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Epigenetic analysis of microRNA genes in tumors from surgically resected lung cancer patients and association with survival

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Abstract

Aberrant microRNA (miRNA) expression is involved in tumorigenesis of several cancers, including non-small cell lung cancer (NSCLC). Furthermore, expression of some miRNAs has been shown to be under epigenetic regulation. However, less is known regarding the role of miRNA methylation in NSCLC development or clinical outcomes. Therefore, we tested miRNA methylation patterns by quantitative real-time methylation specific PCR for a panel of candidate miRNAs in 19 NSCLC paired tumor and adjacent normal tissues. For assessment of survival, methylation was measured in a total of 97 tumor tissues with complete clinical and follow-up data. Analysis was also performed for correlation with age at diagnosis, gender, smoking, and stage. Significant differences in methylation patterns were observed for nine of the 12 miRNAs, all due to hypermethylation in the tumor tissue. Individuals with the highest levels of methylated miR-127 were at a significantly increased risk of dying with a hazard ratio of 1.93 (95% CI, 1.17–3.19; $P=0.010$), in univariate analysis and remained significant after adjusting for age, gender, and stage (HR, 1.97; 95% CI, 1.15–3.40; $P=0.014$). This increase in risk due to increased methylation were accompanied by significant, dramatic difference in survival duration of 17 months ($P=0.0089$). Six of the 12 miRNAs were significantly positively correlated with age at diagnosis. Additionally, methylation of miR-127 significantly greater in higher stage tumors compared to lower stage tumors ($P=0.0039$). However, no significant associations between smoking and gender with miRNA methylation were observed. Our results demonstrate that miRNA methylation plays a role in NSCLC tumorigenesis and prognosis.

Keywords

microRNA; expression; lung cancer; methylation; survival

Introduction

Lung cancer, predominantly non-small-cell lung cancer (NSCLC), is the most common cause of cancer deaths worldwide with a 5-year survival rate of 16% [1,2]. Unfortunately, this has not improved substantially during the past 30 years [2]. It is believed that survival could be improved through earlier diagnosis and improved treatment options. Recently, there has been a focus on microRNAs (miRNAs) and their potential in serving as markers for cancer development and prognosis [3–8].

MiRNAs are small non-coding RNAs that can post-transcriptionally modulate expression of scores of target genes, thereby playing a major role in the regulation of many cellular processes, such as proliferation, differentiation, apoptosis, survival, metabolism, and development [4,9]. Evidence indicates that miRNAs may function as tumor suppressors or oncogenes with altered miRNA expression having critical roles in tumorigenesis and cancer progression [3,6]. In addition, miRNA expression profiles have been shown to be potential markers for cancer diagnosis, prognosis, and therapy [10–15].

Expression patterns of several miRNAs have been shown to be associated with overall survival in NSCLC. For example, high miR-155 or low let-7a-2 expression levels were observed to be associated with worse survival among patients with adenocarcinoma [13]. Similarly, Takamizawa *et al.* also found significantly shorter survival among patients with low let-7 tumor expression [12]. In addition, Yu and colleagues have described a five-miRNA signature (let-7a, miR-221, miR-137, miR-372, miR-182*) associated with survival and relapse in NSCLC patients [16]. It was also found that miR-34a expression was correlated with relapse and overall survival in surgically resected NSCLC [17]. These findings provide strong evidence that miRNA expression is associated with lung cancer survival.

The mechanisms regulating miRNA expression in cancer are becoming more clear. Both genetic and epigenetic alterations can affect miRNA expression, leading to altered target gene expression. Evidence has shown that hypermethylation of miRNAs contributes to the down-regulation of miRNAs and may participate in tumorigenesis. For example, miR-124a has been found to be down-regulated in lung cancers by hypermethylation, leading to the up-regulation of CDK6 (cyclin-dependent kinase 6) and the down-regulation of the tumor suppressor retinoblastoma (RB1) [18]. Similarly, methylation-silencing of several miRNAs has been observed in other cancers. Hypermethylation of miR-124a was observed in gastric cancer patients [19], miR-126 in bladder and prostate tumors [20], miR-137 in colon cancer [21,22], and miR-193a in oral cancer [23]. MiR-127 epigenetic silencing could lead to activation of the proto-oncogene BCL6 in prostate, bladder, and colon tumors [24].

Hypermethylation also plays a role in clinical outcomes. MiR-9, miR-34b/c and miR-148a hypermethylation was demonstrated to be involved in lymph node metastasis [25]. We have previously identified aberrant miRNA-9 methylation linked to renal cell carcinoma metastasis [26]. In lung cancer, recently it was reported that some miRNAs methylation was correlated with clinical outcome in NSCLC patients. For example, miR-34b/c methylation was correlated with a high probability of recurrence and poor overall survival in patients

with stage I NSCLC [27]. miR-34b methylation was also found to be significantly associated with lymphatic invasion of lung cancer [28]. The methylation statuses of miR-9-3, miR-124-2, and miR-124-3 were individually related to recurrence of non-small-cell lung cancer [29].

In this study, we assessed the methylation status of 12 these miRNAs shown to be under epigenetic regulation (miR-9-1, miR-9-2, miR-9-3, miR-34b/c, miR-124a-1, miR-124a-2, miR-124a-3, miR-126, miR-127, miR-137, miR-148a, and miR-193a) in surgically resected NSCLC tumors and adjacent normal tissues. Due to the relationship between aberrant miRNA methylation and cancer, we investigated the methylation status of these candidate miRNAs in lung tumors compared with normal tissue and whether or not methylation patterns were correlated with patient overall survival, age at diagnosis, smoking behavior, gender, and stage.

Materials and Methods

Study population and data collection

The study population consisted of newly diagnosed, histologically confirmed patients with NSCLC who underwent surgery as part of their treatment at The University of Texas MD Anderson Cancer Center. Demographic and epidemiologic variables were collected during an in-person interview using a structured questionnaire. A former smoker was defined as a person who had quit smoking at least 1 year prior to diagnosis. A current smoker was someone who was currently smoking or who had stopped < 1 year prior to being diagnosed. Clinical and follow-up information was obtained from review of patient medical records. This analysis was restricted to Caucasian cases and in total, 19 tumor and 19 adjacent normal lung tissues were included in this study, with an additional 88 tumors for a total of 107 tumor tissues, of which complete clinical and follow-up information was available for 97 patients (38 adenocarcinoma, 36 squamous cell carcinoma, and 23 other NSCLC histology). Staging of the tumors was based on the the International System for Staging Lung Cancer Guidelines (1997). Participants provided written informed consent and approval was obtained from MD Anderson's Institutional Review Board.

DNA extraction, sodium bisulfite modification and quantitative real-time MSP

Snap-frozen tissues contained at least 70% tumor. Tumors were homogenized and genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. For each sample, 500 ng of genomic DNA was treated with sodium bisulfite using the EZ-96 DNA Methylation-gold kit (ZYMO Research, Orange, CA) and incubated for 10 min at 98°C and 2.5 h at 64°C to ensure complete conversion. DNA methylation levels were analyzed by quantitative real-time methylation specific PCR (QMSP) which was performed as described previously, with slight modifications [30–32]. Briefly, PCR reactions were done in 384-well plates using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) with SYBR Green PCR Master Mix (Applied Biosystems). Each amplification mix (10 µl) contained bisulfite-modified DNA (12 ng), 1X SYBR PCR buffer, and 400 nmol/L of each primer. For each selected miRNA, the CpG Island Searcher Program (<http://www.cpgislands.com/>) was used

to identify regions for potential methylation sites in the putative promoter region. Primer sequences for QMSP are in Supplemental Table 1 and PCR conditions have been described previously [19,25]. Human CpGenome Universal Methylated DNA (Chemicon International, Temecula, CA) was used as a positive control and water blanks were included with each plate. Thermal cycling conditions consisted of one cycle for 2 min at 50°C, 10 min at 95°C followed by 40 cycles for 15 s at 95°C, 1 min at 50–60°C for different miRNAs. All reactions were performed in duplicate. Relative levels of methylated DNA (percentage) in each sample were calculated according to the equation, $C_{meth} = 100/[1+2^{(CtCG-CtTG)}]\%$ [33]. CtCG represents the threshold cycle for the methylated CG reaction, and CtTG represents the threshold cycle of the unmethylated reaction.

Statistical and pathway analysis

Comparisons of the characteristics of dead and alive patients were assessed by Pearson χ^2 tests and Student t-tests. Methylation levels for each miRNA in tumor and adjacent normal were compared using the paired t-test. Association between methylation level and overall survival was assessed by Cox regression model while adjusting for age, smoking status, and tumor stage. Overall survival was defined as the duration between data of diagnosis to date of death or last follow-up. The cut-off points for designation of “high” and “low” methylation levels were determined using the median, spline modeling, or 75%/25%-tile, whichever provided the optimal threshold. Survival curves were estimated by the Kaplan-Meier method and compared using the log-rank test. Correlations between smoking behavior, gender, stage, and methylation levels were analyzed with linear regression model. The effect of age on methylation level was analyzed using Spearman's rank correlation coefficient. All the statistical analyses were done using the STATA software (version 10, College Station, TX). Values of $P < 0.05$ were considered significant. Predicted target genes for miR-9 and miR-127 were obtained from TargetScan (human, version 6.1; www.targetscan.org). Ingenuity IPA software was used to determine the top canonical pathways and networks represented by the predicted target genes.

Results

Patient characteristics

Clinicopathological data are shown in Table 1. The median follow-up time was 94.80 (range 0.89–161.33) months for the 97 cases. There was no significant difference between survival status of the patients with respect to age, gender, and smoking status ($P=0.37$, 0.21, and 0.81, respectively). As would be expected, a significant difference was observed for stage at diagnosis ($P<0.01$) with a larger number of patients diagnosed at higher stage in the group of patients who have died than those who are still alive.

Identification of miRNAs differentially methylated in tumor versus adjacent normal lung tissues

As shown in Figure 1, there was a high degree of variation in methylation levels in tumor tissues compared to adjacent normal tissues for nearly all of the microRNAs. In the 19 pairs of tumor and adjacent normal tissues, nine of the 12 microRNAs were differentially methylated between tumor and adjacent normal tissue: miR-9-1 ($P=0.0029$), miR-9-2

($P=0.010$), miR-9-3 ($P=3.6\times 10^{-4}$), miR-34b/c ($P=0.0074$), miR-124a-2 ($P=6.0\times 10^{-4}$), miR-124a-3 ($P=7.7\times 10^{-4}$), miR-126 ($P=8.1\times 10^{-4}$), miR-148a ($P=0.022$), and miR-193a ($P=0.0081$). All of these genes were hypermethylated in the tumor. Interestingly, miR-127 was frequently methylated (>94%) in both tumor and normal. In an exploratory analysis of the 6 adenocarcinoma tumor tissues and the 6 squamous cell carcinoma tissues with their respective adjacent normal paired tissue, patterns of methylation in these two subgroups are very similar to the results for the 19 pairs. For adenocarcinoma, miR-34bc ($P=0.0037$), miR124a2 ($P=0.041$), and miR-123a3 ($P=0.015$) were significant. miR-124a2 was also significant in the squamous cell carcinoma pairs ($P=0.020$) with miR-192a reaching significance as well ($P=0.049$).

Association between miRNA methylation and overall survival of NSCLC patients

Univariate analysis of the methylation patterns of these 12 microRNA genes identified miR-9-2, miR-127, and miR-193 as being significantly associated with overall survival when split into “high” and “low” methylation groups (Table 2). The effect for miR-127 remained significant in multivariable analysis adjusting for age, smoking status, and stage resulting in a nearly 2-fold increase risk of dying (OR, 1.97; 95% CI, 1.15–3.40; $P=0.014$). When patients with the highest 25% miR-127 methylation levels were compared to those with the lowest 75%, they were at an 1.87-fold increase in risk (95% CI, 1.06–3.30; $P=0.031$) and had a significant 17 month reduction in survival from 51.6 months for the low risk group to only 34.6 months ($P=0.0089$). When analyzing methylation as a continuous variable, miR-193 also reached near significance with a $P=0.054$ and also displayed a protective effect as observed for comparison of “high” vs. “low” methylation groups.

Pathway analysis of the predicted target genes for and miR-127 ($N=281$) identified several canonical pathways and networks over-represented by these target genes that are known to play a role in cancer. miR-127 target genes were involved in signaling networks that included terms such as “Cell Death”, “Cellular Development”, “Cellular Function and Maintenance”, and “Gene Expression”.

Effect of smoking, age, gender, and stage on methylation levels of miRNA genes in patients with NSCLC

We next determined the effect of smoking behavior (smoking status and pack-year), age, gender, and stage on miRNA methylation. Increased methylation levels of miR-9-1, miR-9-3, miR-34b/c, miRNA-124a2, miRNA-124a3, and miRNA-148a were significantly correlated with an increase in the age at diagnosis (Table 3). Only miR-127 methylation was found significantly different among the different stages with increased levels of methylation in the higher stages (III and IV) than in the lower stages (I and II) tumors ($P=0.039$). No effect of smoking on miRNA methylation was found and no significant differences in methylation levels were observed between men and women patients.

Discussion

Epigenetic regulation of miRNA genes through methylation in cancer has been described as a potential mechanism to alter miRNA expression that may result in tumorigenesis, cancer

progression, and clinical outcomes [22,25,26]. Recently, the methylation status for several miRNAs has been reported to be correlated with clinical outcomes for NSCLC [17,27–29]. In this study, we analyzed NSCLC tumor and adjacent normal lung tissue samples for differences in miRNA methylation status and determined that a majority of these miRNA genes were significantly hypermethylated in the tumor compared to adjacent normal tissue. Furthermore, hypermethylation of miR-127 was found to be associated with an increased risk of dying and shorter survival time in both univariate and multivariate analyses. These results implicate several miRNAs as playing a role in NSCLC development and suggest that the methylation status of miR-127 could be a biomarker for the prediction of clinical outcome in NSCLC patients.

MiR-127 was shown to be involved in modulating risk of death in NSCLC patients in univariate and multivariate analysis adjusting for age, smoking status, and tumor stage. It has been demonstrated that miR-127 can target BCL-6, a proto-oncogene modulating DNA damage-induced apoptotic responses, with the epigenetic silencing of miR-127 leading to the activation of BCL-6 [24]. Although also highly methylated in adjacent normal tissue, we observed that the level of miR-127 methylation was significantly increased in patients with higher stage disease. Our results suggest that variation in miR-127 within the tumor is an important mediator of survival and tumor development, while methylation in the normal lung tissue is not a predictor of tumor behavior. These results implicate miR-127 hypermethylation as being independently linked to both survival and stage, indicative of poor prognosis.

Several studies have reported hypermethylation of the three genes encoding for miR-9 in multiple cancers resulting in decreased expression of miR-9 in tumor tissue [19,20,22,23,25,26,29]. For example, in renal cell carcinoma, miR-9-1 and miR-9-3 were found to be significantly hypermethylated in tumors compared with adjacent normal tissues, with both miR-9-1 and miR-9-3 expression suppressed in the tumors [26]. Kitano et al. [29] reported the methylated status of miR-9-3 in NSCLC was a significant risk factor for an advanced T status in multivariate regression. Here, we observed similar findings that methylation levels of the miR-9 genes were higher in NSCLC tumors than in normal tissues. MiR-9-1 and miR-9-3 were also observed to be correlated with age, implying miR-9-1 and miR-9-3 hypermethylation could further modulate risk in aged patients with NSCLC. In addition, this is the first study to suggest that miR-9-2 hypermethylation plays a role in patient survival. Pathway analysis of predicted miR-9 target genes indicated a strong role in pathways and networks involved in cancer, including the top pathway being “Molecular Mechanism of Cancer”. Taken together, these results suggest that miR-9 hypermethylation plays a role in NSCLC development and progression.

Of the 12 miRNA genes included in our analysis, nine displayed differential methylation patterns between tumor and adjacent normal tissues. There is existing evidence that the methylation and subsequent downregulation these microRNAs would have an impact on lung tumorigenesis. For example, miR-126 inhibition of *EGFL7* has been shown to reduce proliferation of NSCLC cells [34]. Our results showed that miR-126 is hypermethylated in tumor tissues compared to normal, as would be expected based on this previous finding. Hypermethylation would result in decreased miR-126 levels in the tumor and subsequent

loss of EGFL7 inhibition causing increased cellular proliferation. MiR-34b/c has been previously shown to be often hypermethylated in cancer tumors compared with normal tissues [25], similarly to our findings. This miRNA targets multiple known cancer-related genes, including *CDK6*, *MYC*, and *E2F3*. *CDK6* encodes for a cyclin-dependent kinase that functions as an oncogene involved in cell cycle progression and differentiation. It is often highly expressed in NSCLC, which would be anticipated based on our observed hypermethylation and subsequent downregulation of miR-34b/c, thus reducing the inhibitory effect of this miRNA on *CDK6*. Another of the miRNAs shown to be hypermethylated in lung tumors, miR-124a, has been shown to be silenced in leukemia cells also resulting in the downregulation of *CDK6* [35]. In our NSCLC tumor samples, this was also observed. Higher level of methylated miR-124a, especially miR-124a-2 and miR-124a-3, was evident in tumors compared with normal lung tissues.

The effect of age on gene methylation has been investigated previously [36,37], including a large comparison between DNA methylation profiles in newborns and centenarians [38]. However, less is known regarding the effect of aging on miRNA gene methylation. Significant correlations between age at diagnosis and methylation levels were observed for six miRNA genes. All of these miRNAs were also significantly differentially methylated in tumor tissues, suggesting that the impact of hypermethylation on lung tumorigenesis may be further modulated by age.

Furthermore, we investigated the effect smoking and gender on methylation of miRNAs. Smoking is the most important exogenous risk factor for lung cancer and several studies have reported an association between hypermethylation and cigarette smoking in lung tissue [39,40]. Although no association between miRNA methylation and smoking behavior (smoking status or pack-year) was observed, there was a borderline significance for miR-9-3 ($P=0.091$). This might be due to the moderate sample size of this study that limited our ability to identify the effect of smoking or the potential underreporting of self-reported smoking behavior by study participants. Another possibility is that the mechanism by which smoking contributes to the development of lung cancer may not stimulate aberrant methylation of these miRNAs. Female gender has been demonstrated to be positively associated with DNA methylation for some genes [41,42], but protective for others [39,40,43]. This indicates that risk of hypermethylation for certain genes might vary in a tissue-specific, gender-dependent manner. In our study, none of the methylation levels for these 12 miRNAs were correlated with gender.

In conclusion, the results of this study suggest that hypermethylation of miRNA genes is a relatively common event in NSCLC and plays a role in not only tumorigenesis, but also prognosis. Furthermore, these methylation patterns were modulated by age and stage. These results provide insight into the mechanisms underlying lung cancer development. In addition, they may have prognostic or therapeutic implications for the future management of NSCLC patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

NSCLC	non-small cell lung cancer
miRNA	microRNA

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Figure 1. Methylation levels of a panel of candidate miRNAs between 19 tumor and 19 adjacent normal tissues from NSCLC patients. Inter-quartile range and outlier values are shown.

Figure 2.

Kaplan-Meier overall survival by miR-127 methylation levels in NSCLC tumors. N=number of events/total number of patients, MST=median survival time in months. High and low methylation groupings were based on the 75th and 25th percentiles of methylation in the alive group.

Table 1Distribution of clinical characteristics by survival status in 97 NSCLC cases^{*}

Variable	Alive (%) (N = 27)	Dead (%) (N = 70)	P value [^]
Age at Diagnosis (mean (SD))	62.48 (9.56)	65.09 (13.76)	0.36
Gender			
<i>Female</i>	15 (55.6)	29 (41.4)	0.214
<i>Male</i>	12 (44.4)	41 (58.6)	
Family History of Cancer			
<i>Yes</i>	15 (55.6)	40 (57.1)	0.32
<i>No</i>	11 (40.7)	21 (30.0)	
Smoking History			
<i>Never</i>	2 (7.7)	7 (10.8)	0.81
<i>Former</i>	9 (34.6)	25 (38.5)	
<i>Current</i>	15 (57.7)	33 (50.8)	
Histological Subtype			
<i>Adenocarcinoma</i>	8 (29.6)	30 (42.9)	0.31
<i>Squamous cell carcinoma</i>	10 (37.0)	26 (37.1)	
<i>Other</i>	9 (33.3)	14 (20.0)	
Stage			
<i>I</i>	22 (81.5)	27 (39.1)	0.0027
<i>II</i>	2 (7.4)	14 (20.3)	
<i>III</i>	3 (11.1)	25 (36.2)	
<i>IV</i>	0 (0.0)	3 (4.3)	
Grade			
<i>1</i>	2 (7.4)	3 (4.2)	0.906
<i>2</i>	9 (33.3)	26 (36.6)	
<i>3</i>	14 (51.9)	38 (53.5)	
<i>Unknown/Missing</i>	2 (7.4)	4 (5.6)	

^{*} numbers in some categories do not add up to total due to missing values

Table 2

Significant associations between miRNA methylation levels and overall survival

Gene	Alive N(%)	Dead N(%)	HR(95% CI)	P value	Adjusted HR(95% CI)	P value
miR-127	21(77.8)	44(62.9)	1.00(reference)		1.00(reference)	
	6(22.2)	26(37.1)	1.93(1.17–3.19)	0.010	1.97(1.15–3.40)	0.014
miR-193	14(51.9)	49(70.0)	1.00(reference)		1.00(reference)	
	13(48.1)	21(30.0)	0.55(0.33–0.93)	0.027	0.61(0.32–1.30)	0.088
miR-9-2	7(25.9)	11(15.7)	1.00(reference)		1.00(reference)	
	20(74.1)	59(84.3)	2.13(1.05–4.32)	0.036	1.84(0.88–3.84)	0.11

Adjusted HR: age, smoking status, and tumor stage

Table 3

Significant correlations between methylation level of miRNA genes and age at diagnosis

Gene	N	Spearman's rho	P value
miR-9-1	97	0.26	0.0095
miR-9-3	97	0.30	0.0024
miR-34b/c	97	0.23	0.023
miR-124a-2	97	0.20	0.046
miR-124a-3	97	0.25	0.013
miR-148a	96	0.27	0.0069