Diagnostic Potential of miR-126, miR-143, miR-145, and miR-652 in Malignant Pleural Mesothelioma

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Malignant pleural mesothelioma (MPM) is difficult to distinguish from reactive mesothelial proliferations (RMPs). It is uncertain whether miRNAs are useful biomarkers for differentiating MPM from RMPs. Thus, we screened with a quantitative RT-PCR (RT-qPCR)-based platform the expression of 742 miRNAs in formalin-fixed, paraffin-embedded, preoperative diagnostic biopsy samples, surgically resected MPM specimens previously treated with chemotherapy, and corresponding non-neoplastic pleura (NNP), from five patients. miR-126, miR-143, miR-145, and miR-652 were significantly down-regulated (≥two-fold) in resected MPM and/or chemotherapy-naïve diagnostic tumor biopsy samples. The miRNA expression pattern was validated by RT-qPCR in a cohort of 40 independent MPMs. By performing binary logistic regression on the RT-qPCR data for the four miRNAs, the established four-miRNA classifier differentiated MPM from NNP with high sensitivity and specificity (area under the curve, 0.96; 95% CI, 0.92–1.00). The classifier’s optimal logit(P) value of 0.62 separated NNP and MPM samples with a sensitivity of 0.95 (95% CI, 0.89–1.00), a specificity of 0.93 (95% CI, 0.87–0.99), and an overall accuracy of 0.94 (95% CI, 0.88–1.00). The level of miR-126 in MPM was inversely correlated with that of the known target, the large neutral amino acid transporter, small subunit 1 (r = −0.38; 95% CI, −0.63 to −0.06). Overall, these results indicate that these four miRNAs may be suitable biomarkers for distinguishing MPM from RMPs. (J Mol Diagn 2014, 16: 418–430; http://dx.doi.org/10.1016/j.jmoldx.2014.03.002)

Malignant pleural mesothelioma (MPM) is an aggressive cancer originating from the mesothelial cells lining the pleura. Patients diagnosed with MPM typically have a history of long-term exposure to asbestos2 and a poor prognosis, with a median survival of 12 months from the time of diagnosis.2 Patients with MPM usually present with symptoms of pleural effusion (ie, chest pain and breathlessness), and less commonly with constitutional symptoms such as weight loss and fatigue. Major factors contributing to the poor prognosis are that the symptoms of MPM generally occur at an advanced stage of the disease and that MPM is often difficult to diagnose, which further hampers effective treatment. All this results in only few (<20%) patients eligible for trimodal therapy, which is currently the preferred treatment modality, consisting of chemotherapy, cytoreductive surgery (extrapleural pneumonectomy or pleurectomy/decortication), and radiotherapy.3 MPM is classified histologically as epithelioid, sarcomatoid, or biphasic (mixed epithelioid and sarcomatoid) subtype.

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known to have better, worse, and intermediate prognosis, respectively.\textsuperscript{1} The main diagnostic criterion for MPM is deep invasion in the pleura and underlying fat tissue, but this is often difficult to demonstrate radiologically and histologically in small pleural biopsy samples. Thus, epithelioid MPM may be challenging to distinguish from reactive mesothelial proliferation, whereas sarcomatoid MPM may resemble fibrous pleurisy.\textsuperscript{4} At present, there are no generally accepted diagnostic biomarkers for MPM, and the proposed MPM markers have not shown enough specificity and reproducibility or need further validation.\textsuperscript{5,6}

miRNAs are short, noncoding RNAs of 18 to 24 nucleotides that silence gene expression through base-pairing to complementary sequences, primarily within the 3′-untranslated regions of target mRNAs, which results in mRNA cleavage or translational repression and degradation.\textsuperscript{7} The latest version (release 19) of the miRBase database\textsuperscript{8} (Faculty of Life Sciences, University of Manchester, \url{http://www.mirbase.org}, last accessed May 17, 2013) contains 2042 entries of mature human miRNAs, and it is estimated that they target 3′-untranslated regions in >60% of human mRNAs.\textsuperscript{9} Accordingly, miRNAs are known to regulate several fundamental cellular and cancer-related processes, including proliferation, apoptosis, invasion, metastasis, cell-cycle control, and metabolism.\textsuperscript{10,11} miRNAs are particularly attractive as biomarkers in tissue samples processed for routine pathology, as they are short nucleotide sequences that remain stable and can be quantified in formalin-fixed, paraffin-embedded material by PCR and \textit{in situ} hybridization techniques.\textsuperscript{12} Therefore, several studies of miRNA-expression signatures in other types of human cancers have shown that miRNAs have potential as diagnostic, prognostic, and predictive markers.\textsuperscript{13–16} Others and we have recently reported miRNA signatures that seem to be able to discriminate between MPM and other cancer types or among different histological subtypes of MPM.\textsuperscript{17–20} However, it remains uncertain whether currently published miRNA data may provide candidate biomarkers for differentiating MPM from non-neoplastic reactive mesothelial proliferations (RMPs),\textsuperscript{26} the recurrent differential diagnostic problem in pleural biopsy samples taken from patients with clinical suspicion of MPM.\textsuperscript{4}

With the purpose of identifying more specific diagnostic markers of MPM, we performed quantitative real-time PCR (qPCR) screening and validation of miRNA expression in patient-matched diagnostic pleural biopsy specimens, surgical tissue specimens of MPM, and corresponding non-neoplastic pleura (NNP). We show that miR-126, miR-143, miR-145, and miR-652 are down-regulated in MPM, and we establish a four-miRNA diagnostic classifier that separates MPM and NNP with high overall accuracy.

### Materials and Methods

#### Patients and Tissue Samples

All patients included in this study were diagnosed and treated at Rigshospitalet, Copenhagen University Hospital (Copenhagen, Denmark) during 2004—2012. The miRNA-expression profile of MPM was assessed by qPCR—based screening of total RNA extracted from formalin-fixed, paraffin-embedded tissue specimens collected from five patients (two women and three men), aged 46 to 70 years, with epithelioid MPM (Stages II to IV). For each patient, the specimens included preoperative diagnostic tumor biopsy and tumor tissue sampling, as well as corresponding patient-matched NNP obtained by surgical operation for MPM. The latter was either extra pleural pneumonectomy or pleurectomy/decortication as a part of the trimodal protocol. At our institution, this option is offered to operable (with sufficient cardiopulmonary function and no involvement of N2/N3 lymph nodes) patients diagnosed with epithelioid MPM or biphasic MPM with <50% sarcomatoid component. As an initial part of this protocol, each of the patients had received, before surgery, one to three series of cisplatin and vinorelbine treatments. Thus, to test for any chemotherapy-induced changes in the miRNA expression profile, we included the total RNA preparations of diagnostic biopsy (DB) samples collected from the patients before any treatment was given. The MPM and DB tissue samples used for qPCR screening had 40% to 85% tumor content.

The validation of the obtained miRNA-expression profile was performed on tissue samples (preoperative DB samples, surgically removed tumor tissue, and surrounding NNP) from 40 MPM patients with demographic features and pathological characteristics as specified in Table 1. The DB samples included in the study were collected from a separate group of 12 still—chemotherapy-naïve MPM patients and contained >40% tumor cell content (Supplemental Table S1). The surgical tissue samples included MPM specimens from 40 patients and patient-matched samples of NNP from 14 of these MPM patients. Because of the diffuse growth of MPM, collection of NNP from the other 26 patients was not successful. A representative case of patient-matched specimens of NNP and MPM, characterized on immunohistochemistry (IHC) using anti-cytokeratin and anti-calcitulin antibodies, is depicted in Supplemental Figure S1. Before surgery, all MPM patients in this group received one to three series of cisplatin and pemetrexed treatments. All of the surgical MPM tissue samples used for the validation had >50% tumor cell content (Supplemental Table S1). To further validate the miRNA-expression profile, we included pleural specimens from five patients with non-neoplastic RMP in the pleura unrelated to MPM or any other cancer, but due to pneumothorax (PTHX) (Table 1). Two experienced pathologists (C.B.A. and E.S.-R.) characterized all tissue specimens used in this study according to conventional histological and IHC examination for pleural samples\textsuperscript{5} to verify diagnosis for miRNA and IHC analyses.

#### Ethics

All tissue samples were collected from patients in accordance with the Declaration of Helsinki and with approvals.
miRNA qPCR Analysis

For extraction of total RNA fractions including miRNAs from formalin-fixed, paraffin-embedded tissues, the Ambion RecoverAll Total Nucleic Acid Isolation Kit (item no. AM1975; Life Technologies, Grand Island, NY) was used according to the manufacturer’s instructions. The concentration and integrity of each sample was determined using spectrophotometry on a Nanodrop 2000 UV-Vis spectrophotometer (item no. ND2000; Thermo Fisher Scientific Inc., Waltham, MA). The qPCR screening was performed using the miRCURY LNA Universal RT miRNA Ready-to-Use PCR, Human panels I + II version 2.0 (item no. 203607; Exiqon A/S, Vedbaek, Denmark) according to the manufacturer’s protocol. Data from the qPCR screening experiments were analyzed using the version of the GenEx software version 2.5 (MultiD Analyses AB, Göteborg, Sweden), which was specifically adapted to the miRCURY LNA Universal RT miRNA Ready-to-Use PCR panels. Suitable reference genes were identified using analysis of variance (ANOVA). For the validation of the obtained miRNA-expression profile, we used the TaqMan miRNA Reverse Transcription Kit (item no. 4366596; Life Technologies) and TaqMan miRNA Assays (item no. 4427975; Life Technologies) according to the manufacturer’s instructions. Reverse transcription reactions were performed by using 10 ng total RNA and specific reverse transcription primers (Life Technologies) for homo sapiens (hsa)-miR-126-3p (assay ID 002228), hsa-miR-143-3p (assay ID 002249), hsa-miR-145-5p (assay ID 002278), hsa-miR-193a-3p (assay ID 002250), hsa-miR-193b-3p (assay ID 002367), hsa-miR-652-3p (assay ID 002352), and internal reference gene snRNA, C/D box 49A (SNORD49A) (assay ID 001005). The qPCR amplification was performed on the 7500 Real-Time PCR system (Life Technologies), with enzyme activation at 95°C for 10 minutes, followed by 40 cycles of denaturing for 15 seconds at 95°C and primer annealing/extension for 60 seconds at 60°C. The quantification cycle (Cq) values were determined with related 7500 system software version 2.0.6 (Life Technologies), with default settings. All qPCR data were analyzed comparatively using SNORD49A as a reference gene. All qPCR data for each miRNA are presented as log2-transformed ratio according to the 2−ΔΔCq method, where: 

$$ΔC_q = C_q^{\text{miR}} - C_q^{\text{SNORD49A}}.$$

IHC Analysis

For IHC formalin-fixed, paraffin-embedded tissue sections (2.5 μm thick) mounted on coated glass slides were deparaffinized and immunostained on a BenchMark ULTRA automated slide immunostainer (item no. N750-BMKU-FS; Ventana Medical Systems Inc., Oro Valley, AZ) using Ultra Cell Conditioning solution (CC1) pretreatment (Ventana Medical Systems Inc.) for 64 minutes at 97°C when detecting large neutral amino acid transporter, small subunit 1 (LAT1), adapter molecule Crk (isoform Crk-II) (Crk-II), and calretinin. The pretreatment was followed by incubation for 32 minutes at 36°C with anti-LAT1 rabbit monoclonal antibodies [clone ID, EPR3492(2), dil. 1:2000; Epitomics Inc., Burlingame, CA], anti-Crk-II mouse monoclonal antibodies (clone ID, 3G11C1, dil. 1:3000; Abcam plc., Cambridge, UK), or anti-calretinin rabbit monoclonal antibodies (clone ID, SP65, dil. 1:400; Ventana Medical Systems Inc.). For broad-spectrum cyto keratin (pan-cytokeratin), CC1 pretreatment was 20 minutes, and the slides
were incubated with primary anti-cytokeratin mouse monoclonal antibodies (clone ID, AE1/3 + 8/18, Ready-to-Use; Biocare Medical Inc., Concord, CA) for 20 minutes at 36°C. The reaction was visualized by using ultraView DAB Detection Kit (Ventana Medical Systems Inc.). Afterward the sections were counterstained with hematoxylin (Ventana Medical Systems Inc.) for 8 minutes, following the manufacturer’s recommendations. LAT1 and Crk-II immunostaining was evaluated by light microscopy and scored by a semiquantitative H score originally described by Olaussen et al28 and previously described for other biomarkers in MPM and other cancer types.29–32 As external positive control with moderate protein expression (staining intensity 2), we used liver tissue for LAT1 and colorectal cancer for Crk-II, whereas lymphocytes and endothelial cells were used as internal reference controls for LAT1 and Crk-II, respectively. The H score was determined by counting at least 500 neoplastic or non-neoplastic mesothelial cells per sample. Briefly, the staining intensity of both markers was assessed against their respective positive control and assigned a value of 0, no staining; 1, less than control; 2, equal to control; or 3, more than control. Then the percentage of cells with positive staining was estimated and assigned a proportion score of 0.1 (<10% stained relevant cells), 0.5 (10% to 50% stained cells), or 1 (>50% stained cells). The proportion score was then multiplied by the staining-intensity values to obtain the final semiquantitative H score, with values thereby ranging from 0 to 3. IHC evaluation was performed blinded for clinical and molecular data by two independent observers (E.S.-R. and M.A.), and in discordant cases, consensus was reached by the use of a two-headed microscope. Omission and substitution of the primary antibodies with unspecific IgG were used as negative control.

Statistical Analysis

For hierarchical clustering analysis of the miCURY LNA universal qPCR data, GenEx software (Exiqon A/S) with Euclidean distance and average linkage as parameters was used. For qPCR data, the unpaired Student’s t-test (two-tailed) was used to detect significant differences in miRNA expression between two groups. Significant differences in the miRNA expression in three or more groups overall were detected using one-way ANOVA. P values adjusted for mass significance were obtained using Tukey-Kramer post-tests. Overall survival analysis was performed using the Kaplan-Meier method. Survival curves were tested for differences by using the Mantel-Cox log-rank test. For the analysis of IHC results, the distributions of H scores in three groups were compared using nonparametric Kruskal-Wallis ANOVA and the Dunn post-test. The miRNA expression and H scores of validated miRNA targets were tested for correlation using the Spearman test. Contingency tables were analyzed using the Fisher exact test. The validation RT-qPCR data of the tested miRNAs were used as predictor variables for binary logistic regression. In short, the logistic regression approach provides the probability (P) of a positive outcome using the optimal linear combination of predictor variables. P is expressed by the logit(P) transformation, which is defined as the natural logarithm of the odds, with odds defined as the probability of a positive outcome (P) divided by the probability of negative outcome (1 – P). The logit(P) transformation was predicted by the generalized mathematical expression:

\[ \text{logit}(P) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_n x_n \]  

(1)

This equation links the logit(P) transformation to a number (n) of predictor variables \((x_1, \ldots, x_n)\) and \(n + 1\) regression constants \((\beta_0, \ldots, \beta_n)\). To avoid bias, the logistic regression was performed using a bootstrap of 1000 unrestricted resamples. In all cases, \(P < 0.05\) was considered significant. OriginPro version 8.6.0 (OriginLab Corporation, Northampton, MA) and SAS version 9.4 (SAS Institute Inc., Cary, NC) were used for all statistical calculations.

Results

Screening of miRNA-Expression Profile in MPM

To identify candidate diagnostic miRNA biomarkers of MPM, we analyzed total RNA extracted from five DB samples (DB1 to DB5), five surgical specimens of epithelioid MPM (MPM1 to MPM5), and five corresponding specimens of NNP (NNP1 to NNP5). This learning set of samples was screened by qPCR using the miRCURY LNA Universal RT microRNA Ready-to-Use PCR, Human panels I + II version 2.0 M. In this screening, the expression of 742 human miRNAs was determined. All samples were analyzed successfully, with no signs of inhibition of the real-time amplification due to contaminants. Of 742 analyzed miRNAs, 147 could be detected in all submitted samples. None of the 742 determined miRNAs were expressed exclusively in one or two groups of specimens. The full expression data of the initial qPCR screening are deposited in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; accession number GSE54394).

To identify the miRNAs that are consistently deregulated in MPM compared to NNP, we analyzed the qPCR data with one-way ANOVA and the Tukey-Kramer post-test for direct comparisons of groups. By this approach, we detected 14 miRNAs with statistically significant differential expression between groups (all \(P < 0.05\)). Specifically, we found that miR-378, miR-365a, miR-193a-3p, miR-193b, and miR-210 were up-regulated and that let-7c, miR-99a, miR-126, miR-143, miR-145, miR-144-5p, miR-451a, miR-486-5p, and miR-652 were down-regulated in either DB or MPM samples as compared to those in the NNP samples. Relative to the NNP samples, miR-126 and miR-652 were both significantly down-regulated in the DB and in the MPM specimens as well. Furthermore, when including miRNAs with near-significant
differential expression, four additional miRNAs (miR-21, miR-296-5p, miR-22-5p, and let-7i-5p) were found to be up-regulated, and five miRNAs (miR-30b, let-7g, miR-338-3p, miR-425, and miR-328) were down-regulated in the DB and MPM samples, respectively, as compared to those in the NNP samples. The relative changes in the expression of these 23 miRNAs (significant and near significant) between the tissue groups are illustrated as stack bar charts in Figure 1A. Overall, the largest differences in relative miRNA expression were observed when comparing the DB and NNP tissues, indicating that the chemotherapy reduces the differential expression of these miRNAs (Figure 1A).

The expression data of the miRNAs with significant differential expression between the tissue groups are summarized in Supplemental Table S2. The miRNAs with near-significant (0.05 ≤ P ≤ 0.1) differential expression between tissue groups included miR-21, miR-30b, miR-338-3p, miR-425, miR-328, miR-296-5p, miR-22-5p, let-7i-5p, and let-7g. Their expression data are summarized in Supplemental Table S3.

For the validation of the miRNA-expression profile using TaqMan miRNA assays, we identified stably expressed reference genes using ANOVA of the qPCR data normalized to the global mean of all detected assays. In this way, SNORD49A was identified as the gene with the least variation in expression between the tested tissue groups.

We searched the mirBase release 19 database (Faculty of Life Sciences, University of Manchester, http://www.mirbase.org, last accessed January 31, 2013) for information relating to the genomic location of the 14 identified miRNAs with significant differential expression and discovered that eight miRNAs belonged to the miR-143/145, miR-193b/365a, miR-451/144, and let7c/99a clusters, respectively. The miRNAs that belonged to the same clusters were similarly up- or down-regulated on comparison of their expression in the three groups of analyzed tissue samples.

We used the qPCR data of the 23 identified miRNAs with significant and near-significant differential expression in the three groups of samples to perform hierarchical clustering analysis. The resulting dendrograms to visualize the clustering of analyzed tissue samples and the 23 included miRNAs are shown in Figure 1, B and C. Overall, the DB and MPM samples clustered at the third level and were clearly separated from the NNP samples. The only exception was the MPM2 sample, which clustered with the NNP samples, probably due to the rather low tumor content in this sample (40%).

The miRNAs that we considered the best candidates as diagnostic markers were significantly deregulated in both DB
and MPM tissues when compared to those in NNP tissues. These criteria were fulfilled by miR-126 and miR-652. To test the diagnostic potential of up-regulated miRNAs, we included miR-193a-3p and miR-193b in the validation, although these miRNAs were not significantly up-regulated when comparing the MPM and NNP tissues (Figure 1A). Similarly, miR-143 and miR-145 were not significantly down-regulated when comparing the MPM and NNP tissues. However, considering the well-described regulatory relationship of miR-143 and miR-145 expression and the p53/MDM2 feedback loop, we wanted to explore the down-regulation of miR-126, miR-143, miR-145, and miR-652 when comparing the MPM and NNP tissues (Figure 1A). We determined the robustness of miR-126, miR-143, miR-145, and miR-652 in these samples, we used a paired-samples t-test and the $2^{-\Delta\Delta C_q}$ method. Determining the relative changes in the patient-matched samples, we found a 2.66-fold down-regulation of miR-126 ($P < 0.05$), a 2.33-fold down-regulation of miR-143 ($P < 0.05$), a 6.55-fold down-regulation of miR-145 ($P < 0.001$), and a 2.28-fold down-regulation of miR-652 ($P < 0.01$) (Supplemental Figure S2). Overall, the relative changes in the expression of miR-126, miR-143, miR-145, and miR-652 on analysis of patient-matched samples were consistent only with those obtained when the unmapped MPM-samples were included.

In contrast, we found that miR-193a-3p was equally expressed in the DB and NNP samples and thus was not useful as a diagnostic marker (Figure 2D). Similarly, we could not confirm the preliminary expression pattern of miR-193b observed on miRNA screening (Figure 1), as it was not significantly differentially expressed in the MPM samples relative to those in the NNP samples, and hence miR-193b was disqualified as a potential diagnostic marker (Figure 2E). The validation set of tissue samples comprised 18 epithelioid and 22 biphasic MPMs (Table 1) and when we compared the level of miRNA expression in these two groups, we found significant differences in the expression of miR-126, miR-145, and miR-193a-3p (Figure 3). In particular, biphasic MPM displayed significantly higher miR-126 and miR-145, as well as lower miR-193a-3p, expression previously described, 14 patients in the cohort provided matched samples of NNP and MPM specimens. To compare the expression of miR-126, miR-143, miR-145, and miR-652 in these samples, we used a paired-samples t-test and the $2^{-\Delta\Delta C_q}$ method. Determining the relative changes in the patient-matched samples, we found a 2.66-fold down-regulation of miR-126 ($P < 0.05$), a 2.33-fold down-regulation of miR-143 ($P < 0.05$), a 6.55-fold down-regulation of miR-145 ($P < 0.001$), and a 2.28-fold down-regulation of miR-652 ($P < 0.01$) (Supplemental Figure S2). Overall, the relative changes in the expression of miR-126, miR-143, miR-145, and miR-652 on analysis of patient-matched samples were consistent only with those obtained when the unmatched MPM-samples were included.

Validation of miR-126, miR-143, miR-145, and miR-652 as Diagnostic Biomarkers of MPM

To determine the robustness of miR-126, miR-143, miR-145, miR-193a-3p, miR-193b, and miR-652 as diagnostic biomarkers of MPM, we tested their expression pattern by using TaqMan miRNA assays, in an expanded set of DB-, MPM-, NNP-, and PTHX-induced RMPs, as specified in Table 1. The results of the qPCR validation are summarized in Figure 2 and Table 2. We found that the expression of miR-126, miR-143, miR-145, and miR-652 was significantly down-regulated in the MPM and DB samples as compared to the levels of these miRNAs in the NNP samples (Figure 2 and Table 2). As
levels than those in epithelioid MPM (Figure 3, A, C, and D). Thus, the differentiation pattern of different histological subtypes of MPM seems to affect the levels of these three miRNAs.

The qPCR expression data of miR-126, miR-143, miR-145, and miR-652 in the NNP and MPM samples were used to generate receiver-operating characteristics (ROC) curves for each miRNA and determine the associated areas under the ROC curves (Figure 4 and Table 2). The resulting areas under the ROC curve for miR-126, miR-143, miR-145, and miR-652 ROC curves were 0.78 (95% CI, 0.64–0.92; P < 0.01), 0.76 (95% CI, 0.61–0.90; P < 0.01), 0.93 (95% CI, 0.85–1.02; P < 0.001), and 0.89 (95% CI, 0.80–0.99; P < 0.001), respectively. Taken together, these results suggest that each of these four miRNAs could potentially be used to discriminate MPM from NNP, with a sensitivity or specificity close to 0.80 (miR-126 and miR-143) or >0.80 (miR-145 and miR-652), as recommended by the International Mesothelioma Interest Group for MPM biomarkers. The demographic and clinical features of the patients, including the histological characteristics of the tissue samples used for the validation procedure, are provided in Supplemental Table S1. The raw data sets from the validation procedure are available in Supplemental Tables S4 and S5 contains the normalized expression data. In addition, Supplemental Table S6 lists the range of the raw Cq values for the reference gene SNORD49A across the different tissue groups and experimental platforms (miRCURY LNA qPCR and TaqMan small RNA assays); the data indicate that this reference gene performs equally well on both platforms.

### Four-miRNA Classifier Established by Logistic Regression Analysis as a Diagnostic and Prognostic Biomarker for MPM

To increase the diagnostic performance of the four miRNAs, we then used the qPCR data of miR-126, miR-143, miR-145, and miR-652 expression in the NNP and MPM samples as predictor variables for binary logistic regression (Materials and Methods).

To avoid bias, we furthermore used a bootstrap consisting of a 1000 unrestricted resamples. Using this approach, we obtained a four-miRNA classifier for predicting the probability of a positive MPM diagnosis and determined the value of the intercept (β0) to be 4.38. Similarly, we determined the regression constants of miR-126, miR-143, miR-145, and miR-652 to be 0.53, 0.98, −2.34, and −1.45, respectively (Table 3). Each of the four miRNAs had a significant contribution to the model, but as reflected by their regression constants, miR-145 and miR-652 were the most influential in predicting the outcome. Having determined the regression constants, the logit(P) for each tested NNP and MPM tissue specimen can be calculated by the following mathematical expression:

\[
\text{logit}(P) = 4.38 - 0.53 \times \Delta C_q,\text{miR-126} - 0.98 \times \Delta C_q,\text{miR-143} + 2.34 \times \Delta C_q,\text{miR-145} + 1.45 \times \Delta C_q,\text{miR-652} \quad (2)
\]

We tested the performance of this four-miRNA classifier by calculating the logit(P) of the NNP and MPM samples, respectively, and generated Tukey box plots and the corresponding ROC curves (Figure 5). We found that the MPM samples had a mean logit(P) of 4.49 (95% CI, 3.62–5.36), which was significantly higher than the mean logit(P) of −2.22 (95% CI, −3.71 to −0.74) in the NNP samples (P < 0.001, Student’s t-test). The ROC curve that we generated based on logit(P) had an area under ROC curve value of 0.96 (95% CI, 0.92–1.00; P < 0.001) (Figure 5A), which indicates a high level of accuracy in differentiating the NNP from the MPM samples. By analyzing the ROC curve, we found a logit(P) of 0.62 as the optimal cutoff value for discriminating NNP from MPM (Figure 5B). By using a cutoff value of logit(P) ≤ 0.62, we were able to subdivide the NNP and MPM samples into two groups, resulting in a 2 × 2 contingency
Level of LAT1 Is Inversely Correlated with miR-126 Expression in MPM

We were then interested in assessing the expression patterns of known targets of the four miRNAs in the classifier. To pursue this goal, we started by testing the expression of LAT1 and Crk-II, both of which have been described as validated targets of miR-126.37,38 Using IHC analysis, we determined the expression of Crk-II and LAT1 in the NNP, MPM, and PTHX samples specified in Table 1. Four NNP samples and one MPM sample stained for Crk-II and two NNP samples and one MPM sample stained for LAT1 were not eligible for scoring. Representative IHC stains of Crk-II (H score = 2), LAT1 (H score = 2), and corresponding H&E stain in one MPM specimen are depicted in Figure 7. In general, we observed Crk-II staining to be diffuse throughout the cytoplasm, whereas LAT1 staining primarily was associated with the Golgi apparatus. For the Crk-II staining, the groups of NNP and MPM samples both had a median H score of 2; thus, we did not observe any significant differences in Crk-II expression between these two groups. However, having a median H score of 0.2, the PTHX samples showed significant differences in Crk-II expression as compared to the NNP and MPM samples (Figure 8A). LAT1 immunostaining, instead, resulted in a median H score of 2 in the MPM samples and was significantly higher in these samples than in the NNP samples, which had a median H score of 0.5 (P < 0.01, Dunn post-test). Furthermore, we observed that the PTHX samples had a median H score for LAT1 of 1, but this difference was not significant compared to those in the groups of NNP and MPM samples (Figure 8B). Our results indicate that LAT1 could be a potential diagnostic marker of MPM that might complement the analysis of the miR-126 expression pattern. Thus, based on the LAT1 H scores of the NNP and MPM samples, we generated an area under the ROC curve of 0.77 (95% CI, 0.64—0.90; P < 0.01) (Figure 8C). We used the IHC and qPCR data in the NNP, MPM, and PTHX samples to assess the correlation between LAT1 H score and miR-126 expression (Figure 8D). Using the Spearman test for correlation, we found that LAT1 expression and miR-126 level were significantly inversely correlated (r = −0.38; 95% CI, −0.63 to −0.006; P < 0.05), suggesting that LAT1
is likely to be a target of miR-126 in MPM and might be used as a possible IHC biomarker in MPM diagnostics.

Discussion

Our goal was to identify miRNAs that can aid in the differential diagnosis of MPM from RMPs. By screening the expression of 742 miRNAs and subsequently validating differentially expressed miRNAs in two independent sets of patient-matched preoperative DBs and surgically removed neoplastic and non-neoplastic tissue samples, we identified miR-126, miR-143, miR-145, and miR-652 as the best candidate miRNAs to diagnose MPM. In addition, by combining these four miRNAs, we generated a four-miRNA classifier with enhanced diagnostic performance. Indeed, by using the four-miRNA classifier, we found that cancer tissue and NNP from MPM patients could be correctly classified with an overall accuracy of 0.94. In addition, our IHC data suggest that LAT1 level is regulated by miR-126 in MPM and inversely correlates with the expression of this miRNA and could possibly help in distinguishing MPM from RMPs. However, because of the limited number of samples in the present study, a larger-scale study is needed to confirm these results.

To date, several studies have explored the expression of miRNAs in MPM tumors and in the serum of MPM patients.17–25 Even though the miRNA signatures obtained in these studies can allegedly discriminate between MPM and other cancer types or among different histological subtypes of MPM, they do not seem to provide candidate biomarkers for accurately differentiating MPM from non-neoplastic RMPs.26 In agreement with findings from a previous study focused on serological markers of MPM, we observed significant down-regulation of miR-126 in MPM tissues.19 In contrast, a comparative study in mesothelioma cell lines (MMP-89 and REN) and telomerase reverse transcriptase-immortalized human mesothelial cells (HMC-TERT) reported that miR-143 was up-regulated in the mesothelioma cells,17 but we were unable to confirm this finding in tissue samples. Despite several investigations, a clear consensus is still needed about the nature and identity of the miRNAs that are useful diagnostic biomarkers of MPM. The inconsistencies between previous miRNA studies in MPM are likely caused by different experimental approaches and differing choices of reference materials. For example, as we have previously reported,26 miRNAs elsewhere described as specific for the histological subtypes of MPM17 are not entirely suitable markers for discriminating MPM and NNP.17,26 In this respect, using the experimental approach of an initial broad screen in patient-matched tissue specimens, followed by validation in an independent patient cohort, we have now, for the first time, reported the identity of a four-miRNA classifier (miR-126, miR-143, miR-145, and miR-

### Figure 4

ROC curves of miR-126 (A), miR-143 (B), miR-145 (C), and miR-652 (D). ROC curves were generated using qPCR data of miRNA expression in samples of NNP versus MPM. Areas under the ROC curves (AUC) and 95% CIs are shown. **P < 0.01, ***P < 0.001.

### Table 3

Logistic Regression Constants for miR-126, miR-143, miR-145, and miR-652

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>4.38*</td>
<td>4.22–4.54</td>
</tr>
<tr>
<td>miR-126</td>
<td>0.53*</td>
<td>0.49–0.57</td>
</tr>
<tr>
<td>miR-143</td>
<td>0.98*</td>
<td>0.94–1.02</td>
</tr>
<tr>
<td>miR-145</td>
<td>-2.34*</td>
<td>-2.27 to -2.39</td>
</tr>
<tr>
<td>miR-652</td>
<td>-1.45*</td>
<td>-1.40 to -1.50</td>
</tr>
</tbody>
</table>

ΔCq values for each miRNA are used as input data.

*P < 0.001.
that discriminates NNP and MPM with high accuracy. The International Mesothelioma Interest Group recommends that a diagnostic marker of MPM have sensitivity/specificity >0.80,4 and these criteria are fulfilled by our miRNA classifier. However, further validation in an independent blinded test set is needed to fully establish its diagnostic value. In addition, studies currently under way in our laboratory using in situ hybridization techniques will further corroborate the diagnostic value of the miRNAs described in this paper.

In our opinion, a diagnostic assay that combines ISH and IHC detection of their related targeted gene products could potentially improve the differential diagnosis of RMP and MPM. The present study was conducted with qPCR-based methods using MPM tissue samples with >50% of tumor cell content to allow optimal detection of miRNA expression due to tumor cells. Indeed, our preliminary data with MPM tissue samples having <50% of tumor cell content indicated a significant decrease in accuracy of the assay due to dilution of MPM-derived miRNA-expression profile with miRNAs produced by non-neoplastic tissue (data not shown). In this respect, a combination of ISH and IHC would allow the direct analysis in situ of miRNAs and the expression of their targets also in samples with low tumor cell content. To construct a combined ISH/IHC assay, the related target genes of the classifier miRNAs presented here need to be identified.

Knowledge of the targeted genes of miR-126, miR-143, miR-145, and miR-652 in MPM will furthermore establish their mechanistic impact on mesothelial oncogenesis and their potential relevance as therapeutic targets. Using IHC, we tested the expression of the amino acid transporter LAT1 and the oncogenic adaptor protein Crk-II. LAT1 and Crk-II have both been reported to be experimentally validated targets of miR-126 in lung cancer cell lines.37,38 Crk-II is an oncoprotein that participates in cellular signal transduction and is involved in invasion, survival, proliferation, and migration.39 Although we did not find any significant differences in the expression of Crk-II in the MPM and NNP

652) that discriminates NNP and MPM with high accuracy. The International Mesothelioma Interest Group recommends that a diagnostic marker of MPM have sensitivity/specificity >0.80,4 and these criteria are fulfilled by our miRNA classifier. However, further validation in an independent blinded test set is needed to fully establish its diagnostic value. In addition, studies currently under way in our laboratory using in situ hybridization techniques will further corroborate the diagnostic value of the miRNAs described in this paper.

In our opinion, a diagnostic assay that combines ISH of MPM-deregulated miRNAs and IHC detection of their related targeted gene products could potentially improve the differential diagnosis of RMP and MPM. The present study

miRNA Expression in Mesothelioma

Table 4 Separation of NNP and MPM Samples Using logit(P) = 0.62 as CutOff Value

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NNP</th>
<th>MPM</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test positive*</td>
<td>1</td>
<td>38</td>
<td>39</td>
</tr>
<tr>
<td>Test negative†</td>
<td>13</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>40</td>
<td>54</td>
</tr>
</tbody>
</table>

*Logit(P) > 0.62.
†Logit(P) ≤ 0.62.
NNP, non-neoplastic pleura; MPM, malignant pleural mesothelioma.

Figure 5 Logit(P) values of NNP and MPM specimens. A: Tukey box plots of the logit(P) value in NNP and MPM. The boxes delimit the 25th and 75th percentiles. Horizontal lines inside the boxes mark the medians. Mean expression values are marked with squares. Vertical lines mark the interval between the 5th and 95th percentiles. Significance was determined using Student’s unpaired t-test. B: ROC curve of the logit(P) value in NNP versus MPM. Area under the ROC curve (AUC) and 95% CI is shown. *P < 0.001.

Figure 6 Kaplan-Meier plots of overall survival for MPM patients dichotomized based on high (more than median) versus low (less than or equal to median) logit(P) (A) or on miR-193b expression (B). Overall survival was analyzed in 36 MPM patients; event and censored data were 11 and 25, respectively. Survival curves in each group were tested for differences using the log-rank test. *P < 0.05, **P < 0.01.
samples, we observed a significantly higher expression of Crk-II in these samples compared to that in RMPs unrelated to cancer, such as the one seen after relapsing PTHX. A previous study reported LAT1 up-regulation in 50% of MPMs and associated high LAT1 expression with poor prognosis. Consistently, we observed a significant up-regulation of LAT1 in MPM and an inverse correlation with miR-126 level. An inverse relationship between miR-126 and LAT1, which is a known target gene of miR-126 in small cell lung cancer cells, suggests an equivalent regulatory mechanism of miR-126 in LAT1 expression in MPM cells. However, further studies involving manipulation of miR-126 levels in MPM cell lines are needed to confirm LAT1 as an miR-126 target in MPM.

As for the other three miRNAs in the classifier, miR-652 is the least known. A single publication reports deregulated expression of miR-652 in other tumor types, but to our knowledge no validated targets have been reported yet. Instead, miR-143 and miR-145 appear to be induced by the tumor suppressor p53 and to negatively regulate different oncogenic pathways. Further studies combining the assessment of miR-143 and miR-145 levels with the expression of some of their targets in MPM tissues will clarify whether these regulatory loops are also relevant for mesothelial malignant growth and whether they can be exploited for improving the diagnostics of MPM.

**Conclusion**

In summary, we have shown that the expression data of four miRNAs (miR-126, miR-143, miR-145, and miR-652) can be combined into a diagnostic miRNA classifier for MPM. Furthermore, our preliminary studies by IHC aimed at

Figure 7 Representative serial sections of an epithelioid MPM tissue specimen immunostained for LAT1 (left panel) and Crk-II (right panel), both with a semiquantitative H score of 2 in this case. H&E staining of the same area is shown in the inset. Original magnification, ×200 (left and right panels; inset, left panel).

Figure 8 Scatterplots of LAT1 (A) and Crk-II (B) H scores in MPM, NNP, and PTHX samples. The horizontal lines mark median H scores. Significant differences were determined using Kruskal-Wallis ANOVA and Dunn post-tests. C: ROC curve of LAT1 expression in NNP versus MPM. Area under the ROC curve (AUC) and 95% CI are shown. D: Correlation of miR-126 expression (determined by qPCR) and LAT1 expression (evaluated by IHC) in MPM, NNP, and PTHX. Correlation coefficient (r) was determined with the Spearman test for correlation. *P < 0.05, **P < 0.01.
explore the expression of miRNA targets in MPM illustrate the biological effects of down-regulated miRNAs in MPM and may provide additional tools for MPM diagnostics on tissue sections. In conclusion, we believe that miRNAs may be promising diagnostic biomarkers for MPM and that a diagnostic assay that combines miRNA detection byISH and IHC of corresponding miRNA targets may greatly improve the differential diagnosis between MPM and RMP.

Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.jmoldx.2014.03.002.

References
