CDKN2A, *NF2*, and *JUN* Are Dysregulated Among Other Genes by miRNAs in Malignant Mesothelioma—A miRNA Microarray Analysis

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Malignant mesothelioma (MM) is an aggressive cancer arising from mesothelial cells, mainly due to former asbestos exposure. Little is known about the microRNA (miRNA) expression of MM. miRNAs are small noncoding RNAs, which play an essential role in the regulation of gene expression. This study was carried out to analyze the miRNA expression profile of 17 MM samples using miRNA microarray. The analysis distinguished the overall miRNA expression profiles of tumor tissue and normal mesothelium. Differentially expressed miRNAs were found in tumor samples compared with normal sample. Twelve of them, let-7b*, miR-1228*, miR-195*, miR-30b*, miR-32*, miR-345, miR-483-3p, miR-584, miR-595, miR-615-3p, and miR-885-3p, were highly expressed whereas the remaining nine, let-7e*, miR-144*, miR-203, miR-340*, miR-34a*, miR-423, miR-582, miR-7-1*, and miR-9, were unexpressed or had severely reduced expression levels. Target genes for these miRNAs include the most frequently affected genes in MM such as *CDKN2A*, *NF2*, *JUN*, *HGF*, and *PDGFA*. Many of the miRNAs were located in chromosomal areas known to be deleted or gained in MM such as 8q24, 1p36, and 14q32. Furthermore, we could identify specific miRNAs for each histopathological subtype of MM. Regarding risk factors such as smoking status and asbestos exposure, significantly differentially expressed miRNAs were identified in smokers versus nonsmokers (miR-379, miR-301a, miR-299-3p, miR-455-3p, and miR-127-3p), but not in asbestos-exposed patients versus nonexposed ones. This could be related to the method of assessment of asbestos exposure as asbestos remains to be the main contributor to the development of MM. © 2009 Wiley-Liss, Inc.

INTRODUCTION

A history of exposure to asbestos fibers is a recognized risk factor for malignant mesothelioma (MM), lung cancer, and other non-neoplastic conditions, such as asbestosis and pleural plaques (Wagner et al., 1960; Mossman and Gee, 1989; Mossman et al., 1990, 1996). Since most patients diagnosed with these diseases have a very poor prognosis and MM shows a very poor response to conventional chemotherapy (Robinson and Lake, 2005), any new piece of information on molecular mechanisms behind the malignant transformation is of importance. Many studies focusing on diagnosis and prognosis of MM have been conducted, for example, several gene signature panels for diagnosis/prognosis have been tested for MM (Pass et al., 2004).

Recently, microRNAs (miRNAs), which are small, 19–25 nucleotides long, noncoding RNAs (Bartel, 2004), have generated strong interest in the field of cancer research due to their mutations or aberrant expression. They are currently estimated to be ~1,000, representing ~3% of the currently known genes in the human genome (Bartel, 2004). Using bioinformatic tools, Lewis et al. (2005) have estimated miRNAs to regulate as many as 30% of human genes. Most cancer-related events such as unlimited growth, potential to replicate, evasion of apoptosis, angiogenesis, and ability to metastasize involve changes in the expression of mRNA and proteins. These processes have been shown to be under the regulation of miRNAs (Ambros, 2004; Hwang and Mendell,

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TABLE 1. Clinicopathological Data of the MM Samples

Case	Histotype	Sex	Age	Asb.	Smoking	Date OP	Follow up	Tumor content (%)
1	EP-MM	F	71	Yes	None	04.12.2000	DOD 07.03.2003	70
2	BI-MM	M	72	Yes	Ex	01.07.2002	DOD 05.08.2002	70
3	SA-MM	M	71	Yes	None	01.07.2002	LFU 29.07.2002	80
4	BI-MM	M	63	Yes	None	05.07.2002	DOD 08.03.2003	80
5	BI-MM	M	64	No	None	09.09.2002	LFU 30.10.2006	70
6	EP-MM	M	67	No	Ex	19.09.2002	LFU 31.01.2003	60
7	BI-MM	M	57	Yes	Ex	24.09.2002	DOD 11.01.2003	50
8	EP-MM	M	57	No	Ex	26.11.2002	LFU 31.01.2003	60
9	EP-MM	M	76	Yes	Ex	27.11.2002	DOD 28.11.2002	70
10	EP-MM	M	70	Yes	Ex	13.05.2003	DOD 10.03.2004	90
11	EP-MM	F	56	Yes	None	30.03.2004	DOD 03.09.2004	60
12	DE-MM	M	79	No	Ex	18.10.2001	LFU 23.10.2002	30
13	EP-MM	F	61	Yes	None	01.09.2004	LFU 10.02.2006	70
14	EP-MM	M	71	Yes	Ex	01.09.2004	DOD 04.10.2005	30
15	EP-MM	M	51	Yes	Yes	23.11.2004	LFU 21.12.2004	40
16	EP-MM	F	62	Yes	Ex	23.02.2005	LFU 10.11.2005	60
17	BI-MM	M	57	Yes	None	12.04.2005	DOD 17.09.2005	70

EP, epitheioid; BI, biphasic; SA, sarcomatoid; DE, deciduoid; DOD, dead of disease; LFU, last follow up; OP, operation; Ex, used to smoke.

2006; Fabbri et al., 2008). Significantly, they have also been shown to be present in cancer-associated genomic regions (Calin et al., 2004).

Calin et al. (2002) were the first to show the involvement of two miRNAs, miR-15 and miR-16, in the tumorigenesis of chronic lymphocytic leukemia in 2002. Following this landmark finding, miRNA expression analyses have been carried out in various cancers. In two large-scale miRNA expression analysis studies, Lu et al. (2005) and Volino et al. (2006) concluded that most tumors have miRNA expression signature relating to their pathogenesis. In addition to these, many more studies on individual cancers have reported dysregulation of miRNA expression, including breast cancer (Iorio et al., 2005), colon cancer (Michael et al., 2003), lung cancer (Yanaihara et al., 2006), esophageal cancer (Feber et al., 2008), and serous ovarian cancer (Nam et al., 2008).

It is, therefore, vital to recognize the differentially expressed miRNAs between normal and tumor cells to facilitate the identification of the miRNAs critical for the initiation, progression, and metastases of cancer. The current study presents the analysis of miRNA expression profile of MM, which revealed MM-specific miRNA expression profile.

MATERIALS AND METHODS

Specimens

The fresh frozen specimens used in this study were obtained from patients with MM at the Royal Brompton and Harefield NHS Trust. A histological diagnosis of MM was confirmed by immunohistochemistry using a standard panel of antibodies consisting of calretinin, cyotokeratin 5/6, BerEP4, and CEA (Cury et al., 2000; Addis and Roche, 2009) and the tumor content of each sample was determined by a pathologist at the University Hospital of Helsinki. Normal human pericardium total RNA (Ambion, Austin, TX) was used as a control. Table 1 shows the clinical characteristics of the patients with MM.

Based on the tumor content, ranging between 30 and 90%, 17 samples were chosen for the study. These represented all of the described MM subtypes: 11 epithelioid (including one case of deciduoid variant), 5 biphasic, and 1 sarcomatoid.

RNA Extraction

Total RNA, including miRNA, was extracted using Qiagen's miRNeasy mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The miRNA microarray experiments were performed using Agilent's miRNA microarray system (V2), which contains 723 human and 76 human viral miRNAs catalogued in the Sanger miRNA database v 10 (Agilent technologies, Foster City, CA). Nanodrop-1000 spectrophotometer (Thermo scientific, Wilmington, DE) was used for the quantification of RNA, while quality check on both RNA and miRNA was performed by Agilent bioanalyzer (Agilent Technologies). The RNA 6000 chip was used for total RNA characterization. Total RNA integrity assessment was based on resulting electropherogram, which typically shows two peaks representing the 18S

and 28S ribosomal RNA. Small RNA assay was similarly used for the assessment of miRNA presence. The resulting electropherogram shows many peaks representing small RNAs ranging 10 to 150 nt. The presence of a peak between 10 to 50 nt is an indication of miRNA presence.

Labeling, Hybridization of miRNA, Scanning, and Data Processing

The miRNA expression analysis was carried out using Agilent's microarray-based miRNA platform, which has been shown to produce precise and accurate measurements of miRNA levels (Wang et al., 2007). Agilent's miRNA complete labeling and hybridization kit (Agilent Technologies) was used for labeling and hybridization of the RNA samples. One hundred nanograms of total RNA were treated with calf intestine phosphatase for 30 min at 37°C. 100% DMSO was used for denaturation at 100°C for 5 min, after which the samples were immediately transferred into an ice water bath for the prevention of reannealing. The samples were then labeled with cyanine 3-pCp by incubating with T4 RNA ligase for 2 hr at 16°C. After the labeling reaction, the samples were vacuum dried at medium heat.

According to the manufacturer's instruction, the dried samples were resuspended in nuclease-free water before hybridization. A blocking agent and a hybridization buffer provided by the manufacturer were added to the resuspended samples. These were then hybridized to Agilent human miRNA microarrays (V2) on Agilent SureHyb chambers (Agilent Technologies) for 20 hr at 55°C. The microarrays were then washed with Agilent's prepared washing buffers. Agilent scanner (Agilent Technologies) was used for scanning while Agilent's Feature Extraction software was used to obtain the data.

Agilent's Genespring GX software version 9.0.5 was used for data analysis (Agilent Technologies). The statistical analysis was carried out using the statistical programming language R (http://www.r-project.org). Default parameter settings of the Agilent Feature extraction software (Agilent Technologies) were used in the analysis. In addition to the miRNA expression signal, so-called detected/undetected call is given by the software. The array analysis pipeline provides total gene signal and total gene signal error for each miRNA. If the signal exceeds the estimated measurement noise at least threefold, the gene is considered as detected. Details of the preprocessing protocol are provided in the manufacturer's reference guide for Agilent Feature Extraction version 9.5 (under miRNA analysis). The undetected miRNAs are regarded as absent in the measured sample.

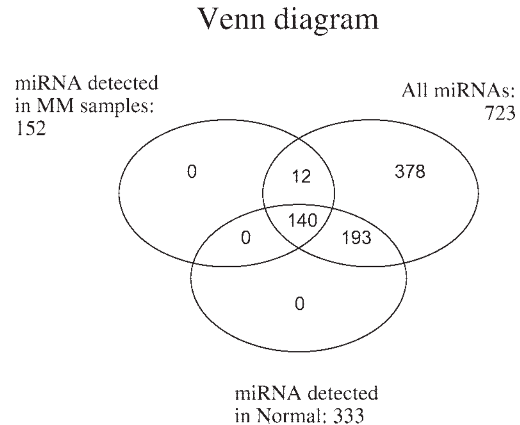

Figure 1. Venn diagram showing the miRNAs detected in tumor and control samples. Three hundred and thirty-three miRNAs were detected in control sample, 140 of them were also detected in tumor samples. In addition to these, 12 miRNAs were exclusively detected in the tumor samples, taking the total miRNAs detected in tumor samples to 152.

The expression values were log-transformed before the analysis. miRNAs that were not detected in any of the samples were excluded from the comparisons. Significance of differential expression between two groups of samples was computed with the *t*-test using the detected signals. miRNAs that were detected in only one of the groups, or had at least twofold expression change between the groups, are considered differentially expressed.

RESULTS

miRNA Expression Profile Differences Between Normal and Tumor Samples

Clear differences in miRNA expression profiles of MM tumor samples and the normal control sample were identified. Significantly, more miRNAs were expressed in the control sample than in MM samples. In the control sample, 333 miRNAs out of 723 human ones were detected, whereas the number of miRNAs for MM samples was 152. One hundred forty of these were also detected in the control sample, suggesting mesothelial tissue specificity (Fig. 1). The remaining 12 miRNAs were expressed exclusively in the MM samples. These are promising candidates for miRNAs with oncogenic function. Some miRNAs detected in the control were also detected in one

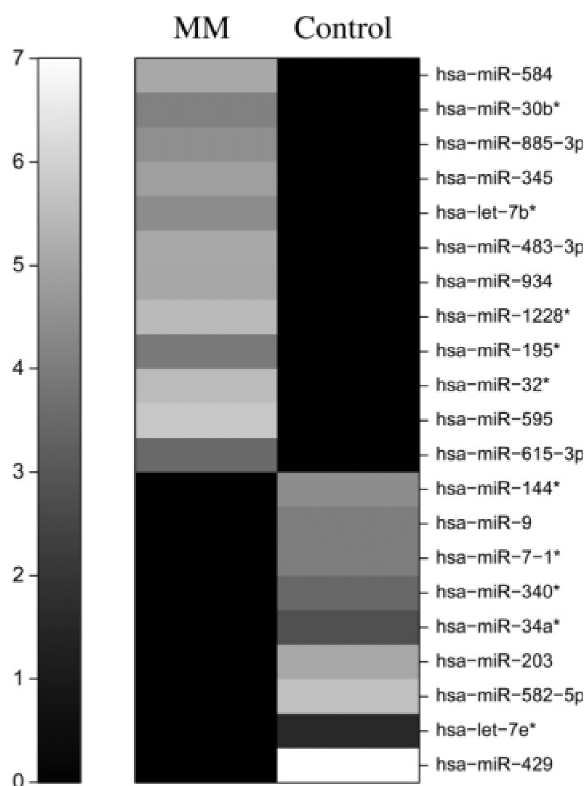

Figure 2. Mean expression levels of tumor-associated miRNAs in tumor (MM) and control samples. The miRNAs were ordered using hierarchical clustering. The 12 miRNAs that were detected exclusively in the tumor sample show increased expression in the tumors, whereas the nine miRNAs exclusively detected in the controls are expressed only in the normal tissue.

or more individual tumor samples and absent in others. However, there were nine miRNAs that were exclusively detected in the control sample and not in any of the tumor samples.

The relationship between the expression level of the tumor-specific miRNAs between control and patients with MM are illustrated with a heatmap in Figure 2. The miRNAs were ordered using hierarchical clustering algorithm in GeneSpring with the default parameters.

miRNA Expression Profile Differences Between Histopathological Subtypes of MM

MM is divided into three subtypes according to their histopathological appearance (Travis et al., 2004), and we additionally looked at miRNA expression profile in relation to these groups. Specific miRNAs were revealed for each subtype, seven for epithelioid, five for biphasic, and three for sarcomatoid. These miRNAs were exclusively detected in their respective histopathological subtypes. These cases were analyzed only in regard to other subtypes and not against the control sample.

Correlation-based analysis revealed remarkably similar miRNA expression profiles for the two most commonly occurring subtypes, epithelioid and biphasic (Fig. 3, Table 2). The sarcomatoid subtype displayed a distinct profile. However, the

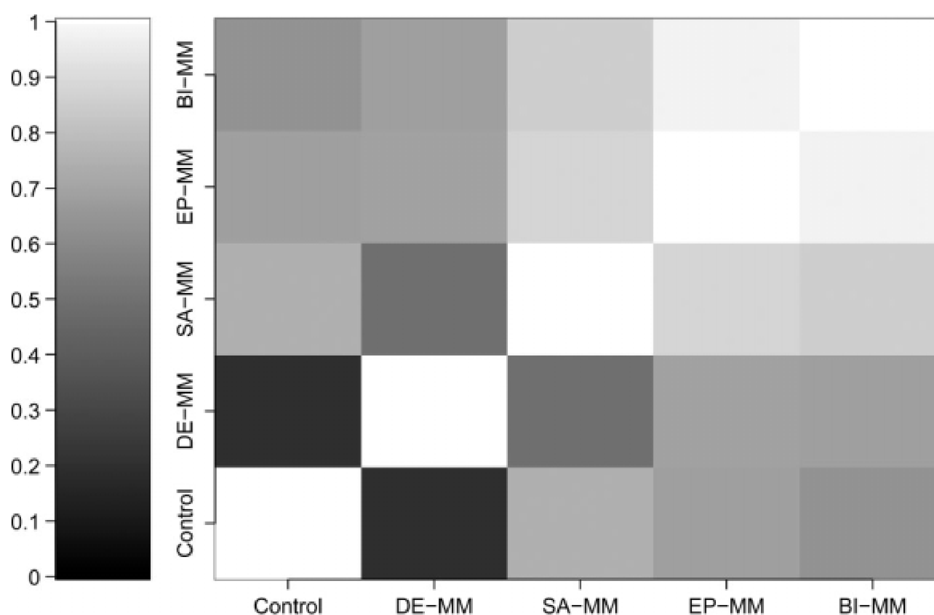

Figure 3. Pearson correlations of the mean miRNA expression levels for the five patient groups, i.e., four histological subtypes of MM and the control sample. Correlations from the pair-wise comparisons are visualized in grayscale (black: no correlation; white: perfect correlation); the actual Pearson correlation values are provided in Table 2. Epithelioid and biphasic profiles have the highest correlation. Desiduoid is a variant of epitheliod and its profile is most closely correlated with that of epithelioid.

TABLE 2. The Actual Pearson Correlation Values of the Mean miRNA Expression Levels for the Five Patient Groups, i.e., Four Histological Subtypes of MM and the Control Sample

	Control	DE-MM	SA-MM	EP-MM	BI-MM
Control	1	0.19	0.73	0.67	0.62
DE-MM	0.19	1	0.48	0.68	0.67
SA-MM	0.73	0.48	1	0.86	0.84
EP-MM	0.67	0.68	0.86	1	0.96
BI-MM	0.62	0.67	0.84	0.96	1

statistical significance of the observations cannot be evaluated in the absence of replicated measurements for this subtype. The deciduoid variant profile was included here separately to explore the correlation with other subtypes; although this case had the lowest tumor content and no replicated measurement, it expectedly correlated closest with epithelioid subtype. Table 3 shows the miRNAs specific for each subtype.

Asbestos Exposure, Smoking Status, Survival Rate, and miRNA Expression

We further analyzed the miRNA expression profile of MM for differentially expressed miRNAs in smokers (including former smokers) versus nonsmokers and asbestos exposed versus nonexposed since, although histopathology subtype and stage are the most used predictors of survival in MM, these other factors have also been proposed as significant predictors of survival (Flores et al., 2007).

If statistically significant differences in the expression levels between two groups of samples exist, the distribution of P values in the comparison is skewed toward small P values (Storey and Tibshirani, 2003). Moreover, the distribution of P values can be used to estimate the q-value, i.e., expected false discovery rate among the most significant findings. No significant differences were observed in asbestos exposed and nonexposed tumor samples, based on asbestos exposure history (Fig. 4A; $q > 0.85$). However, statistically significant differences were observed between smokers and nonsmokers with unexpectedly many miRNAs with small P values in the comparison (Fig. 4B). The most differentially expressed miRNAs ($P < 0.0005$; $q < 0.05$) are listed in Table 4. Furthermore, the smoking status correlated with the survival rate as the nonsmokers were among the longest survivors. The last follow-up data for the only active smoker is only a month from the date of operation. Also the survival rate for asbestos exposed and nonexposed patients were compared, but no significant differences could be detected (data not shown). Larger sets of samples are needed to detect significant differences in survival for diseases with such poor prognosis as MM. The miRNA expression profiles were compared for those who survived over a year versus those confirmed dead of disease within a year.

TABLE 3. Histopathologic Subtype-Specific miRNA

EP-MM	BI-MM	SA-MM
miR-135b	miR-218-2*	miR-301b
miR-181a-2*	miR-346	miR-433
miR-499-5p	miR-377*	miR-543
miR-517b	miR-485-5p	
miR-519d	miR-525-3p	
miR-615-5p		
miR-624		

DISCUSSION

Currently, very little is known about the miRNA expression in MM. In this study, 17 MM samples were analyzed using miRNA microarray to explore miRNA expression profile in tumor tissue compared with a normal mesothelial tissue. The results clearly distinguished the tumor profile from the normal tissue profile, which also displayed higher miRNA expression than the tumor tissues. This is in accordance with previous findings by Lu et al. (2005) who observed in a large set of cancer samples, including eight epithelioid MM, using a bead-based method, a general down-regulation of miRNAs in tumors compared with normal samples.

Our analysis identified 21 miRNAs which were differentially expressed in the tumor samples. Twelve of them, let-7b*, miR-1228*, miR-195*, miR-30b*, miR-32*, miR-345, miR-483-3p, miR-584, miR-595, miR-615-3p, and miR-885-3p, were highly expressed in tumor samples, whereas the remaining nine, let-7e*, miR-144*, miR-203, miR-340*, miR-34a*, miR-423, miR-582, miR-7-1*, and miR-9, were not expressed. Interestingly, miR-203 and miR-9 were also found to be underexpressed by the study of Lu et al. (2005). They performed miRNA expression profiling on a panel of multiple cancer samples including eight epithelioid MM samples. Altogether, the results suggest that the miRNAs identified in the study could potentially have oncogenic or tumor-suppressive function.

Only a few of the miRNAs showing altered expression in our analysis have been reported to

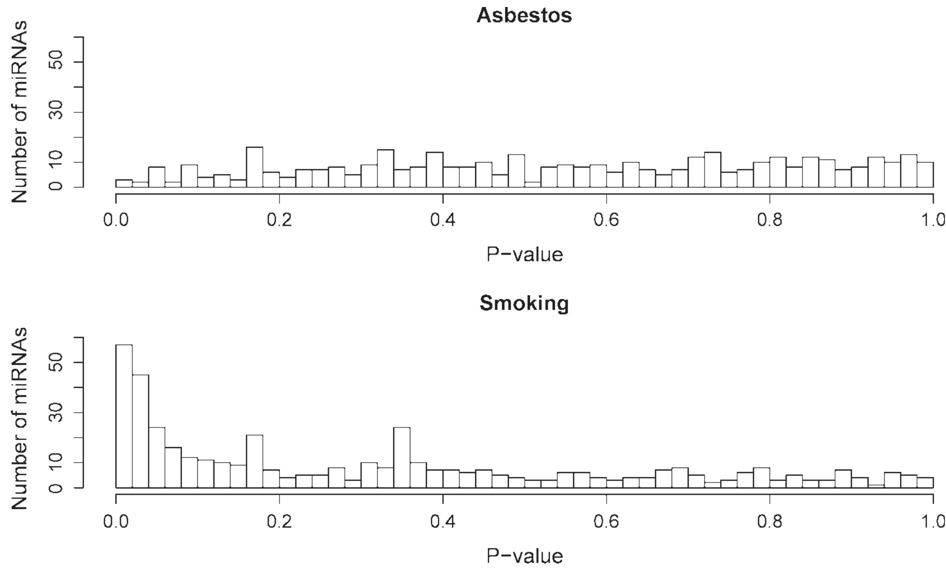

Figure 4. Histogram of *P* values for differential miRNA expression between (A) asbestos exposed versus nonexposed patients and (B) (ex-)smokers versus nonsmokers (See Table 4). The latter comparison reveals unexpectedly many miRNAs with small *P* values, indicating that smoking causes statistically significant changes in miRNA expression. In contrast, no significant changes in miRNA expression are seen in asbestos exposure.

be deregulated in other cancers, a finding which further confirms the specific miRNA signature of MM. Some individual miRNAs could be shared by miRNA signatures of different cancers, but, due to the tissue specificity, cancers of different cellular origins may show different miRNA sets (Volinia et al., 2006).

To evaluate the potential role of the differentially expressed miRNAs in MM, we screened their target genes predicted by web-based computational approaches, Sanger miRBase (www.microrna.sanger.ac.uk/targets/v5/, Griffiths-Jones et al., 2008) and miRanda (www.microRNA.org) for known MM-associated genes including the tumor suppressor genes (TSGs) cyclin-dependent kinase inhibitor 2A (*CDKN2A*, GeneID: 1029) and neurofibromin 2 (*NF2*, GeneID: 4771) and oncogenes hepatocyte growth factor (*HGF*, GeneID: 3082), platelet-derived growth factor (*PDGF*, GeneID: 5154), epidermal growth factor (*EGF*, GeneID: 1950), and jun oncogene (*JUN*, GeneID: 3725). The predicted gene targets for those miRNAs up-regulated in MM samples are expected to be TSGs. Down-regulated miRNA target genes are likely to include oncogenes (Fabbri et al., 2008). Table 5 represents miRNAs, their chromosomal locations, relevant target genes in MM, and their respective chromosomal locations.

CDKN2A, the most commonly affected TSG in MM, inactivated in more than 80% of MMs (Lindholm et al., 2007; Taniguchi et al., 2007; Ivanov et al., 2008), is the putative target of miR-885-3p. Interestingly, the same miRNA also targets the other main TSG in MM, *NF2* (Sekido et al., 1995). This gene has been associated with invasiveness of MM cells (Poulikakos et al., 2006). Although mutations in the *RB1* gene are rare in MM, it is among the targets of miR-30b*, miR-32b*, miR-483-3p, and miR-584. *RB1* mutations are frequently found in other malignancies and, *RB1* is part of the same pathway as *CDKN2A* and, therefore, its noninvolvement in MM has been surprising. One explanation suggested is that due to deletions of *CDKN2A* (and *CDKN2B*) even if *RB1* remains in its wild-type form, it would not function properly (Toyooka et al., 2008).

TABLE 4. Differentially Expressed miRNAs ($P < 0.0005$) in Smokers Versus Nonsmokers Comparison

miRNA with $P < 0.0005$
miR-379
miR-301a
miR-299-3p
miR-455-3p

As for oncogenes, the *JUN* proto-oncogene, reported to be involved in MM by Tanaguchi et al. (2007) is the target of two down-regulated miRNAs, miR-9 and miR-203. This transcriptional factor has a role in cell division, proliferation, and transformation (Angel and Karin, 1991). Several growth factors have been implicated in

TABLE 5. (A) Highly Expressed miRNAs in MM Samples and (B) Unexpressed miRNAs in MM Samples

miRNA Name	Chr. Location	Target gene	Chr. Location
(A) hsa-let-7b*	22q13.31		
hsa-miR-1228*			
hsa-miR-195*	17p13.1		
hsa-miR-30b*	8q24.22	*RB1*	13q14.2
hsa-miR-32*	9q31.3		
hsa-miR-345	14q32.2		
hsa-miR-483-3p	11p15.5	*RB1*	13q14.2
hsa-miR-584	5q33.1	*RB1*	13q14.2
hsa-miR-595	7q36.3		
hsa-miR-615-3p	12		
hsa-miR-885-3p	3p25.3	*CDKN2A*	9p21.3
		NF2	22q12.2
hsa-miR-934	Xq26.3		
(B) hsa-let-7e*	19p13.33		
hsa-miR-144*	17q11.2		
hsa-miR-203	14q32.33	*JUN*	1p32
		HGF	7q21.2
hsa-miR-340*	5q35.3		
hsa-miR-34a*	1p36.22		
hsa-miR-429	1p36.33		
hsa-miR-582-5p	5q12.1		
hsa-miR-7-1*	9q21.32	*EGF*	4q25
		PDGFA	7p22
hsa-miR-9	1q22	*JUN*	1p32

The chromosomal locations of the miRNAs and their target genes indicated. Target gene prediction based on Sanger miRBase and miRanda database.

MM. miR-7-1* targets include *PDGF*. Its A and B chains are thought to be involved in early response to asbestos and increased tumor incidence, and decreased latency period has been associated with the expression of *PDGFA* (Metheny-Barlow et al., 2001). *EGF* is also targeted by the same miRNA, miR-7-1*. Another growth factor implicated in MM, *HGF*, is the target of miR-203 which has been associated with differential cell growth (Jagadeeswaran et al., 2006).

miRNAs have also been shown, by themselves, to be capable of acting as TSGs, oncogenes, or both (Fabbri et al., 2007). In such cases, TSG miRNAs can be inactivated by mutations, deletions, or promoter methylation, and oncogene miRNA can be overexpressed by chromosomal translocations and genomic amplifications (Fabbri et al., 2008). Therefore, we investigated possible correlations between the chromosomal locations of the differentially expressed miRNAs and frequently imbalanced chromosomal regions in MM. miR-30b* is the only potential oncogene that is located at the frequently gained region, at 8q24 (Kivipensas et al., 1996; Taniguchi et al., 2007). As for potential TSGs, miR-34* and miR-429 are located at 1p36 while miR-203 resides at 14q32, which are all well documented to be frequently deleted (Tiainen et al., 1988; Kivipensas et al., 1996; Bjorkqvist et al., 1997; Krismann et al., 2002; Lindholm et al., 2007; Taniguchi et al., 2007). Loss of 14q is common in MM (Balsara et al., 1999; Bjorkqvist et al., 1999; De Rienzo et al., 2000) and as no target gene has yet been identified, one can speculate that miR-203 is that target. As a consequence of miR-203 deletion, its target gene *JUN* oncogene can carry on its oncogenetic properties without control.

Exposure to asbestos has been shown to be one of the main contributors to the development of MM (Mossman and Gee, 1989; Carbone et al., 2002). Recent studies have correlated asbestos exposure with shorter survival in patients with MM (Flores et al., 2007; Christensen et al., 2008), which could suggest differences in differential gene and miRNA signatures between asbestos exposed versus nonexposed patients with MM. However, the analysis made in this study did not reveal any significant differences in miRNA expression between asbestos exposed and nonexposed patients (Fig. 4A). However, exposure was based on the patient's own knowledge, and some nonexposed patients may not have been aware of a potential former exposure to asbestos.

Differentially expressed miRNAs were detected when compared smokers to nonsmokers (Fig. 4B, Table 4). In a large study conducted by Flores et al. (2007), lack of smoking history was found to result in better prognosis. In lung cancer, where smoking is the major cause, some studies have reported smoking-related genomic signatures (Landi et al., 2008; Massion et al., 2008). These comprised mainly genes that regulate chromosomal segregation and mitotic spindle formation. Among them are minichromosome maintenance complex component 2 (*MCM2* GeneID: 4171) and TTK protein kinase (*TTK* GeneID: 7272), which are targets of differentially expressed miRNAs, miR-299-3p and miR-455-3p, respectively.

Finally, our results provide evidence that miRNA expression distinguishes not only tumor tissue from normal tissue but also reveal specific miRNAs for histopathological subtypes of MM. Because the sarcomatoid subtype only comprised one sample, its results must be viewed as preliminary and, therefore, additional investigations are required. Table 3 summarizes the specific miRNAs expressed by each subtype. Also, the

biphasic subtype, which contains at least 10% of both epithelioid and sarcomatoid features (Travis et al., 2004), showed expression pattern that was more closely related to the epithelioid type. This is consistent with survival data which indicate that the biphasic subtype has survival rates closer to the ones of epithelioid subtype, which has the longest survival.

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