Alteration of microRNA expression in vinyl carbamate-induced mouse lung tumors and modulation by the chemopreventive agent indole-3-carbinol

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MicroRNAs (miRNAs) are small, non-protein-coding RNAs that can function as tumor suppressors or oncogenes. Deregelation of miRNA expression has been reported in lung cancer. However, modulation of miRNA expression by chemopreventive agents remains to be defined. In the present study, we examined if the chemopreventive agent indole-3-carbinol (I3C) reversed vinyl carbamate (VC)-induced deregulation of miRNA levels in lung tissues of female A/J mice. Lung tissues were obtained from a previous chemoprevention study, in which mice were treated with VC and given I3C in the diet for 15 weeks. Microarray studies revealed alterations in the expression of a number of miRNAs in lung tumors relative to that of normal lungs. miR-21, miR-31, miR-130a, miR-146b and miR-377 were consistently upregulated, whereas miR-1 and miR-143 were downregulated in lung tumors relative to normal lungs. In mice treated with VC and given I3C in the diet, levels of miR-21, miR-31, miR-130a, miR-146b and miR-377 were reduced relative to the level in mice treated with the carcinogen only. The results of the microarray study were confirmed by quantitative reverse transcription–polymerase chain reaction and gel analysis of polymerase chain reaction products. Further studies with miR-21 indicated that phosphatase and tensin homolog, programmed cell death 4 and rich protein with Kazal motifs are potential targets for the oncogenic effect of miR-21 and the chemopreventive activity of I3C. Taken together, we showed here that miRNAs are deregulated during VC-induced mouse lung tumorigenesis and their levels are modulated by I3C. Therefore, miRNAs and their target genes are promising biomarkers for the diagnosis of lung cancer and efficacy of chemopreventive/chemotherapeutic agents.

Introduction

Lung cancer is the most common cancer-related death worldwide (1). In the USA, an estimated 219,440 new cases and 159,390 deaths are expected in 2009, accounting for 15% of cancer diagnoses and 28% of all cancer deaths, respectively (2). The 5-year survival rate of lung cancer is only 15%, clearly indicating the need for prevention of lung cancer. Epidemiological studies indicate an inverse relationship between consumption of cruciferous vegetables and mortality from lung cancer (3–6). The cancer preventive effect of cruciferous vegetables is attributed to their different phytochemical constituents. One of the most important anticarcinogenic phytochemicals contained in these vegetables is indole-3-carbinol (I3C), an enzymatic breakdown product of indole glucosinolates, sulfur-containing compounds contained in cruciferous vegetables. In the stomach, I3C undergoes condensation reactions to produce various products, the major one being 3,3-diindolylmethane (7), to which most of the biological activities of I3C are attributed (8). Considerable evidence shows that I3C inhibits experimentally induced tumorigenesis in murine models at different sites, including lung, through induction of phase I and phase II enzymes, inhibition of proliferation and induction of apoptosis in tumor cells and modulation of estrogen metabolism (6). In humans, I3C increased the levels of glutathione-S-transferase and P450 1A2, elevated the proportion of estrogen metabolized through the 2-hydroxylation pathway, ‘the good estrogen’ (9) and inhibited cervical intraepithelial hyperplasia (10) and recurrent respiratory papillomatosis (11).

The absence of clinically proven chemopreventive agents against lung cancer is attributed mainly to the incomplete understanding of the molecular mode of action of the agents. Recent advances in gene expression technology enabled identification of novel mediators of the activities of chemopreventive agents. Analysis of messenger RNA (mRNA) expression patterns provided information on effects of chemopreventive agents on a large numbers of genes simultaneously, which is not evident by classical methods. However, despite accumulating data showing the importance of mRNA expression in identifying targets for chemopreventive agents, the posttranscriptional events that also influence cellular behavior are missed. Therefore, alternative approaches are required.

MicroRNAs (miRNAs) are a class of naturally occurring small non-coding RNAs of ~22 nucleotides that have recently emerged as important regulators of gene expression at the posttranscription level. miRNAs frequently undergo aberrant regulation during tumorigenesis, resulting in overexpression or underexpression (12). miRNAs that are overexpressed in tumors may contribute to tumorigenesis by downregulating tumor suppressor genes, whereas underexpression of miRNAs results in oncogene overexpression. Among miRNAs linked to lung cancer, let-7, a negative regulator of the oncogenes KRAS, HMGA2 and c-Myc, is the most studied. Let-7 expression is reduced in a subset of non-small cell lung cancer patients, and this reduction is correlated with poor prognosis (13,14). Recently, several other lung cancer-related miRNAs have been identified (14,15). These studies indicate that miRNAs are attractive targets for lung cancer chemoprevention and chemotherapy.

In the present study, we examined alteration of miRNA expression in vinyl carbamate (VC)-induced lung tumors in A/J mice and assessed if dietary administration of the chemopreventive agent I3C to carcinogen-treated mice modulates miRNA levels. Among carcinogens commonly used to induce lung tumors in A/J mice, VC is the strongest carcinogen, in terms of the incidence of malignant tumors and the multiplicity of pulmonary surface tumors (16). We also determined changes in the expression of genes targeted by miR-21, the most commonly altered miRNA in solid tumors. Our results demonstrate differential changes in the expression of several miRNAs in lung tumors versus normal lungs. Moreover, levels of most of the miRNAs were modulated by I3C. Our study indicates that miRNAs could be potential biomarkers for lung cancer diagnosis and prognosis and efficacy of chemopreventive and chemotherapeutic agents.

Materials and methods

Materials

VC was purchased from Toronto Research Chemicals (Ontario, Canada). I3C was from Sigma (St Louis, MO). The miRNA probe set and Trizol were from Invitrogen (Carlsbad, CA). All chemicals and reagents used for quantitative reverse transcription–polymerase chain reaction (QRT–PCR) were from Qiagen (Valencia, CA).
Table I. miRNA primer sequences used for verification of miRNA microarray results by QRT–PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (F &amp; R 5’ to 3’)</th>
<th>Accession no. or reference article</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td>TAGCCTATCAGACTGATTTGA</td>
<td>MIMAT0000076</td>
<td>8–29</td>
</tr>
<tr>
<td>miR-31</td>
<td>AGGCAAGATCTGGCAGCT</td>
<td>MIMAT000089</td>
<td>8–28</td>
</tr>
<tr>
<td>miR-130a</td>
<td>CAGTGGCAATGGTAAAGGCGAT</td>
<td>MIMAT000425</td>
<td>55–76</td>
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<tr>
<td>miR-146b</td>
<td>TGAGAATCGGATCTCACTGCT</td>
<td>MIMAT0002809</td>
<td>9–30</td>
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<tr>
<td>miR-377</td>
<td>ATTCACAAAGGCCAATCTTGT</td>
<td>MIMAT000730</td>
<td>45–66</td>
</tr>
<tr>
<td>miR-1</td>
<td>TGGAATGGAAGAAGATGATGA</td>
<td>MIMAT000003</td>
<td>61–81</td>
</tr>
<tr>
<td>miR-143</td>
<td>TGAGATGGAGCAGCTTGACTC</td>
<td>MIMAT000435</td>
<td>61–81</td>
</tr>
<tr>
<td>U6</td>
<td>GCCAGCACATATACTAAAATTGGAA</td>
<td>Kalscheuer et al. (18)</td>
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</tr>
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</table>

Table II. Primer sequences for miR-21 target miRNAs used for QRT–PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (F &amp; R 5’ to 3’)</th>
<th>Accession no. or reference article</th>
<th>miRNA size (bp)</th>
<th>Position</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPRY2</td>
<td>TTATTAACAGCGGATGTTG and CACAGAG GCTTGAACACACG</td>
<td>NM_011897</td>
<td>2103</td>
<td>90–260</td>
<td>171</td>
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<tr>
<td>TGFβ1</td>
<td>CAGCAATCTAGGAAACCAA and GAGGTC AAAAAACACCACTCG</td>
<td>NM_011577</td>
<td>2094</td>
<td>1221–1467</td>
<td>247</td>
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<tr>
<td>PDCD4</td>
<td>CAGCTACGCTTTGACACTCTT and CAGATCG GCCACACACCTTGT</td>
<td>NM_011050</td>
<td>2407</td>
<td>1035–1242</td>
<td>208</td>
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<tr>
<td>PTEN</td>
<td>AGTTTTGCGTCTGGCCAGCTA and AGGTTTTC TCTGTCTCGTGA</td>
<td>NM_008960</td>
<td>8229</td>
<td>1509–1727</td>
<td>219</td>
</tr>
<tr>
<td>TPM1</td>
<td>CTCCTCTGAAAGGACTCCTT and CCTCTCT TCAAGGGCTTCTT</td>
<td>NM_024427</td>
<td>1702</td>
<td>338–532</td>
<td>195</td>
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<tr>
<td>RECK</td>
<td>CAAAAGCTCTCTGCAGAGTCG and TCAGGG GTGTCAGGAGCAGAAG</td>
<td>NM_016678</td>
<td>4450</td>
<td>2757–2992</td>
<td>236</td>
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<tr>
<td>CK-18</td>
<td>CGTCAGAGACTGCGCGCCACTTCTT and ATGT CGCTCTCCACAGACTGC</td>
<td>NMNM_010664.1</td>
<td>1386</td>
<td>399–578</td>
<td>180</td>
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<tr>
<td>HPRT1</td>
<td>ATTACGGATGATGAAAGGCTGTATG and TTAT GTCTCCGTTGACTGATC</td>
<td>NM_013556.2</td>
<td>1349</td>
<td>174–489</td>
<td>316</td>
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<tr>
<td>GAPDH</td>
<td>GTGGGATGTTGTCACATCAGC and CAGTG GATGCAGAGATGTGTCTC</td>
<td>Huang et al. (17)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT1, hypoxanthine phosphoribosyltransferase 1; R, reverse; SPRY2, sprouty homolog 2; TGFβ1, transforming growth factor β1; TPM1, tropomyosin 1.

Cells and cell culture

Normal human bronchial epithelial (NHBE) cells were purchased from Clonetics (Palo Alto, CA) and cultured in medium supplemented with defined growth factors and retinoic acid (0.1 ng/ml) contained within the SingleQuot kit of bronchial epithelial cell growth medium provided by Clonetics. Cells were maintained in humidified incubators at 37°C and 5% CO2. Human telomerase (hTERT)-immortalized NHBE cells were kindly provided by Dr O’Grady (Animal Science Department, University of Minnesota) and grown as NHBE gene (hTERT)-immortalized NHBE cells were kindly provided by Dr O’Grady (Animal Science Department, University of Minnesota) and grown as NHBE gene (hTERT)-immortalized NHBE cells were kindly provided by Dr O’Grady (Animal Science Department, University of Minnesota) and grown as NHBE gene (hTERT)-immortalized NHBE cells were kindly provided by Dr O’Grady (Animal Science Department, University of Minnesota) and grown as NHBE gene (hTERT)-immortalized NHBE cells were kindly provided by Dr O’Grady (Animal Science Department, University of Minnesota) and grown as NHBE.

Total RNA from lung tissues or cells was prepared using Trizol extraction technique. All RNA samples were treated with DNase I (QIAGEN) according to the manufacturer’s protocols. Quantity and quality of the RNAs were assessed by A260/A280 nm reading using NanoDrop1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA integrity was determined by running an aliquot of the RNA samples on a denaturing agarose gel stained with ethidium bromide. The ratio of 28S ribosomal RNA to 18S ribosomal RNA was 2:1, indicating that the RNA samples were intact.

Microarray experiments

Microarray technique was used to analyze the miRNA expression profile of mouse lung tissues at a genome scale following the method of Kalscheuer et al. (18). Briefly, a miRNA probe set containing 16 000 oligonucleotides was purchased from Invitrogen and the oligonucleotides were quadruply printed on Corning GAPSII-coated slides by the Microarray Facility at the University of Minnesota. In total, 712 miRNAs (467 human and/or mouse and/or rat miRNAs, 192 mouse and/or rat miRNAs and 53 rat miRNAs) were analyzed. For RNA labeling, 25 μg of total RNA was ligated to 0.5 μg of a synthetic linker, pCU-DY547 (Dharmacon, Lafayette, CO). To control for the hybridization process, reference DNA oligonucleotides complementary to a subset of mammalian miRNAs were combined and labeled with a ULYSIS Alexa Fluor 647 Kit (Invitrogen). Labeled RNAs and DNAs were then mixed and hybridized to microarray slides. Afterward, slides were scanned to quantify pixel intensities and individual spots on the slides were further inspected to exclude abnormal spots from subsequent calculations. Three microarray experiments
were carried out on different days using technical replicates from pooled lung tissue samples of each experimental group. After background subtraction, sample signals were normalized to the signal of a 28S ribosomal RNA and U6 small nuclear RNA. A separate percentile normalization method (GeneSpring) was also employed. Gene expression changes were deemed valid if concurred by both gene-specific and percentile normalization methods. Excel was used to organize and present normalized data. Signals at least 150% higher than background values were considered positive. 

**QRT–PCR assay of miRNAs**

For real-time reverse transcription–polymerase chain reaction (qRT-PCR), 1 μg of DNase-treated total RNA was reverse transcribed to complementary DNA (cDNA) using miScript Reverse Transcription Kit (QIAGEN). During the reverse transcription step, miRNAs, which are not polyadenylated in nature, were polyadenylated by poly (A) polymerase and subsequently converted into cDNA by reverse transcriptase with oligo-dT and random primers. The cycle parameters for the 20 μl reverse transcription reaction per manufacturer’s protocol were 37°C for 30 min and 95°C for 5 min. The protocol enabled efficient reverse transcription of miRNAs into cDNA in a single step. The cDNA was then used as a template for real-time PCR quantification of miRNAs using the designed specific miRNA primers and the miScript Universal primer in combination with the miScript SYBR Green PCR Kit from QIAGEN. Complementary primer sequences for the mature forms of selected miRNAs were designed based on miBase sequence database. All the primer sequences used in this study are presented in Table I. PCR was performed on an Applied Biosystems 7500 system (Applied Biosystems, Foster City, CA) with aliquots used in this study are presented in Table I. PCR was performed on an Applied Biosystems 7500 system (Applied Biosystems, Foster City, CA) with aliquots.

**Identification of potential miR-21 targets**

Among the differentially expressed miRNAs we identified in mouse lung tumors, miR-21 has been shown to be commonly overexpressed in a variety of cancerous tissues. Therefore, we sought to determine the mRNA targets of miR-21 and analyze their expression at mRNA and protein levels in the lung tissues of the different groups of mice. The databases used for target identification were PicTar, TargetScan, miRanda and TarBase. The mRNA levels of target genes identified by all four databases were subsequently quantified using qRT–PCR. The same RNA samples employed for miRNA analysis were used to quantify the mRNA targets for miR-21 and analyze their expression at mRNA and protein levels in the lung tissues of the different groups of mice. Differences in levels of miRNAs among samples were converted into fold changes as follows. First, the miRNA data were normalized against the expression of U6 small nuclear RNA by subtracting the Ct value of U6 from the Ct value of the target gene (ΔCt). The ΔCt was then calculated by subtracting the ΔCt value of normal lung tissues from vehicle-treated mice or VC+I3C-treated mice (animal studies) or ΔCt value of NHBE cells from ΔCt value of hTERT-immortalized NHBE or A549 cells (cell study) or ΔCt value of dimethyl sulfoxide-treated A549 cells from ΔCt value of I3C-treated A549 cells (I3C study in A549 cells). The ΔCt values were converted to approximate fold differences in gene expression by assuming 100% primer efficiency and using the equation 2^−ΔΔCt.

**Western immunoblot analyses**

Aliquots of mouse lung tissues (pooled normal lung tissue, 30 mg/mouse, from six vehicle-treated mice or pooled lung tumors, 30 mg/mouse, six mice/group from VC-treated group or carcinogen plus I3C group) were ground using mortar and pestle on dry ice and the powder suspended into lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM ethyleneglycol-bis(amoethy-l ether)-tetraacetic acid, 1 mM ethylenediaminetetraacetic acid, 20 mM, 1% Triton X-100, pH 7.4) containing protease inhibitors [aprotinin (1 μg/ml), leupeptin (1 μg/ml), pepstatin (1 μM) and phenylmethylsulfonyl fluoride (0.1 mM)] and the phosphatase inhibitors NaVO4 (1 mM) and NaF (1 mM). Lysates from A549 cells (1 × 10^6) were prepared by suspending the cells in lysis buffer on ice for 1 h. Subsequently, the preparations were centrifuged (14 000g for 25 min at 4°C), the supernatants collected, aliquoted and stored at −80°C. For western immunoblotting, 60 μg of protein per sample were loaded onto a 4–12% Novex Tris-glycine gel (Innogenit) and run for 60 min at 200 V. The proteins were then transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) for 2 h at 30 V. Subsequently, membranes were blocked in 5% Blotto non-fat dry milk in Tris buffer containing 1% Tween 20 for 1 h and probed overnight with the following primary antibodies: anti-reversion-inducing cysteine-rich protein with Kazal motifs (RECK; 1:1000), anti-programmed cell death 4 (PDCD4; 1:1000) and anti-phosphatase and tensin homolog (PTEN; 1:1000) from Cell signaling Technology (Beverly, MA) and anti-matrix metalloproteinase (MMP)-2 (1:200) from Santa Cruz Biotechnology (Santa Cruz, CA). After incubating the membranes with a secondary antibody (goat anti-rabbit IgG, 1:20 000, Santa Cruz Biotechnology) for 1 h, chemiluminescent immunodetection was employed. Signal was...
visualized by exposing membranes to HyBolt CL autoradiography film. Membranes were stripped and probed with anti-β-actin to check for differences in the amount of protein loaded in each lane. The experiment was carried out twice using the same tissue lysates.

**Statistical analyses**

All data were reported as mean ± SEM. Statistical significance in miRNA expression of lung tissues as well as pulmonary epithelial cells was assessed by the paired two-tailed student’s t-test. The difference in the expression of the three miR-21 target genes between the VC-treated group and the vehicle control group or the VC plus I3C-treated group was compared by Mann–Whitney unpaired two-tailed t-test. A P value of <0.05 was considered statistically significant.

**Results**

**Microarray analyses of miRNA expression in lung tissues**

Using microarray techniques, we compared miRNA expression levels in normal mouse lung tissues and lung tumors from mice treated with VC alone or VC plus I3C. As shown in Figure 1A, the expression of a number of miRNAs was strongly altered in lung tumors of A/J mice relative to that of normal lungs from vehicle controls. However, levels of only seven miRNAs were altered consistently by ≥2-fold in all three microarray runs: miR-21, miR-31, miR-130a, miR-146b and miR-377 increased but miR-1 and miR-143 decreased in lung tumor tissues from VC-treated mice versus normal lungs from vehicle-treated mice. Dietary administration of I3C to VC-treated mice

![Image](https://academic.oup.com/carcin/article-abstract/31/2/252/2476908)
reversed the effect of the carcinogen as shown by downregulation of miR-21, miR-31, miR-130a, miR-146b and miR-377 levels in VC plus I3C-treated mice versus VC only treated mice (Figure 1B).

Verification of microarray results by QRT–PCR and gel analysis of PCR products

To validate the findings of the microarray studies, all of the miRNAs whose levels were consistently altered by ≥2-fold in all three microarray runs were selected for quantification by QRT–PCR. The trend in the alteration of miRNA levels was generally similar to that observed in microarray studies (Figure 2A). VC treatment significantly increased levels of miR-21, miR-31, miR-130a, miR-146b and miR-377, whereas supplementation of the diet with I3C significantly reduced the effect of the carcinogen with the exception of miR-130a and miR-377, the alterations of which were not significant. Levels of miR-1 and miR-143 were also altered by the carcinogen as well as carcinogen plus I3C, but the changes were not significant. Gel analysis of PCR products confirmed the results obtained in microarray and QRT–PCR studies: miR-21, miR-31, miR-130a, miR-146b and miR-377 were overexpressed in VC-treated mice relative to vehicle-treated mice but underexpressed in VC plus I3C-treated mice versus mice treated with VC alone (Figure 2B).

Analysis of miR-21 targets using QRT–PCR and western immunoblotting

Four commonly used miRNA target prediction programs, PicTar, TargetScan, miRanda and TarBase, suggest the following tumor suppressor genes as potential targets of miR-21: sprouty homolog 2, transforming growth factor β1, PDCD4, PTEN, tropomyosin 1 and RECK. QRT–PCR analysis of the predicted genes in normal lung tissues from vehicle-treated mice and lung tumors from VC- or VC plus I3C-treated mice revealed alterations in the levels of only PTEN, PDCD4 and RECK mRNAs. PDCD4 and RECK levels were significantly downregulated in VC-treated versus vehicle-treated mice, but administration of I3C to carcinogen-treated mice significantly upregulated the message levels of both genes (Figure 3A). The changes in PTEN mRNAs were not statistically significant. Subsequently, we performed western blot analysis of PTEN, PDCD4 and RECK. As shown in Figure 3B, expressions of all three proteins were lower in the carcinogen group (lane 3) relative to the levels in the untreated group (lane 1) and administration of I3C to carcinogen-treated mice restored
the expression of the proteins (lane 2) to that of the vehicle control level (lane 1).

**Differential expression of miR-21, miR-31, miR-130a, miR-146b and miR-377 in A549 cells and modulation by I3C**

In this study, we compared the expression level of miR-21, miR-31, miR-130a, miR-146b and miR-377 in NHBE and A549 cells. As shown in Figure 4, levels of miR-21, miR-130a, miR-146b and miR-377 were significantly increased in A549 cells as compared with the expression in NHBE cells. Indeed, the expression of these miRNAs was also elevated in hTERT-immortalized NHBE cells and the fold change in the level of miR-146b was even much higher than that in A549 cells.

**Effect of I3C on miR-21 and its target proteins in A549 cells**

We then sought to examine if treatment of A549 cells with I3C (100 or 150 μmol) for 24 h modulates levels of miR-21, miR-31, miR-130a, miR-146b and miR-377. I3C significantly reduced the level of miR-21 only; the expression of the other miRNAs was not affected (Figure 5A).

In subsequent studies, we examined if I3C also modulates miR-21 target proteins in A549 cells. As in the immunoblot study with mouse lung tissues, the expressions of PTEN, PDCD4 and RECK were upregulated by I3C (Figure 5B). We also examined the effect of I3C on MMP-2, which is negatively regulated by RECK. Consistent with an increase in the level of RECK, the expression of MMP-2 was reduced by I3C.

**Discussion**

Lung cancer exhibits deregulation of several miRNAs with oncogenic and tumor suppressor activities (14,19). Differential expression of miRNAs and their target genes was also reported in the airway epithelial cells of cigarette smokers (20), lung tissues of cigarette smoke-exposed rats (21) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-treated rats (18). The link between aberrant miRNA expression and lung cancer development indicates that miRNAs could be potential targets for chemopreventive and chemotherapeutic agents. Additionally, deregulated miRNAs can also be valuable lung cancer diagnostic and prognostic markers.

In the present study, using a VC-induced lung carcinogenesis model, we showed that the expression of several miRNAs is altered during the development of lung tumor and the chemopreventive agent I3C modulates the expression of the majority of these miRNAs. All the miRNAs we identified, with the exception of miR-377, are known to be aberrantly expressed in one or more of human cancers. MiR-1 is downregulated in human primary lung cancer tissues and cell lines (22), whereas miR-21 and miR-146 are upregulated in lung cancer relative to normal tissues (14). Increased levels of miR-31 and miR-130a have been found in colon (23) and liver cancer (24), respectively, whereas miR-43 is downregulated in cervical cancer (25). However, the magnitude of change we observed in the present study is mostly weaker than that reported in the above studies. Also, we did not observe changes in the level of let-7, which is the most commonly altered miRNA in lung cancer. One possible explanation for these discrepancies may be that almost all of the lung tumors we observed in A/J mice were at adenoma stage, whereas the studies with human tissues were carried out using advanced stages of cancer. Indeed, miRNA expression was shown to be significantly higher in carcinoma than in precursor stages in several tissues (26).

Among the miRNAs that showed differential expression in our studies, miR-21 is the most frequently upregulated oncomir in solid tumors (27). Increased levels of miR-21 have been reported in cancer of the lung (14), glial cells (28), breast (29), liver (30) and pancreas (31). The link between overexpression of miR-21 and cancer is miR-21-induced changes in the phenotype of cells. Upregulation of miR-21 caused increased cell proliferation, reduced apoptosis and enhanced tumor growth and invasion by downregulating the expression of tumor suppressor genes such as PTEN (30), p53 and transforming growth factor-beta (32), tropomyosin 1 (33), PDCD4 (34) and RECK (35). In our study, levels of miR-21 were inversely related to the expression of PTEN, PDCD and RECK in mouse lung tissues and A549 cells. These findings suggest that miR-21 plays a role in lung tumorigenesis, probably by suppressing the expression of PTEN, PDCD and RECK. Indeed, earlier reports showed decreased expression of PTEN, PDCD and RECK in human lung cancer, although the relationship with miR-21 was not investigated. PTEN, a lipid phosphatase that negatively regulates the phosphatidylinositol 3-kinase/AKT cell survival pathway, and PDCD4, an inhibitor of protein translation that suppresses tumor initiation, progression and invasion, are reduced or lost in lung cancer and downregulation of these proteins is correlated with unfavorable prognosis (36,37). Also, RECK, a negative regulator of MMPs, is an independent and significant factor in predicting a prognosis of lung cancer.

Owing to its regulatory role in cell proliferation, apoptosis and cell migration and invasion, miR-21 is an attractive target for cancer chemoprevention and chemotherapy. In the present study, we showed that the chemopreventive agent I3C downregulated VC-induced overexpression of miR-21. This effect could be causally correlated with our recent observation that I3C inhibited the multiplicity, incidence and size of VC-induced pulmonary adenocarcinoma in A/J mice (38) from which the lung tissues for the present studies were obtained. Interestingly, miR-21-mediated downregulation of PTEN, PDCD4 and RECK was reversed by I3C, suggesting that the lung tumor inhibitory effects of I3C could be mediated, at least partly, through modulation of these target proteins. Consistent with our hypothesis, earlier works have shown a reduction in the expression of PTEN, PDCD4 and RECK during carcinogenesis and reversion of this effect by chemopreventive agents. In mouse cervical cancer model, diminishing PTEN occurred as the cervical disease stages progressed from low-grade dysplasia to carcinoma and I3C administration upregulated PTEN expression (39). Also, retinoic acid receptor agonists, antitumors and the HER-2/neu antagonist herceptin upregulated PDCD4 expression in breast cancer cells and exogenous expression of PDCD4 induced apoptosis of these cells (40). Another study showed upregulation of RECK mRNA and protein in lung cancer cells upon treatment with non-steroidal anti-inflammatory drugs and induction of RECK was associated with reduction of MMP-2 activity (41). The inverse relationship between levels of MMP-2 and RECK, the negative regulator of MMP-2, is in line with what we observed in A549 cells. In the aforementioned studies, up-regulation of PTEN, PDCD4 and RECK by chemopreventive agents might involve suppression of miR-21 levels.

Taken together, our results indicate that modulation of miRNA levels could be used to examine efficacy of chemopreventive agents and the mechanisms through which these chemopreventive agents inhibit carcinogenesis. Another potential use of miRNAs in cancer chemoprevention is to detect and monitor the toxic effects of chemopreventive agents. Indeed, a recent study reported the usefulness of miRNAs as biomarkers for drug-induced toxicity (42).

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**References**


