Genome-Wide miRNA Expression Profiling Identifies miR-9-3 and miR-193a as Targets for DNA Methylation in Non–Small Cell Lung Cancers

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Abstract

Purpose: The major aim of this study was to investigate the role of DNA methylation (referred to as methylation) on miRNA silencing in non–small cell lung cancers (NSCLC).

Experimental Design: We conducted microarray expression analyses of 856 miRNAs in NSCLC A549 cells before and after treatment with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (Aza-dC) and with a combination of Aza-dC and the histone deacetylase inhibitor trichostatin A. miRNA methylation was determined in 11 NSCLC cell lines and in primary tumors and corresponding nonmalignant lung tissue samples of 101 patients with stage I–III NSCLC.

Results: By comparing microarray data of untreated and drug-treated A549 cells, we identified 33 miRNAs whose expression was upregulated after drug treatment and which are associated with a CpG island. Thirty (91%) of these miRNAs were found to be methylated in at least 1 of 11 NSCLC cell lines analyzed. Moreover, miR-9-3 and miR-193a were found to be tumor specifically methylated in patients with NSCLC. We observed a shorter disease-free survival of patients with miR-9-3 methylated lung squamous cell carcinoma (LSCC) than patients with miR-9-3 unmethylated LSCC by multivariate analysis [HR = 3.8; 95% confidence interval (CI), 1.3–11.2, P = 0.017] and a shorter overall survival of patients with miR-9-3 methylated LSCC than patients with miR-9-3 unmethylated LSCC by univariate analysis (P = 0.013).

Conclusions: Overall, our results suggest that methylation is an important mechanism for inactivation of certain miRNAs in NSCLCs and that miR-9-3 methylation may serve as a prognostic parameter in patients with LSCC. Clin Cancer Res; 18(6): 1619–29. ©2012 AACR.

Introduction

DNA methylation (referred to as methylation) is part of the epigenetic gene regulation machinery and describes the covalent addition of a methyl group to the 5' carbon of cytosines within cytosine–guanine (CG) dinucleotides (1). While CG dinucleotides are relatively rare in the mammalian genome, certain genomic regions, called CpG islands (CGI), contain CG dinucleotides at a high density (2). CGIs are found in approximately 60% of the human gene promoter regions, including both protein coding and miRNA coding genes (3, 4). In lung cancer, numerous tumor suppressor genes have already been identified which are frequently methylated and thus, transcriptionally silenced (5–8). Methylation is reversible by demethylating drugs such as the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (Aza-dC) resulting in gene reexpression. A synergistic effect of Aza-dC and the histone deacetylase inhibitor trichostatin A (TSA) in gene reexpression of epigenetically silenced genes was described (9).

miRNAs are small (~22 nucleotides in length) noncoding RNAs which act as posttranscriptional regulators of gene expression. Through regulation of specific target genes a wide variety of biologic processes including cellular differentiation, proliferation, and apoptosis are regulated by miRNAs (10). Thus, deregulated expression of certain miRNAs may lead to alterations of these processes and to the development of a malignant phenotype (11). Recently, it has been reported that downregulated expression of miRNAs occurs frequently in many cancer types including lung cancer.
In our study, we identified DNA methylation (referred to as methylation) as important mechanism for silencing certain miRNAs in non–small cell lung cancers (NSCLC). miR-9-3 and miR-193a were found to be tumor specifically methylated in patients with stage I-III NSCLC. Importantly, we observed that patients with miR-9-3 methylated lung squamous cell carcinoma (LSCC) had a statistically significant shorter disease-free survival as well as shorter overall survival than patients with miR-9-3 unmethylated LSCC in multivariate and univariate analyses, respectively. These findings suggest that miR-9-3 methylation is a prognostic parameter in patients with LSCC. Thus, it can be speculated that the miR-9-3 methylation status might be helpful to more individualize future treatment and follow-up care of patients with LSCC after surgery.

(12, 13). By comparing miRNA expression patterns in primary lung tumors and matching nonmalignant lung tissue samples using miRNA expression microarray analyses, expression of numerous miRNAs was found to be downregulated in primary tumors (13–16). In addition, it has been reported that downregulated expression of certain miRNAs may be of negative prognostic impact for patients with non–small cell lung cancers (NSCLC ref. 13, 17).

Besides chromosomal loss and alterations of the miRNA processing machinery, methylation has been identified as a mechanism which may cause downregulation of miRNA gene expression in cancer cells (12, 18). Examples for methylated miRNA-encoding genes (referred to as methylated miRNAs) in NSCLCs are members of the miR-34 family, miR-124a, miR-126, and miR-200c (19–23). However, knowledge about methylation-mediated miRNA silencing in NSCLCs is still very limited to date. To gain more information about the role of methylation in miRNA silencing, we conducted a microarray-based screen for miRNAs whose expression was upregulated after treatment of NSCLC cells with Aza-dC and/or a combination of Aza-dC/TSA. Moreover, we determined miRNA methylation in NSCLC cell lines and in primary tumors and corresponding nonmalignant lung tissue samples of a large number of patients with stage I-III NSCLC by methylation-sensitive high resolution melt (MS-HRM) analysis and confirmed our methylation results by bisulfite genomic sequencing (BGS). In addition, we compared methylation and expression of certain miRNAs. Finally, we investigated possible associations between miRNA methylation and clinicopathologic characteristics of the patients with NSCLC.

Materials and Methods

Tumor cell lines and tissue samples

Genomic DNA of NSCLC cell lines NCI-H23, NCI-H266, NCI-H358, NCI-H1703, and NCI-H1792 was kindly provided by Dr. Adi F. Gazdar and John D. Minna (Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center at Dallas, Dallas, TX) and was used as reported previously (5). NSCLC cell line NCI-H1975 was kindly provided by Dr. Walter Berger (Institute of Cancer Research, Medical University of Vienna, Vienna, Austria). NSCLC cell lines A427, A549, NCI-H1915, NCI-H1993, and NCI-H2073 were purchased from the American Type Culture Collection during the last 5 years, stored in liquid nitrogen until use and grown as recommended. A total of 2 × 10^5 A549 cells/mL were treated either with 0.5 μmol/L Aza-dC for 6 days or with the combination of 0.5 μmol/L Aza-dC for 6 days and 100 ng/mL TSA for additional 24 hours as described previously (5). Control cells were not drug treated.

Frozen primary tumors and corresponding nonmalignant lung tissue samples of 101 patients with stage I-III NSCLC who underwent surgical resection of their tumor in a curative intent have been collected during the years 2000–2004. In addition, formalin-fixed, paraffin-embedded (FFPE) primary tumors and corresponding nonmalignant lung tissue samples of 2 of these patients were available. Clinicopathologic data including gender, age, histology, tumor stage, lymph node stage, and stage of disease (according to 6th edition of the TNM classification) were available from all 101 patients with NSCLC. Information about disease recurrence and disease-free survival (DFS)/overall survival (OS) were available from 98 and 97 patients with NSCLC, respectively. None of the patients received adjuvant chemotherapy. Follow-up of patients with NSCLC was done according to ACCP evidence–based clinical practice guidelines (24). Suspected disease recurrences by imaging were also proven by biopsies in more than 85% of cases. The median follow-up time of the study population was 54 months. This study was approved by the local ethics committee.

Genomic DNA was isolated from tumor cell lines and frozen tissue samples as reported and was stored at −80°C until use for methylation analyses (5). Total RNA was isolated from NSCLC cell lines by the miRNeasy Kit (Qiagen). FPPE samples were macrodissected followed by total RNA extraction by the miRNeasy FFPE Kit (Qiagen).

miRNA gene expression microarray analyses

Total cellular RNA was isolated from A549 cells and afterwards size fractionated. The miRNA microarray analyses were carried out with the μParaflo Microfluidic Biochip technology from LC Sciences according to miRBase version 12.0 containing 856 miRNAs. Data obtained by miRNA microarrays were background subtracted and then normalized using a cyclic loess filter (locally weighted regression). For 2 color experiments, the ratio of the 2 sets of detected signals (log2 transformed, balanced) and P values of the t test were calculated. Differentially detected signals were those with fold changes 1.5 or more and P values of less than 0.1. All statistical tests were carried out by LC Sciences. Microarray data were submitted to the GEO database (accession number GSE31579).
miRNA target prediction and functional annotation

Predicted gene targets of miRNAs whose expression was upregulated after drug treatment of A549 cells and which are associated with a CGI were identified using 6 different open source programs including DIANA-MicroT (25), miRanda (26), miRBase (27), miRDB (28), PicTar (29), and TargetScanS (30). Predicted targets which were identified by at least 4 of these programs were categorized into different functional classes with GENESIS software version 1.7.6. (31) and Ontologizer (32).

MS-HRM analyses

Genomic DNA was modified by treatment with sodium bisulfite using EpiTect Bisulfite Kit from Qiagen. Primer design for MS-HRM analyses was conducted with the Methyl Primer Express v.1.0 software. Primer sequences are shown in Supplementary Table S1. MS-HRM analyses were conducted by EpiTect HRM PCR kit in a RotorGene Q Cycler (Qiagen). A description of selected MS-HRM assays is shown in Fig. 2A. Water blanks were used as negative controls.

Bisulfite genomic sequencing

Primer sequences for BGS were designed with Methyl Primer Express v1.0 software and are listed in Supplementary Table S1. PCR products were gel purified and cloned by TOPO TA Cloning Kit for Sequencing (Invitrogen). PCR amplification for sequencing was carried out directly on clones with M13 primers. For each gene, 4 clones of a primary tumor and of a corresponding nonmalignant lung tissue sample were sequenced. Percentage of methylation was calculated as reported previously (33).

Figure 1. Summary of results of miRNA expression microarray analyses. A, Aza-dC and Aza-dC/TSA treatment of A549 cells resulted in upregulated expression of 46 and 45 miRNAs, respectively, with an overlap of 25 miRNAs. B, Heatmap summarizing expression values of 66 miRNAs in untreated and in drug-treated A549 cells. Colors range from red (low expression) to green (high expression). C, GO analysis of predicted targets of 33 miRNAs. Subcategories of “biologic process” and “cellular process” are shown.
Quantitative real-time PCR

Total RNA was reverse transcribed by miScript II RT Kit (Qiagen). Real-time PCR (RT-PCR) was carried out by miScript SYBR green PCR kit and miScript Primer Assays (Qiagen) for precursor miR-9-3 and miR-193a. RNU1A and RNU5A were used as internal expression controls. PCR reactions were carried out with a RotorGene Q cycler (Qiagen) and data were analyzed using the comparative C_{t} method (ΔΔC_{t} method; ref. 34).

Statistical analysis

Wilcoxon signed rank tests were used to calculate methylation differences between primary tumors and corresponding nonmalignant lung tissue samples obtained by MS-HRM analyses. The χ² tests and Fisher exact tests were used to test for differences between groups. t tests were used to calculate differences between means. Comparisons of methylation results with DFS and OS of patients with NSCLC were carried out using Kaplan–Meier log-rank testing. The Cox proportional model was used for multivariate analyses on DFS and on OS. Factors included in the multivariate analyses were gender, age, tumor stage, lymph node stage, and stage of disease. Statistical analyses were conducted with the statistics software PASW (version 18). Heatmaps were generated by Flexarray 1.6 software and GENESIS software.

Results

miRNA expression in untreated and in drug-treated A549 cells

To identify miRNAs whose expression is epigenetically regulated in A549 cells, we conducted miRNA expression microarray analyses of untreated, with Aza-dC–treated and with the combination of Aza-dC/TSA–treated A549 cells. In total, expression of 856 miRNAs was analyzed. By comparing miRNA expression profiles of untreated and Aza-dC–treated A549 cells, we identified 46 unique miRNAs whose expression was upregulated (fold change ≥1.5 and P values < 0.1) after Aza-dC treatment. The level of upregulated miRNA expression ranged from 1.52- to 45.3-fold (mean, 3.8-fold). Expression of 45 unique miRNAs was found to be upregulated after treatment of A549 cells with Aza-dC/TSA compared with untreated cells. The level of upregulated miRNA expression ranged from 1.52- to 98.2-fold (mean, 12-fold). Interestingly, expression of 25 miRNAs upregulated after Aza-dC treatment was also upregulated after Aza-dC/TSA treatment of A549 cells (Fig. 1A). Overall, expression of 66 unique miRNAs was found to be upregulated after Aza-dC and/or Aza-dC/TSA treatment of A549 cells (Supplementary Table S2). A heatmap summarizing expression values of these miRNAs before and after drug treatment is shown in Fig. 1B.

Genomic sequences of genes encoding for miRNAs whose expression was upregulated after drug treatment were obtained from ENSEMBL database (release 60). A search for CGIs revealed that 33 of 66 (50%) miRNA genes are associated with a CGI suggesting that expression of these genes might be epigenetically regulated. Detailed information about these genes is shown in Table 1.

miRNA target prediction and functional characterization of predicted miRNA targets

We were also interested to obtain information about potential targets of the 33 miRNAs described earlier. Thus, we conducted in silico miRNA target prediction followed by Gene Ontology (GO) analysis. Overall, 1,169 different miRNA targets were predicted. GO analysis identified several molecular pathways whose deregulation may contribute to a malignant phenotype (Fig. 1C). Examples for miRNA targets involved in apoptosis, cell proliferation, transcription, cell adhesion, cell differentiation, cell fate determination, and angiogenesis are shown in Supplementary Table S3. However, these findings need to be investigated in more detail in additional functional studies.

Methylation of miRNAs in NSCLC cell lines and in NSCLC patients

We developed MS-HRM assays to determine methylation of miRNAs whose expression was found to be upregulated after drug treatment of A549 cells and which are associated with a CGI. A description of some MS-HRM assays is shown in Fig. 2A.

Thirty of 33 (91%) miRNAs were observed to be methylated in at least 1 of 11 NSCLC cell lines of different histologies. An overview of these results is shown in Fig. 2B.

In addition, we conducted MS-HRM analyses of miR-9-3 and miR-193a also in primary tumors and corresponding nonmalignant lung tissue samples of 101 patients with stage I-II NSCLC. Differences in methylation between tumors and corresponding nonmalignant lung tissue samples were statistically significant for both miRNAs (miR-9-3, \( P = 4.3 \times 10^{-14} \); miR-193a, \( P = 1.9 \times 10^{-3} \)) suggesting that these miRNAs are tumor specifically methylated (Fig. 2C).
The mean% of methylation of miR-9-3 and miR-193a in primary tumors was 25 (range, 2%–93%) and 33 (range, 7%–103%), respectively. The mean% of methylation of these genes in corresponding nonmalignant lung tissue samples was 11 (range, 1%–36%) and 21 (range, 8%–38%), respectively. Mean $R^2$ values resulting from 8 MS-HRM runs were 0.995 (range, 0.984–0.999) in the case of miR-9-3 and 0.994 (range, 0.986–0.998) in the case of miR-193a, respectively, indicating high linearity and reproducibility of the MS-HRM assays.

Additional miRNAs analyzed for methylation in the majority of these patients with NSCLC included miR-29c, miR-140, miR-424, and miR-1203. However, no statistically significant differences in methylation between primary tumors and corresponding nonmalignant lung tissue samples of these patients were observed (data not shown).

In a next step, for each patient T/N ratios of miR-9-3 and of miR-193a methylation were calculated as the percentage of methylation in the primary tumor/percentage of methylation in the corresponding nonmalignant lung tissue sample.
Patients with a T/N ratio 1.5 or more were considered methylated. Sixty-nine of 101 (68%) patients with NSCLC and 42 of 101 (42%) patients with NSCLC were methylated for miR-9-3 and miR-193a, respectively. Seventy-seven of 101 (76%) patients with NSCLC were methylated for miR-9-3 and/or miR-193a.

Confirmation of MS-HRM data by BGS

To confirm data obtained by MS-HRM analyses, we, in addition, conducted BGS of a part of the 5’ regions of miR-9-3 and miR-193a in a primary NSCLC sample and in the corresponding nonmalignant lung tissue sample. Genomic regions selected for BGS contained regions analyzed by MS-HRM. In total, 39 CpG sites were analyzed for miR-9-3 and for miR-193a, respectively, in the corresponding nonmalignant lung tissue samples only 5% and 1% of CpG sites were methylated for miR-9-3 and for miR-193a, respectively. Differences in methylation between tumor and corresponding nonmalignant lung tissue sample were statistically significant for both genes.

Comparison of miRNA methylation with miRNA expression

In addition, we compared methylation of miR-9-3 and miR-193a with expression of precursor miR-9-3 and miR-93a in 3 NSCLC cell lines found to be methylated for these miRNAs at different percentages. Because miR-9 is encoded by 3 different genes (miR-9-1, miR-9-2, and miR-9-3), only expression of precursor miR-9-3 is
comparable with miR-9-3 methylation. Expression of precursor miR-9-3 was increased 2.7- and 3.8-fold in the cell lines A549 (41% methylated) and NCI-2073 (44% methylated) compared with expression in the cell line NCI-H1993 (98% methylated), respectively. Similar results were observed for expression of miR-193a which was increased 1.6- and 2.6-fold in the cell lines A427 (36% methylated) and A549 (41% methylated) compared with expression in the cell line NCI-H1993 (94% methylated), respectively. These results are shown in Supplementary Fig. S1. Moreover, miR-193a expression was investigated in macrodissected FFPE samples of 2 patients with miR-193a tumor specifically methylated NSCLC. Expression of miR-193a was downregulated (−6.5- and −2.3-fold, respectively) in the primary tumors compared with the corresponding nonmalignant lung tissue samples of both patients (Supplementary Fig. S1C).

Overall, these results confirm that methylation is indeed responsible for downregulation of precursor miR-9-3 and miR-193a expression.

**Comparisons of miR-9-3 and of miR-193a methylation results with clinicopathologic characteristics of NSCLC patients**

We compared miR-9-3 and miR-193a methylation results determined by MS-HRM analyses with clinicopathologic characteristics including gender, age, histology, tumor stage, lymph node stage, stage of disease, disease recurrence, DFS, and OS of the patients with NSCLC. For these analyses, T/N ratios of methylation, as described earlier, were used. An overview about methylation frequencies of these miRNAs in subgroups of patients with NSCLC and clinicopathologic characteristics is shown in Table 2.

### Table 2. Clinicopathologic characteristics of patients with NSCLC and results of miR-9-3 and miR-193a MS-HRM analyses

| Variables         | N   | miR-9-3 methylated, n (%) | miR-193a methylated, n (%) | miR-9-3 and/or miR-193a methylated, n (%) |
|-------------------|-----|--------------------------|---------------------------|------------------------------------------|
| Gender            |     |                          |                           |                                          |
| Male              | 61  | 40 (66)                  | 25 (41)                   | 44 (72)                                  |
| Female            | 40  | 29 (73)                  | 17 (43)                   | 33 (83)                                  |
| Age, y            |     |                          |                           |                                          |
| ≤60               | 50  | 36 (72)                  | 20 (40)                   | 39 (78)                                  |
| >60               | 51  | 33 (65)                  | 22 (43)                   | 38 (75)                                  |
| Histology         |     |                          |                           |                                          |
| ADC               | 57  | 46 (81)\(^a\)            | 21 (37)                   | 49 (86)\(^a\)                           |
| SCC               | 40  | 19 (48)                  | 20 (50)                   | 24 (60)                                  |
| LCC               | 3   | 3 (100)                  | 0 (0)                     | 3 (100)                                  |
| NSCLC, NOS        | 1   | 1 (100)                  | 1 (100)                   | 1 (100)                                  |
| T stage           |     |                          |                           |                                          |
| T1                | 21  | 17 (81)                  | 7 (33)                    | 18 (86)                                  |
| T2                | 51  | 32 (63)                  | 19 (37)                   | 37 (72)                                  |
| T3                | 23  | 15 (65)                  | 13 (56)                   | 17 (74)                                  |
| T4                | 6   | 5 (83)                   | 3 (50)                    | 5 (83)                                   |
| N stage           |     |                          |                           |                                          |
| N0                | 60  | 42 (70)                  | 24 (40)                   | 47 (78)                                  |
| N1                | 24  | 15 (63)                  | 12 (50)                   | 17 (71)                                  |
| N2                | 17  | 12 (71)                  | 6 (35)                    | 13 (76)                                  |
| Disease stage     |     |                          |                           |                                          |
| I                 | 49  | 35 (71)                  | 18 (37)                   | 39 (80)                                  |
| II                | 24  | 15 (63)                  | 12 (50)                   | 17 (71)                                  |
| III               | 28  | 19 (68)                  | 12 (43)                   | 21 (75)                                  |
| Disease recurrence|     |                          |                           |                                          |
| Yes               | 36  | 24 (66)                  | 14 (39)                   | 48 (77)                                  |
| No                | 62  | 42 (68)                  | 28 (45)                   | 26 (72)                                  |

Abbreviations: ADC, adenocarcinomas; LCC, large cell carcinomas; N, lymph node stage; NOS, not otherwise specified; SCC, squamous cell carcinomas; T, tumor stage.

\(^a\)Statistically significant differences of miR-9-3 and miR-9-3 and/or miR-193a methylation in primary ADCs compared with tumors of other histologies.
Interestingly, patients with miR-9-3 methylated lung squamous cell carcinoma (LSCC) had a statistically significant shorter DFS than patients with miR-9-3 unmethylated LSCC (median survival: 35 months vs. not reached, \( P = 0.013 \)). A similar association was observed regarding OS of patients with LSCC and miR-9-3 methylation status. The median survival was 39 months for patients with miR-9-3 methylated LSCC compared with 69 months for patients with miR-9-3 unmethylated LSCC (\( P = 0.013; \) Fig. 4).

Multivariate analysis revealed that methylation of miR-9-3 is an independent prognostic factor for shorter DFS of patients with LSCC [HR = 3.8; 95% confidence interval (CI), 1.3–11.2; \( P = 0.017 \)]. Compatible to our findings about DFS, disease recurrence was observed more frequently in patients with miR-9-3 methylated LSCC than patients with miR-9-3 unmethylated LSCC, although this difference did not reach statistical significance (\( P = 0.088 \)). Whereas 10 of 16 (63%) patients with LSCC whose tumor was methylated for miR-9-3 had disease recurrence, only 6 of 16 (38%) patients with LSCC whose tumor was not methylated for miR-9-3 had disease recurrence. Moreover, miR-9-3 and miR-9-3 and/or miR-193a methylation was found more frequently in adenocarcinomas than in tumors of other histologies (\( P = 0.002 \) and \( P = 0.009 \), respectively; Table 2). However, patients with adenocarcinomas whose tumor was observed to be miR-9-3 or miR-9-3 and/or miR-193a methylated did not show a worse DFS or OS.

**Discussion**

It has been reported that deregulated expression of miRNAs may occur in many cancer types including NSCLCs and methylation has been suggested as one of the mechanisms which may be relevant for miRNA silencing (12, 13, 19, 36, 37). To obtain more knowledge about the role of methylation in miRNA silencing, we carried out a high-throughput screen for miRNAs whose expression is upregulated after treatment of A549 cells with epigenetically active drugs. In a second step, we investigated methylation of several miRNA-encoding genes in NSCLC cell lines and tissue samples of patients with NSCLC using MS-HRM analysis.

We identified 66 miRNAs whose expression was upregulated after drug treatment. Thirty-three (50%) of them are associated with a CGI suggesting that these genes indeed may be regulated by methylation and that upregulation of gene expression does not occur due to secondary effects of Aza-dC and TSA.

From the vast majority of miRNAs whose expression was found to be upregulated in our microarray approach and which are associated with a CGI the finding that they may be epigenetically regulated in NSCLCs is new. Interestingly, among them are some miRNAs, whose expression was reported to be downregulated in various cancer types (i.e., miR-7, miR-22, miR-125a, miR-193a, miR-194, and miR-494) and especially in NSCLCs (i.e., mir-9, mir-34a, and mir-200c; refs. 21–23, 38).

To analyze methylation of the 33 miRNAs which were found to be upregulated after drug treatment of A549 cells and which are associated with a CGI, we used the MS-HRM approach, a relatively new method to detect methylation quantitatively. We found 91% of these miRNAs to be methylated in at least 1 of the NSCLC cell lines analyzed. Selected miRNAs were also investigated for methylation in primary tumors and corresponding nonmalignant lung tissue samples of a large number of patients with NSCLC. MiR-9-3 and miR-193a were found to be tumor specifically methylated. However, because our clinical samples were not microdissected, we cannot exclude “contamination” of the corresponding nonmalignant lung tissue samples with tumor cells leading to detection of some methylation in this sample type. Tumor-specific methylation of miR-9-3 and miR-193a was confirmed by BGS.

It is noteworthy, that the vast majority (88%) of miRNAs whose expression was found to be upregulated after drug
treatment of A549 cells were also found to be methylated in A549 cells indicating that they are indeed epigenetically regulated. These findings are confirmed by results showing that expression of precursor miR-9-3 and miR-193a is downregulated in NSCLC cell lines with high miR-9-3 and/or miR-193a methylation levels. In the case of miR-193a, we, in addition, observed downregulated expression in the methylated tumors compared with corresponding nonmalignant lung tissue samples of 2 patients with NSCLC. Because of some technical problems, we were not able to show these results also for precursor miR-9-3. Overall, our data confirm that methylation of certain miRNAs is associated with their transcriptional regulation.

Recently, Lujambio and colleagues (38) used a similar approach as we to identify miRNAs whose expression was upregulated after treatment of lymph node metastatic cancer cells of different origin with Aza-dC. The authors reported that 57 of 389 miRNAs analyzed were found whose expression was upregulated 2-fold or more compared with untreated cells and that 27 of them were associated with a CGI including miR-9 family. Although there are certain differences in the conception of these 2 studies, the results about the number of miRNAs whose expression was found to be upregulated after drug treatment and of those associated with a CGI were very similar. In addition, also the frequencies of miR-9-3 methylation in primary lung tumors (53% reported by Lujambio and colleagues; ref. 38; and 68% in our study) were similar in both studies. However, the study by Lujambio and colleagues (38) was not focused on NSCLCs but used tumor samples of different malignant diseases. Thus, the number of primary lung cancers was relatively small. In addition, no potential clinical associations were investigated in their study.

It has been reported that miR-193a regulates expression of certain oncogenic factors (39–41). Downregulated miR-193a expression was reported in LSCC, malignant melanomas, oral squamous cell carcinomas (OSCC), and acute myelogenous leukemia (AML; refs. 40–43). Moreover, methylation of miR-193a was identified as mechanism for downregulated miR-193a expression in OSCC and in AML (40, 41).

Methylation of certain miRNAs in NSCLCs was also investigated in the studies by Kitano and colleagues (44) and by Watanabe and colleagues (23). Kitano and colleagues (44) observed miR-9-3 methylation in 65% of primary NSCLCs. Watanabe and colleagues (23) identified miR-34b and miR-126 to be silenced by methylation in NSCLCs. We found an almost identical frequency of miR-9-3 methylation in primary NSCLCs (68%), however, we did not observe upregulation of miR-34b and miR-126 expression after drug treatment which might be explained by different methods used. In addition, Gallardo and colleagues (22) and Ceppi and colleagues (21) identified methylation as mechanism for silencing miR-34a, a member of the p53 network, and miR-200c, a putative metastasis suppressor gene, in NSCLCs. In concordance to these results, we observed upregulation of miR-34a and miR-200c expression after drug treatment suggesting that these miRNAs are regulated by methylation.

Finally, we compared our methylation results with clinicopathologic characteristics of the patients with NSCLC. We observed that miR-9-3 methylation is associated with a shorter DFS and a shorter OS of patients with LSCC in multivariate and univariate analyses, respectively. Because none of the patients included in our study received adjuvant chemotherapy after surgery, the DFS is a strong parameter. Although most patients with disease recurrence received platin-based chemotherapy, we cannot exclude a potential influence of treatment after disease recurrence on the OS. Compatible to our findings, we detected miR-9-3 methylation more frequently in patients with LSCC with disease recurrence compared with patients with LSCC with no disease recurrence. However, this association did not reach statistical significance. We are aware of that the numbers of patients in our survival analyses are low, nevertheless, we believe that miR-9-3 methylation is of prognostic impact for patients with LSCC. However, prospective studies are necessary to confirm our results. Overall, our findings could be potentially helpful for a more personalized treatment and follow-up care of patients with LSCC after surgery.

Similar to findings that methylation of certain protein-encoding genes is tumor type–specific, we observed that the miRNA-encoding gene miRNA-9-3 is more frequently methylated in adenocarcinomas than in nonadenocarcinomas (8).

In conclusion, using a genome-wide approach to investigate miRNAs which respond to epigenetically active drugs, we identified several miRNAs which may be targets for methylation in NSCLCs. From many of them, epigenetic regulation was unknown so far. In addition, miR-9-3 and miR-193a were found to be tumor specifically methylated in patients with NSCLC. Moreover, our data suggest that miR-9-3 methylation is a prognostic factor for patients with LSCC. All together, the results of our study stress the importance of methylation for miRNA silencing in the pathogenesis of NSCLCs.

Disclosure of Potential Conflicts of Interest

A. End-Putzungerreuter has honoraria from speakers’ bureau, European Respiratory Society. No potential conflicts of interest were disclosed by the other authors.

Acknowledgments

The authors thank Dr. Klaus Kirchbacher and Alexandra Cee for providing and collecting clinical information about patients.

Grant Support

This work has been funded by the Vienna Science and Technology Fund (WWTF) through project LS07-019 to S. Zachbauer-Müller.

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Received September 22, 2011; revised January 3, 2012; accepted January 14, 2012; published OnlineFirst January 26, 2012.
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