Research Article

Cancer Epidemiology, Biomarkers & Prevention

Four-miRNA Signature to Identify Asbestos-Related Lung Malignancies

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Abstract

Background: Altered miRNA expression is an early event upon exposure to occupational/environmental carcinogens; thus, identification of a novel asbestos-related profile of miRNAs able to distinguish asbestos-induced cancer from cancer with different etiology can be useful for diagnosis. We therefore performed a study to identify miRNAs associated with asbestos-induced malignancies.

Methods: Four groups of patients were included in the study, including patients with asbestos-related ($NSCLC^{Asb}$) and asbestos-unrelated non–small cell lung cancer (NSCLC) or with malignant pleural mesothelioma (MPM), and disease-free subjects (CTRL). The selected miRNAs were evaluated in asbestos-exposed population.

Results: Four serum miRNAs, that is miR-126, miR-205, miR-222, and miR-520g, were found to be implicated in asbestos-related malignant diseases. Notably, increased expression of miR-126 and miR-222 were found in asbestos-exposed subjects, and both miRNAs are involved in major pathways linked to cancer development. Epigenetic changes and cancer-stroma cross-talk could induce repression of miR-126 to facilitate tumor formation, angiogenesis, and invasion.

Conclusions: This study indicates that miRNAs are potentially involved in asbestos-related malignancies, and their expression outlines mechanism(s) whereby miRNAs may be involved in an asbestos-induced pathogenesis.

Impact: The discovery of a miRNA panel for asbestos-related malignancies would impact on occupational compensation and may be utilized for screening asbestos-exposed populations.

Introduction

Although asbestos use has been banned or restricted, exposure to asbestos is still widespread around the world. Besides the occupational exposure, a great number of people are affected by environmental or domestic exposure to asbestos (1). This results in serious risk of developing malignant pleural mesothelioma (MPM) and lung cancer (LC; refs. 2–4). The association between asbestos exposure and lung adenocarcinoma is well established (5). Nevertheless, precise histopathologic data are poorly understood when investigating the asbestos-cancer link.

Although the Helsinki criteria for identifying individuals with high occupational risk of asbestos exposure have been accepted, it is insufficient, and specific asbestos-related parameters are needed. The presence of pleural plaques is not considered a precancerous condition, while asbestos and smoking are associated with increased risk of lung cancer. Various candidates for biomarkers of asbestos-related malignancies have been proposed (6–8). However, most of the tumor markers are derived from molecules produced and secreted from cancer cells. This means that when the tumor mass is relatively small, the levels of certain tumor markers in serum are initially low and gradually increasing as the tumor develops.

Recently, miRNAs have become attractive entities for profiling cancer. miRNA expression is altered soon after exposure to occupational and environmental carcinogens (9). It therefore appears of importance to identify a novel asbestos-related profile able to distinguish asbestos-induced cancer from cancer with different etiology. Here, a sequential phase study has been performed to identify a panel of serum miRNAs associated with development of asbestos-induced thoracic malignancies (LC and MPM). The identified miRNA panel has potential clinical value for early detection of asbestos-induced malignancies and may be utilized for screening high-risk populations exposed to asbestos.

Materials and Methods

Ethics statement

All subjects filled a questionnaire including their informed written consent. The study was carried out according to the

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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Helsinki Declaration and the samples were processed under approval of the written consent statement by Ethical committee of the University Hospital of Marche, N. 51/DG 05/02/2009, Italy.

Study population

The LC cohort included 105 patients affected by non–small cell lung cancer (NSCLC) (squamous, adenocarcinoma, and large cell carcinoma) recruited from January 2011 to March 2017 at the Clinic of Pneumology and Thoracic Surgery of the Hospital of Ancona, Italy. Tumor staging was performed according to the Sixth Edition of American Joint Commission on Cancer tumor–node–metastasis (TNM) staging system. Based on the evidence of occupational and nonoccupational (environmental, familial, domestic) exposure to asbestos, NSCLC patients were stratified into a group without previous exposure to asbestos ($n = 60$) and an asbestos-exposed NSCLC group ($NSCLC^{Asb}$, $n = 45$). This group included patients who submitted a complaint of occupational disease to INAIL (National Institute for Insurance Against Workplace Accidents and Occupational Disease), encompassing shipbuilders, machine operators, electricians, pipe fitters, and construction workers.

Patients with MPM ($n = 74$) were recruited from November 2008 to January 2013 at the Clinics of Oncology, Pneumology and Thoracic Surgery of the University Hospital of Ancona, Italy. Pathological diagnosis was performed on pleural biopsies obtained by thoracoscopy or thoracotomy. According to Ferrante and colleagues (10), a "fiber-year" exposure metric was calculated for each asbestos-exposed patient, assigning to each person an arbitrary coefficient of "inhaled fibers (ff)" indicating the occupational hazard. The "cumulative fibers" (Cf) are interpreted as the cumulative dose of asbestos fibers of the workplace of (ff/liter) × years.

The patients affected by sinonasal cancer (SNC) were enrolled at the UOC-ORL Budrio-Metropolitan Hospital, Bologna, Italy.

The asbestos-exposed cohorts consisting of 80 subjects free from cancer (age 57.5±12.2) with a history of asbestos exposure (asbestos cement, rolling stock, shipbuilding) were enrolled at the Occupational Medicine, University of Trieste, Trieste, Italy. The population was stratified as currently exposed (commercial asbestos occurs predominantly during maintenance operations and remediation of older buildings containing asbestos) and former exposed with and without benign asbestos-related diseases (ARD).

The control group consisted of healthy subjects ($n = 78$) recruited from November 2008 to January 2017 at the Occupational Medicine, Polytechnic University of Marche, Ancona, Italy, and Occupational Medicine of University of Trieste, Trieste, Italy. The subjects were undergoing screening radiography at the Pneumology Clinic of the University Hospital of Ancona, Italy. None of them had ever been exposed to asbestos as documented by their occupational histories, and they presented with normal chest radiographs. The participants were interviewed by trained personnel and answered a detailed questionnaire that included information on the gender, age, histology, neoadjuvant chemoradiation and therapy administration (before surgery), smoking status, and the pathologic staging, as well as the duration of asbestos exposure and occupational tasks. The demographic and pathological characteristics of the subjects are summarized in Table 1. Blood samples were collected at the time of interview, and serum samples isolated and stored at −80°C until use.

Table 1. Demographic characteristics and clinical parameters of study population

	FFPE			SERUM									
	Discovery			Verification				Training				Validation			
	NSCLC ($n = 4$)	$NSCLC^{Asb}$ ($n = 4$)	MPM ($n = 4$)	CTRL ($n = 14$)	NSCLC ($n = 16$)	$NSCLC^{Asb}$ ($n = 11$)	MPM ($n = 19$)	CTRL ($n = 21$)	NSCLC ($n = 24$)	$NSCLC^{Asb}$ ($n = 19$)	MPM ($n = 22$)	CTRL ($n = 36$)	NSCLC ($n = 20$)	$NSCLC^{Asb}$ ($n = 15$)	MPM ($n = 33$)	SNC ($n = 16$)
Age (years)	65 ± 7	69 ± 11	72 ± 8	41 ± 11	68 ± 12*	70 ± 7*	73 ± 8*	56 ± 17	71 ± 10*	74 ± 9*	71 ± 7*	56 ± 9	71 ± 9*	69 ± 7*	71 ± 7*	64 ± 14
Gender (M/F %)	100/0	100/0	100/0	92/8	69/31	64/36	84/16	79/21	60/40	80/20	83/17	89/11	55/45*	80/20	82/18	69/31
Smoking (%)																
No	25	25	25	40	38	33	50	42	29	20	38	22	16	13	29	62
Yes	75	75	75	20	50	42	0	26	26	35	12	25	21	33	12	38
Former	0	0	0	40	12	25	50	32	45	45	50	53	63	53	59	0
Asb-exp (%)																
No	100	0	0	100	100	0	0	100	100	0	0	100	100	0	0	100
Occupational	0	100	100	0	0	100	100	0	0	45	59	0	0	67	74	0
Environmental	0	0	0	0	0	0	0	0	0	55	41	0	0	33	26	0
Cf (ff/l) × years										6.4 ± 3.7	4.2 ± 3.3	0	0	6.6 ± 4.3	8.0 ± 7.3	0
ARDs (%)	0	100		0	0	40	83	0	0	55	81				74	
Histotypes (%)	0 (SQ)	25 (SQ)	90 (EP)		43 (SQ)	40 (SQ)	90 (EP)		35 (SQ)	36 (SQ)	75 (EP)		13 (SQ)	67 (SQ)	59 (EP)	
	0 (LC)	0 (LC)	0 (BF)		0 (LC)	20 (LC)	0 (BF)		10 (LC)	18 (LC)	0 (BF)		12 (LC)	0 (LC)	11 (BF)	
	100 (AD)	75 (AD)	10 (BF)		57 (AD)	40 (AD)	10 (BF)		55 (AD)	46 (AD)	25 (SA)		75 (AD)	33 (AD)	30 (SA)	

NSCLC-histotypes: SQ, Squamous; LC, Large Cell; AD, Adenocarcinoma.
MPM-histotypes: EP, Epithelioid; BF, Biphasic; SA, Sarcomatoid.

Microarray analysis

Total RNA was extracted from formalin-fixed, paraffin-embedded (FFPE) sections (10 μg) using the Ambion Recover All Total Nucleic Acid Isolation Kit (Life Technologies), according to the manufacturer's instructions. The concentration and integrity of RNA samples were determined by Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). Human Cancer Pathway Finder 384HC miScript miRNA PCR Array (MIHS-3102Z; Qiagen) was used for screening 384 miRNAs. A set of controls (six housekeeping genes, three control genes for quality of the retro-transcription, and three control genes for qPCR) included in this array enables data analysis using the ΔΔCT method of relative quantification, assessment of reverse transcription performance, and assessment of PCR performance. The gene fold changes were investigated in the study groups (NSCLC, NSCLC^{Asb}, MPM) by evaluating ΔΔCt between the cancerous and noncancerous FFPE tissues according to the $2^{-\Delta\Delta Ct}$ method. MiScript miRNA PCR Array Data Analysis Web Portal (Qiagen) was used to determine differentially expressed miRNAs ($P < 0.01$; fold change > 2.0).

Quantitative RT-PCR

Total RNA was isolated from serum samples as previously described (11). The miRNAs were purified from total RNA using the miRNA Isolation Kit (PureLink miRNA Isolation Kit; Thermo Fisher Scientific). miRNAs were eluted in the final volume of 40 μL RNase-free water, and 2 μL of the preparation were used for reverse-transcription to cDNA using the TaqManAdvanced miRNA cDNA Synthesis Kit (Applied Biosystems; Life Technologies) according to the manufacturer's instructions. The qRT-PCR reactions were carried out using TaqMan Fast Advanced Master gene expression (Applied Biosystems; Life Technologies) by using Realplex Mastercycler epgradient S (Eppendorf). Exogenous control (Cel miR-39) were used for normalization and the results were expressed as $2^{-\Delta Ct}$. Alternatively, the ratios between the expressions of all miRNAs were also calculated.

Statistical analysis

Results are expressed as mean ± SD unless indicated otherwise. Comparisons among groups of data were made using one-way ANOVA with Tukey *post hoc* analysis. The two-tailed Student *t* test was used to compare two groups. Differences with $P < 0.05$ were considered statistically significant. Correlations were performed according to the Spearman's test. Backward stepwise logistic regression model with Wald statistical analysis was used to select asbestos-related miRNA biomarkers based on the training dataset. The predicted probability of being diagnosed with NSCLC and MPM related to asbestos exposure was used as surrogate biomarkers to construct ROC curves. The area under curve (AUC) indicates the accuracy for evaluating the performance of selected miRNA panel. All data generated in this study were analyzed using the SPSS software.

Results

Selection and validation of candidate miRNAs

To uncover potentially differentially expressed miRNAs in relation to asbestos-induced thoracic malignancies such as LC and MPM, a multiphases study has been performed (Supplementary Fig. S1). The discovery phase was performed on FFPE tissue samples from patients affected by NSCLC ($n = 4$), NSCLC^{Asb} ($n = 4$), and MPM ($n = 4$) collected from the Pathological Anatomy Unit of the Hospital of Ancona, Italy. To ensure that NSCLC^{Asb} patients were exposed to asbestos, tissue samples from patients financially compensated by occupational INAIL were used. These samples were screened for 384 miRNAs using the microarray platform. By comparing the cancerous versus noncancerous tissue from patients with NSCLC, NSCLC^{Asb}, and MPM deregulated miRNAs were detected (Supplementary Fig. S2).

Based on the fold change expression and significance, a miRNA panel was obtained. It includes four deregulated miRNAs in NSCLC (miR-204, miR-519a, miR-485, and miR-205), three specific miRNAs in NSCLC^{Asb} (miR-520g, miR-504, and miR-34a), and four deregulated miRNAs in MPM (miR-222, miR-126, miR-100, and miR-145). To verify whether the candidate miRNAs uncovered in the FFPE samples via microarray analysis can be detected in serum, an independent cohort of 60 serum samples were analyzed by qRT-PCR. Of eleven candidate miRNAs detected by the microarray analysis, only four were stably detected in serum samples and were found differently expressed among groups (Supplementary Fig. S3). miR-205 was specific for NSCLC, miR-126 for MPM, whereas miR-222 and miR-520g were representative of asbestos-related NSCLC.

Next, the expression profiles of candidate miRNAs were evaluated with qRT-PCR using the training dataset, an independent cohort of 86 serum samples including patients with NSCLC, NSCLC^{Asb}, and MPM and healthy controls (CTRL). Low expression levels of miR-126 were observed in the NSCLC and even

Table 2. Deregulated miRs in NSCLC, NSCLC^{Asb}, and MPM groups (A) and logistic regression analysis for asbestos-associated risk in training phase (B)

	CTRL	NSCLC	NSCLC^{Asb}	MPM
A				
miR-126	12.79 [0.36–260.8]	6.99 [0.09–53.36][a,b]	15.56 [1.77–129.6]	4.56 [0.02–45.81][a,b]
miR-205	1.13 [0.03–24.89][b]	2.50 [0.01–70.9][b]	3.83 [0.05–232.0][a]	1.29 [0.01–61.72][b]
miR-222	0.41 [0.01–3.03][b]	0.66 [0.05–5.77][b]	1.19 [0.16–32.85][a]	0.39 [0.03–8.92][b]
miR-520g	0.41 [0.02–22.0]	0.52 [0.01–20.36][b]	1.01 [0.01–30.44]	0.60 [0.00–7.99][b]
variables		**OR**	**95 % CI**	***P* value**
B				
Age		1.066	1.018–1.116	0.006
miR-222		1.28	1.014–1.616	0.038

NOTE: The data are expressed as median [min–max]. Backward stepwise logistic regression model with Wald statistical analysis of biomarkers (miRNAs and their ratio) adjusted for age, gender, and smoking. Data were presented as OR, 95% CI, and *P* value. Significance was determined by logistic regression analysis and *P* value <0.05 was considered significant.

[a]CTRL vs. NSCLC, NSCLC^{Asb}, MPM.

[b]NSCLC^{Asb} vs. CTRL, NSCLC, MPM.

more in the MPM group as compared with those in the control group. In contrast, high level of expression of miR-126, miR-205, miR-222, and miR-520g was found in asbestos-related NSCLC (Table 2, top). A backward stepwise logistic regression model with Wald statistical analysis was applied to estimate the probability of being asbestos-exposed with thoracic malignancies (NSCLC^{Asb} and MPM) using the training dataset with four significant miRNAs expressed either as relative level or as their ratio values including age, gender, and smoking as confounding variables. A classifier's optimal logit (*P*) model was obtained in order to discriminate the asbestos-related malignancies from cancers not associated with asbestos (Table 2, bottom).

The predicted probability from the logit model based on miR-222 and age was used to construct the ROC curve. The AUC was 0.706 (0.598–0.814), $P = 0.001$, which was further increased in the independent validation dataset, that is AUC = 0.767 (0.675–0.858), $P = 0.0005$ (Fig. 1A). In the validation phase, patients affected by SNC, an occupational malignancy associated with wood dust and leather exposure, was included to evaluate the prediction specificity. Taking into account a cut-off of 0.466 (80% sensitivity and 70% specificity), only 10% to 15% of asbestos-related malignancies (NSCLC^{Asb} and MPM) were depicted as asbestos-unrelated, whereas 40% to 60% of asbestos-unrelated NSCLC and SNC patients were detected as associated with asbestos (Fig. 1B). The addition of the miR-222/miR-126 ratio in the predicted model based on the training dataset significantly reduced the false-positive cases in the NSCLC group but generated about 50% of false-negative cases in the NSCLC^{Asb} group (Supplementary Fig. S4A–S4C). However, both miR-126 and miR-222 significantly correlated with asbestos Cf, further supporting their relationship with asbestos-related malignancies (Figs. 2A and 3A), and their expression were associated with TNM stage in NSCLC (Fig. 2B).

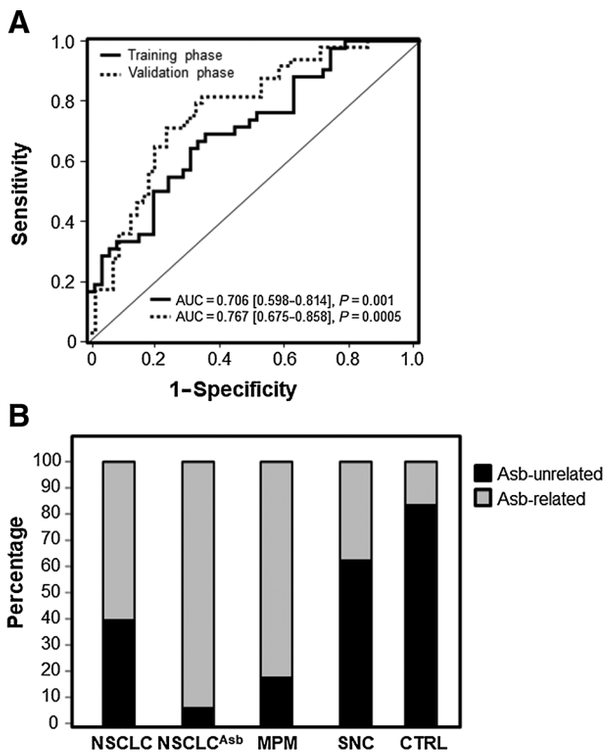

Figure 1.
ROC curve analysis. AUC estimation of the logit model with miR-222 and age using the training and validation dataset to discriminate the asbestos-related from non-asbestos-related malignancies (**A**). Distribution of the study population according to the predicted model (**B**).

A logistic regression model was performed to evaluate the risk of being diagnosed with NSCLC using the four miRNA panel. Two of the four miRNAs turned out to be significant predictors, that is only miR-205 and miR-222 were included in the logit model showing an ROC curve of 0.865 (0.792–0.937), $P = 0.0005$ (Fig. 2C–E).

The diagnostic performance of the four-miRNA panel to discriminate MPM from healthy controls was also evaluated. The miR-222/miR-126 ratio significantly distinguishes the two groups. However, the predicted logit model included miR-126 and miR-205 best discriminates patients with MPM from healthy controls (Fig. 3B–D).

miRNA panel as biomarker of early effect of carcinogens

To investigate whether the selected miRNAs were early-responsive genes to asbestos exposure, their expression was evaluated in serum samples of the population exposed to asbestos. The enrolled subjects were stratified as currently exposed subjects being working at the maintenance and remediation of older buildings containing asbestos, and former exposed subjects with a history of past asbestos exposure with and without benign ARDs (Supplementary Table S1). As shown in Figure 4, high levels of miR-126 and miR-222 were found in currently asbestos exposed subjects. No changes in miRNA level have been observed in subjects exposed to asbestos in the past, regardless of the ARD status. This supports their role as early responsive miRNAs to the asbestos.

Discussion

Four asbestos-related miRNAs, that is miR-126, miR-205, miR-222, and miR-520g that are implicated in thoracic malignant diseases such as LC and MPM, were detected in patient serum. Because the normalization of miRNA data in serum samples is still a controversial issue (12, 13), the ratios between the expression of all miRNAs consistently expressed in serum were also calculated. Each value of a single miRNA as relative expression and their ratio values were used for class prediction of asbestos-related malignancies in the training and validation sets. Class comparison analysis was initially performed in the training set to identify a group of miRNA showing a predictive model to discriminate asbestos-related malignancies (NSCLC^{Asb} and MPM) from asbestos-unrelated lung cancer (NSCLC), and disease-free patients (CTRL). The asbestos signature obtained was then used to calculate specificity and sensitivity in an independent validation set. In the predictive model, miR-222 and age best depicted asbestos-related malignancies (cf. Fig. 1).The association of miR-222 with miR-222/miR-126 best characterized the asbestos-unrelated NSCLC group.

Both miR-126 and miR-222 were strongly associated with asbestos exposure, and both miRNAs were involved in major pathways linked to cancer (cf. Figs. 2 and 3). In particular, miR-126 is an endothelial-specific miRNA essential for regulation of vascular integrity and angiogenesis. miR-126 could promote angiogenesis by repressing the inhibitors of VEGF signaling

Spred1 and PIK3R2 (14) or suppress tumor growth and tumor angiogenesis by direct targeting VEGF-A signaling (15, 16). Both miR-126 and miR-222 were associated with lung cancer aggressiveness (17–19). miR-222 was upregulated in NSCLC samples and was associated with advanced clinical stage and lymph node metastases (17). By analyzing miR-221/-222 in normal and malignant lung tissues, overexpressed miR-221 and miR-222 were found in aggressive NSCLC (20). Conversely, Boeri and colleagues (12) found that some miRNAs, including miR-126 and miR-221, were downregulated in plasma of patients with lung cancer with

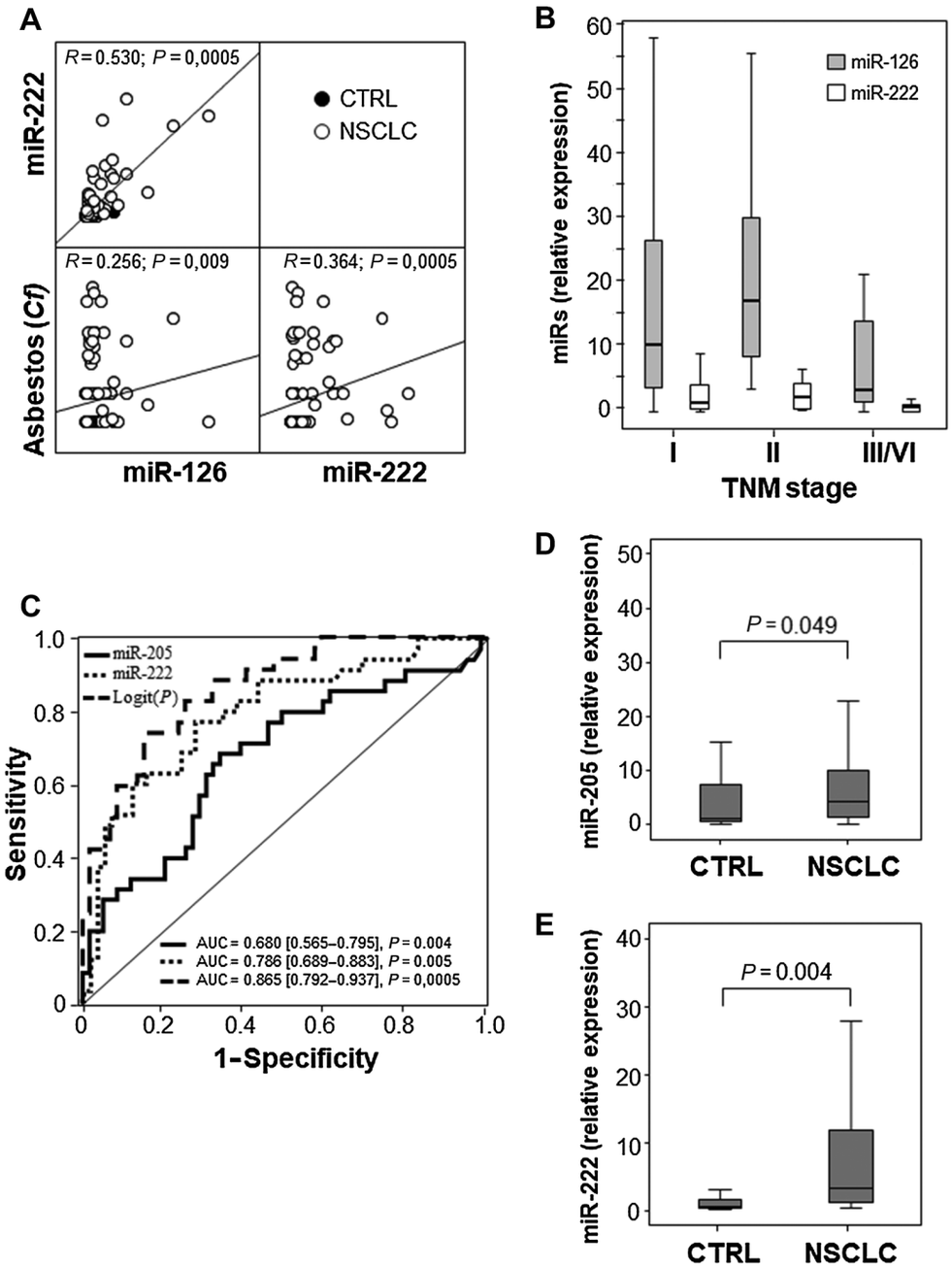

Figure 2.

Relationship between asbestos fibers and selected miRNAs, their correlation with tumor progression, and performance to discriminate NSCLC from CTRL. **A,** Correlation between cumulative asbestos fibers (*Cf*) and miRNA are shown. The correlation coefficient (*R*) was determined by means of the Spearman test. **B,** Correlation between miR-126 and miR-222 expression and tumor stage (stage I, $n = 44$; stage II, $n = 17$; stage III/IV $n = 10$). ROC curve of Logit (*P*) value in NSCLC and CTRL are documented. Area under the ROC curves (AUC) and 95% CI are shown (**C**). Distribution of miR-205 (**D**) and miR-222 (**E**) in NSCLC and control group are presented. Significant differences were determined using Student *t* test.

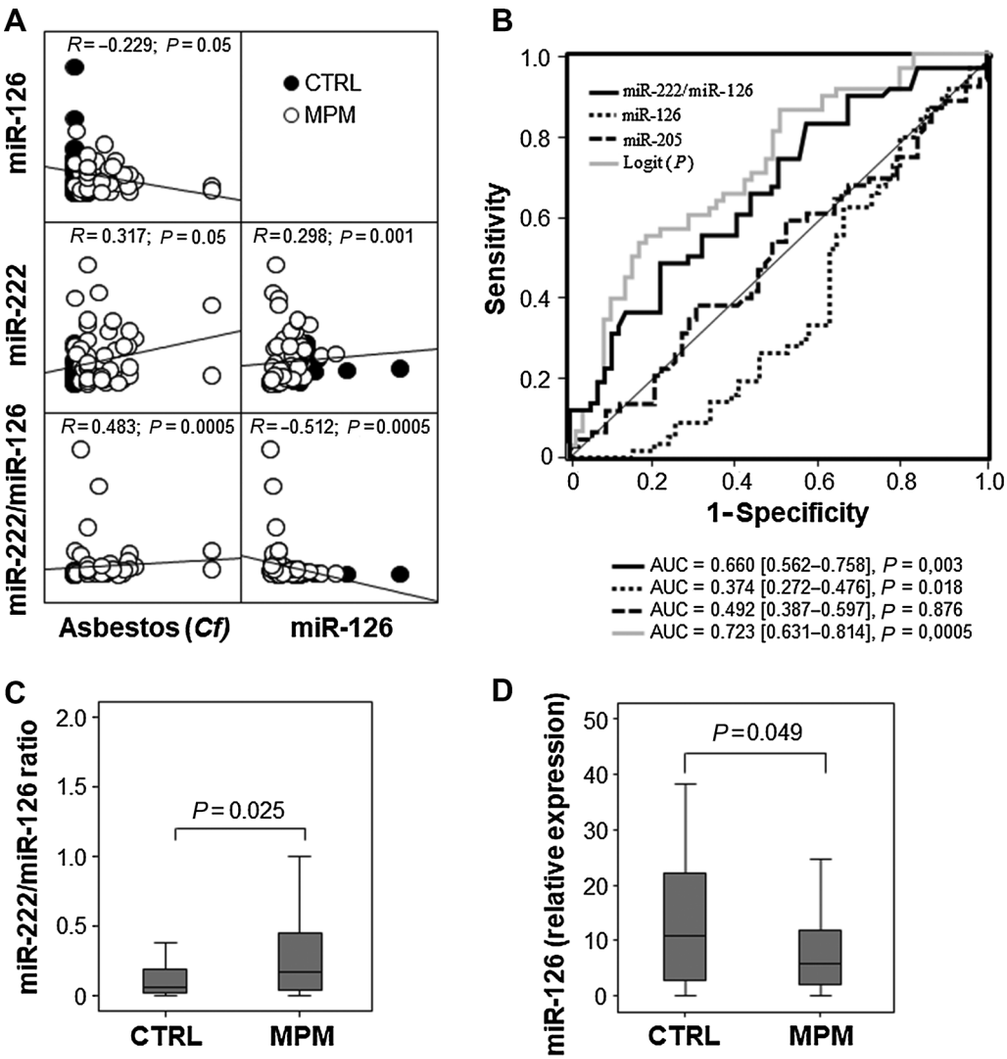

Figure 3.

Relationship between asbestos fibers and miRNAs, and their performance to discriminate MPM from CTRL. Correlation between cumulative asbestos fibers (*Cf*) and miRNAs. Correlation coefficient (*R*) was determined using the Spearman test (**A**). ROC curve of miR-126, miR-222/miR-126 ratio and Logit (*P*) value in MPM and CTRL are documented. Area under the ROC curves (AUC) and 95% CI are shown (**B**). Distribution of miR-222/miR-126 ratio (**C**) and miR-126 (**D**) in MPM and CTRL group are presented. Significant differences were determined using Student *t* test.

unfavorable prognosis. In this study, we found that both miR-126 and miR-222 were downexpressed in advanced clinical stage of NSCLC (cf. Fig. 2B). Downexpression of miR-126 has been observed in several cancers (21). There was lack of relationship between miR-222 tissue expression and miR-222 serum level, suggesting a predictive role of circulating miRNAs independent from tissue specimens.

To elucidate the role of these miRNAs in the association between asbestos exposure and tumor development, a disease-free population exposed to asbestos was evaluated for the selected miRNAs. This population included workers with current and past exposure to asbestos. Notably, increased expression of miR-126 and miR-222 were found only in currently exposed subjects (cf. Fig. 4). Studies indicate that aggregation of EGFR by long fibers may initiate cell signaling cascades in asbestos-induced carcinogenesis (22, 23). In this context, regulation of miR-222 via EGFR activation has been reported (24, 25). Increased expression of miR-222 was also observed in subjects exposed to air pollution and metal-rich particles (26–28). miRNA changes may therefore be sensitive indicators of the biological effects of acute and chronic environmental exposure. Results from animal studies suggest that changes in miRNA expression in response to exposure to environmental carcinogens are transient and revert to normal levels after recovery from the exposure (29, 30). Accordingly, subjects exposed to asbestos in the past did not show any changes in miRNA expression. We can thus postulate that irreversible alterations of miRNA expression can result in carcinogenesis when accompanied by other molecular changes. Epigenetic changes play an important role in inactivating tumor suppressor genes in cancer (31). DNA methylation in cancerous tissue has been

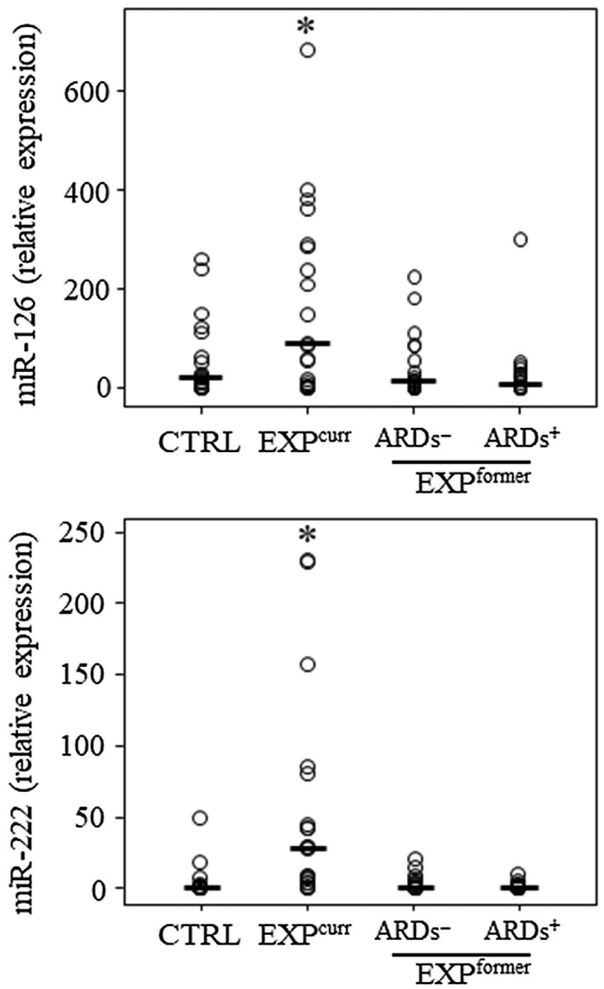

Figure 4.

Distribution of miR-126 and miR-222 in serum of healthy CTRL and asbestos-exposed subjects stratified as currently exposed (EXP^{curr}), earlier exposed (EXP^{former}) with ($ARDs^{+}$) and without ($ARDs^{-}$) benign ARDs. Significant differences were determined using one-way ANOVA and Tukey–Kramer *post hoc* test. *, CTRL vs. asbestos-exposed groups.

shown to cause miRNA silencing located in the vicinity of CpG sites (32). In particular, miR-126 was silenced by the DNA methylation of its gene, *EGFL7* both in NSCLC and MPM (33, 34). Huang and colleagues demonstrated that the miR-126 was downregulated during cancer progression, particularly in stroma cells. They hypothesized that the low levels of miR-126 found in cancer was linked to a cancer-stroma cross-talk, inducing repression of miR-126 to facilitate angiogenesis and invasion (35). The expression of miR-126 and its host gene was reduced in most cancers with a high frequency of *KRAS* mutations including lung cancer (36). A subset of miR-126–regulated genes selectively required for the survival and clonogenicity of *KRAS*-Mutant cells have been identified, supporting the role of miR-126 in tumor formation and progression (37).

Although the low number of enrolled subjects, as well the difficulties to estimate asbestos exposure and to attribute asbestos as the etiologic agent of NSCLC, are the major limitations, this study uncovers miR-126 and miR-222 that are likely involved in asbestos-related malignancies. It also outlines the mechanism of their expression, further pointing to the involvement of these miRNAs in asbestos-induced pathogenesis.

Disclosure of Potential Conflicts of Interest

M. Bovenzi is a consultant/advisory board member and expert witness for the public prosecutor in criminal trials on asbestos-related cancers. No potential conflicts of interest were disclosed for the other authors.

Authors' Contributions

Conception and design: L. Santarelli, M. Tomasetti, M. Bovenzi
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Bracci, M. Valentino, M. Amati, A. Sabbatini, E. Pasquini, N. Zanotta
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Santarelli, S. Gaetani, M. Valentino, J. Neuzil, M. Tomasetti, M. Bovenzi
Writing, review, and/or revision of the manuscript: M. Comar, J. Neuzil, M. Tomasetti, M. Bovenzi
Study supervision: L. Santarelli, M. Bovenzi
Other (WHO classification of lung tumors): C. Rubini

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References

1. Wolfe C, Buck B, Miller A, Lockey J, Weis C, Weissman D, et al. Exposure to naturally occurring mineral fibers due to off-road vehicle use: a review. Int J Hyg Environ Health 2017;220:1230–41.
2. Markowitz S. Asbestos-related lung cancer and malignant mesothelioma of the pleura: selected current issues. Semin Respir Crit Care Med 2015;36: 334–46.
3. Nielsen LS, Bælum J, Rasmussen J, Dahl S, Olsen KE, Albin M, et al. Occupational asbestos exposure and lung cancer-a systematic review of the literature. Arch Environ Occup Health 2014;69:191–206.
4. Liu B, van Gerwen M, Bonassi S, Taioli E, International association for the study of lung cancer mesothelioma task force. Epidemiology of environmental exposure and malignant mesothelioma. J Thorac Oncol 2017;12: 1031–45.
5. Uguen M, Dewitte JD, Marcorelles P, Loddé B, Pougnet R, Saliou P, et al. Asbestos-related lung cancers: a retrospective clinical and pathological study. Mol Clin Oncol 2017;7:135–9.
6. Tomasetti M, Santarelli L. Biomarkers for early detection of malignant mesothelioma: diagnostic and therapeutic application. Cancers (Basel) 2010;2:523–48.
7. Ying S, Jiang Z, He X, Yu M, Chen R, Chen J, et al. Serum HMGB1 as a potential biomarker for patients with asbestos-related diseases. Dis Markers 2017;2017:5756102.

8. Comar M, Zanotta N, Zanconati F, Cortale M, Bonotti A, Cristaudo A, et al. Chemokines involved in the early inflaMPMatory response and in pro-tumoral activity in asbestos-exposed workers from an Italian coastal area with territorial clusters of pleural malignant mesothelioma. Lung Cancer 2016;94:61–7.
9. Izzotti A, Pulliero A. The effects of environmental chemical carcinogens on the microRNA machinery. Int J Hyg Environ Health 2014;217:601–27.
10. Ferrante D, Mirabelli D, Tunesi S, Terracini B, Magnani C. Pleural mesothelioma and occupational and non-occupational asbestos exposure: a case-control study with quantitative risk assessment. Occup Environ Med 2016;73:147–53.
11. Tomasetti M, Staffolani S, Nocchi L, Neuzil J, Strafella E, Manzella N, et al. Clinical significance of circulating miR-126 quantification in malignant mesothelioma patients. Clin Biochem 2012;45:575–81.
12. Boeri M, Verri C, Conte D, Roz L, Modena P, Facchinetti F, et al. MicroRNA signatures in tissues and plasma predict development and prognosis of computed tomography detected lung cancer. Proc Natl Acad Sci USA 2011;108:3713–18.
13. Fortunato O, Boeri M, Verri C, Conte D, Mensah M, Suatoni P, et al. Assessment of circulating microRNAs in plasma of lung cancer patients. Molecules 2014;19:3038–54.
14. Fish JE, Santoro MM, Morton SU, Yu S, Yeh RF, Wythe JD, et al. miR-126 regulates angiogenic signaling and vascular integrity. Dev Cell 2008;15: 272–84.
15. Chen H, Li L, Wang S, Lei Y, Ge Q, Lv N, et al. Reduced miR-126 expression facilitates angiogenesis of gastric cancer through its regulation on VEGF-A. Oncotarget 2014;5:11873–85.
16. Tomasetti M, Nocchi L, Staffolani S, Manzella N, Amati M, Goodwin J, et al. MicroRNA-126 suppresses mesothelioma malignancy by targeting IRS1 and interfering with the mitochondrial function. Antiox Redox Signal 2014;21:2109–25.
17. Mao KP, Zhang WN, Liang XM, Ma YR. MicroRNA-222 expression and its prognostic potential in non-small cell lung cancer. Sci World J 2014; 908326:1–4.
18. Shang AQ, Xie YN, Wang J, Sun L, Wei J, Lu WY, et al. Predicative values of serum microRNA-22 and microRNA-126 levels for non-small cell lung cancer development and metastasis: a case-control study. Neoplasma 2017;64:453–9.
19. Chen Q, Hu H, Jiao D, Yan J, Xu W, Tang X, et al. miR-126–3p and miR-451a correlate with clinicopathological features of lung adenocarcinoma: the underlying molecular mechanisms. Oncol Rep 2016;36:909–17.
20. Garofalo M, Di Leva G, Romano G, Nuovo G, Suh SS, Ngankeu A, et al. miR-221&222 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3 downregulation. Cancer Cell 2009;16:498–509.
21. Dong Y, Fu C, Guan H, Zhang Z, Zhou T, Li B. Prognostic significance of miR-126 in various cancers: a meta-analysis. Onco Targets Ther 2016;9: 2547–55.
22. Pache JC, Janssen YM, Walsh ES, Quinlan TR, Zanella CL, Low RB, et al. Increased epidermal growth factor-receptor protein in a human mesothelial cell line in response to long asbestos fibers. Am J Pathol 1998;152: 333–40.
23. Carbonari D, Campopiano A, Ramires D, Strafella E, Staffolani S, Tomasetti M, et al. Angiogenic effect induced by mineral fibres. Toxicology 2011;288: 34–42.
24. Teixeira AL, Gomes M, Medeiros R. EGFR signaling pathway and related-miRNAs in age-related diseases: the example of miR-221 and miR-222. Front Genet 2012;3:286.
25. Garofalo M, Romano G, Di Leva G, Nuovo G, Jeon YJ, Ngankeu A, et al. EGFR and MET receptor tyrosine kinase-altered microRNA expression induces tumorigenesis and gefitinib resistance in lung cancers. Nat Med 2011;18:74–82.
26. Vriens A, Nawrot TS, Saenen ND, Provost EB, Kicinski M, Lefebvre W, et al. Recent exposure to ultrafine particles in school children alters miR-222 expression in the extracellular fraction of saliva. Environ Health 2016; 15:80.
27. Vrijens K, Bollati V, Nawrot TS. MicroRNAs as potential signatures of environmental exposure or effect: a systematic review. Environ Health Perspect 2015;123:399–411.
28. Bollati V, Marinelli B, Apostoli P, Bonzini M, Nordio F, Hoxha M, et al. Exposure to metal-rich particulate matter modifies the expression of candidate microRNAs in peripheral blood leukocytes. Environ Health Perspect 2010;118:763–8.
29. Rager JE, Moeller BC, Doyle-Eisele M, Kracko D, Swenberg JA, Fry RC. Formaldehyde and epigenetic alterations: microRNA changes in the nasal epithelium of nonhuman primates. Environ Health Perspect 2013;121: 339–44.
30. Rager JE, Moeller BC, Miller SK, Kracko D, Doyle-Eisele M, Swenberg JA, et al. Formaldehyde-associated changes in microRNAs: tissue and temporal specificity in the rat nose, white blood cells, and bone marrow. Toxicol Sci 2014;138:36–46.
31. Morgan AE, Davies TJ, McAuley MT. The role of DNA methylation in ageing and cancer. Proc Nutr Soc 2018;77:412–22.
32. Joyce BT, Zheng Y, Zhang Z, Liu L, Kocherginsky M, Murphy R, et al. miRNA-Processing gene methylation and cancer risk. Cancer Epidemiol Biomarkers Prev 2018;27:550–7.
33. Watanabe K, Emoto N, Hamano E, Sunohara M, Kawakami M, Kage H, et al. Genome structure-based screening identified epigenetically silenced microRNA associated with invasiveness in non-small-cell lung cancer. Int J Cancer 2012;130:2580–90.
34. Andersen M, Trapani D, Ravn J, Sørensen JB, Andersen CB, Grauslund M, et al. Methylation-associated silencing of microRNA-126 and its host gene EGFL7 in malignant pleural mesothelioma. Anticancer Res 2015; 35:6223–9.
35. Huang TH, Chu TY. Repression of miR-126 and upregulation of adrenomedullin in the stromal endothelium by cancer-stromal cross talks confers angiogenesis of cervical cancer. Oncogene 2014;33:3636–47.
36. Ebrahimi F, Gopalan V, Smith RA, Lam AK. Mir-126 in human cancers: clinical roles and current perspectives. Exp Mol Pathol 2014;96:98–107.
37. Hara T, Jones MF, Subramanian M, Li XL, Ou O, Zhu Y, et al. Selective targeting of KRAS-mutant cells by miR-126 through repression of multiple genes essential for the survival of KRAS-mutant cells. Oncotarget 2014; 5:7635–50.