Endobronchial miRNAs as Biomarkers in Lung Cancer Chemoprevention


Abstract

Lung cancers express lower levels of prostacyclin than normal lung tissues. Prostacyclin prevents lung cancer in a variety of mouse models. A randomized phase II trial comparing oral iloprost (a prostacyclin analog) with placebo in high-risk subjects showed improvement in bronchial histology in former, but not current, smokers. This placebo-controlled study offered the opportunity for investigation of other potential intermediate endpoint and predictive biomarkers to incorporate into chemoprevention trials.

Matched bronchial biopsies were obtained at baseline and at 6-month follow-up from 125 high-risk individuals who completed the trial: 31/29 and 37/28 current/former smokers in the iloprost and placebo arm, respectively. We analyzed the expression of 14 selected miRNAs by Real Time PCR in 496 biopsies.

The expression of seven miRNAs was significantly correlated with histology at baseline. The expression of miR-34c was inversely correlated with histology at baseline (P < 0.0001) and with change in histology at follow-up (P = 0.0003), independent of treatment or smoking status. Several miRNAs were also found to be differentially expressed in current smokers as compared with former smokers. In current smokers, miR-375 was upregulated at baseline (P < 0.0001) and downregulated after treatment with iloprost (P = 0.0023). No miRNA at baseline reliably predicted a response to iloprost.

No biomarker predictive of response to iloprost was found. MiR-34c was inversely correlated with baseline histology and with histology changes. Mir-34c changes at follow-up could be used as a quantitative biomarker that parallels histologic response in formalin-fixed bronchial biopsies in future lung cancer chemoprevention studies. Cancer Prev Res; 6(2); 100–8. ©2012 AACR.

Introduction

Smoking exposes the respiratory mucosa to carcinogens in a "field cancerization" process (1). Smokers develop bronchial lesions in a multistep set of preinvasive stages preceding the development of invasive lung cancer. The lesions are multiple and occur throughout the bronchial airways of smokers, putting the entire bronchial epithelium at risk. The administration of chemopreventive agents may, thus, be more effective than local treatment in reducing the risk of developing lung cancer. Smoking cessation is the only intervention proven to reduce the risk of lung cancer; however, the risk for former smokers remains elevated for many years compared with never smokers. Therefore, chemoprevention is particularly attractive for former smokers.

Epidemiologic studies have shown decreased rates of certain cancers with the chronic administration of anti-inflammatory medication such as COX-2 inhibitors that alter prostaglandin production (2, 3). Downstream of the COX-1 and COX-2 enzymes, levels of prostacyclins are determined by the expression of prostacyclin synthetase and prostaglandin E2 synthetase. In normal lung, prostacyclin synthetase is active and, thus, prostacyclin (PGI2) levels are high. In the majority of lung cancers, prostacyclin levels are low while prostaglandin E2 synthetase and prostaglandin E2 synthetase. In normal lung, prostacyclin synthetase is active and, thus, prostacyclin (PGI2) levels are high. In the majority of lung cancers, prostacyclin levels are low while prostaglandin E2 synthetase and prostaglandin E2 (PGE2) levels are high. High PGE2 levels and low PGI2 levels are associated with worse prognosis in lung cancer (4, 5). Selective pulmonary overexpression of prostacyclin synthase and supplementation of oral iloprost, a stable prostacyclin analog, prevents the development of lung cancer in a variety of murine models, including cigarette smoke exposure (6–8). On the basis of this promising preclinical data, we developed a phase II chemoprevention...
trial comparing the oral prostacyclin analog iloprost to placebo. This double-blind phase II multicenter chemoprevention trial met the predetermined primary endpoint of improvement in endobronchial histology in former, but not current, smokers (9). Although there are no established intermediate biomarkers, histology is a logical intermediate endpoint for early chemoprevention trials (10). We have exploited the unique opportunity offered by the iloprost chemoprevention trial to explore additional potential intermediate and/or predictive biomarkers for chemoprevention trials.

MicroRNAs (miRNA) are highly stable and exhibit tissue specificity, making them attractive biomarkers (11). Moreover, we have shown that miRNAs are correlated with the earliest steps in lung squamous cell carcinogenesis (12). The latter study analyzed the expression of 374 miRNAs in biopsies representing various stages of endobronchial premalignancy and showed that 69 miRNAs were differentially expressed in the course of squamous cell carcinogenesis (12). This earlier work yielded a number of candidate biomarkers that discriminate between squamous cell lung cancer and varying degrees of premalignancy. Thus, as they are modified at the earliest steps of lung carcinogenesis, we hypothesized that miRNAs could be both potential intermediate endpoints and/or predictive biomarkers for chemoprevention trials. We tested this hypothesis in biospecimens from the phase II iloprost lung cancer chemoprevention trials. We selected for study inclusion. We stratified all analyses by smoking status. Using data from our preliminary studies, we found that the 90th percentile of the SDs over miRNA and smoking status. Using data from our preliminary studies, we found that the 90th percentile of the SDs over miRNA and 31 current smokers in the iloprost arm, and 28 former smokers (9). Although there are no established intermediate biomarkers, histology is a logical intermediate endpoint for early chemoprevention trials (10). We have exploited the unique opportunity offered by the iloprost chemoprevention trial to explore additional potential intermediate and/or predictive biomarkers for chemoprevention trials.

Sample size calculation and sample selection for the present biomarker study

Power was greater than 80% to detect a 2-fold or greater change in miRNA expression between 2 groups with a false discovery rate (FDR) of 0.10 across miRNAs. The biopsy sites corresponding to the best (lowest histology score) and worst (highest histology score) at baseline were sampled. The corresponding biopsy sites at follow-up were also selected for study inclusion. We stratified all analyses by smoking status. Using data from our preliminary studies, we found that the 90th percentile of the SDs over miRNA and histology groups was approximately 0.71 on the log 2 scale. Assuming that a log 2 difference in miRNA expression between groups (a fold change of 2) is the smallest biological difference of interest, and that there are 2 truly differentially expressed miRNAs in the set, we estimated that a minimum of 13 subjects per group would be needed, while for a smaller difference of 0.7 on the log scale a minimum of 24 subjects per group would be needed. For analyses where subjects are the unit of analysis, 125 subjects – 29 former and 31 current smokers in the iloprost arm, and 28 former smokers (9).

Material and Methods

Population

The iloprost lung cancer chemoprevention study was a multicenter, phase II, randomized, double-blind, placebo-controlled trial of iloprost in subjects at increased risk for lung cancer (defined as current or former smokers with ≥20 pack-years or more of smoking history, at least mild sputum cytologic atypia, or a history of biopsy-proven endobronchial dysplasia). The characteristics of the population and the results have been reported in detail previously (9). Autofluorescence and/or white-light bronchoscopy was carried out before randomization and after 6 months of treatment, with 6 standard endobronchial sites biopsied (bronchial carinae, RUL, RML, RB6, LUL, LL1DB, and LB6), together with all other visually suspicious areas. The trial sample size was 152 subjects. After obtaining written informed consent, participants were randomized on the basis of smoking status (current vs. former) and study center. Following randomization, subjects were treated with iloprost or placebo at an initial dose of 1 tablet BID (50 µg iloprost clathrate per tablet) that was escalated to 3 tablets (150 µg) BID as tolerated. Following 6 months of treatment, a second bronchoscopy was carried out with repeat biopsies at all of the baseline sites, as well as any new suspicious areas.

Pre- and posttreatment bronchial biopsies were available for all 125 subjects completing the trial. All biopsies were graded according to the World Health Organization (WHO) classification for bronchial epithelium (13) and scored according to the following system: (i) normal histology; (ii) reserve cell hyperplasia; (iii) squamous metaplasia; (iv) mild dysplasia; (v) moderate dysplasia; (vi) severe dysplasia; (vii) carcinoma in situ; and (viii) invasive carcinoma. All the histopathologic classifications were carried out by 1 pathologist (W.A. Franklin) and a subset was repeated by W. A. Franklin and by a second pathologist (D.T. Merrick) to determine inter- and intraobserver reproducibility. Figure 1 illustrates normal tissue and dysplasia. Histologies from the same sites were compared before and after treatment. Six to 12 biopsies, with an average of 10, were obtained for each subject.

Sample size calculation and sample selection for the present biomarker study

Power was greater than 80% to detect a 2-fold or greater change in miRNA expression between 2 groups with a false discovery rate (FDR) of 0.10 across miRNAs. The biopsy sites corresponding to the best (lowest histology score) and worst (highest histology score) at baseline were sampled. The corresponding biopsy sites at follow-up were also selected for study inclusion. We stratified all analyses by smoking status. Using data from our preliminary studies, we found that the 90th percentile of the SDs over miRNA and histology groups was approximately 0.71 on the log 2 scale. Assuming that a log 2 difference in miRNA expression between groups (a fold change of 2) is the smallest biological difference of interest, and that there are 2 truly differentially expressed miRNAs in the set, we estimated that a minimum of 13 subjects per group would be needed, while for a smaller difference of 0.7 on the log scale a minimum of 24 subjects per group would be needed. For analyses where subjects are the unit of analysis, 125 subjects – 29 former and 31 current smokers in the iloprost arm, and 28 former smokers (9).
and 37 current smokers in the placebo arm were available. We also correlated changes in histology with changes in miRNA expression at the biopsy level. To identify a predictive signature of response, we compared the association between expression and response in the 2 treatment arms by smoking status. Finally, we correlated changes in histology at the biopsy level with changes in expression of miRNAs.

For each biopsy, a slide with 8 sections from a formalin-fixed paraffin embedded (FFPE) block was used for the extraction of the miRNAs. The slide adjacent to the pathological diagnostic slide was used and histology was confirmed by the study pathologist (W.A. Franklin).

Selection of the miRNA analyzed

Fourteen miRNAs were selected from the list of 69 miRNA identified as being differentially expressed during lung squamous carcinogenesis (12) using the following criteria: (i) the miRNA were statistically differentially expressed in at least 1 comparison involving high-grade lesions after correction for multiple testing (CMT); or (ii) the miRNA were statistically differentially expressed in at least 1 comparison involving high-grade lesions (but not necessarily after CMT) and reported as modified in response to lung inflammation (14) and, thus, biologically relevant for iloprost. In total, 14 miRNA met these criteria and were analyzed, including: miR-9, miR-34c, miR-101, miR-135a, miR-142-3p, miR-196a, miR-196b, miR-199a, miR-214, miR-224, miR-9, miR-34c, miR-101, miR-135a, miR-139, miR-142-3p, miR-196a, miR-196b, miR-199a, miR-214, miR-224, miR-375, miR-452, and miR-487b.

Nucleic acid extraction and miRNA analyses

Total RNA was extracted from a slide stored at room temperature and containing eight 4-μm sections. The 8 FFPE sections were pulled off the slides and dropped into a 1.5-mL microfuge tube. Total RNA was extracted using the MasterPure RNA Purification kit (Epicentre kit; Biozym) according to the manufacturer’s protocol. RNA was stored at −80°C.

The maximum available yield of total RNA (estimated using a standard curve as ranging between 0.11 and 135.19 ng, with a median at 9.65 ng) was used for every sample. miRNA were retrotranscribed and amplified (PCR) using the TaqMan MicroRNA Reverse transcription kit (Applied Biosystem). MiRNA expression was analyzed by TaqMan MicroRNA assays (Applied Biosystem) using the following assays: hsa-miR-9 (Assay ID 00583), hsa-miR-34c (000428), hsa-miR-101 (002253), hsa-miR-135a (000460), hsa-miR-139-5p (002289), hsa-miR-142-3p (000464), hsa-miR-196a (241070-mat), hsa-miR-196b (002215), hsa-miR-199a (000498), hsa-miR-214 (002306), hsa-miR-224 (002099), hsa-miR-375 (000564), hsa-miR-452 (002329), and hsa-miR-487b (001285). The small nuclear housekeeping RNA, RNU148 (SNORD48; 001006), served as the control and was run in triplicate for the large majority of the samples (451 – 91%) or in duplicate for 45 samples (9%). In our previous study, RNU148 proved to be very stable in bronchial biopsies (12). The amount of RNA from each sample was calibrated to the average value of the cycle threshold (Ct) values for RNU148 in the same sample. This normalized value gives a delta Ct (ΔCt) value for each miRNA relative to the RNU148 (miRNA Ct value – RNU148 average Ct value). The average ΔCt was then calculated for each group of samples and the delta ΔCt (ΔΔCt), which corresponds to the differences between 2 groups, was obtained by subtracting the average ΔCt of the second group from that of the first group. Fold differences for upregulated miRNAs were calculated using $2^{\frac{-\Delta C_t}{50}}$, as a decrease in 1 Ct value is equivalent to a 2-fold increase in the amount of starting cDNA. For the downregulated miRNAs, fold differences were calculated using $1/2^{\frac{-\Delta C_t}{50}}$. By definition, a higher ΔΔCt value represents a lower expression and vice versa.

In a sample, exhibiting a Ct value either more than 37 or undetermined for RNU148 would be considered as non-valuable. All samples were deemed evaluable. Undetermined ΔΔCt values for the miRNA assays were discarded from further analyses as recommended (15).

Statistical analyses

Welch (i.e., unequal variance) t test P values were used to assess the statistical significance of the difference in miRNA expression between specific groups, that is, between iloprost- and placebo-treated subjects, before and after treatment and between responders and non-responders treated with iloprost (16). We stratified all analyses by smoking status (current vs. former). Logistic regression models were used to analyze the relationship between baseline or change in miRNA expression and response status. All logistic regression models were adjusted for age, sex, and pack-years. Models for former smokers were also adjusted for number of years since smoking cessation. Assessment of the relationships between baseline histology and ΔCt and change in histology and ΔΔCt was assessed using Spearman correlation coefficients.

The FDR was set to 0.1 for all analyses. FDR adjustments to P values were applied across the 14 miRNAs for each set of comparisons (e.g., responder vs. non-responder).

Histologic response was evaluated using 2 different criteria. The first criterion termed the "worst histology score criterion" used 1 definition from the clinical trial: a decrease of 1 or more in worst histology score from the same patient after treatment as compared with before (9). The second is the "paired sample based criterion", and response is defined by a decrease of 1 histologic grade within matched-paired biopsies taken at the same site before and after treatment.

The analyses for this study were generated by 2 biostatisticians (W.J. Feser and A.E. Baron) using SAS software, Version 9.2 of the SAS System for Windows Copyright 2011 SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA.

Results

Characteristics of the study population

A total of 496 out of the 500 bronchial biopsies requested from the Colorado SPORE Tissue Bank had sufficient tissue...
for this study: 248 samples at baseline and 248 samples at follow-up. The distribution of bronchial histologies at baseline and follow-up based on treatment arms and stratified by smoking status is shown in online Supplementary Table S1. In the whole cohort, the distribution of the histology was: 37% normal mucosa (39% and 36% of baseline and follow-up samples, respectively), 23% hyperplasia (19%/26%), 3% metaplasia (1%/5%), 14% mild dysplasia (13%/14%), 21% moderate dysplasia (22%/19%), and 3% severe dysplasia (6%/1%). Moderate and severe dysplasias were more frequent in the current smokers (38/226, 17%; P = 0.001) and in baseline biopsies (69/248, 28%) compared with follow-up biopsies (49/248, 20%; P = 0.0448). The distribution of histologies within the pairs of samples [best (lowest score) or worst (highest score) histology at baseline] is shown in online Supplementary Table S2. The very large majority of biopsies (93%) with the best histology at baseline have either a normal histology (78/125, 63%) or a reserve cell hyperplasia (38/125, 30%).

The histologic response rates per arm, stratified by smoking status, are reported in online Supplementary Table S3. Based on the "worst histology score criterion" for response, there were more responders in former than in current smokers (48% vs. 33%) in the iloprost arm and inversely (15% vs. 24%) in the placebo arm. The response rate was lower in the placebo arm than in the iloprost arm as presented previously (9). Based on the "paired sample based criterion", the response rate was much higher in the pairs of biopsies with the worst histology at baseline than in those with best histology at baseline, both in former smokers (response rate 55% vs. 7% for pairs of biopsies with the worse or the best histology at baseline) and in current smokers (67% vs. 6%) in the iloprost arm. We observed the same in the placebo arm with response rate of 37% versus 7% in former smokers and 49% versus 11% in current smokers. This is at least partially explained by the large number of samples with normal histology in the pairs with best histology at baseline that makes response impossible, as the histologic score cannot improve. As shown above, the response rates are slightly better and the differences are slightly larger in the iloprost than in the placebo arm.

miRNA expression associations with baseline characteristics

Association with baseline histology. At baseline, 7 miRNA were found significantly correlated to histology stages after FDR adjustment: miR-452, miR-224, miR-101, miR-139-5p, miR-199a, miR-214, and miR-34c. The expression of 2 miRNA was directly correlated with the stage of histology from lower to higher (Cv values inversely correlated; thus, expression positively correlated): miR-452 (r = −0.43; P < 0.001) and miR-224 (r = −0.54; P < 0.001). The expression of the 5 other miRNAs was inversely correlated (Cv values directly correlated thus expression inversely correlated) with the stage of histology: miR-101 (r = 0.20; P = 0.0013), miR-139-5p (r = 0.18; P = 0.0042), miR-199a (r = 0.20; P = 0.0024), miR-214 (r = 0.18; P = 0.0060), and miR-34c (r = 0.36; P < 0.0001).

Association with current versus former smoker status. Several miRNAs showed a significantly different expression at baseline in current smokers compared with former smokers. Three miRNAs had a significantly higher expression in current than in former smokers after FDR adjustment: miR-224 (P < 0.0001), miR-375 (P < 0.0001), and miR-452 (P < 0.0001). Two miRNAs had a lower expression in current compared with former smokers after FDR adjustment: miR-142-3p (P = 0.0069) and miR-34c (P = 0.0550). We further restricted comparison of miRNA expression between current and former smokers to biopsies with a normal histology at baseline, in order to control for the more frequent dysplasia in current smokers. In biopsies with normal histology, the upregulation of miR-224 (P = 0.0003), miR-375 (P < 0.0001), and miR-452 (P < 0.0013) was confirmed and miR-9 (P = 0.0278) and miR-487b (P = 0.0290) were also found significantly upregulated in current smokers compared with former smokers, all after FDR adjustment. The downregulation of miR-142-3p and miR-34c in current smokers was not found to be significant in normal biopsies.

Association with treatment arm. There was no significant difference in miRNA expression at baseline between the samples of the iloprost arm and those of the placebo arm for any of the miRNA tested.

Expression changes between baseline and follow-up biopsies

Changes specific to iloprost administration. The expression of miR-375 was not correlated with histology at baseline or histologic changes at follow-up but was significantly upregulated in current compared with former smokers. In current smokers, the expression of miR-375 was significantly downregulated after treatment by iloprost (P = 0.0023 after FDR adjustment; Table 1). Furthermore, the downregulation of miR-375 in current smokers after treatment by iloprost was significantly different (P = 0.0011 after FDR adjustment) from its change in current smokers who received the placebo, where a slight but not significant upregulation was observed. Thus, the downregulation of miR-375 in current smokers appears to be specific to iloprost treatment.

Association with histologic changes. We assessed the correlation between changes in miRNA expressions and changes in histology stages between baseline and follow-up biopsies. In the whole cohort, the changes in expression of 2 miRNAs, miR-34c and miR-224, were significantly correlated after FDR adjustment with histologic changes between baseline and follow-up. Including all patients in the analyses, the expression of miR-224 was upregulated in higher histologic grades (r = −0.22; P = 0.0006; Fig. 2). This was also shown in 1 subgroup, the former smokers in the iloprost arm (r = −0.37; P = 0.0044; Fig. 2). The changes in expression of miR-34c were inversely correlated with histologic changes, as miR-34c expression was downregulated in...
higher histologic grades. This correlation between miR-34c expression and histology changes is shown when including all samples ($r = 0.23; P = 0.0003$). A significant correlation (but not after FDR adjustment) was also consistently seen in most subgroups (iloprost arm: current smokers, $r = 0.26; P = 0.041$; former smokers, $r = 0.24; P = 0.0666$; placebo arm: current smokers, $r = 0.23; P = 0.0466$). These results are illustrated in Table 2.

### Table 1. Significant changes in miRNA expression in follow-up samples as compared with baseline samples

<table>
<thead>
<tr>
<th>miRNA</th>
<th>All</th>
<th>Both smoker groups</th>
<th>Current smokers</th>
<th>Former smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Iloprost</td>
<td>Placebo</td>
<td>Iloprost</td>
</tr>
<tr>
<td>miR-9</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
</tr>
<tr>
<td></td>
<td>$P &lt; 0.0001$</td>
<td>$P = 0.0007$</td>
<td>$P = 0.001$</td>
<td>$P = 0.0022$</td>
</tr>
<tr>
<td>miR-375</td>
<td>Not significant</td>
<td>Not significant</td>
<td>Not significant</td>
<td>Down</td>
</tr>
<tr>
<td></td>
<td>$P = 0.0023$</td>
<td>$FDR$</td>
<td>$FDR$</td>
<td>$P = 0.0023$</td>
</tr>
</tbody>
</table>

NOTE: This table reports the significant modification of miRNA expression in follow-up samples as compared with baseline samples matched from the same site. Only miR-9 and miR-375 showed significant changes in expression level at follow-up. The table shows up- or downregulation at follow-up compared with baseline. The significant $P$ values using Welch $t$ test are reported. “FDR” indicates that the $P$ value is significant after adjustment for FDR.
Downregulation of miR-9 expression after bronchial biopsy. MiR-9 expression was not correlated with histology at baseline (r = 0.01, P = 0.8600) or with histologic changes between baseline and follow-up (r = 0.04, P = 0.5074). However, miR-9 was found to be significantly downregulated in follow-up as compared with baseline biopsies. The downregulation of miR-9 expression in follow-up compared with baseline samples was significant after FDR adjustment when analyzing all samples (Welch t test, P < 0.0001) and in most subgroups (Table 1): in the iloprost arm, all patients (P = 0.0007) and current smokers (P = 0.0022) and, in the placebo arm, all patients (P = 0.001), and, former smokers (P = 0.0071). In current smokers in the placebo arm (P = 0.0476) and in the former smokers in the iloprost arm (P = 0.0868), the same trend was observed, but did not achieve statistical significance after FDR adjustment. Thus, miR-9 is consistently downregulated in follow-up biopsies 6 months later at the same site independently of histology, treatment, or smoking status.

Comparison of miRNA expression between histologic responders and nonresponders

Significant differences at baseline between responders and nonresponders in both the iloprost and placebo arms were shown for the expression of 2 miRNAs that are correlated with histology. A lower expression of miR-34c at baseline was detected in responders compared with nonresponders, both in the iloprost and placebo arms, and was very consistent with OR ranging between 1.38 and 1.49. A significantly higher expression of miR-224 at baseline was also shown in responders compared with nonresponders in several subgroups. Detailed results and OR in different subgroups are illustrated in online Supplementary Table S4.

In current smokers in the placebo arm, a lower expression of miR-375 at baseline predicted response using the "worst histology score criterion" in all samples (t test = 0.0012 after FDR adjustment with OR = 1.93 [95% confidence interval (CI), 1.10–3.39, P = 0.0219] as well as in the "paired sample based criterion" (t test = 0.0027 after FDR adjustment and OR = 3.74; 95% CI: 1.27–10.98, P = 0.0165).

We tested the predictive value of miRNA expressions using interaction terms between baseline expression and treatment arm in the logistic regression models of response. No individual miRNAs were found to be predictive of response to iloprost. We examined the group of miRNAs to determine if they, together or as subsets, were associated with response overall and, then, if they were differentially associated with response in the iloprost versus placebo group. We did this in the former smokers only because that was the group in which iloprost showed a beneficial effect on histology. We evaluated 3 models: 1 in which the definition of response for each biopsy was at the participant level, 1 in which the definition of response was at the biopsy level, and 1 in which the response was defined at the biopsy level and excluded the biopsies that were normal at baseline. In the first model, 1 miRNA was associated with response after adjusting for all of the design variables (treatment, pack-years of smoking, years since quitting, age, and sex) but it was not predictive (P = 0.98 for the 1 df test of the interaction between miRNA and treatment.

### Table 2. Correlation between miRNA expression changes and histology changes at follow-up

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Baseline samples</th>
<th>Paired samples: follow-up expression compared with baseline</th>
<th>Spearman correlation between miRNA expression changes and histology change (upgrading) at follow-up compared with baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spearman correlation between miRNA expression and histology grade (low to high)</td>
<td>All</td>
<td>Iloprost</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-34c</td>
<td>Down</td>
<td>Down</td>
<td>Down</td>
</tr>
<tr>
<td></td>
<td>r = 0.36</td>
<td>r = 0.23</td>
<td>r = 0.26</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.0001, FDR</td>
<td>P = 0.0003, FDR</td>
<td>P = 0.041</td>
</tr>
<tr>
<td>miR-224</td>
<td>Up</td>
<td>Up</td>
<td>Up</td>
</tr>
<tr>
<td></td>
<td>r = 0.54</td>
<td>r = 0.22</td>
<td>r = 0.15</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.001, FDR</td>
<td>P = 0.0006, FDR</td>
<td>Not significant</td>
</tr>
</tbody>
</table>

**NOTE:** This table reports the correlations between miRNA expression and histology at baseline and between miRNA expression changes and histology changes at follow-up for miR-34c and miR-224 as these 2 miRNA are the ones whose expression changes are correlated with the histology changes. The table shows up- or downregulation with regard to histology upgrading at follow-up compared with baseline. The Spearman correlation coefficient (R) is negative when the C value is inversely correlated with histologo upgrading and, thus, reflect a positively correlated expression. The Spearman correlation coefficient is positive when the C value are positively correlated with histology upgrading and, thus, reflect an inversely correlated expression. Significant P values are reported. "FDR" indicates that the P value is significant after adjustment for FDR.
group). In the second model, there were 4 miRNA that were together independently associated with response after adjustment but were not predictive ($P = 0.4$ for the 4 df test of the interaction between miRNA and treatment group), and in the third model, no miRNA was associated with response after adjustment. Thus, we conclude that the miRNAs applied are not predictive of response to iloprost, neither as single markers nor in combinations.

**Discussion**

In the current study, we hypothesized that miRNA expression could be used as an intermediate endpoint or as a predictive biomarker in a recent lung cancer chemoprevention trial. We tested this hypothesis in the phase II iloprost lung cancer chemoprevention trial by analyzing the expression of 14 selected miRNA in matched bronchial biopsies before and after treatment with iloprost or placebo. MiRNA analyses were successfully carried out from small FFPE endobronchial biopsies, providing an extremely low amount of starting RNA. Despite this technical challenge, the reproducibility of the data was very high with a low variability in RNU48 Ct value triplicates.

Seven of the miRNAs analyzed in this study were correlated with histology at baseline. The direct or inverse correlation to histology stage of expression of 6/7 of these miRNAs is concordant with our previously reported data (12). This study did not aim nor was it designed to validate the data of the previous study. However, the concordant results support the validity of the present study’s results.

In the current study, biopsies were collected at multiple sites before and after treatment, providing unique paired data. We analyzed the change in miRNA expression in biopsies at the same site before and after treatment. The expression of miR-34c and miR-244 correlated (inversely or directly, respectively) with histology at baseline and histologic changes. However, the association with histology and histologic changes was stronger and more consistently seen for miR-34c. In mice, miR-34a is ubiquitously expressed, while miR-34b/c is mainly expressed in the lung. The members of the miR-34 family are direct targets of p53 and, thus, are key players in apoptosis and cell-cycle arrest (17). The promoter of miR-34c has been shown to be methylated and miR-34c is, thus, downregulated in several cancers including non–small cell lung cancer (18–22). DNA hypermethylation of miR-34b/c also has a prognostic value for stage I NSCLC (21). Downregulation was shown in rats in after exposure to cigarette smoke (23) or the tobacco-associated carcinogen NNK (24). We previously showed that miR-34c is downregulated in normal human bronchial biopsies from smokers compared with never smokers and is further downregulated during the successive steps of lung carcinogenesis (12). In the current study, we showed that changes in miR-34c expression between baseline and follow-up correlate with histology changes in both the iloprost and placebo arms. As miR-34c expression is a reproducible and quantitative laboratory assay reflecting histologic changes and is independent of treatment, miR-34c could be a biomarker for the quantitative measure of histologic response and a potential intermediate endpoint in lung cancer chemoprevention trials. However, the present study does not show that miR-34c is an intermediate endpoint, or a biomarker that could replace histology or add to histology for evaluation of response. Prospective studies designed to investigate these questions are warranted.

In addition, the results showed that a lower expression of miR-34c and a higher expression of miR-224 were detected in responders in both treatment and placebo arm. As miR-34c and miR-224 expressions are correlated, inversely and directly, respectively, with the stages of histology at baseline, lower miR-34c and higher miR-224 expressions at baseline in responders are likely to be related to higher histology at baseline, which allows response (histology downgrading) while normal histology does not. Therefore, these miRNAs cannot be considered as a predictor of response.

MiR-9 was reproducibly downregulated in follow-up independently of histology, smoking status, and treatment. Thus, the downregulation of miR-9 expression at follow-up at the same site might be related to the biopsy and subsequent repair. Mir-9 is highly evolutionarily conserved (25) and regulates the expression of genes involved in cell motility and invasion. It downregulates SNAIL-1 and NF-kB and upregulates E-cadherin (26). MiR-9 also plays a role in cytoskeletal organization (26) and its downregulation increases cell motility (26). This could, perhaps, explain its potential role in tissue repair, but this hypothesis requires further study.

To our knowledge, differences in miRNA expression between current and former smokers have not been previously examined. There are previous data in animal models (23, 24, 27) and in human airways analyzing miRNA profiles between never smokers and smokers (12, 28). We found that several miRNAs were up- or downregulated in current smokers as compared with former smokers. The miRNAs that were upregulated in current smokers in this study have not been previously described as related to smoking. The 2 miRNAs, miR-142-3p and miR-34c, that were downregulated in current smokers as compared with former smokers were described earlier. Mir-142-3p and miR-34c were observed to be downregulated in the normal bronchial mucosa of smokers as compared with never smokers in our previous study (12). In addition, miR-34c has been shown to be downregulated in rats exposed to cigarette smoke (23).

MiR-375 is upregulated in airways by current smoking and downregulated after iloprost treatment in current smokers. The expression of miR-375 has not been shown to be related to smoking in animal studies or in studies using human tissue and comparing never smokers to smokers. In normal lung, miR-375 has been shown to inhibit surfactant secretion by altering cytoskeletal reorganization (29). We showed in our previous study that miR-375 was downregulated in invasive squamous carcinoma of the lung compared with noninvasive bronchial lesions (12). MiR-375 has been shown to be downregulated in other squamous tumors (30, 31) as well as other cancers (32–36). Its upregulation in active smokers as compared with former smokers could be related to the secretion of miR-375 and to the upregulation of SNAIL-1 (37), which increases cell motility (26). This could, perhaps, explain its potential role in tissue repair, but this hypothesis requires further study.

MiR-375 has been shown to be related to histology, smoking status, and treatment. Thus, the downregulation of miR-9 expression at follow-up at the same site might be related to the biopsy and subsequent repair. Mir-9 is highly evolutionarily conserved (25) and regulates the expression of genes involved in cell motility and invasion. It downregulates SNAIL-1 and NF-kB and upregulates E-cadherin (26). MiR-9 also plays a role in cytoskeletal organization (26) and its downregulation increases cell motility (26). This could, perhaps, explain its potential role in tissue repair, but this hypothesis requires further study.
smokers and its downregulation in current smokers by iloprost treatment are of interest and cannot be explained by any previous functional data on miR-375.

This study failed to identify predictive markers of response to iloprost. The phase II Iloprost Trial was not designed for finding predictive biomarkers to histologic response. In addition, other miRNAs, which we did not study could potentially be predictive of response to iloprost. We could not study a larger panel of miRNAs because the amount of available RNA was limited. Therefore, we selected miRNAs on the basis of our previous findings in similar bronchial biopsies. This work must be considered as being exploratory, and further investigation is required in subsequent chemoprevention trials. As no predictive biomarkers were found, it will be necessary to carry out additional similar studies with a larger panel of miRNAs.

In conclusion, no biomarkers predictive of response to iloprost were discovered. However, some interesting results were obtained. MiR-375 was downregulated in current smokers receiving iloprost. MiR-9 was downregulated in follow-up biopsies compared with baseline biopsies and may represent a tissue repair response. Several novel miRNAs with expression modulated by current versus former smoking status were identified. The expression of miR-34c was inversely correlated with histology at baseline and with changes in histology between baseline and follow-up, independent of the treatment arm or smoking status. Thus, the change in miR-34c expression between baseline and follow-up biopsies may be a quantitative biomarker of histologic response. Additional lung chemoprevention trials are in progress and we plan to validate the role of miR-34c and others in these trials.

Disclosure of Potential Conflicts of Interest
PAB is a consultant for Bayer. YEM and RLK have applied for a patent regarding prostacyclin for the chemoprevention of cancer. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: C. Mascaux, A.E. Baron, W.A. Franklin, P.A. Bunn, Y.E. Miller, R.L. Keith, F.R. Hirsch
Development of methodology: C. Mascaux, J.I. Eckelberger, W.A. Franklin, F.R. Hirsch
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Mascaux, M.T. Lewis, D.T. Merrick, T.C. Kennedy, J.I. Eckelberger, P.A. Bunn, Y.E. Miller, R.L. Keith, F.R. Hirsch
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Mascaux, W.J. Fezer, A.E. Baron, C.D. Coldren, L.M. Rezeboom, P.A. Bunn, F.R. Hirsch
Writing, review, and/or revision of the manuscript: C. Mascaux, W.J. Fezer, A.E. Baron, D.T. Merrick, L.M. Rezeboom, W.A. Franklin, J.D. Minna, P.A. Bunn, Y.E. Miller, R.L. Keith, F.R. Hirsch
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W.J. Fezer, J.I. Eckelberger, M.T. Lewis, F.R. Hirsch
Study supervision: Y.E. Miller, R.L. Keith, F.R. Hirsch

Grant Support
This work was financially supported by a Specialized Program of Research Excellence (SPORE) Career Development Award (C. Mascaux) and by Lung Cancer Biomarkers and Chemoprevention Consortium (LCBCC) funds (C. Mascaux and F.R. Hirsch). C. Mascaux was also supported by a fellowship from the International Association for the Study of Lung Cancer (IASLC), a grant from the Louisiana Chapter of the National Lung Cancer Partnership (NLCP), and a grant from the Gary L. and Thelissa Zollinger Early Detection of Lung Cancer Endowment Fund. Additional support was provided from the Colorado SPORE in Lung Cancer NCI P50 CA58187, NCI RO1 CA164780, Texas SPORE in Lung Cancer (P50 CA079097), Department of Veterans Affairs Merit Review Program (to R.L. Keith).

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Received September 10, 2012; revised December 3, 2012; accepted December 7, 2012; published OnlineFirst December 26, 2012.

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Endobronchial miRNAs as Biomarkers in Lung Cancer Chemoprevention

Celine Mascaux, William J. Feser, Marina T. Lewis, et al.


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