The Human *let-7a-3* Locus Contains an Epigenetically Regulated MicroRNA Gene with Oncogenic Function

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Abstract

MicroRNAs (miRNAs) are small noncoding RNAs that repress their target mRNAs by complementary base pairing and induction of the RNA interference pathway. It has been shown that miRNA expression can be regulated by DNA methylation and it has been suggested that altered miRNA gene methylation might contribute to human tumorigenesis. In this study, we show that the human *let-7a-3* gene on chromosome 22q13.31 is associated with a CpG island. Let-7a-3 belongs to the archetypal let-7 miRNA gene family and was found to be methylated by the DNA methyltransferases DNMT1 and DNMT3B. The gene was heavily methylated in normal human tissues but hypomethylated in some lung adenocarcinomas. Let-7a-3 hypomethylation facilitated epigenetic reactivation of the gene and elevated expression of let-7a-3 in a human lung cancer cell line resulted in enhanced tumor phenotypes and oncogenic changes in transcription profiles. Our results thus identify let-7a-3 as an epigenetically regulated miRNA gene with oncogenic function and suggest that aberrant miRNA gene methylation might contribute to the human cancer epigenome. [Cancer Res 2007;67(4):1419–23]

Introduction

MicroRNAs (miRNAs) are small noncoding RNAs that repress their target mRNAs by complementary base pairing and induction of the RNA interference pathway (1). Several miRNAs have been shown to play specific roles in development and differentiation and, correspondingly, their expression patterns seem to be highly regulated. miRNA expression profiling has provided indications for aberrant expression patterns in human cancer cell lines and in primary cancers, which suggested that aberrant miRNA expression might contribute to tumorigenesis (2–4). Consistent with this notion, the expression pattern of 200 human miRNA genes can be of greater prognostic value than the expression pattern of 13,000 protein-encoding genes (3).

Human tumorigenesis is characterized by specific changes in genomic DNA methylation patterns. Compared with nonmalignant cells, tumor cells show hypermethylation or hypomethylation in CpG islands of genes that are functionally important for tumor development (5–7). Hypermethylation-induced gene silencing can be reverted by DNA methyltransferase inhibitors, such as 5-aza-2′-deoxycytidine (DAC). Treatment of cancer cells with these compounds followed by expression profiling has been used to systematically unmask tumor suppressor genes that are hypermethylated in human cancer cells (8, 9). Recently, Saito et al. (10) have used a similar approach to identify miRNA genes that are affected by DNA hypermethylation: combinatorial treatment of T24 bladder cancer cells with DAC and a second epigenetic drug, the histone deacetylase inhibitor 4-phenylbutyrate, caused >3-fold up-regulation in 17 of 313 miRNA genes analyzed. The strongest effects (49-fold up-regulation) were observed for *miR-127* and the corresponding gene was found to be embedded in a CpG island. Epigenetic activation of *miR-127* resulted in a detectable down-regulation of the *BCL6* proto-oncogene, which suggested a tumor suppressor function for this miRNA (10). However, the *miR-127* gene was found to be methylated in many human tissues and no methylation changes could be detected in the three primary human tumor samples that were analyzed in this study.

We have analyzed the methylation of the human *let-7a-3* gene that belongs to the archetypal family of *let-7* miRNA genes. We found that *let-7a-3* methylation is prevalent in normal human tissues but can be lost in lung cancers. The characterization of the mechanisms mediating *let-7a-3* methylation and the function of *let-7a-3* expression in a human lung adenoma cell line suggests that epigenetic activation of *let-7a-3* might contribute to human lung tumorigenesis.

Materials and Methods

Cell culture and patient samples. HCT116 and HCT116 knockout cells (11) were cultured in McCoy's 5A medium supplemented with 10% FCS. A549 cells were cultured in DMEM supplemented with 10% FCS. For inhibitor treatment, cells were incubated for 72 h with 500 nmol/L 5-aza-2′-deoxycytidine (DAC; Calbiochem), and/or 1 mmol/L valproic acid (VPA; Merck). Patient DNA was prepared from fresh-frozen tissues after institutional review board approval. For all experiments, genomic DNA was prepared using the DNeasy kit (Qiagen).

DNA methylation analysis. Genomic DNA was deaminated with sodium bisulfite using standard procedures and *let-7a-3* was amplified using nested primers as follows: let7a_out_for (GTTAGAATTAGGGTTTTTGGGGAGG) and let7a_out_rev (ACCTATCAAACTTCTCAATATAAAC), 95°C for 3 min, followed by 34 cycles (95°C for 30 s, 54°C for 45 s, and 72°C for 1 min), and 72°C for 4 min. Primers and PCR conditions for the second amplification were let7a_in_for (GGGAGGGATGTTTGTTTGTTTAGTG) and let7a_in_rev (AACTACCCCCAAACCTAACCCTACC), 95°C for 3 min, followed by 34 cycles (95°C for 30 s, 64°C for 30 s, and 72°C for 45 s), and 72°C for 4 min. The PCR product (723 bp) was gel purified and digested with *Bst*UI (New England Biolabs). Digested PCR products were separated on agarose gels and visualized by ethidium bromide staining. For bisulfite sequencing, PCR products were gel extracted and cloned using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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***Let-7a-3* expression analysis.** Total RNA was isolated from cells using Trizol (Invitrogen) and cDNA was synthesized using the ThermoScript reverse transcription-PCR (RT-PCR) system (Invitrogen). Twenty microliters of PCRs contained 2 μL cDNA template, 1× ReddyMix buffer (Abgene), 1 μmol/L of each primer, 1 mmol/L deoxynucleotide triphosphates (Stratagene), and 1.5 units of Thermoprime polymerase (Abgene). The primers and PCR conditions for let-7a-3 cDNA amplification were let7a_RT2_for (CTCTGGAAGCCACGGAGTC) and let7a_RT2_rev (GTTCCAGACGCTCTGTCCAC), 95°C for 3 min, followed by 34 cycles (95°C for 30 s, 62°C for 30 s, and 72°C for 30 s), and 72°C for 3 min. Primers and PCR conditions for tissue inhibitor of metalloproteinase-3 and β-amyloid have been described elsewhere (12). PCR amplicons were separated on agarose gels and visualized by ethidium bromide staining.

Establishment of stably transfected let-7a-3 cell lines. The precursor sequence encoding *let-7a-3* was PCR amplified from human genomic DNA. A product of 179 bp was subcloned into the expression vector pZeoSV2− (Invitrogen) by using *Eco*RI and *Kpn*I restriction sites contained in the PCR primers and verified by DNA sequencing. The primers and PCR conditions for let-7a-3 amplification were let-7a_forcl (ATGAATTCCTCTGGAAGCCACGGAGTC) and let7a_revcl (ATGGTACCGTTCCAGACGCTCTGTCCAC), 95°C for 3 min, followed by 34 cycles (95°C for 30 s, 62°C for 30 s, and 72°C for 30 s), and 72°C for 3 min.

Constructs were transfected into A549 cells using Fugene 6 transfection agent (Roche), according to the manufacturer's protocol. Cells were grown for 3 days in transfection medium and then selected in cell culture medium containing 200 μg/mL zeocin (Invitrogen).

Colony formation assays. Cells (2×10^5) in 1.5 mL medium supplemented with 0.3% agarose were layered on a 3-mL base of 0.5% agarose with medium. Soft agar assays were done in 60-mm dishes and in triplicate. After colonies became visible, cells were stained with 200 μL *p*-iodonitrotetrazolium violet solution (5 mg/mL).

Microarray experiments. Hybridizations were done on genome-wide cDNA microarrays. Gene expression analysis comprised four microarray hybridizations: four let-7a-3–transfected cell culture replicates and four parental cell culture replicates (controls). Each let-7a-3–expressing replicate was hybridized against a control replicate of the same cell line, including a dye swap design. One-round linear amplification of 2 μg total RNA was done using the Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies) according to the manufacturer's instructions. Hybridization and washing were done as described previously (13). Hybridized arrays were scanned with the GenePix 4000B microarray scanner (Axon Instruments), and analyzed using GenePix Pro 4.1. Microarray raw data processing was done using software ArrayMagic (14) and the data set was deposited to National Center for Biotechnology Information Gene Expression Omnibus (GEO) database[4] by GEO series accession number GSE6474. We considered only genes that fulfilled the cutoff criteria of a *P* value ≤0.05 and a linear fold change ≥1.5. Functional annotation of the differentially expressed genes was done using the Gene Ontology software GOstat.

Results and Discussion

Recently, 17 of 313 human miRNA genes were found to be up-regulated in T24 human bladder cancer cells that had been treated with the DNA methyltransferase inhibitor DAC and the histone deacetylase inhibitor 4-phenylbutyric acid (10). The observation that gene reactivation required a DNA methyltransferase inhibitor raised the possibility that a larger proportion of miRNA genes might be methylated in human cells. We noticed that the *let-7a-3* gene on chromosome 22q13.31 is associated with a well-defined CpG island (200 bp length, 55% CG content, 0.65 observed/expected CG ratio; Fig. 1*A*). We used bisulfite sequencing to analyze the methylation status of 33 CpG dinucleotides of the *let-7a-3* CpG island in HCT116 cells and the results showed that the gene was densely (90%) methylated (Fig. 1*B*). The analysis of genomic DNA from isogenic DNA methyltransferase knockout cell lines (11) showed that methylation seemed unchanged in DNMT3B knockout cells (Fig. 1*B, 3BKO*). *Let-7a-3* methylation seemed somewhat lower in DNMT1 knockout cells (Fig. 1*B, 1KO*) and the gene was almost completely demethylated in DNMT1;DNMT3B double knockout cells (Fig. 1*B, DKO*). This showed that *let-7a-3* methylation is cooperatively maintained by the DNA methyltransferases DNMT1 and DNMT3B and suggests that miRNA genes are methylated by the same mechanisms that govern methylation of other genomic regions (11).

We then sought to further characterize the methylation pattern of *let-7a-3* in human tissues by combined bisulfite restriction analysis (COBRA). COBRA detects DNA methylation by probing restriction enzyme sites in PCR amplicons from bisulfite-deaminated DNA. Methylated genes generate small restriction fragments, whereas unmethylated genes generate PCR fragments that cannot be cut by restriction enzymes. *Let-7a-3* COBRA analysis of genomic DNA from normal placenta, brain, bone marrow, blood, colon, and skin samples revealed strong methylation of *let-7a-3* in all tissues analyzed (Fig. 2*A*). Because let-7 miRNAs have been linked to lung tumorigenesis (15–18), we also analyzed *let-7a-3* methylation in a set of eight lung adenocarcinomas and eight matched nonneoplastic lung tissue samples from the same patients. The results

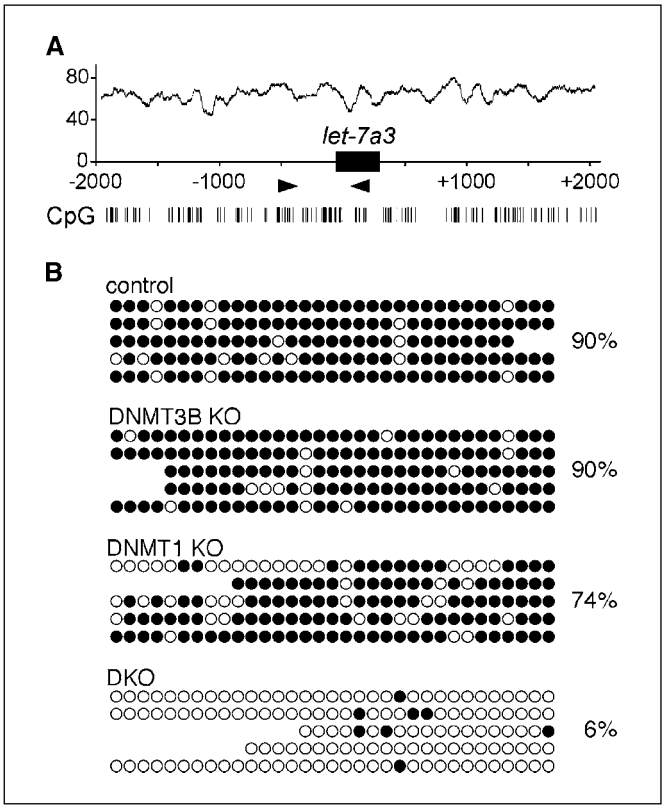

Figure 1. Methylation of *let-7a-3* is cooperatively maintained by DNMT1 and DNMT3B. *A, let-7a-3* is embedded in a CpG island. The percentage of G+C over a 100-bp window. *Vertical bars,* position of individual CpG dinucleotides; *black box,* position of the *let-7a-3* pri-miRNA; *arrowheads,* bisulfite PCR primers. *B,* bisulfite sequencing analysis of *let-7a-3* methylation with genomic DNA from HCT116 cells and isogenic DNA methyltransferase knockout cell lines. ○, unmethylated CpG dinucleotides; ●, methylated CpG dinucleotides. Percentages indicate the fraction of methylated CpG dinucleotides.

[4] http://www.ncbi.nlm.nih.gov/projects/geo/

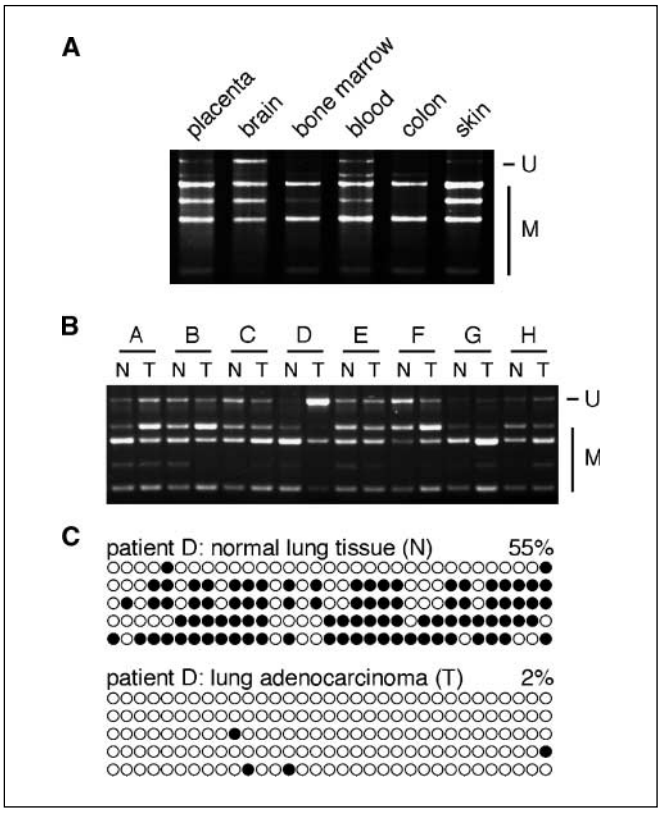

Figure 2. *Let-7a-3* is methylated in normal human tissues and can be hypomethylated in lung cancer. *A,* COBRA analysis of *let-7a-3* methylation in normal human tissues. *B,* COBRA analysis of *let-7a-3* methylation in eight sample pairs from lung adenoma patients. *N,* samples from normal lung tissue; *T,* samples from tumors. *C,* bisulfite sequencing analysis of *let-7a-3* in patient D. Percentages indicate the fraction of methylated CpG dinucleotides.

indicated that *let-7a-3* was methylated in all normal lung samples, with a methylation pattern similar to other human tissues (Fig. 2*B*). Patients A and D showed a clear difference between tumor and control tissue and the restriction pattern indicated strong hypomethylation in the tumor (Fig. 2*B*). This observation was confirmed by bisulfite sequencing of multiple independent clones, which showed almost complete *let-7a-3* demethylation (2%) in a lung adenoma sample from patient D, whereas the locus was substantially methylated (55%) in normal lung tissue from the same patient (Fig. 2*C*). We therefore concluded that *let-7a-3* is strongly methylated in normal human tissues and that the gene can be hypomethylated in lung tumors.

To analyze the role of DNA hypomethylation in the regulation of *let-7a-3*, we treated A549 lung adenocarcinoma and HCT116 cells with the DNA methyltransferase inhibitor DAC. COBRA analysis of *let-7a-3* showed that drug treatment caused effective gene demethylation (Fig. 3*A*). Bisulfite sequencing of multiple independent *let-7a-3* clones also showed a decrease from 92% methylation in untreated control cells to 42% in DAC-treated cells (Fig. 3*B*). Combinatorial treatment with DAC and the histone deacetylase inhibitor VPA caused a significant up-regulation of let-7a-3 expression, whereas treatment with either DAC or VPA alone had little effect (Fig. 3*C*). These results agree with the observations made for the human *miR-127* gene (10) and suggest that hypomethylation facilitates epigenetic reactivation of *let-7a-3* expression.

Having shown that *let-7a-3* can be hypomethylated in human lung cancer and that hypomethylation can lead to epigenetic activation of the gene, we then sought to determine the consequences of *let-7a-3* expression in a human lung cancer model. *Let-7a-3* is strongly methylated and transcriptionally silenced in the A549 adenocarcinoma cell line (Fig. 4*A*). To restore let-7a-3 expression, we stably transfected A549 cells with a construct that drives let-7a-3 pri-miRNA expression from a cytomegalovirus promoter (L7-A549). For controls, we also transfected A549 cells with the empty vector (Co-A549). Let-7a-3 expression was analyzed by semiquantitative RT-PCR in all transgenic cell lines and was found to be clearly increased in let-7a-3–transfected cells (Fig. 4*A*). To explore the functional consequences of let-7a-3 expression, we analyzed the anchorage-independent cell growth in soft agar assays. Staining and counting of colonies from several independent experiments showed an 11-fold increase in colony numbers for let-7a-3–expressing cells when compared with controls (Fig. 4*B*). These data provide a strong indication that *let-7a-3* can have oncogenic functions in lung cancer cells.

To further substantiate this observation, we obtained gene expression profiles from L7-A549 and Co-A549 cells. Hybridization of total RNA from both cell lines to cDNA microarrays revealed a substantial let-7a-3–associated transcriptional deregulation. Linear

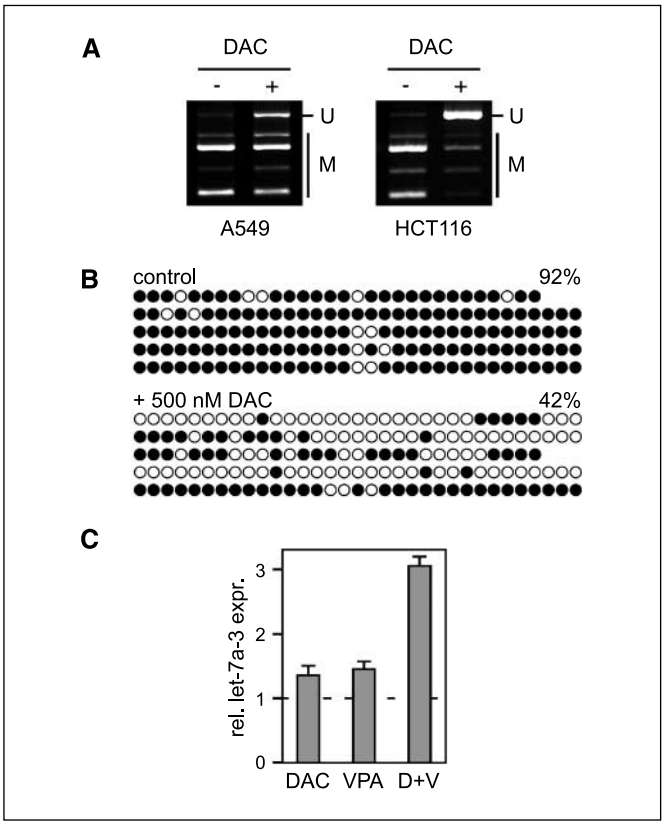

Figure 3. Hypomethylation of *let-7a-3* facilitates epigenetic reactivation. *A,* COBRA analysis of *let-7a-3* methylation in A549 and HCT116 cells indicates effective demethylation following DAC treatment. *B,* bisulfite sequencing analysis of *let-7a-3* methylation with genomic DNA from untreated and DAC-treated cells, respectively. ○, unmethylated CpG dinucleotides; ●, methylated CpG dinucleotides. Percentages indicate the fraction of methylated CpG dinucleotides. *C,* epigenetic reactivation of let-7a-3 expression by combinatorial treatment with DAC and VPA. The results were derived from three independent experiments, and all values were normalized to the expression level of β-amyloid in untreated HCT116 cells. *Columns,* relative let-7a-3 expression; *bars,* SD.

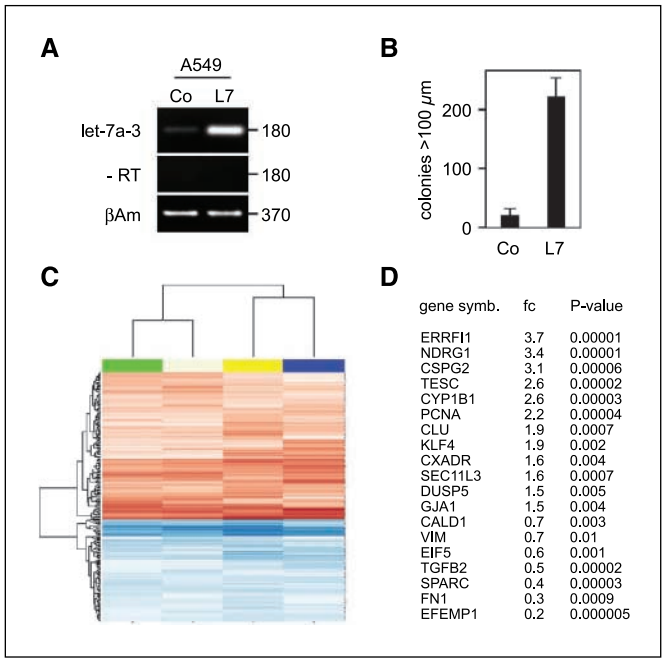

Figure 4. Increased *let-7a-3* expression causes oncogenic changes in human cancer cells. *A,* semiquantitative RT-PCR analysis of let-7a-3 pri-miRNA expression in let-7a-3–transfected (*L7*) and empty vector-transfected (*Co*) A549 cells. *B,* colony numbers of control and *let-7a-3*–overexpressing (*L7*) A549 cells in soft agar assays. The results were derived from three independent experiments. *Columns,* colonies > 100 μm; *bars,* SD. *C,* gene expression profiling of L7-A549 and Co-A549 cells using cDNA microarrays. Hierarchical clustering of 197 differentially expressed genes in four replicates. *D,* let-7a-3–dependent deregulation in the expression of RAS-responsive genes. *fc,* fold changes in transcript levels in L7-A549 versus Co-A549 cells.

modeling of microarray data with cutoff values at $P \leq 0.05$ and linear fold change ≥ 1.5 revealed that let-7a-3 expression caused deregulation of 197 genes (Fig. 4*C*; Supplementary Table S1). Gene ontology statistics revealed overrepresentation of genes involved in cell proliferation, adhesion, and differentiation (Supplementary Table S2). In agreement with a previous study showing a role of let-7 in the regulation of RAS (17), we found 19 RAS-responsive genes to be deregulated in L7-A549 cells (Fig. 4*D*). RAS mRNA levels were only weakly affected by let-7a-3 overexpression (data not shown), which is consistent with previous data, suggesting that let-7–dependent RAS regulation occurs at the translational level (17). We also identified several potentially oncogenic genes that were up-regulated by let-7a-3 expression in A549 cells and that have been described previously to be associated with lung cancer progression. These genes include *CDK6, PCNA, PRDX1*, and *CXCL5*. In addition, down-regulation was observed for several genes that have been described to inhibit lung cancer cell proliferation, such as *PPARG, TGFB2*, and *SFRP1*. In addition, genes that are functionally related to cell adhesion processes, a gene ontology category, which is relevant for tumor progression and metastasis formation, were predominantly down-regulated in our experiments (Supplementary Table S2). The gene expression profiles are therefore in agreement with the increased anchorage-independent growth of let-7a-3–expressing A549 cells and provide further confirmation for an oncogenic role of let-7a-3 in lung carcinogenesis.

Various reports have linked let-7 miRNAs to human cancers (15–18). These observations, combined with the fact that the human *let-7a-3* gene was embedded in a well-defined CpG island, prompted us to analyze the methylation and the function of this gene in detail. It should be noted that the human *let-7* family encompasses at least 12 genes with distinct and potentially diverse contributions to tumorigenesis and that only some of the previous studies have discriminated between individual human *let-7* genes. This might explain why we have found a consistent oncogenic role of let-7a-3, whereas others have described tumor-suppressing activities for other let-7 miRNAs (15–18). An oncogenic function of *let-7a-3* is also supported by our gene expression profiling results that indicate increased oncogenic characteristics following expression of let-7a-3 in A549 cells. Our microarray data support a role of let-7a-3 in the regulation of RAS signaling, as described by others (15–18). However, the observed effects were complex and not limited to down-regulation of RAS effector genes.

In agreement with an oncogenic function of let-7a-3 in a lung cancer model, we found *let-7a-3* to be substantially hypomethylated in some lung cancer samples. To our knowledge, this finding represents the first example for an epigenetic mutation affecting a miRNA gene. Global hypomethylation of genomic DNA has been the first epigenetic change described in human cancers, and several oncogenes, including *BCL-2* (19) and *R-RAS* (20), are hypomethylated in primary human tumors. More extensive studies will be required to comprehensively address the significance of aberrant miRNA gene methylation for human tumorigenesis.

Acknowledgments

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