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Downregulated microRNAs in the differential diagnosis of malignant pleural mesothelioma

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Malignant pleural mesothelioma (MPM) is a rapidly fatal disease whose diagnosis, particularly through less invasive techniques such as analysis of pleural effusion, can be challenging. Currently, a commercially available diagnostic test based on microRNA (miRNA) expression patterns is purported to distinguish between mesothelioma and lung adenocarcinoma. Yet, the biological basis of this technology has not been reported in the literature, and little research has been aimed at determining how differential miRNA expression contributes to the differences in pathogenesis between these diseases, both of which can be caused by asbestos exposure. We sought to illuminate the molecular differences between mesothelioma and lung adenocarcinoma by using miRNA microarrays to identify patterns in the most differentially expressed miRNAs. From this, we identified a panel of miRNAs, including members of the miR-200 gene family, that were all downregulated in MPM compared to lung adenocarcinoma. Using the more sensitive detection method of quantitative RT-PCR on an independent series of tumors, we validated the specificity of these alterations in 100 MPMs and 32 lung adenocarcinomas. Statistical analysis reveals that these miRNAs exceed the current recommendations for biomarkers and could greatly aid in the differential diagnosis. Further examination led us to predict that they act as redundant regulators of wnt signaling and suggests a role for this pathway in tumor progression. This research points to novel approaches using miRNAs whose decreased expression is unique to mesothelioma as potentially suitable for rapid diagnosis and reveals prospective new targets for the treatment of this deadly disease.

Key words: mesothelioma, microRNA, wnt signaling, lung adenocarcinoma

Abbreviations: AUC: area under the curve; FDR: false discovery rate; miRNA/miR: microRNA; MPM: malignant pleural mesothelioma; mRNA: messenger RNA; OOB: out-of-bag; QRT-PCR: quantitative RT-PCR; ROC: receiver-operating characteristic; RF: random forest; UTR: untranslated region

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Malignant pleural mesothelioma (MPM) is an aggressive tumor that develops in the parietal pleura and is generally attributable to asbestos exposure. Although there is a long latency period between exposure to the carcinogen and disease, once detected it is rapidly fatal, with the median survival time being less than a year after diagnosis.[1] MPM can be difficult to distinguish from adenocarcinoma arising in the periphery of the lung, and epidemiological evidence suggests asbestos and smoking are shared risk factors for these diseases.[2–4] Accurate diagnosis can require multiple, time-consuming tests, and the "golden standard" in identification of MPM relies on light microscopy, in conjunction with a panel of immunohistochemical stains.[1,5–7] Investigation into the molecular differences between these 2 diseases should lead to a better understanding of the mechanistic differences as well as provide biomarkers useful in diagnosis.

Increasingly, microRNAs (miRNAs) are being recognized as key players in cancer incidence and progression, and a number of reports have suggested that they may hold significant clinical utility in cancer diagnosis and prognosis.[8,9] miRNAs are small noncoding regulatory RNA elements that can bind to messenger RNA (mRNA) transcripts and suppress

protein expression. They are formed in the nucleus from a stem-loop pre-miRNA precursor, which is then transported to the cytoplasm and processed into the 19–22 nucleotide double-stranded mature miRNA. Generally, the functional mature miRNA is loaded into the RNA-induced silencing complex, while the minor, complementary strand, referred to as the miRNA* sequence, is often degraded.[10] The miRNA can then pair with the 3′ UTR of a target gene, leading to either degradation of the mature mRNA if there is complete complementarity or with incomplete complementarity, reversible sequestering of the transcript and inhibition of protein translation.[11,12] It is clear that miRNAs play an essential regulatory role in healthy cells, and miRNA gene clustering and families ensure functional redundancy and safeguards against single mutations resulting in a dysregulation of key pathways.[13–15] By examining the relationship between miRNA expression and their targets, we should be able to predict the outcome of loss or gain of expression and be able to design novel treatments accordingly.

Currently, there is a commercially available miRNA-based diagnostic test, marketed as ProOnc Mesothelioma^{Dx} by Rosetta Genomics and distributed by Prometheus Laboratories, that claims to be a highly accurate test.[16] Yet, little work has described the basis of this test, nor have these miRNA differences been examined in regards to disease pathogenesis. Although miRNA profiles of lung adenocarcinoma have been investigated, there has not been a comprehensive analysis of how these changes distinguish adenocarcinoma of the lung from mesothelioma, and it is not clear how these miRNAs influence the progression of disease.[17]

We used a relatively large number of histologically confirmed, primary human clinical tumor samples to gain preliminary insight into the key differences in the pattern of miRNA expression between MPM and lung adenocarcinoma. We found these miRNAs can be used as highly sensitive and specific biomarkers of MPM, which suggested that their loss may act in concert to lead to disease. Using *in silico* approaches, we sought to better understand how the biology of these diseases may be differentially impacted by the expression patterns of key miRNA.

Material and Methods

Tissue ascertainment

For preliminary miRNA analysis by microarray, tissues were obtained from the CREST Biorepository of the National Cancer Research Institute, Genova, Italy. The samples consisted of pleural biopsies from lung adenocarcinoma ($n = 10$) patients and nonhistologically defined mesothelioma patients ($n = 15$).

The validation series consisted of a total of 100 mesothelioma tumor samples obtained from 2 sources: mesothelioma tumors obtained from surgical resection at Brigham and Women's Hospital from an incident case series beginning in 2005 ($n = 23$) and tumor samples obtained from the National Mesothelioma Virtual Bank ($n = 77$). These samples consisted of 32 uncharacterized mesotheliomas, 39 epithelioid mesotheliomas, 19 biphasic mesotheliomas and 10 sarcomatoid mesotheliomas. For comparison, we studied 32 lung adenocarcinoma tumor samples from the tumor bank at Rhode Island Hospital ($n = 32$). Nondiseased lung tissue samples were obtained from the National Disease Research Interchange ($n = 4$). Small RNA from nondiseased tissue was extracted, pooled and used as a relative baseline for comparison in qRT-PCR validation experiments.

RNA isolation

RNA extractions were performed using the Ambion *mir*Vana^{TM} microRNA Isolation Kit (cat # AM1561) according to the manufacturer's instructions for total RNA and small RNA-enriched fractions. RNA quality was assessed using an Agilent 2100 bioanalyzer. RNA was used immediately or aliquoted and stored at −80°C until use.

Microarray analysis

The enriched small RNA fraction obtained from the isolation was labeled using the mirVana miRNA Labeling kit (Ambion cat # AM1562) and biotin-labeled UTP (Ambion cat # AM8452). Labeling controls were RNA oligonucleotides homologous to the *C. elegans* miRNAs, cel-mir-2 and cel-lin-4. Labeled RNA was immediately processed for microarray work. Experiments were performed using custom Affymetrix gene chip miRNA microarrays (TGmirV1b520432f) provided by Tony Godfrey (University of Rochester Medical School). These arrays contain 3,231 individual sequences made up of 2,564 human, 357 mouse and 238 rat and 72 nonendogenous control miRNAs. The microarray work was carried out in the Core Facility of the Center for Genomics and Proteomics at Brown University following standard procedures used in the core for the Affymetrix platform. The resulting array data were assessed for quality using Expression Console and processed using Partek Genomics Suite software with normalization performed using the RMA procedure. Unsupervised hierarchical clustering analysis using the Euclidean distance metric and Ward linkage as parameters was carried out on unadjusted data using the statistical software program "R" (http://www.r-project.org/).

Quantitative RT-PCR

TaqMan miRNA Assays (Applied Biosystems) were used to quantify mature miRNA expression for the chosen candidate miRNAs: hsa-miR-200a* (part # 4373273), hsa-miR-200c (part # 4395411), hsa-miR-200b (part # 4395362), hsa-miR-203 (part # 4373095), hsa-miR-141 (part # 4373137), hsa-miR-429 (part # 4373203) and hsa-miR-205 (part # 4373093) and the reference noncoding RNAs RNU44 (part # 4373384) and RNU48 (part # 4373383). Five nanograms of RNA enriched for small RNAs was used as the template. All cDNA synthesis and qRT-PCR were performed according to the manufacturer's recommended conditions using the

TaqMan® MicroRNA Reverse Transcription Kit and TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems). QRT-PCR reactions were run on an Applied Biosystems 7900 Real-Time PCR System using the 384-well plate configuration. We used a nonparametric Wilcoxon signed rank test to test for differential expression between the MPM and lung adenocarcinoma samples among the 7 biologically relevant miRNAs in both qRT-PCR data sets.[18] Furthermore, we used the Benjamini and Hochberg false discovery rate to correct for multiple testing.[19]

ROC/AUC analysis

To access the individual ability of each miRNA to differentiate between tumor types, we used receiver-operating characteristic (ROC) curves and computed the area under the curve (AUC).[20] The ROC curve is a graphical plot of the sensitivity by 1-specificity as the discrimination threshold is varied, whereas AUC is a measure of the similarity of the 2 classes, where a class in the context of our problem represents the tumor type. The closer the AUC is to 1 for a particular miRNA, the better the individual ability that miRNA has for differentiating between tumor types. Asymptotic normal approximation was used to compute 95% confidence intervals for each of the AUCs to determine the range of plausible AUCs for each miRNA. Reported data are based on optimal cutoff points.

Random forest procedure

To determine how well, collectively, the 7 candidate differentially expressed miRNAs classify tumor type, we used the random forest (RF),[21] a supervised method of classification, which we implemented using the RandomForest package in R, version 4.5-25 by Liaw and Wiener. The RF classifier is essentially a collection of classification trees, where each classification tree is developed using a bootstrap sample (sampling with replacement) of the data, and on an average, about one-third of the data is not sampled [out-of-bag (OOB)]. The observations that were sampled are used as training data to develop the classification trees, where at each node of the tree, m out of the total M predictors are chosen, and the best split among these m predictors is found. The default value of m in the RandomForest R-package is $\sqrt{M}$, which we have found in many cases gives the lowest prediction error. The OOB data for each tree are used as test data to obtain an estimate of the OOB error rate, the percentage of times the RF prediction is incorrect.

Here, we used the independent qRT-PCR data as training data to develop the RF classifier and use the qRT-PCR data from the original samples as test data. As both data sets had some missing values, we used a RF method of imputing the missing data.[21] This method imputes missing data based on the proximity matrix from the RF.

Target prediction

TargetScan Human 5.1 (http://www.targetscan.org/) and TargetScan Custom 4.2 (http://www.targetscan.org/vert_42/seedmatch.html) were used to predict miRNA targets based on a 6 or 7 nucleotide sequence, and the presence of an "anchoring" adenosine in the 3′ UTRs of genes that bind to the seed sequence (nt 2–7) of the miRNAs.[22] Targets are analyzed for evolutionary conservation across vertebrates (broadly conserved), across placental mammals (conserved) or without regard to conservation (not conserved). TargetScan Human 5.1 was used to search for miRNA families that targeted known genes involved in wnt signaling, and TargetScan Custom 4.2 was used to search the seed sequence of miR-200a* for predicted human gene targets. miRNA-target interactions were then queried using the PicTar algorithm (http://pictar.mdc-berlin.de/) and MicroCosm Targets version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/). PicTar uses its own algorithm,[23] and MicroCosm uses the miRanda algorithm to identify potential miRNA-binding sites in genomic sequences.[24]

Results

Unsupervised hierarchical clustering using Euclidean distance and Ward linkage among the top 250 most variable miRNAs demonstrated imperfect clustering of mesothelioma from lung adenocarcinoma, as evidenced by the heatmap (Fig. 1*a*). This is reflective of the fact that these 2 diseases have similar etiologies and presentations and suggest a much more subtle molecular distinction. Careful analysis of the microarray results revealed the mesothelial-specific downregulation of 4 of 5 members of the miR-200 miRNA family (miR-200c, miR-200b, miR-141 and miR-429) and the downregulation of miR-200a*, the minor miRNA sequence of another miR-200 family member. We also noted reduced expression of 2 other cancer-related miRNAs, miR-203 and miR-205, in MPM compared to lung adenocarcinoma (Fig. 1*b*).

QRT-PCR measurement of miRNA isolated from the original tumor biopsies confirmed array-based measured downregulation of each of the 7 miRNAs in MPM compared to lung adenocarcinoma. We calculated average relative expression plus or minus the standard error and found the reduced expression to be statistically significant ($p \leq 0.05$) in 4 of the miR-200 family members (miR-200c, miR-200b, miR-141 and miR-429) as well as in miR-203 and miR-205 (Table 1).

To validate our findings and increase our statistical power, we investigated the expression of these 7 miRNAs in a much larger, independent data set using quantitative PCR. Consistent with array results, all tested miRNAs had significantly lower expression in MPM relative to lung adenocarcinoma ($p \leq 0.004$, Fig. 2). The average expression of miRNAs in MPM compared to lung adenocarcinoma ranged from 6-fold reduction (miR-203) to 42-fold reduction (miR-205). MiR-141 is reduced 11-fold and miR-200a* is down 9-fold. MiR-200c/200b and 429, which, based on seed sequence are all

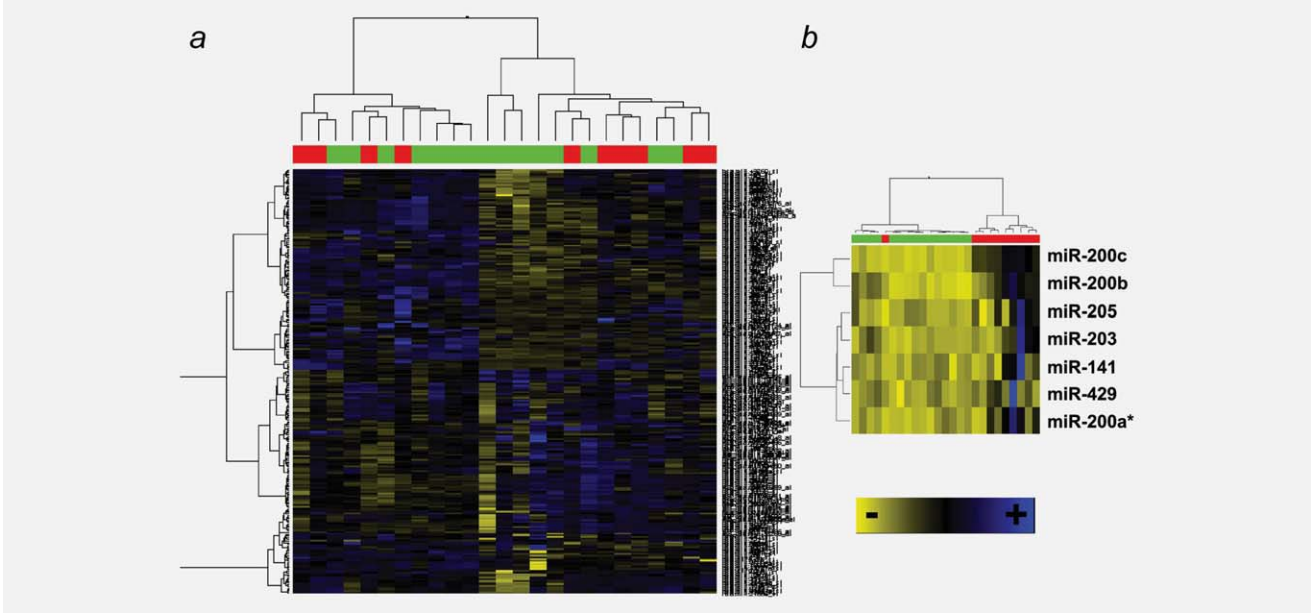

Figure 1. (*a*) Heatmap of the top 250 most variable microRNAs between 15 mesothelioma (green) and 10 lung adenocarcinoma tumors (red). Unsupervised hierarchical clustering using Euclidean distances and Ward linkage did not distinguish between the 2 tumor types well. Blue indicates increased expression and yellow indicates decreased expression. (*b*) Heatmap showing the microarray expression levels of 7 miRNAs of interest that were reduced in mesothelioma biopsies compared to lung adenocarcinoma biopsies.

Table 1. Average relative expression levels of original tumors measured by qRT-PCR

	miR-200c	miR-141	miR-200b	miR-200a*	miR-429	miR-203	miR-205
Lung adenocarcinoma relative expression	4.38 ± 2.0	2.0 ± 0.9	3.16 ± 1.3	1.5 ± 0.6	1.03 ± 0.5	1.34 ± 0.6	0.91 ± 0.2
Mesothelioma relative expression	0.07 ± 0.05	0.1 ± 0.07	0.17 ± 0.1	0.35 ± 0.2	0.02 ± 0.01	0.13 ± 0.08	0.01 ± 0.003
p-Value	0.003	0.015	0.016	0.129	0.003	0.009	0.003
Benjamini and Hochberg FDR	0.007	0.02	0.019	0.129	0.01	0.015	0.019

predicted to target the same genes, were reduced 30-, 8- and 7-fold, respectively, in MPM compared to lung adenocarcinoma. The levels of each of these miRNAs were compared among the 3 histological types of mesothelioma, epithelioid, sarcomatoid and biphasic and found not to be statistically significantly different.

Next, we wanted to evaluate the individual ability of these miRNAs to distinguish between sample types within these datasets. To achieve this, we generated ROC curves using the qRT-PCR data from the independent data set and computed the AUC with asymptotic normal 95% confidence intervals, for each of the 7 miRNAs (Fig. 3). All 7 miRNAs prove to be good discriminators of disease, but 4 of the miR-200 family member miRNAs (miR-200c, miR-141, miR-200b and miR-429) are excellent at discrimination, with AUC values greater than 0.9 (Table 2). In fact, all 7 of these miRNAs exceed the recommendation of The International Mesothelioma Interest Group for identifying markers that either the sensitivity or specificity be greater than 80%.[5]

To examine how these miRNA may interact to distinguish between MPM and lung adenocarcinoma, we developed a RF classifier using the qRT-PCR data on the independent tumor samples with all 7 miRNAs (miR-200c, miR-141, miR-200b, miR-200a* miR-429, miR-203 and miR-205). This worked extremely well, resulting in a 10% misclassification error when used to classify the qRT-PCR data from the original samples with 1 mesothelioma misclassifying as lung adenocarcinoma and 1 lung adenocarcinoma misclassifying as mesothelioma. The null value represents the expected misclassification error by randomly assigning a subject to 1 of the 2 classes (Table 3).

RF classification demonstrated that the combination of miRNAs we investigated is predictive of tumor type. This reflects a convergent biological quality of this group of miRNAs and suggests that they may target a common pathway. We hypothesized that their phenotypic action would delineate critical pathways in mesothelioma tumorigenesis. To investigate this, we used target prediction software to identify proteins predicted to be downregulated by these miRNAs. We then queried our results with 2 different predictive algorithms to find targets with higher probability of interactions (Table 4). As the miR-200 miRNA family has been shown to

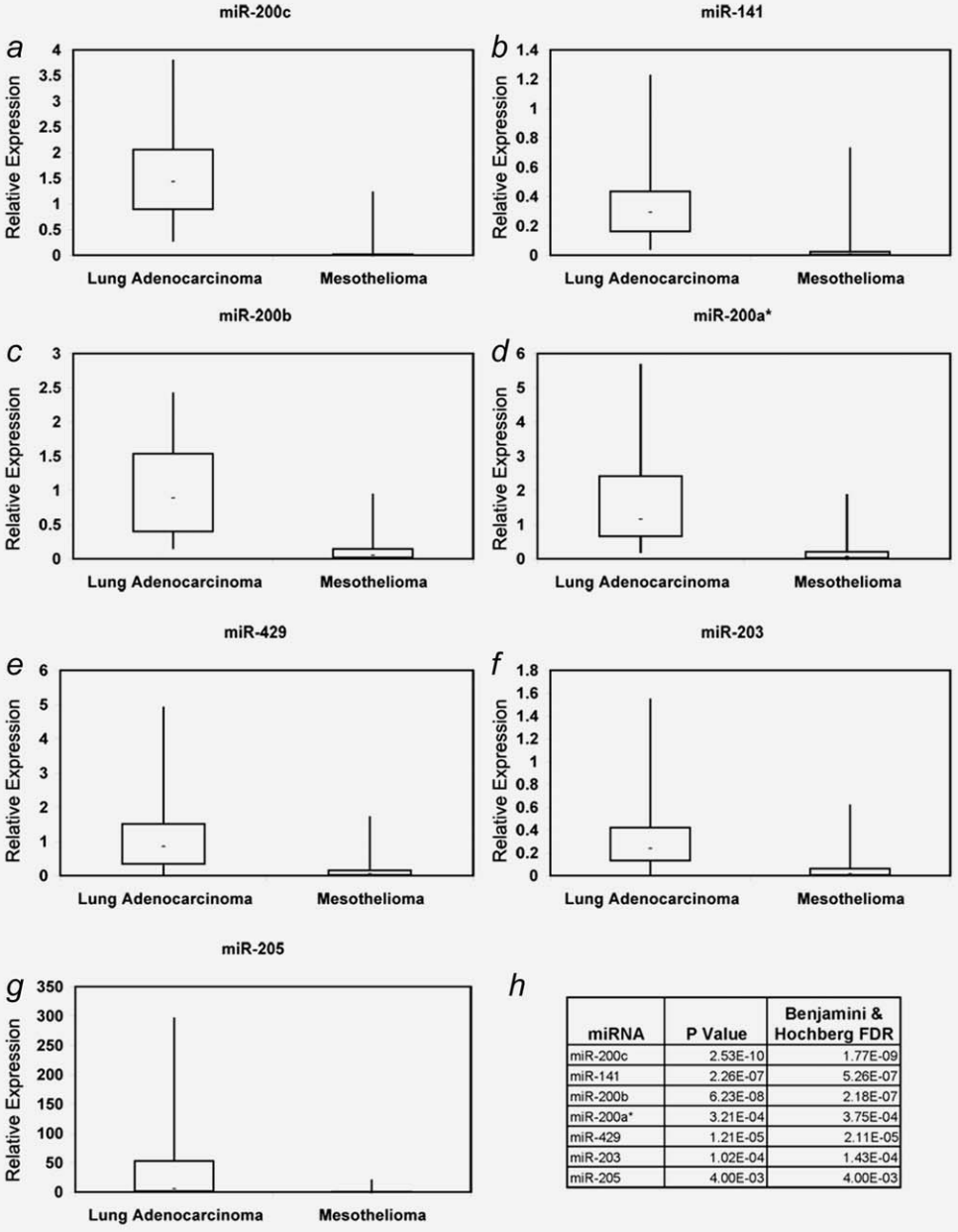

Figure 2. Quantitative PCR was used to measure the expression of miR-200c, miR-141, miR-200b, miR-200a*, miR-429, miR-203 and miR-205 in an independent sample set. We obtained and isolated miRNA from an independent sample set, which consisted of 100 mesothelioma and 32 lung adenocarcinoma fresh frozen biopsies. Data are reported with simplified boxplots and (–) represents the median value. *p* values are based on a Wilcoxon signed rank test. Benjamini and Hochberg false discovery rate was used to adjust the error rate for multiple testing. (*a*) miR-200c, (*b*) miR-141, (*c*) miR-200b, (*d*) miR-200a*, (*e*) miR-429, (*f*) miR-203, (*g*) miR-205 and (*h*) corresponding *p* value and corrected *p* values for quantitative PCR measurement of miRNA levels.

be negative regulators of wnt signaling in invertebrates,[25] we searched the 3′ UTRs of multiple proteins involved in this pathway for predicted miRNA targets. This approach, summarized in Table 4, revealed that the downregulated miRNAs are predicted to redundantly target multiple downstream modulators of the canonical wnt signaling pathway, such as *MYC* and *JUN*, as well as effectors of the noncanonical wnt pathways: the planar cell polarity pathway (*VANGL1*, *RHOA*, *ROCK2*) and the wnt/Ca^{2+} pathway (*WNT5A* and *PLCB1*).[26]

We investigated the nature of the potential miRNA-target duplexes and found the strengths of these interactions occur in varying degrees. Stronger pairing, indicated by either an additional match or an adenosine after the seed sequence at nucleotide 8, has been shown to increase the likelihood of an

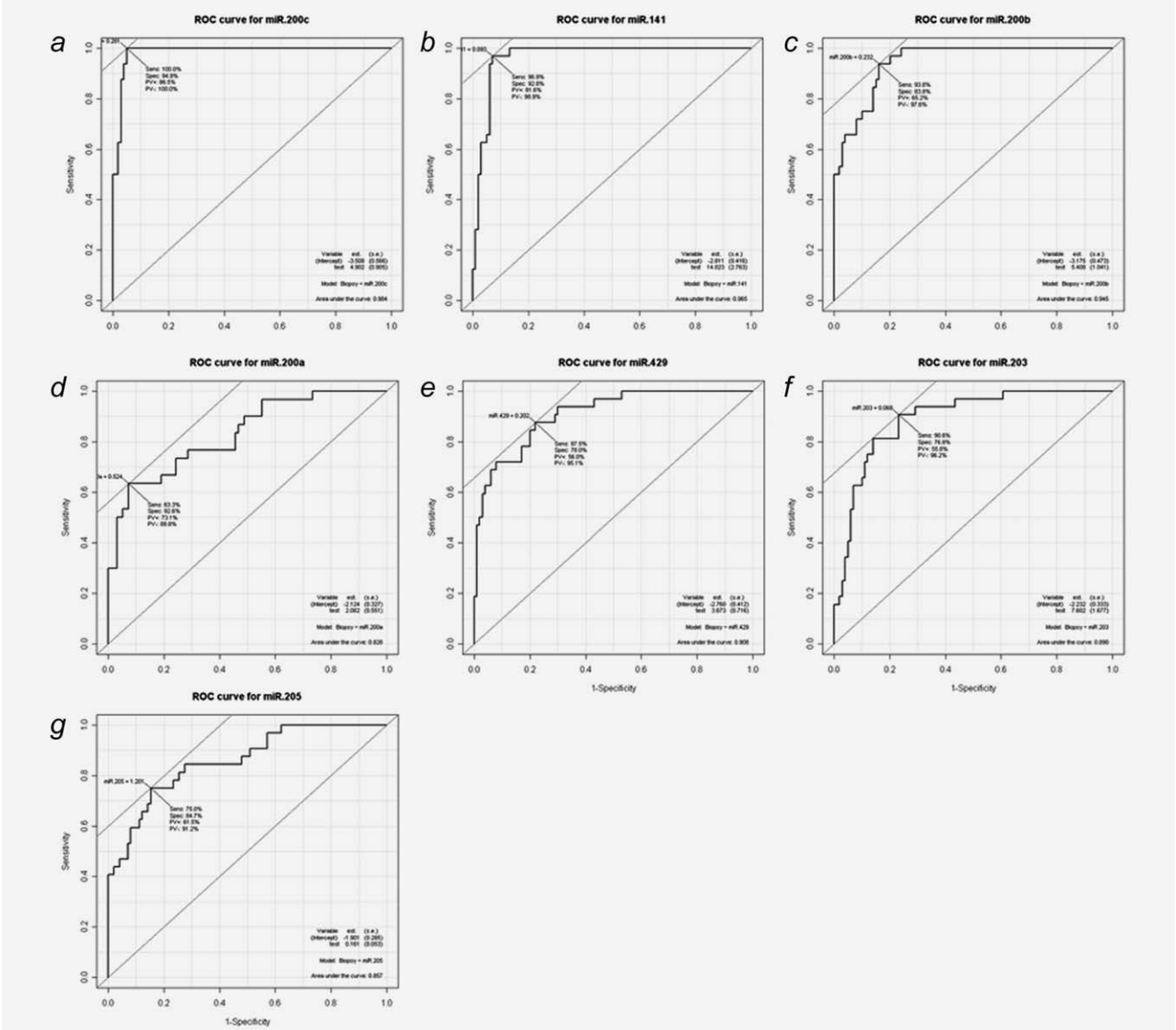

Figure 3. ROC curves were created using the quantitative PCR data from the independent data set. Asymptotic normal approximation was used to compute 95% confidence intervals. (*a*) miR-200c, (*b*) miR-141, (*c*) miR-200b, (*d*) miR-200a*, (*e*) miR-429, (*f*) miR-203 and (*g*) miR-205.

Table 2. Characteristics of ROC curves: AUC (95% CI)

miRNA	AUC	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
miR-200c	0.98 (0.97, 1.00)	100.0	94.9	85.5	100.0
miR-141	0.96 (0.94, 0,99)	96.9	92.8	81.6	98.9
miR-200b	0.95 (0.91, 0.98)	93.8	83.8	65.2	97.6
miR-200a*	0.83 (0.74, 0.92)	63.3	92.6	73.1	88.8
miR-429	0.91 (0.85, 0.96)	87.5	78.0	56.0	95.1
miR-203	0.89 (0.83, 0.95)	90.6	76.8	55.8	96.2
miR-205	0.86 (0.78, 0.93)	75.0	84.7	61.5	91.2

Table 3. Random forest classifier

Overall misclassification error	0.1
Misclassification error for mesothelioma	0.1
Misclassification error for lung adenocarcinoma	0.1
Null value	0.32

interaction. Notably, increasing degrees of complementarity in these duplexes are linked to stronger degrees of protein repression.[12,27] We also found that a number of the 3′ UTR target recognition sites are conserved in vertebrates, suggesting that they are functionally active. Most strikingly, *in silico* approaches predict that a number of wnt signaling proteins could be repressed by more than one of our miRNAs. From the predictions of 3 separate algorithms, we can reason that the loss of multiple, redundant miRNA regulators would result in aberrant wnt signaling at the level of posttranscriptional gene silencing.

Discussion

MPM is a rapidly fatal disease that is often difficult to diagnose pathologically and can be confused with other cancers, often lung adenocarcinoma. Treatment of MPM relies on a rapid diagnosis, and it would be advantageous to have specific biomarkers to differentiate it from other tumors.[6] miRNAs are being used clinically to aid in the diagnosis of this disease, although there is little experimental literature detailing the identification of differentially altered miRNA or the biological pathways predicted to be affected by miRNA expression alterations. We identified a panel of miRNAs that are specifically downregulated in MPM compared to lung adenocarcinoma. Expression of these miRNAs was not significantly different in the histological types of mesothelioma, suggesting that their downregulation is, in fact, a general characteristic of mesothelioma and distinguishes this disease from its main differential diagnosis, adenocarcinoma. Analysis of these miRNAs reveals that they can act as biomarkers, which exceed the current guidelines for the diagnosis of mesothelioma (Table 2). In fact, miR-200c has a 100% negative predictive value, indicating that detection of this miRNA is enough to rule out a diagnosis of mesothelioma. When pathological tests are inconclusive, measuring a combination of these miRNAs could lead to an accurate diagnosis, which can greatly influence the treatment and outcome of the patient.

Understanding the normal, collective role of these miRNA can inform on the consequences of their loss in mesothelioma and suggests novel targets for therapeutic intervention and disease screening, both of which are crucial to patient survival. Previous studies relied on cultured tumor cell lines to identify a panel of miRNAs, which may be associated with disease.[28] Although most research on miRNAs done in cell lines can provide clues to human cancers, because of the inherent artificiality of *in vitro* systems, conclusions drawn from experimental systems alone must be corroborated with data from human tumors. Experiments in our laboratory as well as published reports have shown that cultured cell lines are inappropriate for miRNA biomarker identification and often have no relation to the expression patterns in primary tumors.[29] Our approach using human tumors allowed us to identify a group of miRNAs whose downregulation in MPM is predictive of disease. Analysis of the overlapping targets of these miRNAs reveals the biological mechanisms through which they lead to mesothelial carcinogenesis. The gene clustering and familial pattern of these miRNAs indicated that they affect common pathways[13] and led us to predict aberrant wnt signaling as a mechanism for disease, providing potential new targets for treatment.

Experiments in simple organisms that show most miRNAs are not essential, and individually often have subtle effects on their downstream targets, provide evidence for the theory of miRNA redundancy.[14,15] Five of 7 of our miRNAs are members of the same gene family (miR-200). This family is encoded in 2 clusters on chromosomes 1 (miR-200a/b/429) and 12 (miR-200c/141) and is evolutionarily conserved. Forty-two percent of human miRNAs is encoded in clusters less than 3,000 nucleotides apart, suggesting that they are coregulated and that their expression levels can be indicative of certain diseases and syndromes.[13] These miRNAs are grouped into a family due to homology in their seed sequences and which messenger RNAs they will target. In fact, miR-200b/c/429 have identical seed sequences, indicating that they are redundant regulators of the same genes. The loss of expression of these closely related miRNAs by at least 2 separate events suggests they have redundant functions that are absent in the diseased state. By comparing overlapping targets of all of our classifying miRNAs, we predicted that absence of these miRNAs would result in the loss of multiple levels of posttranscriptional gene regulation of the wnt signaling pathways.

Our observation of the downregulation of 7 potential miRNA wnt antagonists in mesothelioma suggests that dysregulation of this pathway, through the loss of miRNA expression, is an essential process in disease progression. The mechanism for this is not known but may occur in various ways. Two of the miRNAs are encoded individually; miR-203 on chromosome 14 and miR-205 on chromosome 1, and loss of their expression seems to be unrelated. Downregulation of the miR-200 gene family may be coordinate, however, as the genes for the miRNAs miR-200c/141 are clustered on chromosome 12, whereas the genes for miR-200a/b/429 are clustered on chromosome 1. This clustering, combined with evidence that distinct methylation alterations predict MPM, indicates epigenetic silencing as a possible mechanism of repression.[30] Indeed, the expression of miR-203 from a fragile region of chromosome 14 has been shown to be modulated by loss of heterozygocity and through hypermethylation in some acute lymphoblastic leukemias.[31] It is of interest to investigate the mechanisms for downregulation of these specific miRNAs in MPM to gain further insight into the genesis

Table 4. Predicted miRNA target seed sequences in the 3′ UTRs of genes involved in wnt signaling

	miR-200b/c/429	miR-200a*	miR-200a/141	miR-203	miR-205
APC				**7mer-m8**[#]	7mer-1A
CTNNB1			**7mer-1A**[#]		
CTNNBIP1				**7mer-1A**	
DIXDC1	**8mer***		**7mer-m8***,[#]	8mer (2)	
DKK1				**7mer-m8**	
DKK2			**7mer-m8**	**7mer-1A** **7mer-m8**	
DKK3	*		**7mer-m8 (2)**		
DKK4				**7mer-1A**	
DVL3				**7mer-m8** **7mer-1A**	
EGR1				7mer-m8	
EP300	***7mer-1A**				
FBXW2	[#],*7mer-m8		**8mer*** 8mer 7mer-m8 (2)		
FBXW11	**7mer-m8**[#]		7mer-m8		
FGF4		7mer-1A*			*
FSHB			7mer-m8	7mer-m8	7mer-1A
FZD1		(*)7mer-m8		**7mer-m8**	**7mer-1A**
FZD2				**7mer-m8**	
FZD4	**7mer-m8**		**7mer-1A**	**7mer-m8** **7mer-1A (2)**	
FZD5	**7mer-1A**			**7mer-m8**	
FZD6	**7mer-1A**	*		**7mer-m8**	
FZD8			**7mer-m8**		
JUN	**8mer**		7mer-m8[#]	8mer	
LRP6		*	**7mer-m8**		**8mer** **7mer-1A**
MYC	7mer-1A*			7mer-1A	
NLK				**8mer**[#] 7mer-1A	
NKD1	7mer-1A*				**8mer**
PLCB1	[#]		7mer-m8	7mer-1A	8mer[#] 7mer-m8
PPP2CA	**7mer-m8**[#]		**7mer-m8**[#] 7mer-1A		
PPP2R5C	7mer-1A **7mer-m8**[#]		7mer-m8	7mer-1A	
PPP2R5E	**8mer*** **7mer-1A**		7mer-m8	[#]	
RHOA	**7mer-m8**[#]				
ROCK2	**8mer*** 7mer-1A 7mer-m8*				
SOX17	*		**8mer***		
TCF4	**7mer-m8** **7mer-1A**		**8mer** **7mer-m8** **7mer-1A**	**8mer (2)** **7mer-m8** **7mer-1A (2)**	**7mer-1A (2)**

Table 4. Predicted miRNA target seed sequences in the 3′ UTRs of genes involved in wnt signaling (Continued)

	miR-200b/c/429	miR-200a*	miR-200a/141	miR-203	miR-205
VANGL1	7mer-m8 (2)		8mer 7mer-1A	7mer-1A (3)	
VEGF A	**7mer-m8**			**7mer-1A**	**8mer***
WIF1	8mer*			7mer-m8	
WNT3A					**7mer-1A**
WNT4	**7mer-1A**			**7mer-m8**	
WNT5A	7mer-m8		**7mer-m8**		***,#**
WNT5B					***7mer-m8**
WNT8A				7mer-m8	
WNT16	**8mer** **7mer-m8**		**7mer-m8**		

8mer: An exact match to positions 2–8 of the mature miRNA (the seed + position 8) followed by an "A," 7mer-m8: an exact match to positions 2–8 of the mature miRNA (the seed + position 8), 7mer-1A: an exact match to positions 2–7 of the mature miRNA (the seed) followed by an "A." Sites in bold indicate that the site is broadly conserved in vertebrates. Parentheses indicate the number of same type sites within the 3′ UTR the gene. *Indicates site predicted by microcosm, and #indicates predicted by PicTar. When symbol appears after TargetScan site, both programs predict the same target.

of this disease as well as to develop novel modes of treatment.

Out of the 7 miRNAs that we describe as underexpressed in MPM compared to lung adenocarcinoma, 2 (miR-203 and miR-205) have previously been shown to be upregulated in lung adenocarcinoma compared to matched, noncancerous tissue.[17] Even higher expression of miR-205 has been suggested as a biomarker for squamous cell carcinoma, as its upregulation can distinguish this tumor from adenocarcinoma.[32] The miRNAs miR-203 and miR-429 have recently been shown to be downregulated in MPM.[33] We found a panel of miRNAs (miR-200c/141/200b/200a*/429/203/205) downregulated in MPM that we propose as biomarkers for disease and predict that these miRNAs play overlapping redundant roles in modulation of proteins associated with wnt signaling. This is significant, as it has been shown that activation of the wnt pathway, specifically the nuclear translocation of β-catenin, occurs in mesothelioma cell lines.[34] In fact, we identified multiple wnt pathway gene products that have been shown to be upregulated in MPM, including Jun, Myc, EGR1 and Wnt5B, which are predicted to modulated but our panel of miRNAs.[35] (Table 4).

The importance of wnt signaling has long been studied in development. In the canonical wnt pathway, wnt binding to its receptor leads to the stabilization of β-catenin. β-catenin can then translocate to the nucleus and interact with the transcription factors LEF/TCF (lymphoid enhancer-binding factor/T cell factor) to activate target genes.[36] In *Drosophila*, the members of the miR-8 gene family have been shown to antagonize this signaling pathway in multiple ways, including through the inhibition of TCF translation, a positive regulator of wnt signaling, as well as by targeting effectors of the pathway.[25] In human cancers, reduction in expression of the orthologous miR-200 gene family and its target E-cadherin, as well as lower miR-205 expression, is associated with metastases.[37,38] Specifically, it has been demonstrated that miR-200c can block the translation of the zinc finger transcription factor ZEB1 (TCF8/dEF1) and result in an increase in E-cadherin.[39] Other evidence indicates that decreased expression of the miR-200 gene family results in the upregulation of the E-cadherin repressors ZEB1 and ZEB2, promoting the epithelial-to-mesenchymal transition (EMT) that is indicative of malignant tumor progression.[40] This pathway was revealed to be part of a regulatory loop when it was shown that ZEB1 can directly repress miR-200c and miR-141.[41] The effect of the loss of cadherins on the wnt signaling pathway is still being investigated, but it is known that cadherin can tether β-catenin to the cellular membrane. In conjunction with a disruption in the β-catenin degradation pathway, the loss of this adhesion molecule may elevate wnt signaling by allowing translocation of β-catenin to the nucleus.[42]

Interestingly, dysregulation of non-miRNA regulators of the wnt signaling pathway has been reported in the metastases lung adenocarcinoma. In this case, disruption of the WNT/TCF signaling occurs through the transcription factors LEF1 and HOXB9 and promotes invasion and colonization of multiple organ types and is not associated with primary lung adenocarcinoma.[43] Target prediction software indicates that the miRNAs downregulated in MPM have conserved and redundant roles in the regulation of the wnt signaling pathway. Our data combined with previously published observations provide evidence that wnt signaling dysregulation in MPM occurs through the miRNAs, but aberrant wnt signaling in lung adenocarcinoma occurs through other mechanisms.

In summary, our data shown that MPM can be distinguished from lung adenocarcinoma through the downregulation of miRNAs: miR-141, miR-200a*, miR-200b, miR-200c,

miR-203, miR-205 and miR-429. The downregulation of these miRNAs is characteristic of mesothelioma regardless of histological subtype. We propose that these miRNAs can be used as biomarkers to develop an assay for detection of MPM and aid in distinguishing it from lung adenocarcinoma. In addition, analysis of the cumulative loss of these miRNAs identified aberrant regulation of the wnt signaling pathway, effected through the loss of its miRNA antagonists, as a key component of MPM. Further study of this phenomenon should lead to a better understanding of the mechanism of MPM as well as provide new strategies for the treatment of this disease.

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