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Prognostic Value of Mature MicroRNA-21 and MicroRNA-205 Overexpression in Non–Small Cell Lung Cancer by Quantitative Real-Time RT-PCR

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BACKGROUND: microRNA (miRNA) expression profiles are being intensively investigated for their involvement in carcinogenesis. We evaluated the prognostic value of mature microRNA-21 (miR-21) and mature microRNA-205 (miR-205) overexpression in non–small cell lung cancer (NSCLC).

PATIENTS AND METHODS: We studied 48 pairs of NSCLC fresh frozen tissue specimens collected at time of surgery and before chemotherapy. Highly specific amplification and quantification of mature miR-21 and mature miR-205 was achieved using looped real time RT-PCR.

RESULTS: miRNA expression, determined by real time RT-PCR, was defined by ΔΔCt measurements. We detected overexpression of mature miR-21 in 25 (52.0%) of the 48 NSCLC paired specimens and overexpression of miR-205 in 31 (64.6%). Overexpression was assessed after comparison of miRNA expression in NSCLC tissues and in their corresponding noncancerous tissues with respect to U6 expression. During the follow-up period, 29 of 48 (60.4%) patients relapsed, and 23 of 48 died (47.9%). Mature miR-21 was upregulated in 16 of 29 (55.2%) patients who relapsed and 15 of 23 (65.2%) patients who died. Mature miR-205 was overexpressed in 19 of 29 patients who relapsed (65.5%) and 15 of 23 patients who died (65.2%). Mature miR-21 overexpression correlated with overall survival (OS) of the patients ($P = 0.027$), whereas overexpression of mature miR-205 did not.

CONCLUSIONS: Our results suggest that overexpression of mature miR-21 is an independent negative prognostic factor for OS in NSCLC patients.

Lung cancer is the leading cause of cancer-related deaths worldwide, and non–small cell lung cancer (NSCLC)[5] accounts for approximately 80% of all cases *(1)*. Emerging evidence suggests that microRNAs (miRNAs) may control lung cancer development and play a critical role in its pathogenesis *(2)*.

miRNAs are endogenous, small [approximately 22 nucleotides (nt) long], noncoding RNA molecules that silence gene expression by either cleaving target mRNAs or inhibiting their translation *(3)*. Bioinformatic data indicate that each miRNA can control hundreds of gene targets, underscoring the potential influence of miRNAs on almost every genetic pathway *(3, 4)*. Mature miRNAs are generated from precursor molecules that are 80 nt–long hairpin structures (pre-miRNAs). The human genome is predicted to encode as many as 1000 miRNAs, which comprise approximately 3% of the total number of human genes *(3, 5)*.

Abnormal cell proliferation is a hallmark of human cancers, and miRNAs seem to be important factors for the development or maintenance of the neoplastic state *(3, 6)*. Studies in model systems have demonstrated that specific miRNAs contribute to the regulation of cellular differentiation, proliferation, and apoptosis *(4)*. Recent studies have shown an altered expression of miRNAs in cancer relative to corresponding normal tissues, and specific expression signa-

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[5] Nonstandard abbreviations: NSCLC, non–small cell lung cancer; miRNA, microRNA; nt, nucleotide; miR-21, microRNA 21; miR-205, microRNA 205; VEGF, vascular endothelial growth factor; DFI, disease-free interval; OS, overall survival.

tures have been correlated with prognosis, indicating that miRNAs are determinants of clinical aggressiveness *(6–11)*. A potential role for miRNAs in cancer has been suggested by the location of the genes for several miRNAs at sites of translocation breakpoints or detection linked to human leukemias *(7)*. miRNAs, whose expression is increased in tumors, may function as a novel class of oncogenes or tumor suppressor genes. These oncogene miRNAs, called oncomirs, usually promote tumor development by negatively inhibiting tumor suppressor genes and/or genes that control differentiation or apoptosis. The amplification or overexpression of an oncogenic miRNA could eliminate the expression of a miRNA-target tumor suppressor gene and lead to cancer progression *(12)*.

Recent work has clearly shown that miRNAs repress the expression of important cancer-related genes and that miRNA expression profiles can classify human cancers *(13)*. In this way, miRNAs might prove useful in the diagnosis and treatment of cancer. There is extensive diversity in miRNA expression across human cancers, and a large amount of diagnostic information is encoded in a relatively small number of miRNAs *(13)*. Researchers are now using miRNA expression signatures to classify human cancers and to define miRNA markers that might predict favorable prognosis *(13)*. Abnormalities in miRNA expression could contribute to the generation or maintenance of cancer stem cells *(13)*, recently proposed to be responsible for cancerous growth in leukemia and solid tumors *(14)*. Deregulation of miRNA expression is a frequent occurrence in diverse types of cancer, and these findings highlight the potential utility of miRNA profiling for diagnostic and prognostic applications *(13, 15 ,16)*.

MicroRNA 21 (miR-21), located in 17q23.2, has been shown to be upregulated in breast cancer *(11, 17)*, lung cancer *(9)*, and glioblastoma *(18)*. Antisense studies have shown that it controls cell growth by inhibiting apoptosis *(19)* but does not affect cell proliferation. The notion that miR-21 functions as an oncogene was very recently supported by showing that suppression of miR-21 can inhibit tumor growth through downregulation of tumor suppressor tropomyosin I *(20)*. Using presently available computational approaches to predict gene targets, Iorio et al. *(11)* have shown that *TGF-β, TRM2*, the protooncogene *SKI*, and the RAS homologs *RAB6A* and *RAB6C* were predicted to be miR-21 targets in breast cancer.

MicroRNA 205 (miR-205), located in 1q32.2, was shown to be overexpressed in head and neck cancer compared with other cancer lines from lung, breast, colorectal, prostate, and pancreas *(21)*. Yanaihara et al. *(22)* have shown for the first time that miRNA expression profiles are diagnostic and prognostic markers of lung cancer. Upregulation of miR-205 was observed in bladder cancer *(23)* as well as in adult mouse corneal and footpad epithelium *(24)*.

A variety of platforms have recently been developed for miRNAs expression analyses. Higher-throughput expression approaches can be classified as hybridization-based methods using microarrays *(25–27)*, cloning *(28)*, or sequencing approaches *(29)* and bead-based flow cytometry *(13)*. Examination of individual miRNAs has been also performed by Northern hybridization *(30)*. Despite the fact that these methodologies are high throughput, reliable, and well established in a research setting, they are very expensive, and neither quantitative nor suitable for a clinical laboratory setting, where many samples have to be analyzed. A novel real-time PCR quantification approach for miRNAs, the stem-looped real time RT-PCR, seems to overcome these problems and is suitable for the accurate and sensitive quantification of miRNAs *(31)*.

In this work, we have modified a looped real-time RT-PCR methodology in the LightCycler platform to quantify the expression of mature miR-21 and mature miR-205 in NSCLC tissues and their surrounding noncancerous lung tissues. We report for the first time that overexpression of mature miR-21 is an independent negative prognostic factor for overall survival for NSCLC patients.

Materials and Methods

PATIENTS AND TISSUE SAMPLES

Lung carcinoma and adjacent noncancerous tissues were obtained sequentially at the time of surgery and before chemotherapy from 48 NSCLC patients surgically resected at the "Sotiria" General Hospital for Chest Diseases from 2004 to 2005. All patients gave their informed consent, and the Ethical and Scientific Committees of the participating institutions approved the study. Tumor types and stages were determined according to WHO classification. At the time of surgery, all tissue samples were immediately flash frozen in liquid nitrogen and stored at −80 °C until use. We analyzed all samples histologically to assess the amount of tumor component (at least 70% tumor cells) and the quality of material (i.e., absence of necrosis). The majority of patients (44 of 48, 91.7%) were smokers and suffered from mild to moderate chronic obstructive pulmonary disease according to pulmonary function tests that were included as part of the standardized preoperative evaluation of the patients. All patients were treatment naive when the samples were collected, but after surgery all patients received standard chemotherapy protocols for adjuvant NSCLC—gemcitabine plus taxanes (90%) or platinum-based chemotherapy (10%). The majority of patients changed stage to IIIB

after disease relapse. None of these relapses was a new second primary lesion.

ISOLATION OF TOTAL RNA

We isolated total cellular RNA with the Trizol LS reagent (Invitrogen) according to the manufacturer's instructions and as described *(32, 33)*. All preparation and handling steps of RNA were performed in a laminar flow hood under RNase-free conditions. We dissolved the isolated RNA in RNA storage buffer (Ambion) and stored it at −70 °C before use. We measured RNA concentration using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

REAL-TIME RT-PCR FOR miRNA QUANTIFICATION

We modified a previously described *(34)* looped real-time RT-PCR methodology to quantify the expression of mature miR-21 and mature miR-205 in the LightCycler platform. The sequences of mature miR-21, mature miR-205, and U6 primers and probes used in this study were the same as described *(34)*. We performed extensive optimization of the primers and probe concentrations, $MgCl_2$ concentration, and reaction temperatures and times (data not shown). All PCR experiments were run in duplicate after we carefully evaluated the between-run and within-run imprecision of our measurements. We also performed separate experiments for 10 samples starting over from the total RNA step. The results were not significantly different with respect to overexpression of studied variables. The final optimized procedure includes the following 3 main steps.

Reverse transcription with a miRNA-specific looped primer. We used 1 μg total RNA to perform reverse transcription of RNA with the ThermoScript RT-PCR System (Invitrogen) in a total volume of 10 μL (according to the manufacturer's instructions). For RT-PCR, we used a gene-specific looped primer (0.5 μmol/L) as described *(34)*.

Initial PCR step to amplify the cDNAs (pre-PCR). We used RT-PCR product (2 μL) as the template for a 25-μL PCR reaction. Briefly, we added 0.5 μL miRNA-specific primers (10 μmol/L), 2.5 μL PCR Buffer (10×), 0.75 μL $MgCl_2$ (50 mM), 0.5 μL dNTPs (10 mM; Fermentas), and 0.2 μL *Taq* DNA polymerase (5 U/μL, Platinum DNA Polymerase; Invitrogen), followed by 18 cycles of 95 °C for 1 s and 65 °C for 1 min in a PCR thermal cycler (PTC-200 DNA Engine; MJ Research). The cycling protocol consisted of an initial 10-min denaturation step at 95 °C and 55 °C for 2 min, followed by 18 cycles of denaturation at 95 °C for 1 s, and annealing at 65 °C for 1 min.

Quantification of mature miR-21, miR-205, and U6 expression by real-time RT-PCR. Real-time PCR was performed in a total volume of 10 μL per reaction. We placed 1 μL of 1:400 diluted pre-PCR product into a 9 μL reaction mixture that contained 0.1 μL *Taq* DNA polymerase (5 U/μL, Platinum DNA Polymerase; Invitrogen), 1 μL supplied 10× PCR buffer, 0.5 μL $MgCl_2$ (50 mM), 0.2 μL of dNTPs (10 mM; Fermentas), 0.15 μL bovine serum albumin (10 g/L, Serva), 0.5 μL of the appropriate forward primer for each miRNA (10 μM), 0.5 μL of the universal reverse primer (10 μM), and 0.5 μL of the corresponding TaqMan probe (3 μM, TIB MOL); finally, we added diethylpyrocarbonate (DEPC)-treated H_2O to a final volume of 10 μL. The cycling protocol consisted of an initial 10-min denaturation step at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and extension at 72 °C for 20 s. Real-time PCR for U6 gene was performed in the same way, by using the corresponding primers and Taqman probe.

NORMALIZATION OF DATA

Careful normalization is essential for accurate quantification of miRNAs. We evaluated all our data with respect to miR-21 and miR-205 overexpression by normalizing to the expression of U6 and using the $2^{-\Delta\Delta C_t}$ method, as described in detail by Livak and Schmittgen *(35)*. We used a cutoff value of 2.00, with samples having a $2^{-\Delta\Delta C_t}$ value >2.00 considered positive for overexpression. This number for the cutoff is in accordance to the Relative Quantification Software (Roche Molecular Diagnostics) recommendations for the LightCycler. More specifically, the expression of mature miR-21 and mature miR-205 is expressed as a relative ratio to U6 used as a reference gene, which is then normalized to the expression of the same microRNA, as measured in the corresponding adjacent tissue used as a calibrator. Thus, using the $2^{-\Delta\Delta C_t}$ approach, we have related the real-time PCR analytical signal (Cp) of the target transcript (e.g., miR-21) in each NSCLC tumor sample to its corresponding adjacent noncancerous sample.

REAL-TIME PCR FOR TOTAL VEGF

We performed real-time PCR for total vascular endothelial growth factor (VEGF) with the LightCycler as described *(32, 33)*. Overexpression of VEGF was evaluated by relative quantification with respect to *PBGD* gene expression and the $2^{-\Delta\Delta C_t}$ method as described above.

STATISTICAL ANALYSIS

We assessed the correlations among mature miR-21 and mature miR-205 overexpression and the clinicopathological features of the patients using χ^2 test. Sur-

vival times were calculated from the date of surgery to the occurrence of cancer-related events. Survival curves were estimated by the Kaplan–Meier method and P values ≤0.05 were considered to be statistically significant. We analyzed all data using SPSS software (version 11.0, SPSS). Statistical analysis was performed in all cases by using the Wilcoxon nonparametric test.

Results

REAL-TIME RT-PCR QUANTIFICATION OF MATURE miR-21 AND MATURE miR-205 IN NSCLC PAIRED TISSUE SAMPLES

We quantified mature miR-21 and mature miR-205 in 48 pairs of NSCLC tissues and their adjacent noncancerous tissues using real-time RT-PCR. Both mature miR-21 and mature miR-205 were expressed in all tissues, and their expression was normalized with respect to U6 gene expression and by using the relative quantification approach described by Livak and Schmittgen *(35)*. We observed that mature miR-21 was overexpressed in 25 of 48 (52.0%) NSCLC tissues. More specifically, as can be seen in Fig. 1A, mature miR-21 relative expression (expressed as $2^{-\Delta C_t}$) was significantly higher ($P = 0.005$) in the 29 tumor tissues from the NSCLC patients who relapsed (median 4.68) compared with the corresponding noncancerous tissues (median 1.00). Additionally, in the 23 patients who died from the disease, the difference between the tumor tissues (median 5.43) and the adjacent tissues (median 0.57) was statistically significant ($P = 0.006$). On the other hand, in the 19 patients who were relapse free (tumors median 3.39 vs adjacent median 1.32), this difference was not significant ($P = 0.231$). Mature miR-205 was found to be overexpressed in 31 of the same 48 (64.6%) NSCLC tissues. More specifically, as shown in Fig. 1B, mature miR-205 relative expression (expressed as $2^{-\Delta C_t}$) was significantly higher ($P = 0.025$) in the 29 tumor tissues from the NSCLC patients who relapsed (median 0.22) compared with the corresponding noncancerous tissues (median 0.02). In the 22 patients who died from the disease, the difference between the tumor tissues (median 0.14) and the adjacent tissues (median 0.02) was not statistically significant ($P = 0.053$); however, in the 19 patients who were relapse free (tumors median 0.82 vs adjacent median 0.035; $P = 0.040$), it was.

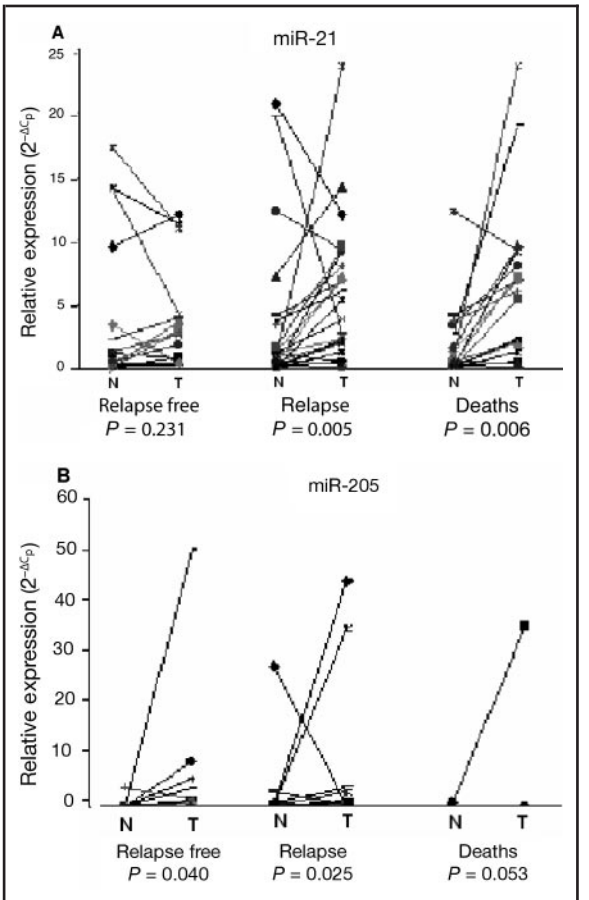

Fig. 1. Real-time RT-PCR analysis of mature miR-21 and miR-205 expression in lung cancer. N, noncancerous lung tissues; T, cancerous tissues prepared from the same patients.

(A), Relative Expression of mature miR-21 ($2^{-\Delta C_p}$) in NSCLC patients who relapsed (n = 29) in comparison with relapse-free patients (n = 19) and patients who had already relapsed and died (n = 23). (B), Relative expression of mature miR-205 ($2^{-\Delta C_p}$) in NSCLC patients who relapsed (n = 29) in comparison with relapse-free patients (n = 19) and patients who had already relapsed and died (n = 23).

MATURE miR-21 AND MATURE miR-205 OVEREXPRESSION IN RELATION TO CLINICOPATHOLOGICAL FEATURES

Patient characteristics with respect to overexpression of mature miR-21 and miR-205 are shown in Table 1; the primary tumor size was ≤3.0 cm in 31.2% of the patients, and 52.1% of the patients had lymph node–positive tumors. The majority of the patients were smokers and ex-smokers, whereas only 8.3% were true nonsmokers. As can been seen in Table 1, mature miR-21 overexpression did not correlate ($P > 0.05$) with age, histology grade, tumor size, lymph node status, smoking history, VEGF expression, tumor type, or clinical recurrences for the patients in the population studied; the same was found for mature miR-205 expression ($P > 0.05$) as quantified by real time RT-PCR. Also, mature miR-21 and mature miR-205 expression did not differ between adenocarcinoma specimens and squamous cell carcinoma specimens.

Table 1. NSCLC patient characteristics.

	All patients		Patients with miR-21 overexpression			Patients with miR-205 overexpression		
	n	%	n	%	P^a	n	%	P^a
All patients	48		25	52.1		31	64.6	
Age, years					0.382			0.585
<60	23	47.9	13	52.0		15	48.4	
≥60	25	52.1	12	48.0		16	51.6	
Histology grade					0.766			0.222
I/II	32	66.7	17	68.0		21	67.7	
III/IV	16	33.3	8	32.0		10	32.3	
Tumor size					0.808			0.379
T1 (0–3 cm)	15	31.2	7	28.0		9	29.0	
T2 (>3 cm)	33	68.8	18	72.0		22	71.0	
Lymph node					0.391			0.077
Positive	25	52.1	14	56.0		19	61.3	
Negative	23	47.9	11	44.0		12	38.7	
Smoking					0.517			0.215
Nonsmokers	4	8.3	2	8.0		1	32.2	
Current smokers	40	83.3	22	88.0		27	87.9	
Ex-smokers	4	8.3	1	4.0		3	9.7	
VEGF					0.217			0.587
Overexpression	21	43.8	13	52.0		14	45.2	
Underexpression	27	56.2	12	48.0		17	54.8	
Type of tumor					0.544			0.252
Adenocarcinoma	25	52.1	14	56.0		14	45.2	
Squamous	23	47.9	11	44.0		17	54.8	
Recurrence					0.062			0.453
Local	12	25.0	4	16.0		7	22.6	
Distant	13	27.1	8	32.0		8	25.8	
Both	4	8.3	4	16.0		4	12.9	
None	19	39.6	9	36.0		12	38.7	

[a] χ^2 test.

PROGNOSTIC SIGNIFICANCE OF MATURE miR-21 AND MATURE miR-205 OVEREXPRESSION IN NSCLC

Relapse. During the follow-up period of 39 months, 29 of 48 NSCLC patients (60.4%) developed metastases. During the follow-up period, these 29 patients presented with a distant (n = 13; 44.8%) and/or local (n = 16; 55.2%) regional recurrence. Clinical recurrence was not significantly more frequent in patients with (60.4%) than without (56.5%) overexpression of mature miR-21 ($P = 0.171$) (Table 2) or in patients with (61.3%) than without (58.8%) overexpression of mature miR-205 ($P = 0.565$) (Table 2). The Kaplan–Meier estimates of the cumulative disease-free interval (DFI) for the group of patients with overexpressed mature miR-21 and those without overexpressed mature miR-21 were not significantly different ($P = 0.172$, log rank test) (Fig. 2A). The Kaplan–Meier estimates of the cumulative DFI for the group of patients with overexpressed mature miR-205 and those without were not significantly different ($P = 0.565$, log rank test) (Fig. 2B).

Overall survival. During the follow-up period, 23 of 48 (47.9%) NSCLC patients died as a result of disease progression. Fifteen patients of the 25 who had overexpression of mature miR-21 died from NSCLC (15/25; 60%). In the group of patients without mature miR-21 overexpression, 8 died from the disease (8 of 23; 34.8%). Patients with overexpression of mature

Table 2. Incidence of clinical relapses and deaths in NSCLC patients according to expression of mature miR-21 and mature miR-205.

	Clinical relapse			Death		
miRNA	n	%	*P*	n	%	*P*
miR-21			0.171			0.027
Overexpression (n = 25)	16	64.0		15	60.0	
No overexpression (n = 23)	13	56.5		8	34.8	
miR-205			0.565			0.602
Overexpression (n = 31)	19	61.3		15	48.4	
No overexpression (n = 17)	10	58.8		8	47.1	
Total (n = 48)	29	60.4		23	47.9	

miR-21 had a significantly shorter overall survival compared with patients with no overexpression of mature miR-21. The Kaplan–Meier estimates of the cumulative overall survival (OS) for the group of patients with overexpression of mature miR-21 and those without were significantly different ($P = 0.027$, log rank test) in favor of those patients without overexpression of mature miR-21 (Fig. 2C).

We then analyzed the relationship between mature miR-205 overexpression and OS. Fifteen of the 31 patients (49.4%) with overexpression of mature miR-205 died, in comparison to 8 out 17 (41.7%) without miR-205 overexpression who died from disease relapse. Thus, as shown in Table 2, overexpression of mature miR-205 was not significantly associated ($P = 0.602$) with overall survival in this group of patients. The Kaplan–Meier estimates of the cumulative OS for the group of patients with overexpression of mature miR-205 and those without were not significantly different ($P = 0.602$, log rank test) (Fig. 2D).

UNIVARIATE AND MULTIVARIATE ANALYSIS

We tested mature miR-21 expression, mature miR-205 expression, VEGF expression, age, smoking history, tumor size, tumor grade, and lymph nodes in univariate analysis for association with DFI and overall survival. None of these parameters was significantly associated with reduced DFI. The corresponding Kaplan–Meier curves are shown in Supplementary Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue10. Univariate analysis showed that only the detection of mature miR-21 ($\chi^2 = 4.883$, $P = 0.027$) was associated with decreased overall survival. Subsequently, we performed multivariate analysis with overall survival. Overexpression of mature miR-21 was found to be an independent prognostic factor for overall survival (hazard ratio 2.533, 95% CI 1.066–6.020, $P = 0.035$).

Discussion

Lu et al. *(13)* recently used a bead-based flow cytometric methodology to study the expression profile of 217 mammalian miRNAs across a large panel of tumor samples and tissues. According to their study, miRNA expression profiles were highly informative, reflecting the development lineage and differentiation state of the tumors. MicroRNA expression profiles were used to identify the tissue's origin of poorly differentiated tumors with greater accuracy than expression profiles constructed using approximately 16 000 mRNAs.

For lung cancer, it has been recently shown that the expression of miRNAs of the let-7 family were frequently reduced both in vivo and in vitro, and that reduced let-7 expression was significantly associated with shortened postoperative survival, independent of disease stage *(36)*. In contrast to miRNA let-7, the expression of miRNA cluster miR-17–92 was substantially increased in lung cancer, especially in small cell lung cancer *(37)*. Finally, in a recent study that investigated the involvement of miRNA in lung carcinogenesis, miRNA microarray analysis identified statistical unique profiles that could discriminate lung cancers from noncancerous lung tissues as well as molecular signatures that differ in tumor histology. This was the first study showing that miRNA molecular profile of lung adenocarcinoma correlates with patient survival *(22)*.

We studied here the expression profile of mature miR-21 and mature miR-205 in a quantitative aspect in 48 pairs of NSCLC fresh frozen tissues and their corresponding adjacent noncancerous tissues. According to our results, overexpression of mature miR-21 had a prognostic implication for NSCLC patients, since it was associated with reduced OS ($P = 0.027$), but did not correlate with any histopathological feature of the patients in the population studied. On the contrary, overexpression of mature miR-205 had no prognostic

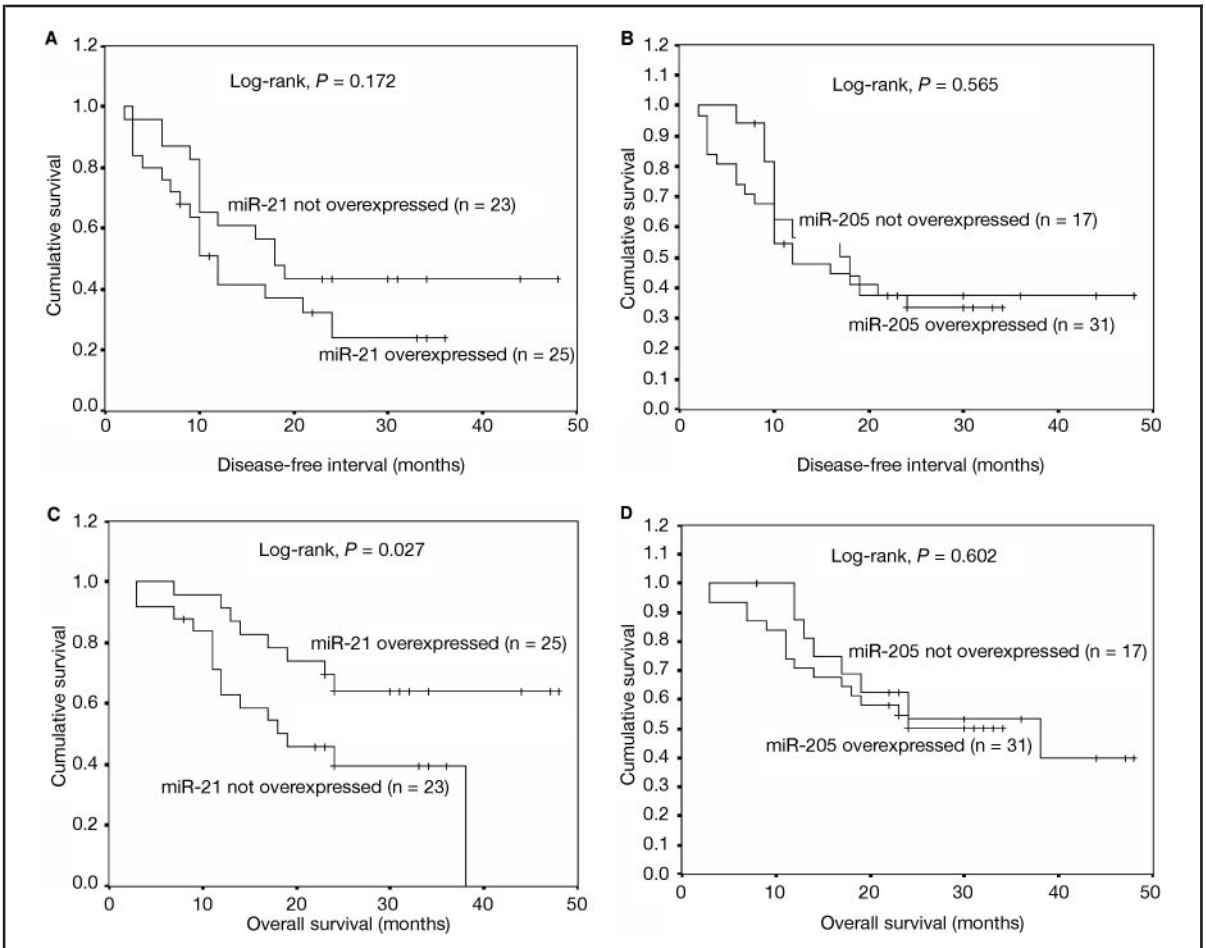

Fig. 2. (A), Kaplan–Meier estimates of DFI for the NSCLC patients with or without miR-21 overexpression (P = 0.172, n = 48).

(B), Kaplan–Meier estimates of DFI for the NSCLC patients with or without miR-205 overexpression (P = 0.565, n = 48). (C), Kaplan–Meier estimates of OS for the NSCLC patients with or without miR-21 overexpression (P = 0.027, n = 48). (D), Kaplan–Meier estimates of OS for the NSCLC patients with or without miR-205 overexpression (P = 0.602, n = 48). +, censored results

implication for NSCLC patients, since it was not associated with reduced DFI ($P = 0.565$) or OS ($P = 0.602$) and also did not correlate with any clinicopathological feature of the patients in the population studied. The results presented in the current study demonstrate, for the first time, that overexpression of mature miR-21 in NSCLC patients, estimated by using a real-time PCR assay, is associated with high incidence of death resulting from lung cancer. Moreover, miR-21 overexpression was revealed in the multivariate analysis to be an independent prognostic factor for disease-specific death. Because of the relatively small number of events, however, further confirmation of these results is required, and these results should be interpreted with caution.

Very recently, miR-21 has been reported to be highly expressed in breast tumors in comparison to their matched normal breast tissues, suggesting that it may potentially act as an oncogene *(17)*. Moreover, Volinia et al. *(9)* reported widespread overexpression of miR-21 in diverse tumors, including those derived from breast, colon, lung, pancreas, stomach, and prostate. Yanaihara et al. *(22)* reported that precursor miR-21 was significantly upregulated in lung cancer tissues by quantifying precursor miRNAs using real-time PCR. Mechanisms by which microRNA-21 alters cancer cell phenotype, such as downregulation of the tumor suppressor PDCD4, have been very recently identified in colorectal and breast cancer cell lines *(38, 39)*.

In conclusion, our study has revealed a different expression profile for mature miR-21 and mature miR-205 in NSCLC tissue specimens with respect to their nonmalignant paired tissues. We show for the first time that overexpression of mature miR-21 is of prognostic significance in NSCLC patients. It would be of interest to extend this study in a larger number of paired fresh frozen NSCLC samples and in an independent cohort of clinical samples as well as in different cancers, since most studies so far are based on the expression of premature microRNAs, and moreover not in paired fresh frozen tissues. Taking into account the great importance of microRNAs in cancer, and their promising potential as new specific prognostic biomarkers as well as molecular targets for the development of novel cancer therapeutics, we strongly believe that our results will be of importance for both clinical researchers and those who design microRNA-based novel cancer therapeutics. The question remains, however, as to what the biological relevance is for mature miR-21 overexpression in lung cancer and how high expression of miR-21 may alter approach to treatment and surveillance.

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