SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 12: 4829-4837, 2016

Evaluation of plasma microRNA levels to predict insensitivity of patients with advanced lung adenocarcinomas to pemetrexed and platinum

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Received July 17, 2015; Accepted September 9, 2016

DOI: 10.3892/ol.2016.5295

Abstract. Pemetrexed combined with platinum is a first-line therapy used to treat patients with advanced non-small cell lung cancer (NSCLC) that exhibit negative or unknown epidermal growth factor receptor (EGFR) mutational status or anaplastic lymphoma kinase (ALK) rearrangements. Lung adenocarcinoma (LAC) is the primary type of NSCLC. In order to prevent overtreatment, it is necessary to identify patients with LAC who may not benefit from certain chemotherapies. Patients recruited in the present study (n=129) were diagnosed with advanced LAC and received first-line pemetrexed and platinum-based chemotherapy. A microRNA (miR) microarray was used to screen the plasma miR expression profiles in a screening set of eight patients prior to and following treatment. Specifically, plasma miR-25, miR-21, miR-27b, miR-326, miR-483-5p and miR-920 were selected for reverse transcription-quantitative polymerase chain reaction analysis in a training set (n=44) prior to treatment. The screening and training set patients were all non-smokers with no prior history of serious or chronic disease. The ΔΔCq values of these miRs were compared between the group that showed benefit from pemetrexed and platinum treatment and the group that did not. Consequently, the ΔΔCq values of miR-25, miR-21, miR-27b and miR-326 were further determined in a validation set (n=77). The results of the present study demonstrate that plasma expression levels of miR-25, miR-21, miR-27b and miR-326, in the training and validation sets prior to treatment, were significantly different between the benefit and non-benefit groups (P≤0.001). The expression of miR-25, miR-21, miR-27b and miR-326 was upregulated in the non-benefit group and this elevation was positively correlated with decreased progression-free survival (PFS; P≤0.001). In addition, the predictive power of each miR was evaluated through receiver operating characteristic curves, in which miR-25 exhibited the highest degree of accuracy (area under the curve, 0.926; 95% confidence interval, 0.881-0.971). These results indicate that overexpression of plasma miR-25, miR-21, miR-27b and miR-326, prior to treatment, in patients with advanced LAC is predictive of non-benefit from first-line pemetrexed and platinum-based chemotherapy, and is associated with decreased PFS. Among these four miRs, miR-25 exhibited the highest degree of accuracy in predicting insensitivity, suggesting it is the most promising biomarker.

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Key words: advanced lung adenocarcinoma, pemetrexed, platinum, circulating microRNA, predictive markers

Introduction

Lung cancer is the primary cause of cancer-associated mortality worldwide (1) and non-small cell lung cancer (NSCLC) accounts for ~85% of all lung cancer cases. NSCLC is divided into two major types, squamous cell carcinoma and non-squamous cell carcinoma. Non-squamous cell carcinoma is comprised of a number of subtypes, including adenocarcinoma and large-cell carcinoma. Adenocarcinoma is the most frequent non-squamous cell carcinoma and its prevalence is increasing (2).

For patients with advanced NSCLC that exhibit a negative or unknown EGFR mutational status or ALK rearrangements, platinum-based double chemotherapy is the typical first-line therapy (3,4). In the treatment of non-squamous cell carcinoma, pemetrexed combined with platinum demonstrates a significantly improved toxicity profile and OS, compared with taxane- or gemcitabine-based regimes (3,5,6). Nevertheless, a number of patients do not benefit from pemetrexed and platinum-based therapy, due to insensitivity or high toxicity to the combination (7-9). Therefore, it is necessary

to identify patients unlikely to benefit from this treatment in order to avoid unnecessary treatment. Previous studies have demonstrated that excision repair cross-complementation group 1 (ERCC-1), breast cancer type 1 susceptibility protein (BRCA-1), ribonucleotide reductase catalytic subunit M1 (RRM-1) and thymidylate synthase (TS) are predictive markers of the chemotherapeutic effect of platinum agents, taxanes, gemcitabine and pemetrexed (10-14). However, the predictiveness of ERCC-1, RRM-1 and BRCA-1 has not been reproducible (15-18). There is a particular need for further phase III studies of TS to validate the reproducibility of the applied immunohistochemistry and scoring systems for its use as a predictive marker (18). Consequently, it is important to identify novel biomarkers for the prediction of chemotherapeutic benefit.

MicroRNAs (miRs) are small noncoding RNAs, of between 19 and 25 nucleotides in length, which bind to the 3'-untranslated region of mRNAs to regulate their translation. miRs are involved in numerous essential biological processes and are associated with a number of types of cancer and disease (19). Numerous studies have demonstrated that miRs are dysregulated in cancer, such as that of the lung (20-23). As circulating miRs are stable in blood plasma, they have potential applications as diagnostic markers (24,25). Increasing evidence has shown circulating miR profile analysis can aid in early diagnosis, staging, tracking and prognosis of different types of cancer (26-32). In addition, circulating miR profile analysis is useful in predicting the response to chemotherapy (33,34).

The present study aimed to determine which plasma miRs may be used to predict the clinical outcome of pemetrexed and platinum treatment for advanced lung adenocarcinoma (LAC). In contrast with previous research, patient benefit to the treatment was examined instead of tumor response. Seidman *et al* (35) observed that the overall survival (OS) of patients with stable metastatic breast cancer resembled that of patients with complete remission (CR) or partial remission (PR), indicating that similar benefits to remission may come from stable disease (SD). Therefore, benefit (CR+PR+SD) may be deemed a more accurate indicator of treatment efficacy than tumor response (36). A miR microarray and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were used to identify and verify potential markers in training and validation sets following screening. It was demonstrated that high plasma expression levels of miR-25, miR-21, miR-27b and miR-326 may predict non-benefit from chemotherapy, and that increased levels of these miRs was inversely correlated with progression-free survival (PFS).

Materials and methods

Study participants. A total of 129 participants (Table I) diagnosed with stage IIIB-IV LAC were recruited from the Jiangsu Cancer Institute and Hospital (Nanjing, China) between September 2010 and January 2013. All patients had histological or cytological confirmation of their tumor diagnosis. Tumors were staged based on the Seventh Edition Tumor-Node-Metastasis Staging System of the American Joint Committee on Cancer (37). All patients received first-line chemotherapy, pemetrexed (500 mg/m^2) on day 1 with either cisplatin (75 mg/m^2) or carboplatin [area under the curve (AUC)=5] on day 2 of a 21-day treatment cycle. All patients experienced ≥2 cycles of chemotherapy. Therapeutic response was evaluated by computed tomography following 2 cycles of treatment, according to Response Evaluation Criteria in Solid Tumors 1.1 (38). Response was classified as PR, CR, SD or progressive disease (PD). Patients classified as CR, PR or SD for ≥4 weeks were assigned to the benefit group. Conversely, patients classified as PD were assigned to the non-benefit group.

Plasma samples from all patients were collected prior to chemotherapy, between September 2010 and January 2013. A miR microarray was used to screen the plasma miR expression profiles of a screening set of eight patients prior to and following treatment. Specifically, plasma miR-25, miR-21, miR-27b, miR-326, miR-483-5p and miR-920 were selected for analysis in a training set (n=44) prior to treatment. The screening and training set patients were all non-smokers with no prior history of serious or chronic disease. Consequently, the ΔΔCq values of miR-25, miR-21, miR-27b and miR-326 were further determined in a validation set (n=77), which included smokers and patients with a prior history of serious or chronic disease. Patients were observed until December 2014. The present study was approved by the Medical Ethics Committee of Jiangsu Cancer Institute and Hospital and all participants provided informed consent.

Total RNA isolation. Whole blood samples (5 ml per patient) were collected in anticoagulant tubes and centrifuged at 1,811 x *g* for 5 min at 4˚C. The supernatant was subsequently transferred into an RNase/DNase-free 1 ml microfuge tube and immediately stored at -80˚C until required. As there is no established endogenous miR control for blood plasma, prior to the isolation process, cel-miR-238 (UUUGUACUCCGA UGCCAUUCAGA; Takara Biotechnology Co., Ltd., Dalian, China), a synthetic *Caenorhabditis elegans* miR, was mixed with every sample as an internal control, at a final concentration of 25 fmol. Separation of total RNA from the plasma was performed using the NucleoSpin miRNA Plasma kit (Machery-Nagel GmbH, Düren, Germany), according to the manufacturer's instructions. RNA purity and quantity was determined using a NanoDrop spectrophotometer (ND-1000; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

miR microarray analysis. The isolated RNAs were labeled using the miRCURY Hy3/Hy5 Power Labeling kit (cat. no. 208520), and the Hy3TM-labeled samples were then hybridized, following the manufacturer's protocol, to the miRCURYTM LNA Array (cat. no. 208031-A) (both Exiqon A/S, Vedbaek, Denmark). This array covered miRs annotated in miRBase version 18.0 (www.mirbase.org), comprising of 3,100 capture probes, including all human, rat and mouse miR genes. Subsequently, the Axon GenePix 4000B Microarray Scanner (Molecular Devices, LLC, Sunnyvale, CA, USA) was used to scan the slides at a wavelength of 532 nm. Image files were then transferred to GenePix Pro version 6.0 software (Molecular Devices, LLC) for further analysis. Replicated miR readings were averaged and miRs with intensities ≥50 were selected for normalization using the median normalization method (39). Subsequently, fold change filtering identified differentially expressed miRs.

Table I. Demographic and clinical characteristics of the non-benefit and benefit groups.

	Profiling set		Testing set			Validation set		
Characteristic	Non-benefit	Benefit	Non-benefit	Benefit	P-value	Non-benefit	Benefit	P-value
Number of patients	4	4	15	29		26	51	
Mean age, (years ± standard error)	43±1.2	45±4.4	55±10.1	56±8.8	0.717[a]	60±10.1	55±11.2	0.159[a]
Gender (no.)								
Male	3	3	10	18	0.764[b]	18	31	0.466[b]
Female	1	1	5	11		8	20	
Stage of LAC								
IIIB	0	0	2	3	1.000[c]	3	7	0.617[c]
IV	4	4	13	26		23	44	
Smoking status								
Yes	0	0	0	0		10	21	0.818[b]
No	4	4	15	29		16	30	
EGFR genotype								
Wild-type	1	0	1	3	1.000[c]	4	5	0.477[c]
Unknown	3	4	14	26		22	46	
Prior serious or chronic disease history								
Yes	0	0	0	0		11	22	0.945[b]
No	4	4	15	29		15	29	
KPS score								
≥80	4	4	15	29		26	51	
<80	0	0	0	0		0	0	
Relative dose intensity (%)								
Pemetrexed	100.0	97.0	98.0	97.7		98.5	97.6	
Carboplatin	71.5	62.5	91.0	78.7		87.2	86.1	
Cisplatin	87.0	100.0	93.2	92.6		94.1	91.6	
Maintenance therapy								
Yes	0	2	0	19		0	24	
No	4	2	15	10		26	27	

[a]Two-sided χ^2 test; [b]Pearson's χ^2 test; [c]Fisher's exact test. LAC, lung adenocarcinoma; EGFR, epidermal growth factor receptor; KPS, Karnofsky performance status.

All miR microarray analysis was performed by KangChen Bio-tech (Shanghai, China).

Quantification and confirmation of candidate miRs using RT-qPCR. Plasma sample miRs were quantified using RT-qPCR. Briefly, 1.67 μl RNA (50 ng) was included in a 5 μl reaction mixture using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and a RT primer (TaqMan® MicroRNA Assay; Applied Biosystems; Thermo Fisher Scientific, Inc.) to convert miRNA to cDNA. The RT thermocycling conditions were as follows: 30 min at 16˚C; 30 min at 42˚C; 5 min at 85˚C; and 4˚C until required. Subsequently, qPCR was carried out in a volume of 10 μl containing 4.5 μl diluted cDNA (1:15), 5 μl TaqMan Universal PCR Master Mix (No AmpErase UNG), AmpliTaq Gold® DNA Polymerase and 0.5 μl PCR probes (TaqMan® MicroRNA Assay; Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed using a 7900 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR thermocycling conditions were as follows: 10 min at 95˚C; 40 cycles of 95˚C for 15 sec; and 60˚C for 1 min. Each sample was run in duplicate and the mean Cq value was determined. The relative miRs expression level (Log_2 relative expression level) was calculated using the $2^{-\Delta\Delta Cq}$ method (40), where ΔCq was calculated as follows: Cq (target miR)-Cq (cel-miR-238).

Statistical analysis. Fisher's exact test, a two-sided χ^2 test and Pearson's χ^2 test were used to evaluate differences in the clinicopathological factors between groups. To compare the expression levels of different miRs between the benefit and non-benefit groups, the Mann-Whitney U test was used. To estimate the diagnostic potential of plasma miRs, receiver-operating characteristics (ROC) curves were produced. The predictive power was estimated by calculating the AUC of the ROCs, and the maximum value of the Youden's index was used as a criterion

Table II. Relative plasma expression of miRNAs from microarray analysis.

	Relative plasma expression (fold change)		
miR type	B prior to treatment: NB prior to treatment	B prior to treatment: B following treatment	NB prior to treament: NB following treatment
miR-21	0.018830	16.524080	0.474396
miR-27b	0.013600	40.662910	<2.000000
miR-326	0.030405	<2.000000	0.483120
miR-25	0.093175	12.106610	<2.000000
miR-483-5p	17.768710	0.109332	4.362240
miR-920	22.678370	0.027606	2.048308

miR, microRNA; B, benefit; NB, non-benefit.

for selecting the optimum cut-off point (41). The Kaplan-Meier estimator was used to evaluate PFS and compare log rank statistics. SPSS software (version 19.0; IBM SPSS, Armonk, NY, USA) was used for statistical analysis. Results are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Patient characteristics. Patient characteristics are presented in Table I. The demographics and clinical characteristics of patients in the benefit and non-benefit groups were similar in regards to age, gender, smoking status, tumor stage, EGFR/ALK state, prior serious/chronic disease history and Karnofsky performance status score. The relative dose intensity of each drug was the real dose (actual dose used): Ideal dose (the dose planned) ratio. PFS time was calculated from the date of initiation of chemotherapy to the date of the last follow-up, to the date of detected progression or date of death owing to any cause. Among the patients, 13 could not be evaluated for PFS due to requiring further radiotherapy or being untraceable for follow-ups.

miR expression profiles in the plasma of patients with LAC prior to and following chemotherapy. miR expression profiles were assessed in 8 patients (benefit group, n=4; non-benefit group, n=4), prior to and following treatment, in duplicate, to give a total of 16 plasma samples. The results showed that, prior to treatment, there were 312 upregulated miRs (fold change ≥2.0) in the benefit group and 233 upregulated miRs in the non-benefit group. Following treatment, there were 213 upregulated and 186 downregulated miRs in the benefit group, and 188 upregulated and 157 downregulated miRs in the non-benefit group. The miRs selected for further confirmation by RT-qPCR displayed the following: A 10-fold change in expression between the benefit and non-benefit groups; >2-fold change in expression in the same group between pre- and post-treatment; and an association with cancer in published literature. A total of 6 of the differentially expressed miRs (miR-483-5p, miR-920, upregulated in the benefit group; miR-21, miR-27b, miR-326, miR-25, upregulated in the non-benefit group) were further analyzed. Relative expressions of these 6 miRs are listed in Table II.

miR expression in the training set. RT-qPCR was used to confirm the expression levels of 6 candidate miRs in a training set (benefit, n=29; non-benefit, n=15). To avoid the effect of compounding factors, all patients recruited were non-smokers with no prior history of serious or chronic disease. The ΔΔCq values and relative expression levels of the candidate miRs was calculated (Fig. 1A), confirming the screening results of miR-25, miR-27b, miR-21 and miR-326. However, the relative expression levels of miR-483-5p and miR-920 were inconsistent with the results of the microarray (data not shown). As shown in Fig. 1A, the ΔΔCq values of miR-25, miR-27b, miR-21 and miR-326 in patient plasma prior to treatment were significantly increased in the benefit group compared with the non-benefit group (P<0.001). Therefore, miR-25, miR-27b, miR-21 and miR-326 were selected for further validation.

Confirmation of miR expression in the validation set. As shown in Fig. 1B, the ΔΔCq values of plasma miR-25, miR-27b, miR-21 and miR-326 prior to treatment were compared between the benefit and non-benefit groups in a validation set (benefit, n=51; non-benefit, n=26). The ΔΔCq values of these 4 miRs were significantly upregulated in the benefit group compared with the non-benefit group (P≤0.001; Fig. 1B). In addition, the trend in the alteration of relative expression levels was similar to that seen in the training set.

Evaluation of the diagnostic potential of the candidate miRs. ROC curve analysis was performed on miR-25, miR-27b, miR-21 and miR-326 in 121 patients to evaluate their suitability as predictive biomarkers of pemetrexed and platinum insensitivity (Fig. 2). The AUC of miR-25 was 0.926 [95% confidence interval (CI), 0.881-0.971], which was the highest of the four miRs tested. The AUCs of miR-21, miR-27b and miR-326 were 0.905 (95% CI, 0.845-0.964), 0.817 (95% CI, 0.733-0.900) and 0.803 (95% CI, 0.717-0.890), respectively. The optimal cut-off points were-0.171 (sensitivity, 91.3%; specificity, 80.5%) for miR-25, 0.568 (sensitivity, 82.5%; specificity, 73.2%) for miR-27b, -1.85 (sensitivity, 82.5%; specificity, 85.4%) for mir-21 and 3.05 (sensitivity, 90.0%; specificity, 63.4%) for mir-326. miR-25 exhibited the most accurate predictive power.

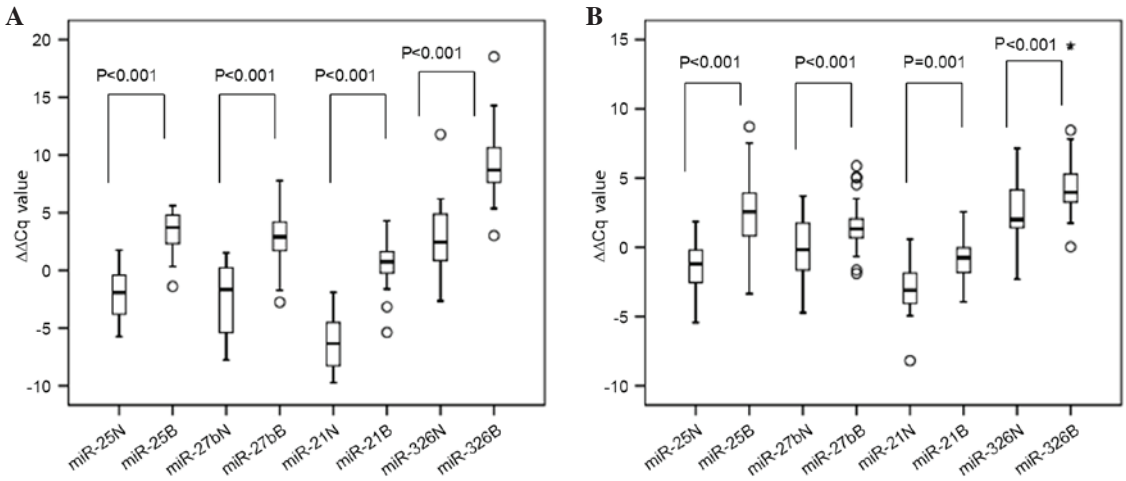

Figure 1. Box-plots of plasma miR-25, miR-27b, miR-21 and miR-326 expression ΔΔCq values of the non-benefit and benefit groups in the (A) training set (n=44), and (B) validation set (n=77). Plasma miR expression levels were analyzed by reverse transcription-quantitative polymerase chain reaction analysis and ΔΔCq values were compared using the Mann-Whitney U test. ΔΔCq values for the 4 plasma miRs, prior to treatment, were significantly upregulated in the benefit group compared with the non-benefit group, in the training set and validation sets (o, outlier; *, abnormal value). Outliers: the deviation between the measured value and the average value is less than two times the standard deviation; abnormal: the deviation between the measured value and the average value is more than two times the standard deviation. miR, microRNA; N, non-benefit group; B, benefit group.

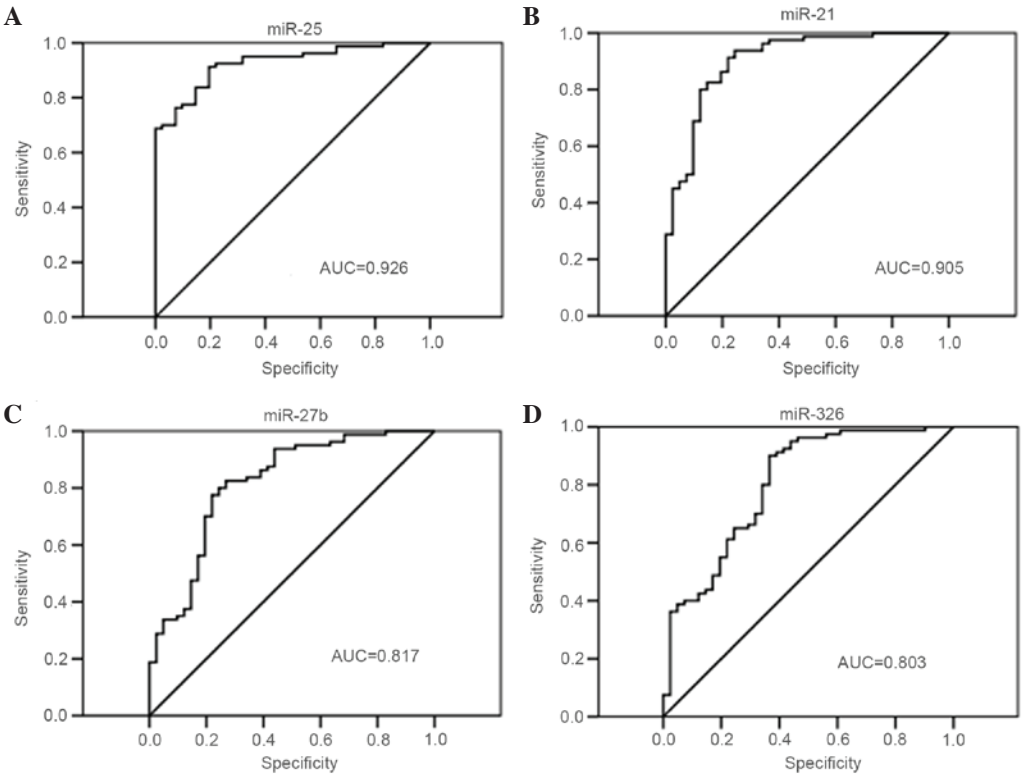

Figure 2. Receiver operating characteristic curves of (A) miR-25, (B) miR-21, (C) miR-27b and (D) miR-326, of the non-benefit and benefit groups. miR-25 showed the highest degree of accuracy (AUC, 0.926). miR, microRNA; AUC, area under the curve.

Prediction of PFS rate using plasma miR expression levels. The association between plasma miR expression levels and PFS rate in 108 patients (benefit group, n=67; non-benefit group, n=41) was investigated. As shown in Fig. 3, the expression levels of miR-25, miR-27b, miR-21 and miR-326 had a significant effect on the PFS rate (all P≤0.001, high miR expression vs. low miR expression). Plasma miR expression levels were inversely correlated with PFS, indicating that increased expression of these miRs is associated with decreased PFS.

Discussion

It is well established that miRs are stable in the blood, and that circulating miRs may act as biomarkers for early diagnosis and prognosis of human cancer (24-32). Likewise, clinical studies

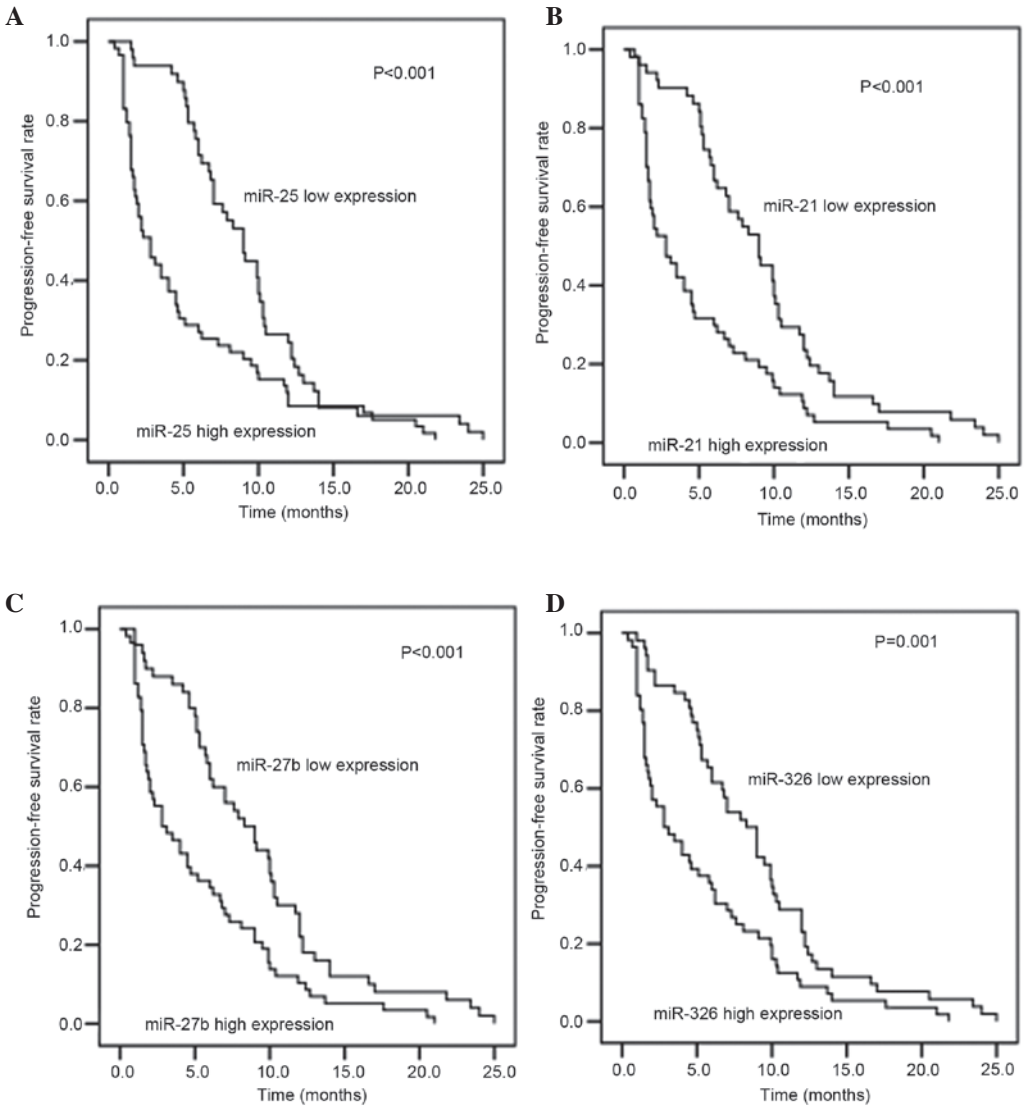

Figure 3. Kaplan-Meier estimation of the progression-free survival of patients with advanced lung adenocarcinoma according to the plasma expression of specific miRs in 108 patients. (A) miR-25, (B) miR-21, (C) miR-27b and (D) miR-326. Increased miR expression levels were associated with decreased progression-free survival. miR, microRNA.

have demonstrated that circulating miRs may serve as predictors of resistance to anticancer agents (33,34). In the present study, plasma miR profiles were compared between benefit and non-benefit groups in order to identify candidate circulating biomarkers that may be used to predict non-benefit from first-line pemetrexed and platinum therapy in patients with advanced LAC.

The expression profile of circulating miRs differs markedly between individuals, disease states, types of cancer and tissues. In the present study, to prevent the influence of this diversity, patient plasma was compared prior to and following chemotherapy using a microarray to measure target miR expression levels. To avoid the compounding effect of nicotine, which may alter the miR expression (42), and comorbidities, including diabetes and hypertension, patients exhibiting these characteristics were excluded from the screening and the training sets. The results in the training set were repeatable in the validation set.

From the results of the miR microarray screening, 6 miRs were selected for further analysis using RT-qPCR. It was observed that in the training and validation sets, the plasma expression levels of miR-25, miR-21, miR-27b and miR-326 prior to treatment were significantly upregulated in the non-benefit group compared with the benefit group. Furthermore, increased expression of these four miRs was associated with poor PFS. The predictive power of each miR was evaluated using ROC curves, in which miR-25 exhibited the highest degree of accuracy (AUC, 0.926; 95% CI, 0.881-0.971). Among these four miRs, miR-21 is recognized as a marker of reduced therapeutic response and decreased survival in patients with lung cancer (43-45). In addition, miR-21 is associated with multidrug resistance (46), particularly to platinum in NSCLC (47). In the present study, plasma miR-21 expression levels were upregulated in the non-benefit group compared with the benefit group, and high expression of miR-21 was associated with poor PFS.

miR-25 is known to be dysregulated in various types of cancer, and to have oncogenic and tumor suppressive functions (48,49). In NSCLC cell lines and human tissue, miR-25

expression is significantly increased compared with normal lung cells or adjacent non-cancerous tissues, respectively (50). Downregulation of miR-25 may increase cisplatin sensitivity and suppress the growth of NSCLC cells *in vivo* (51). Furthermore, increased miR-25 expression has been associated with poor OS in non-smoking females with LAC (52). The results of the present study demonstrate that miR-25 is significantly upregulated in the blood plasma of patients with LAC in the non-benefit group compared with the benefit group, and that high plasma miR-25 expression is associated with decreased PFS.

The expression level of miR-27b varies between different types of cancer. miR-27b may be upregulated or downregulated in chemoresistant cancer cells and tumor samples (53-55). High expression levels of miR-27b in a number of cancer samples have been reported to be associated with good or bad prognoses (56,57). In previous studies miR-27b was found to be downregulated in several NSCLC cell lines and lung cancer tissues (58,59). In the present study, plasma miR-27b was demonstrated to be significantly upregulated in patients with LAC in the non-benefit group compared with the benefit group, and high plasma expression of miR-27b was associated with decreased PFS. Shen *et al* (60) reported that a number of miRs did not exhibit similar expression patterns in plasma and tumor tissue samples, suggesting that miR expression may be altered by host-derived factors in response to the tumor and metastases, in addition to by the tumor directly.

miR-326 is a suppressor of the Hedgehog, Notch and mitogen-activated protein kinase signaling pathways that are associated with brain tumors (61-63), and may block expression of multidrug resistance-associated proteins in breast cancer (64). Low expression levels of miR-326 are correlated with poor OS in patients with pathological grade III-IV glioma (65). In addition, miR-326 expression was shown to be downregulated in metastatic compared with non-metastatic primary loci in nude mouse NSCLC cells (66). Therefore, it may be possible to use miR-326 expression levels to monitor bone metastasis in patients with LAC (67). The results of the present study are consistent with a previous study, which demonstrated that high plasma expression of miR-27b, miR-148a and miR-326 prior to treatment with 5-fluorouracil and oxaliplatin was correlated with decreased PFS in patients with metastatic colorectal cancer (68).

In the current study, the EGFR genotype of the majority of patients was unknown. Previous studies have identified an interaction between EGFR and miRs (69). For instance, miR-21 is positively regulated by EGFR in cancer cells (70) and the gene encoding EGFR is a potential target of miR-23b/27b (71). Therefore, EGFR genotype status may influence miR expression levels. In the present study, the EGFR genotype status (known or unknown) was similar in the non-benefit and benefit groups, which may have reduced this effect to a certain extent.

In conclusion, the results of the present study demonstrate that overexpression of plasma miR-25, miR-21, miR-27b and miR-326 in patients with advanced LAC is predictive of non-benefit to pemetrexed and platinum therapy, and that increased expression of these miRs is associated with decreased PFS. Among these miRs, miR-25 exhibited the highest degree of accuracy in predicting non-benefit, indicating that it is the most promising biomarker. The results of the current study suggest that plasma miRs may be used as minimally invasive independent molecular biomarkers to predict non-benefit from chemotherapy and PFS rates in patients with advanced LAC.

Acknowledgements

The authors thank Professor Tingting Wang of the Immunology and Reproduction Biology Laboratory of Nanjing University for her advice.

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