes	51	36	0.741
No	61	41	
TNM stage			
I+II	39	26	0.881
III+IV	73	51	
Lymph node metastasis			
Yes	87	70	0.017*
No	25	7	

$*P < 0.05$.

analyzed by multivariate analysis, and the results demonstrated that miR-638 (HR = 2.258; 95% CI, 1.031–3.456; $P = 0.04$) and regional lymph node metastasis (HR = 3.196; 95% CI, 1.938–15.869; $P = 0.001$) remained significant as independent prognostic factors for overall survival rates (Table 2).

Dynamic changes in miR-638 levels during chemotherapy

To monitor the dynamics of serum miR-638 during chemotherapy, serum samples from NSCLC patients who received several cycles of chemotherapy were collected after different cycles of chemotherapy (Figure 6). For each individual, serum samples were collected on the last day of each administration of medication, and the relative fold change of 1 represented the serum miR-638 expression level before chemotherapy. Figure 6 shows the dynamic changes in the serum miR-638 expression levels of four representative patients after chemotherapy. Patient A (Figure 6a) received six cycles of chemotherapy, and CT scans showed that the tumor shrank significantly, with decreased levels of tumor markers that were inversely associated with serum miR-638 expression. Indeed, the serum miR-638 level after the last administration of medication was 2.5-fold greater than that before chemotherapy, and the patient was alive at 15 months of follow-up. Patient B (Figure 6b) displayed a stable condition after three cycles of chemotherapy, as determined by CT scans, along with decreased levels of tumor markers, such as CEA, NSE and CYFRA21-1, in the reference range and gradually increased serum miR-638 expression levels. At the time of our most recent follow-up, 27 months after treatment, patient B was still alive. By contrast, patient C (Figure 6c) received four cycles of chemotherapy,
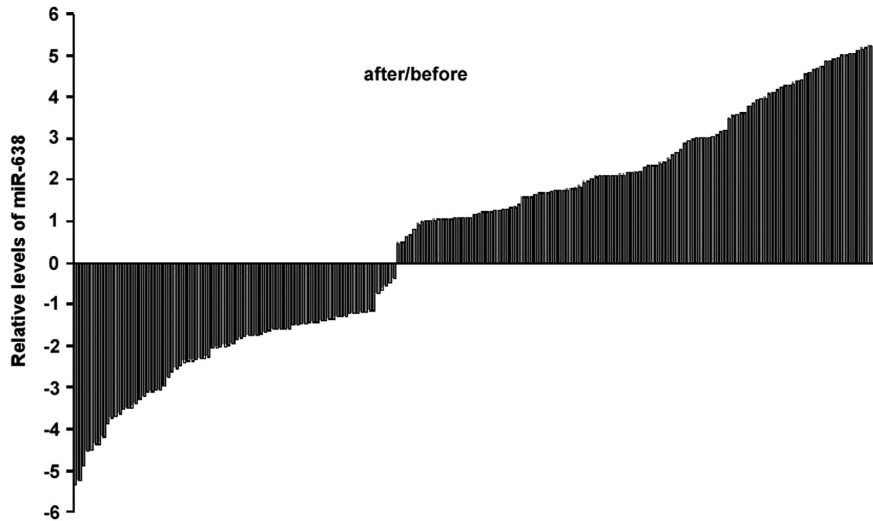
Figure 4 Expression levels of miR-638 in NSCLC patients before and after chemotherapy. Fold changes of serum miR-638 levels in serum of each individual paired NSCLC patient sample before and after chemotherapy. Relative data are represented as $\log_2$ (fold change) (after/before), in which a 'Y' value >1 was defined as an increase in miR-638 levels, whereas a value <-1 was defined as a decrease in levels.
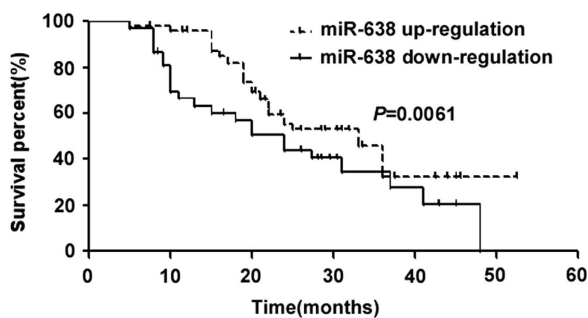

Figure 5 Kaplan–Meier survival curve for overall survival of NSCLC patients ($n=183$). Kaplan–Meier analysis was performed to evaluate the overall survival of NSCLC patients according to miR-638 expression patterns after one cycle of first-line chemotherapy, in which 109 patients had higher miR-638 expression levels, and 74 patients had lower miR-638 expression levels.

during which CT scans determined that the patient's condition had deteriorated because of tumor metastasis. Throughout treatment, the patient's serum miR-638 levels were lower after chemotherapy, and the patient lived for only 9 months after cessation of treatment. Likewise, patient D (Figure 6d) received three cycles of chemotherapy and displayed decreased serum miR-638 levels together with tumor progression and metastasis, as determined by CT scans. Additionally, serum levels of CEA, CYFRA21-1 were observed to be higher than before chemotherapy. In particular, serum miR-638 levels decreased significantly after the second and third cycle of chemotherapy in patient D. The relative fold change in serum miR-638 level after the last cycle compared to the pre-treatment level was

0.06, trending to 0. The patient died 5 months after the final diagnosis.

DISCUSSION

miRNAs have been shown to function in the regulation of cell proliferation, apoptosis and cell transformation at a posttranscriptional level.[16–18] In lung cancer, studies have shown that miRNAs play an important role in the development of chemosensitivity or chemoresistance.[19,20] Indeed, miRNAs have been found to have a crucial role in the regulation of tumor biological behavior, and their expression patterns have been linked with tumor development. In the field of oncology, miRNA detection has been confirmed to be applicable in early diagnosis, prognosis and therapeutic evaluation.[21–24]

Accumulating studies have revealed the dysregulation of several miRNAs in NSCLC, suggesting that they play important roles in tumorigenesis and the progression of NSCLC. For instance, the downregulation of miR-497 in NSCLC promotes cell proliferation and angiogenesis.[25] miR-149 is also significantly downregulated in NSCLC cells and is inversely correlated with invasion through the inhibition of epithelial mesenchymal transition by targeting FOXM1.[26] Similarly, miR-181b is markedly downregulated in NSCLC and significantly correlates with tumor size, p-TNM/TNM stage, and the status of lymph node metastasis.[27] Moreover, miR-449a has been shown to inhibit both cell migration and invasion by directly targeting the c-Met gene in NSCLC, downregulation of which has been associated with poor prognosis.[28] Here, we aimed to investigate the clinical significance of miR-638 in NSCLC.

A previous expression microarray analysis illustrated that SPC-A1 cells treated with NJ001 exhibited the most

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Table 2 Univariate and multivariate COX regression model analysis of overall survival in NSCLC patients

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Age	1.014	0.984–1.044	0.366			
Gender	0.908	0.415–1.986	0.809			
Smoking	1.733	0.800–3.750	0.163			
TNM stage	2.017	0.686–5.934	0.202			
Lymph node metastasis	6.035	2.041–17.845	0.001*	3.196	1.938–15.869	0.001*
miR-638 expression status	2.142	1.130–4.060	0.02*	2.258	1.031–3.456	0.04*

Abbreviations: HR, hazard ratio; CI, confidence interval; NSCLC, non-small cell lung cancer.
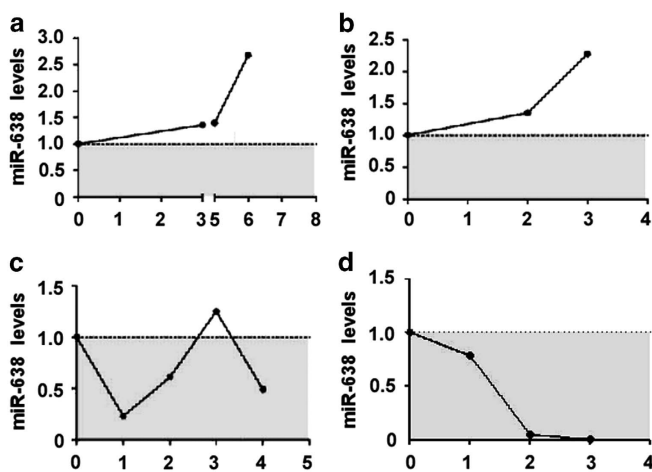*$P<0.05$, $n=183$.

Figure 6 Expression levels of miR-638 at different time points following chemotherapy. (**a–d**) Serum miR-638 expression levels in four patients who received several cycles of chemotherapy were monitored by qRT–PCR. A relative $\log_2$ (fold change) of 1 was considered the initial miR-638 expression level before chemotherapy.

significantly increased expression of miR-638 in a time-dependent manner. To further elucidate the potential role and clinical value of miR-638 in NSCLC, we treated SPC-A1 cells with different concentrations of cisplatin for different periods of time *in vitro* and measured the apoptosis rates and the expression levels of miR-638 in culture supernatants. The results showed that cisplatin induced SPC-A1 apoptosis in a dose- and time-dependent manner. Importantly, this positively correlated with a time-dependent increase in miR-638 expression in the culture supernatant when the concentration of cisplatin was 2.5 or 5.0 µg ml^{-1}. Moreover, miR-638 expression gradually increased following prolonged exposure times and increased apoptosis rates, indicating a possible positive role for miR-638 in apoptosis. Furthermore, *in vivo* tests demonstrated that cisplatin-induced upregulation of miR-638 in the sera of nude mice xenograft models of NSCLC could be detected 24 h after administration, suggesting that miR-638 expression is increased at an early stage in response to cisplatin.

We also measured the serum miR-638 levels of 189 NSCLC patients by qRT–PCR analysis and found that the 1-year cumulative survival rate of patients with increased serum miR-638 levels was 61.4% compared with 49.2% in patients who had decreased serum miR-638 levels after one standard chemotherapy cycle. Survival analyses further indicated that NSCLC patients with higher serum miR-638 expression after chemotherapy had a longer mean survival time than those with lower serum miR-638 expression. Serum expression patterns of miR-638 after chemotherapy were also associated with lymph node metastasis, suggesting that expression patterns may be inversely related to cell invasion in NSCLC. In addition, monitoring the dynamic changes in miR-638 expression before and after chemotherapy in the sera of representative patients demonstrated that those who had positive responses to chemotherapy showed increased serum miR-638 levels, whereas those who displayed stable or progressive disease exhibited decreased serum miR-638 levels.

The value of miRNAs as prognostic tools has previously been demonstrated.[29–32] Indeed, as non-invasive biomarkers of disease and therapy response, miRNAs could serve as prognostic indicators in some solid tumors. For example, serum miR-221 is closely related to ovarian cancer prognosis, and its elevated expression indicates a poor prognosis.[23] Additionally, colorectal cancer patients with higher serum expression of miR-92a have a poorer prognosis.[22]

It has been reported that miR-638 is down-regulated in several types of cancer, including lung cancer. miR-638 is dramatically down-regulated in gastric cancer tissues, and it has been shown to inhibit the expression of cyclin D1 by inhibiting specificity protein 2 (Sp2) expression, thus suppressing gastric cancer cell proliferation.[33] Downregulation of miR-638 expression has been confirmed in NSCLC tissues and is reported to be involved in promoting the transformation of normal cells into tumor cells.[14] Moreover, downregulated miR-638 has been shown to induce cell invasion and proliferation by regulating SRY-box 2, which is related to epithelial-to-mesenchymal transition in the development of NSCLC, and the effect is reversed by high expression of miR-638 or silencing of SRY-box 2.[34] A similar functional role has also been found in colorectal carcinoma cells.[35] However, over-expression of

miR-638 in vascular smooth muscle cells has been shown to inhibit cell proliferation and migration by targeting the NOR1 receptor,[36] which might partially explain the correlation between the increased expression of miR-638 and favorable prognosis. In our previous study, over-expression of miR-638 *in vitro* induced apoptosis of lung cancer cells by regulating P21-activated protein kinase. However, a clinically significant link between levels of miR-638 in serum and NSCLC prognosis has not been previously reported.

In conclusion, we have revealed that serum miR-638 expression levels varied in NSCLC patients after chemotherapy, which was associated with NSCLC prognosis. More specifically, upregulation of serum miR-638 was correlated with a favorable prognosis for NSCLC patients. This is also the first study to indicate that miR-638 expression patterns correlate with lymph node metastasis and clinical outcome. Serum miR-638 is a useful independent factor for NSCLC prognosis and could be used as a potential prognostic biomarker for overall survival of NSCLC patients. However, further studies are required to explore the underlying molecular mechanisms of miR-638 in lung cancer tumorigenesis and progression and its possible use as a therapeutic target for NSCLC.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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