Increased Circulating miR-625-3p

A Potential Biomarker for Patients With Malignant Pleural Mesothelioma

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Introduction: We investigated the ability of cell-free microRNAs (miRNAs) in plasma and serum to serve as a biomarker for malignant mesothelioma (MM).

Methods: Using miRNA microarrays, we profiled plasma samples from MM patients and healthy controls. miRNAs with significantly different abundance between cases and controls were validated in a larger series of MM patients and in an independent series of MM patients using quantitative real-time polymerase chain reaction. Levels of candidate miRNAs were also quantified in MM tumor samples.

Results: We compared cell-free miRNA profiles in plasma from MM patients with healthy controls. Reviewing 90 miRNAs previously reported to be associated with MM, we found that the levels of two miRNAs, miR-29c* and miR-92a, were elevated in plasma samples from MM patients. In addition, we identified 15 novel miRNAs present at significantly higher levels in the plasma of MM patients. Further analysis of candidate miRNAs by real time-quantitative polymerase chain reaction confirmed that one of them, miR-625-3p, was present in significantly higher concentration in plasma/serum from MM patients and was able to discriminate between cases and controls, in both the original and the independent series of patients. MiR-625-3p was also found to be up-regulated in tumor specimens from a group of 18 MM patients, who underwent extrapleural pneumonectomy.

Conclusion: Our data confirm the potential of miR-29c* and miR-92a as candidate tumor markers and reveal that miR-625-3p is a promising novel diagnostic marker for MM.

Key Words: Mesothelioma, MicroRNA, Plasma, Biomarker, Diagnosis.

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Maligant mesothelioma (MM) is a highly aggressive tumor arising from the mesothelial surfaces of the pleural and peritoneal cavities. The global burden of this neoplasm that in most cases has direct causal relationship with exposure to asbestos has increased significantly in the last two decades and is still on the rise.[1,2] The diagnosis of tumors arising in the pleura or peritoneum is difficult,[3,4] and the accuracy of the MM diagnosis depends on the availability of sufficient biopsy material. Some centers use cytological analysis of pleural fluid for differential diagnosis but this approach has not received universal acceptance.[4] Finding a reliable diagnostic marker in plasma or serum would represent a significant advancement and might at the same time provide the opportunity for detecting the disease at an earlier stage. Although the application of new molecular biological techniques has led to the identification of serum proteins associated with the presence of MM, markers such as mesothelin, megakaryocyte potentiating factor, and osteopontin have failed to achieve the specificity and sensitivity required for an accurate diagnosis.[5–8]

MicroRNAs (miRNAs) are a class of short (~25 nucleotides) noncoding RNAs that regulate gene expression post-transcriptionally through sequence-specific interaction with target sites in messenger RNAs.[9] Characteristic alterations in miRNA expression profiles have been found in a number

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of tumor types,[10] including MM. Their stability in archival tumor blocks and body fluids such as serum and plasma[11,12] makes them attractive candidate biomarkers. Elevated plasma miRNA levels have been reported in patients with diffuse large B-cell lymphoma,[13] ovarian cancer,[14] colorectal cancer,[15,16] prostate cancer[12], and lung cancer.[11] Other studies have revealed an association between reduced serum levels of particular miRNAs and the presence of a tumor.[17]

In this study, we have used microarrays to compare the plasma miRNA profiles of MM patients with control subjects. After reverse transcription-quantitative polymerase chain reaction (RT-qPCR) validation in two independent series of patients, miR-625-3p was found to be present at significantly higher levels in plasma/serum of MM patients, and this miRNA was also present at elevated levels in tumor samples.

MATERIALS AND METHODS

Blood Collection and Preparation of Plasma and Serum

Plasma of the test cohort was collected in 10 ml Vacutainer Plus K_3ethylenediaminetetraacetic acid tubes (BD Biosciences, Franklin Lakes, NJ). Samples from MM patients or control subjects (patients with coronary artery disease or healthy subjects) were collected before treatment (Table 1 with details provided in Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/JTO/A284). Within 30 minutes of collection, the tubes were centrifuged for 20 minutes at 2500 *g* at room temperature. Levels of free hemoglobin were measured by spectral analysis as previously described,[18] and samples with hemoglobin levels corresponding to an A414 reading of greater than 0.25 were excluded from analyses. Plasma was stored at −80°C until further processing. All samples were collected from individuals who had given written informed consent and participated in studies approved by the Human Research Ethics Committees at Concord Repatriation General Hospital and Royal Prince Alfred Hospital (RPAH), Sydney, New South Wales, Australia. Sera of the validation cohort (Table 1 with details provided in Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/JTO/A284) were collected from patients presenting with symptoms at the respiratory clinics of either Sir Charles Gairdner Hospital or the Hollywood Specialist Centre in Perth, Western Australia (WA). The serum samples form part of the Australian Mesothelioma Tissue Bank, a member bank of the Australasian Biospecimen Network, which is supported in part by the Australian National Health and Medical Research Council and the National Centre for Asbestos-Related Diseases. Blood samples collected by routine venipuncture were stored in BD Vacutainer serum tubes (BD Bioscience). Serum samples were allowed to clot at room temperature for at least 2 hours, or at 4°C overnight, before processing. Samples were centrifuged at 1200 rpm for 10 minutes, and then the supernatant was removed, aliquoted, and stored at −80°C until assay. Written informed consent was obtained from all participants. This study was approved by the Human Research Ethics Committees of Sir Charles Gairdner Hospital and Hollywood Specialist Centre.

TABLE 1. Demographic and Pathological Characteristics of Study Patients (Plasma/Serum)

	Test Cohort		Validation Cohort	
	MM (N = 15)	Control (N = 14)	MM (N = 30)	Asbestosis (N = 10)
Median age (range)	68 (51–83)	58.5 (21–78)	72.5 (47–86)	70.5 (67–86)
Sex				
Male	13	9	30	10
Female	2	5	0	0
Histotype				
Epithelioid (%)	9 (60)	—	29 (96.66)	—
Biphasic (%)	3 (20)	—	0	—
Sarcomatoid (%)	2 (13.33)	—	0	—
Unspecified (%)	1 (0.07)	—	1 (0.33)	—

MM, malignant mesothelioma.

Tumor and Pericardial Tissue Samples

The tumor samples used in this study were part of a previously reported series of samples from MM patients who underwent extrapleural pneumonectomy between 1994 and 2009 at RPAH or Strathfield Private Hospital, Sydney, Australia.[19] The specimens were all formalin-fixed paraffin-embedded (FFPE) tumor blocks (Table 2, with details provided in Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/JTO/A284). The tumor content was marked on whole sections to guide laser-capture microdissection. This work was conducted as part of a larger study aimed at identifying prognostic factors in MM, and was approved by the Human Research Ethics Committee at Concord Repatriation General Hospital, Sydney. Normal pericardial tissue was collected from consenting patients without history of cancer, undergoing cardiac or aortic surgery at RPAH (Table 2, with details provided in Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/JTO/A284). The tissues were fixed in formalin. All pericardial samples were collected as part of a study approved by the Human Research Ethics Committee at RPAH, Sydney, Australia.

TABLE 2. Demographic and Pathological Characteristics of Study Patients (Tissue Samples)

	MM (N = 18)	Control (N = 7)
Median age (range)	55 (37–66)	68 (57–76)
Sex		
Male	14	5
Female	4	2
Histotype		
Epithelioid	15 (83.33 %)	—
Biphasic	3 (16.66 %)	—
Sarcomatoid	0	—

MM, malignant mesothelioma.

RNA Isolation

Total RNA was isolated from plasma using the mirVana PARIS miRNA isolation kit (Ambion/Applied Biosystems, Foster City, CA) according to the manufacturer's instructions for isolation of total RNA, with modifications as previously described.[18] RNA from microdissected FFPE tumor samples or formalin-fixed pericardium was isolated using the miRNeasy FFPE extraction kit (Qiagen, Valencia, CA) following the manufacturer's instructions. All RNAs were quantified using a Nanophotometer (Implen, Munich, Germany) with readings at 260 and 280 nm. RNA samples were stored at −80°C until further processing.

Microarray Analysis

The miRNA content in plasma from five patients with MM and from three healthy controls was profiled using the Human 8x15K miRNA Microarray Kit (V3, miRBase V12.0, Agilent Technologies, Santa Clara, CA). RNA (77 ng per sample) was labeled following the manufacturer's instructions, and hybridized to the array slide for 21.5 hours. After washing, the array slide was stored in N_2 gas for scanning within 24 hours. The slide was scanned at the Ramaciotti Centre for Gene Function Analysis, UNSW, Sydney, using an Agilent Microarray Scanner (Agilent Technologies, Santa Clara, CA). Data were extracted using Agilent Feature Extraction FE10.5 software and then analyzed for differential expression of miRNAs using GeneSpringGX 11.0 software (Agilent Technologies). Data processing was performed applying thresholding of the signal values to 1, followed by normalization to the 75th percentile without baseline transformation.

Real-Time RT-qPCR

Quantification of miRNA content in RNA from plasma of the test cohort was performed using stem-loop primers for reverse transcription and TaqMan primers/probes specific for each miRNA (Applied Biosystems, see Supplemental Data Table 2, Supplemental Digital Content 2, http://links.lww.com/JTO/A285, for TaqMan Assay IDs). For reverse transcription we used the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) with the following reaction conditions: 30 minutes at 16°C, followed by 30 minutes at 42°C, and 5 minutes at 85°C. For plasma samples the reaction was carried out in a total reaction volume of 5 µl with a fixed volume of 1.67 µl isolated RNA as template, whereas for FFPE samples, 100 ng total RNA was reverse transcribed in a total reaction volume of 10 µl. All complementary DNA was used immediately in qPCR reactions or stored at −20°C for use within 1 week.

After reverse transcription, as recommended by the manufacturer, the complementary DNA was further diluted by addition of 28.9 µl or 57.8 µl H_2O, respectively, to achieve a final dilution of 1:15 of the RT product. From the diluted RT product, 2.25 µl was used as template in a triplicate qPCR with a total reaction volume of 10 µl. Amplification was performed using miRNA TaqMan primers/probes specific for each miRNA together with TaqMan 2x Universal PCR MasterMix, No AmpErase UNG (Applied Biosystems) with enzyme activation for 10 minutes at 95°C followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C. No-template and no-RT samples were included as negative controls. The qPCR reactions were set up manually and run on a Stratagene Mx3000P instrument. C_q (quantification cycle) values were determined using adaptive-baseline and background-based threshold (cycle range, 5–8) using the MxPro Mx3000P v4.10 software (Stratagene/Agilent Technologies).

As we have shown previously that miR-16 levels vary little between patient and control plasma,[18] this miR was used as endogenous reference for data normalization. miR-16 Cq values ranged from 19.54 to 21.72 with a standard deviation of 0.66. Relative expression levels of candidate miRNAs were calculated using the $2^{-\Delta\Delta Cq}$ method[20] with normalization to miR-16 (plasma/serum) or RNU6B (FFPE) as endogenous control, respectively, and calculation of relative expression as compared with the average ΔCq of the respective control samples.

OpenArray Analysis

Quantification of miRNAs in serum samples from the validation cohort was performed on the OpenArray platform (Applied Biosystems) following the manufacturer's instructions. Briefly, for each sample two RT reactions with 3 µl or a maximum of 100 ng input RNA were performed using the MegaPlex RT primer pools A and B (Applied Biosystems). The RT reactions were performed on a StepONE PLUS real-time thermocycler (LifeTechnologies) for 40 cycles of 2 minutes at 16°C, 1 minute at 42°C, and 1 second at 50°C, followed by 5 minutes at 85°C. The entire RT reaction was then used to perform the preamplification reaction using the MegaPlex preamp primer Pools A and B together with MegaPlex preamp Master Mix (Applied Biosystems). The preamplification reactions were performed on the StepONE PLUS thermocycler under the following conditions: 10 minutes at 95°C, 2 minutes at 55°C, 2 minutes at 72°C, followed by 16 cycles of 15 seconds at 95°C, and 4 minutes at 60°C. Preamplification reactions were then incubated at 99.9°C for 10 minutes and cooled to 4°C. The samples were then frozen at –20°C overnight. The preamplification reactions were combined with the OpenArray real-time PCR Master Mix and loaded on to the OpenArray miRNA panel plates (Applied Biosystems) using the AccuFill autoloader. The loaded OpenArray plates were run on the OpenArray real-time PCR instrument (at the LotteryWest State Biomedical Facility Genomics, Royal Perth Hospital, WA) using the default protocol for reaction conditions.

Statistical Analysis

Differences in relative expression levels were analyzed using two-tailed Student's *t* test. Receiver operating characteristic curve of relative expression levels for hsa-miR-625-3p was plotted to assess the diagnostic value of plasma miRNAs to differentiate between patients and controls. Sensitivity and specificity were determined using the 75th percentile of the control group values (relative miRNA expression of 2.19) of the test cohort as cutoff. *p* values are unadjusted and a value of less than or equal to 0.05 was considered significant. Analyses were conducted using Predictive Analysis Software (version 8; SPSS Inc., Chicago, IL).

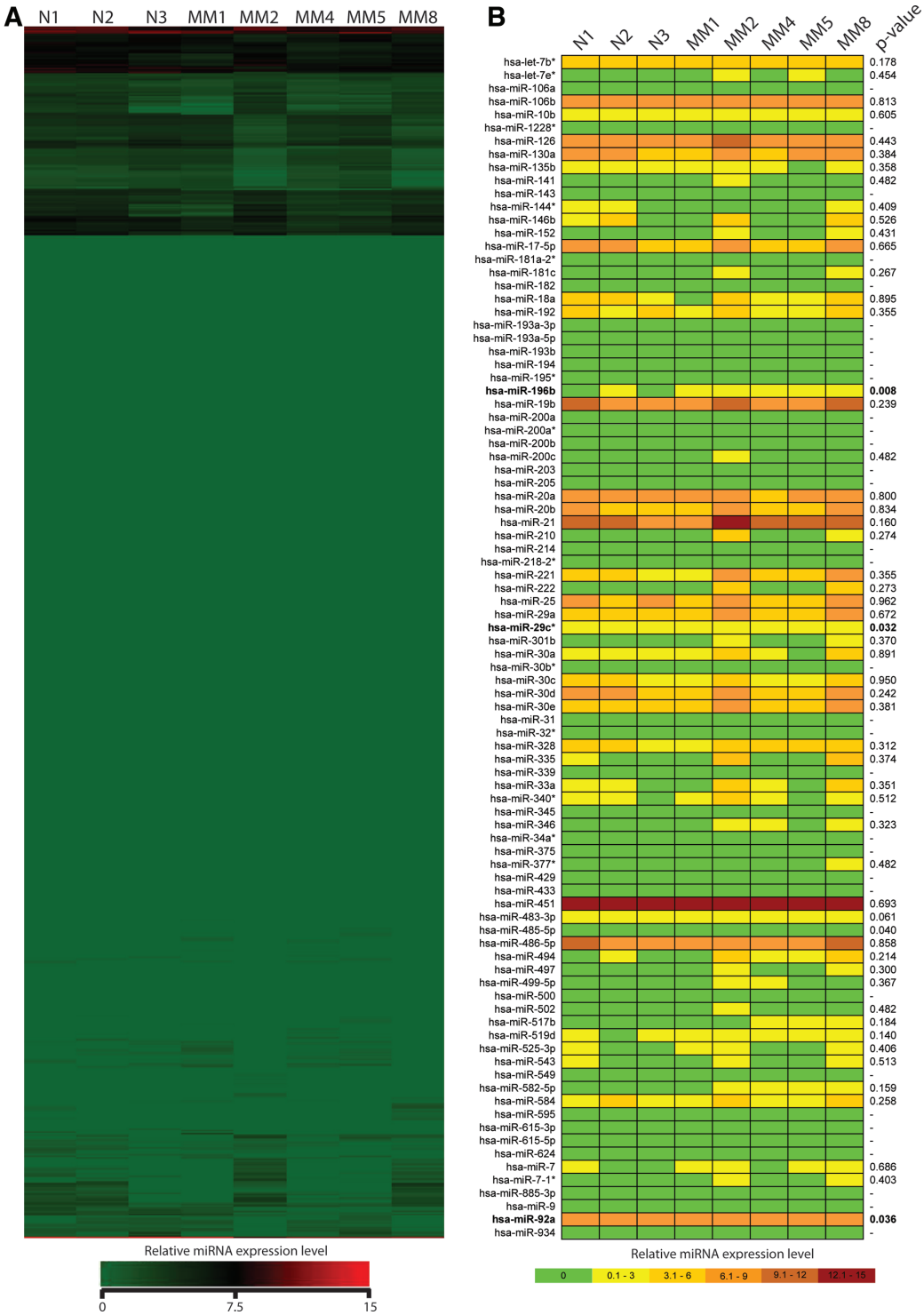

FIGURE 1. Microarray analysis of plasma from MM patients and controls. *A,* Relative expression of miRNAs in plasma from mesothelioma patients and healthy controls. Unsupervised clustering of the relative expression of 854 miRNAs detected on Agilent 8x15k microRNA microarrays in plasma from three healthy controls and five MM patients. The most abundant miRNAs detected on the array were present at similar levels in each sample. Most of the variation between samples was detected in those miRNAs present at low levels. *B,* Presence/absence of 90 MM-associated miRNAs as plotted as relative intensities per sample. Relative miRNA expression levels were divided into six categories depicting absence (green) or different expression levels (yellow = low expression and red = high expression). Those miRNAs previously associated with MM [23–30] and found at statistically significant elevated levels the plasma of mesothelioma patients in this study are highlighted in bold. Two-tailed *t* test *p* values were calculated on miR-16 normalized expression levels. MM, Maligant mesothelioma; miRNA, MicroRNAs.

RESULTS

Analysis of Plasma miRNA Profiles in MM Patients and Controls

In our search to identify new markers for MM, we used microarrays to profile the miRNA content of plasma from 5 MM patients (three epithelioid [MM one, two, and eight], two sarcomatoid [MM four and five], see Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/JTO/A284, for details) and three healthy controls (N1-3). Unsupervised hierarchical clustering (euclidian and centroid linkage) of the processed intensity values (background corrected, normalized by shift to 75th percentile without baseline transformation) confirmed previous observations that the majority of miRNA species were present at similar levels in the plasma of patients and controls, and this included the common reference miRNA miR-16 (Fig. 1*A*).[11,21,22] To compensate for differences in the quantities of miRNA isolated from plasma, we normalized the processed intensity data to the miR-16 levels in each sample, and then compared normalized levels of miRNAs of MM patients with that of controls. In this way, we identified 15 miRNAs present at significantly higher levels in patients (Table 3). We further analyzed levels of all 90 miRNAs previously reported to be associated with MM.[23–30] Of these, 24 were detected in all samples, 36 were undetectable, and the remaining 30 were detected in one to seven samples (Fig. 1*B*). Only three miRNAs previously found to be associated with MM, miR-29c* [29], miR-92a,[23] and miR-196b,[23] were significantly higher in plasma of patients when compared with controls.

RT-qPCR Validation of miRNA Candidates

To validate miRNA candidates identified in the microarray analysis, the 12 miRNAs with the most significant elevation levels in the plasma of MM patients were assessed in plasma from a series of 15 MM patients and 14 controls (Table 1) using TaqMan assay-based real-time RT-qPCR. After normalization to miR-16, the levels of miR-625-3p were 3.8-fold higher in MM patients when compared with controls (p = 0.004, Fig. 2*A*). Levels of miR-29c* and miR-92a were 1.64- and 1.25-fold higher, respectively, without reaching significance (Fig 2A, p = 0.098 and p = 0.147), whereas levels of the 9 other miRNA candidates were not different between cases and controls (miR-26a-2-3p, -196b, -454-5p, and -1914-3p, data not shown) or were below the limits of detection (miR-186-3p, -520a-3p, 548-3p, 767-3p, and -575, data not shown). Receiver operating characteristics curve analysis showed that plasma miR-625-3p levels discriminated between patient and control plasma with an accuracy of 82.4 % (area under the curve, 0.824; confidence interval, 0.669–0.979), a sensitivity of 73.33 % and a specificity of 78.57 % (Fig. 2*B*). Logistic regression was used to investigate the performance of combinations of the three miRs in terms of accuracy; however, miR-625-3p on its own provided the best and most simple classification (data not shown).

After confirmation in the test cohort, we compared miR-625-3p levels in an independent validation cohort consisting of serum from 10 patients with asbestosis and 30 with MM from WA (Table 1) using the RT-qPCR-based OpenArray platform. Data from this cohort showed that miR-625-3p level was significantly elevated in serum from MM patients compared with asbestosis patients (Fig. 2*C*, p = 0.023), with an accuracy of 79.3% (area under the curve, 0.793; confidence interval, 0.657–0.930), a sensitivity of 70%, and a specificity of 90% (Fig. 2*D*).

Analysis of miRNAs in MM Tumors

The miRNAs present at higher levels in plasma of MM patients were further analyzed in RNA isolated from 18 archival MM tumor blocks. RNA extracted from seven pericardial tissue samples served as control. For miR-625-3p, the average increase in tumor compared with normal mesothelium was 4.35-fold (p = 0.012), with a greater than twofold increase in 14 tumor samples (Fig. 3). Although the levels of the previously identified MM-associated miRNA, miR-29c*, showed a trend toward an increase in patient plasma, this miR was down-regulated twofold or more in 15 of 18 tumor specimens (average-fold decrease of –2.65; p = 0.005). Levels of miR-16, present at very similar levels in the plasma of patients and controls, were strongly down-regulated by −36.7-fold (p = 0.008) in all tumors (Fig. 3). The level of miR-92a, which tended to increase in patient plasma, was elevated in only one sample, whereas it was either unchanged or decreased (average −1.8-fold, p = 0.082) in the remaining tumors (Fig. 3). The other miRNAs tested all showed lower expression in tumors (Fig. 3), with miR-196b (−3.78-fold, p = 0.014), miR-26a-2-3p (−4.53-fold, p = 0.014), and miR-1914-3p (−2.22-fold, p = 0.021) being expressed significantly lower than in pericardium, whereas the −3.42-fold lower level of miR-575 was not significantly (p = 0.174) different from the levels in normal tissue.

TABLE 3. Significantly Different miRNAs Detected by Microarray

	N1	N2	N3	MM1	MM2	MM4	MM5	MM8	p Value
hsa-miR-186-3p	0.00	0.00	0.00	0.10	0.12	0.08	0.06	0.13	0.002
hsa-miR-520a-3p	0.05	0.04	0.04	0.14	0.16	0.16	0.21	0.11	0.002
hsa-miR-625-3p	0.22	0.22	0.21	0.25	0.27	0.27	0.28	0.24	0.002
hsa-miR-26a-2-3p	0.07	0.01	0.03	0.13	0.14	0.12	0.11	0.08	0.006
hsa-miR-196b	0.00	0.07	0.00	0.12	0.18	0.09	0.11	0.15	0.008
hsa-miR-454-5p	0.00	0.00	0.07	0.10	0.18	0.22	0.18	0.11	0.008
hsa-miR-1914-3p	0.18	0.10	0.25	0.28	0.45	0.33	0.47	0.35	0.019
hsa-miR-548c-3p	0.00	0.00	0.00	0.13	0.13	0.02	0.06	0.08	0.025
hsa-miR-767-3p	0.18	0.19	0.19	0.20	0.25	0.27	0.25	0.21	0.033
hsa-miR-575	0.31	0.32	0.32	0.34	0.48	0.37	0.47	0.49	0.034
hsa-miR-29c*	0.04	0.08	0.01	0.11	0.26	0.09	0.19	0.15	0.035
hsa-miR-92a	0.75	0.74	0.73	0.74	0.83	0.80	0.81	0.79	0.039
hsa-miR-483-5p	0.31	0.25	0.42	0.41	0.43	0.45	0.52	0.43	0.040
hsa-miR-22	0.74	0.79	0.68	0.74	0.99	0.92	0.92	0.88	0.045
hsa-miR-335-3p	0.06	0.18	0.13	0.17	0.22	0.22	0.20	0.17	0.049

*Passenger strand.

MiR-16–normalized signal intensity levels of miRNA detected on the microarray. Statistical differences between groups (N vs. MM) were calculated by two-tailed t test.

MM, malignant mesothelioma; miRNA, MicroRNAs.

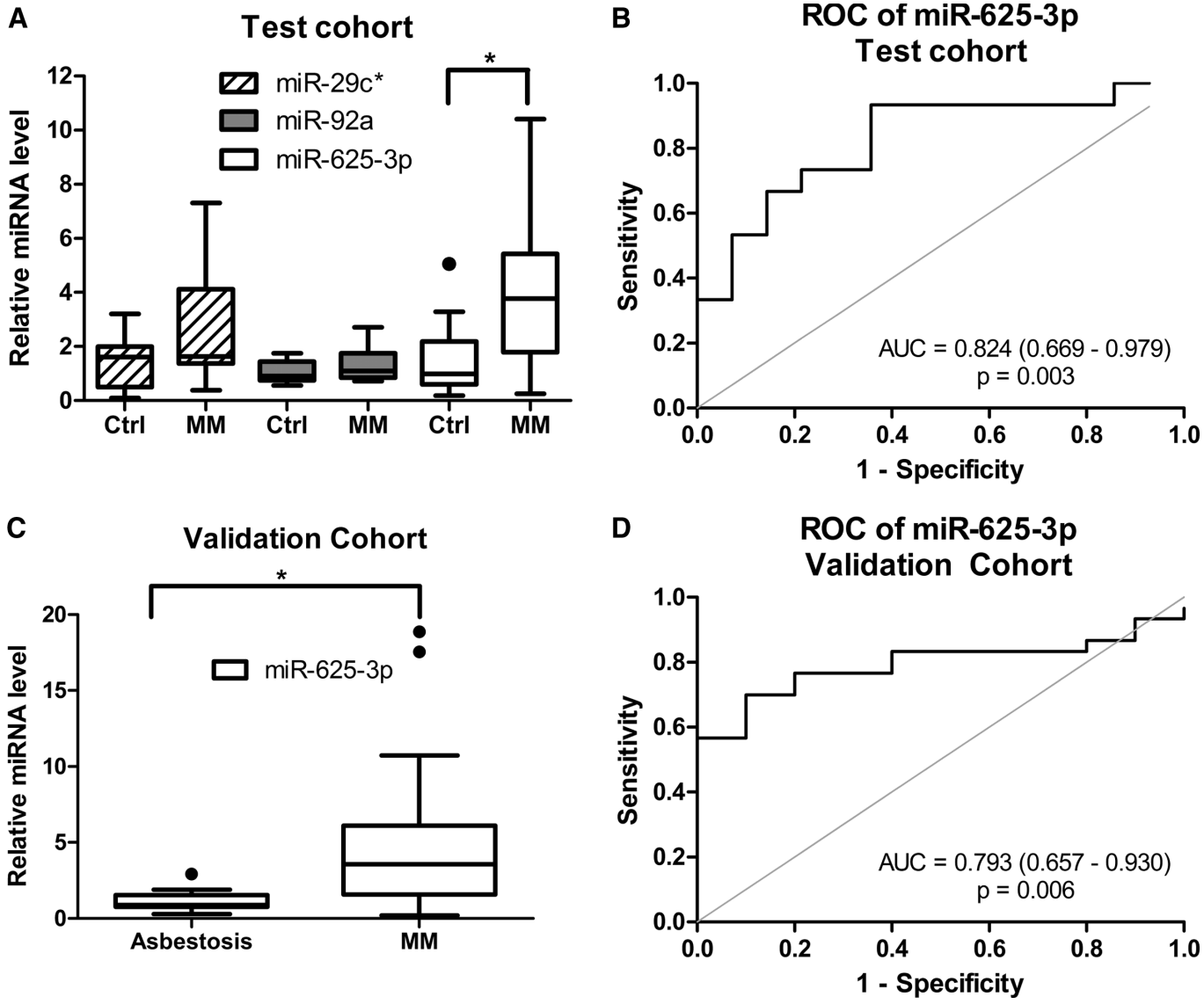

FIGURE 2. RT-qPCR validation reveals significant differences in miRNAs in plasma/serum from MM patients and controls. Levels of candidate miRNAs measured in plasma/serum were normalized to miR-16 expression, and their relative expression as compared with the average level in the control samples was calculated using the $2^{-\Delta\Delta Cq}$ method. In the New South Wales test cohort miR-625-3p level was significantly elevated in the plasma of 15 MM patients, whereas both miR-29c* and miR-92a showed a trend toward higher levels in patients without reaching statistical significance (*A*). MiR-625-3p level was also significantly elevated in 30 MM patients of the independent validation cohort (*C*). The Tukey box plots have median values represented by the line within the boxes and the lower and upper limits of the boxes denote the 25th and 75th percentile. ROC curve analyses of plasma miR-625-3p levels for discrimination of MM in the test (*B*) and validation (*D*) cohort, respectively. *$p < 0.05$ (two-tailed *t* test). qPCR, quantitative polymerase chain reaction; miRNA, MicroRNAs; MM, maligant mesothelioma; ROC, receiver operating characteristic.

DISCUSSION

Since the discovery of tumor-specific miRNAs in the plasma of leukemia patients,[13] numerous studies have linked changes in circulating miRNAs to the presence of cancer.[17] Most of these studies used miRNA profiles derived from tumors to preselect miRNAs for screening. To identify potential miRNA biomarkers for MM, and to avoid bias in our screening, we profiled the miRNA content of plasma from MM patients and compared it with that of normal healthy controls. Consistent with previous reports, almost all the miRNA species detected were common to samples from patients and controls,[11,21,22] supporting the idea that circulating miRNAs have an important function in homeostasis and are released by cells of the circulatory system.[21] Moreover, the most abundant miRNAs were the same in patients and controls, and although we detected more than 200 distinct miRNA species, just two dozen account for more than 90% of the miRNA content of plasma (Fig. 1), suggesting that any changes in circulating miRNAs are likely to be found in the rarer species.

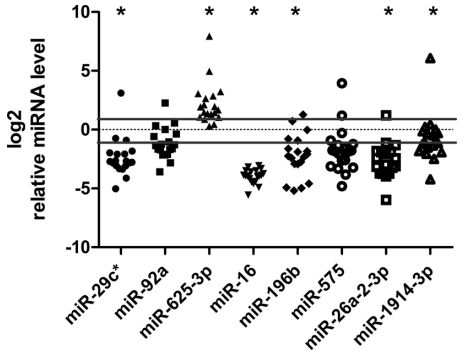

FIGURE 3. Expression of plasma miRNA candidates in MM tumor samples. Levels of miRNAs were normalized to RNU6B and are expressed relative to the levels in normal pericardial tissue as calculated using the $2^{-\Delta\Delta Cq}$ method. Solid lines indicate ± twofold changes in tumor as compared with normal control samples. *$p < 0.05$ (two-tailed t test). miRNA, MicroRNAs; MM, maligant mesothelioma.

So far, a limited number of studies have investigated miRNA expression in MM. Busacca et al.[25] compared two MM cell lines with immortalized mesothelial cells using microarrays, whereas Guled et al.[27] compared miRNA expression in different histopathological subtypes of MM with miRNAs in normal pericardium. The last approach identified a number of miRNAs related to MM and its histopathological subtypes. Most recently, miRNA profiles of MM cell lines were compared with those in normal human mesothelial cells, revealing strong up-regulation of the oncogenic miR-17-92 cluster and its paralogs.[23] When comparing these three profiling studies, almost no overlap of the specific MM-associated miRNAs was noted (Supplemental Data Table 3, Supplemental Digital Content 3, http://links.lww.com/JTO/A286) and this may well be the consequence of different profiling approaches (including normalization techniques) and different control samples.

The selection of a control population is an important consideration for studies of potential biomarkers in MM patients. Although we used healthy controls in our initial discovery phase, individuals with documented asbestos exposure are generally regarded a better control group. However, there is evidence to suggest that many people in Australia, a country with unparalleled presence of asbestos in the built environment, are either unknowingly exposed or are exposed to low-level asbestos in a nonoccupational setting.[31,32] Consequently, we assume that this phenomenon must also have translated into our control series. As even low levels of exposure to asbestos can elicit mesothelioma, it is difficult to define "healthy" subjects. Nevertheless, a formal comparison of circulating miRNA levels in MM patients with those in individuals with a documented history of asbestos exposure remains an important next step.

The use of miRNAs preselected by profiling of cell lines or tumor tissue to detect changes in plasma miRNAs is based on the assumption that changes in circulating miRNAs derive solely from changes in the expression of miRNAs in the tumor. This strategy is unlikely to identify miRNAs present at reduced levels in plasma. As the miRNA profile in serum is also thought to reflect miRNAs predominantly released by blood cells rather than tumor,[11,21] changes in miRNA levels in serum and other fluids may reflect a systemic response to the presence of a tumor, rather than simply being released by tumors. Several studies have demonstrated an association between reduced serum levels of particular miRNAs and the presence of tumor[33,34] or other conditions.[35–37] Reduced levels of miRNAs in general (and specific miRNAs in particular) have been shown in a number of tumors, including MM.[24,26,29] Although likely to have important biological consequences, low levels of (down-regulated) miRNAs are not ideal as a tumor-derived biomarker in plasma/serum.[38,39]

Reduced levels of miR-29c* were associated with poor prognosis, and loss of miR-31 expression was associated with deletion at 9p21, a frequently observed mutation in MM [40]. Re-expression of either miR-29c* or miR-31 was able to attenuate proliferation, migration, and invasion of MM in vitro, suggesting a role for these miRNAs as potential tumor-suppressor genes.[28,29]

The trend toward higher levels of miR-92a in plasma from MM patients is in line with results from studies of colorectal and ovarian carcinoma, which reported higher miR-92a in patients.[14–16] They are also consistent with findings of a recent study of miRNA expression profiles in MM, which found miR-92a and others of the miR-17-92 cluster to be significantly higher in tumor cell lines when compared with immortalized mesothelial lines.[23]

Most recently, the down-regulation of miR-126 in MM tumor biopsies has been reported.[30] In addition, it was suggested that reduced levels of miR-126 in plasma of patients were associated with elevated mesothelin levels and increased MM risk. However, we were unable to observe any difference in plasma miR-126 levels between patients and controls, and more evidence is needed to establish a causal relationship between miR-126 levels and the diagnosis of MM.

Our finding that miR-625-3p is present at significantly higher levels in plasma and serum of MM patients is the first such report of elevated levels of a circulating miRNA with potential value as a biomarker for MM. This miRNA is yet to be functionally characterized, although its complement miR-625-5p was identified in genome-wide screens.[41] Identification of putative targets with microscan, targetscan, or other algorithms has revealed multiple candidates, and we are currently determining the functional significance of miR-625-3p up-regulation in MM.

Regardless of their origin, circulating miRNAs have great potential to serve as new biomarkers.[17] In the present study, we have identified a previously uncharacterized miRNA— miR-625-3p—which is elevated in plasma or serum from MM patients and up-regulated in archival tumor samples. Taken together, these data from two independent series of patients suggest that miR-625-3p represents a promising biomarker for MM.

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