

Reduced Expression of the *let-7* MicroRNAs in Human Lung Cancers in Association with Shortened Postoperative Survival

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Abstract

In this study, we report for the first time reduced expression of the *let-7* microRNA in human lung cancers. Interestingly, 143 lung cancer cases that had undergone potentially curative resection could be classified into two major groups according to *let-7* expression in unsupervised hierarchical analysis, showing significantly shorter survival after potentially curative resection in cases with reduced *let-7* expression ($P = 0.0003$). Multivariate COX regression analysis showed this prognostic impact to be independent of disease stage (hazard ratio = 2.17; $P = 0.009$). In addition, overexpression of *let-7* in A549 lung adenocarcinoma cell line inhibited lung cancer cell growth *in vitro*. This study represents the first report of reduced expression of *let-7* and the potential clinical and biological effects of such a microRNA alteration.

Introduction

Cells contain a variety of noncoding RNAs, which perform a multitude of functions. Recently, microRNAs (miRNAs), an abundant class of small noncoding RNAs of about 22 nucleotides in length, have been recognized as being numerous and phylogenetically well conserved (1). The miRNA species are encoded by genes that are presumably transcribed into single or clustered miRNA precursors, which are converted to mature forms of miRNAs through stepwise processing including generation of ~70 nucleotide pre-miRNA with a characteristic hairpin structure from the longer nascent transcripts (pri-miRNA) and the following Dicer-mediated processing into mature forms (2–5). Although thus far over 300 miRNA genes have been discovered in various organisms (6–10), including humans, their precise physiological functions are largely unknown except for a handful of miRNAs (11–17), and their potential pathological involvement including oncogenesis is yet to be explored.

The *Caenorhabditis elegans let-7* miRNA is to date the best-studied example along with *lin-4* of the same worm (11–15), both of which were initially identified by genetic analysis of the developmental timing defects of mutants. The *let-7* miRNA, which starts to be expressed during the late developmental stage, acts as a post-transcriptional repressor of *lin-41*, *hbl-1/lin-57* and perhaps other genes that contain sequences imprecisely complementary to the miRNA in their 3' untranslated regions. The expression levels of the human *let-7*

gene have been shown to vary among various adult tissues, lung being one of the tissues with most abundant expression of *let-7* (18).

In this study, we show for the first time that expression levels of *let-7* are frequently reduced in lung cancers both *in vitro* and *in vivo*. Furthermore, lung cancer patients with reduced *let-7* expression were found to have significantly worse prognosis after potentially curative resection, and this prognostic impact of reduced *let-7* expression appears to be independent of disease stage in multivariate COX regression analysis. In addition, we show that overexpression of *let-7* inhibits growth of lung cancer cells *in vitro*.

Materials and Methods

Study Population. This study dealt with 159 nonsmall cell lung carcinoma (NSCLC) tissue specimens collected with the approval of the institutional review board of the Aichi Cancer Center. The specimens from 143 cases (105 adenocarcinomas, 25 squamous cell carcinomas, 9 large cell carcinomas, and 4 adenosquamous cell carcinomas), which had been followed up for >5 years after potentially curative resection, were used specifically for studying the prognostic significance of *let-7*. These 143 cases consisted of 90 female and 53 male patients with a median age of 62 (range, 32–84), and with 75 in stage I, 19 in stage II, and 49 in stage III.

Preparation of Cell Line and Tissue Samples. All of the human NSCLC cell lines analyzed were cultured with 5% (v/v) FCS-containing RPMI 1640 at 37°C with 5% CO₂. BEAS-2B and HPL1D (19) cells were cultured with 1% (v/v) FCS-containing Ham's F-12 supplemented with bovine insulin (5 µg/ml), human transferrin (5 µg/ml), 10⁻⁷ M hydrocortisone, 2 × 10⁻¹⁰ M triiodo thyronine, penicillin (100 IU/ml), and streptomycin (100 µg/ml) at 37°C with 5% CO₂. The tumor specimens were homogenized in guanidine isothiocyanate homogenization buffer immediately after resection and stored at -30°C until use with the approval of the institutional review board. Processing of all cell lines and tissue samples for RNA extraction were performed according to the standard procedures.

Northern Blotting. Ten µg of RNA were separated on a 15% denaturing polyacrylamide gel. The RNA was then transferred to Zeta-Probe GT Blotting Membranes electrophoretically overnight. Probes (*let-7*; 5'-TACTATACAACCTACTACCTCAATTTGCC and 5S; 5'-TTAGCTTCGAGATCA-GACGA) were generated by T4 polynucleotide kinase (New England Biolabs, Beverly, MA) mediated end-labeling of DNA oligonucleotides with [³²P]ATP. Prehybridization and hybridization were carried out using hybridization buffer (0.25 M sodium phosphate (pH 7.2), 7% SDS, 0.5% sodium PP_i). The most stringent wash was carried out in 2× SSC and 1% SDS at 37.5°C.

Real-Time Reverse Transcription-PCR. Real-time reverse transcription-PCR was performed using an ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA), the SYBR Green PCR Master Mix (Perkin-Elmer Applied Biosystems), and random-primed cDNAs (corresponding to 20 ng of total RNA extracted from tissue samples).

The primer pairs used were *let-7a-1S* (sense; 5'-CCTGGATGTTCTCT-TCACTG) and *let-7a-1AS* (antisense; 5'-GCTTGATGCAGACTTTTCT); *let-7a-2S* (sense; 5'-TTCCAGCCATTGTGACTGCA) and *let-7a-2AS* (antisense; 5'-CTCACCATTGTTTGTAGTGC); *let-7a-3S* (sense; 5'-ACCAA-GACCCACTGCCCTTT) and *let-7a-3AS* (antisense; 5'-CTCTGTCCACCG-CAGATATT); *let-7f-1S* (sense; 5'-TGTACTTTCCATTCCAGAAG) and *let-7f-1AS* (antisense; 5'-TAATGCAGCAAGTCTACTCC); *let-7f-2S* (sense; 5'-TGAAGATGGACACTGGTCT) and *let-7f-2AS* (antisense; 5'-

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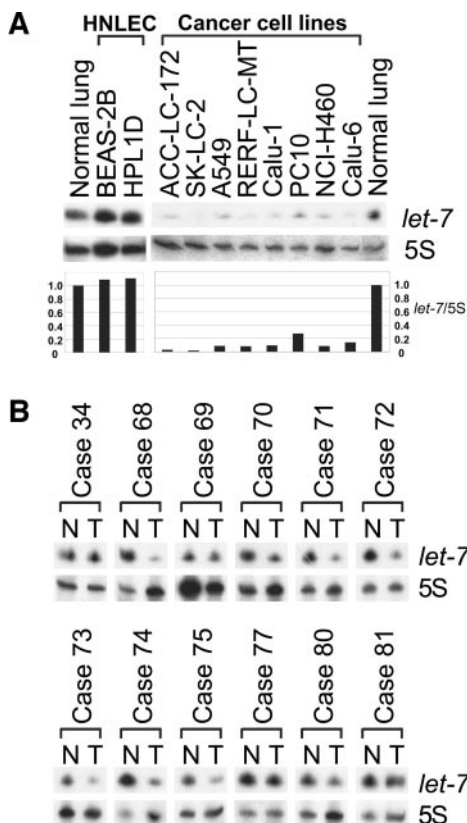


Fig. 1. Northern blot analysis of *let-7* expression in primary lung cancers. A, representative Northern blot analysis in lung cancer cell lines *in vitro*. BEAS-2B and HPL1D, immortalized human normal bronchial and peripheral lung epithelial cell lines, respectively. HNLEC, human normal lung epithelial cell lines. B, representative Northern blot analysis of primary lung cancer specimens *in vivo*. 5S rRNA served as a loading control. N, normal lung; T, lung cancer.

CAGTCCGAGAAGAAGTGTAC); and 5S-S (sense; 5'-TACGGCCATAC-CACCCTGAA) and 5S-AS (antisense; 5'-TAACCAGGCCCGACCCTGCT). To quantify the expression level of the *let-7* genes, standard curves were made using serially diluted pBluescriptIIISK (-) inserted with each PCR product into the *EcoRV* site. PCR amplification consisted of 55 cycles (95°C for 30 s, 56°C to 60°C optimized for each primer set for 30 s and 72°C for 15 s) after the initial denaturation step (95°C for 10 min). Expression levels of the *let-7* genes were based on the amount of the target message relative to the 5S rRNA control, to normalize the initial input of total RNA.

Hierarchical Clustering. We used the Eisen CLUSTER and TREEVIEW programs for hierarchical clustering and visualization of data sets. Before applying the clustering algorithm, we log-transformed the fluorescence ratio for each expression and then average centered the data for all samples. Agglomerative hierarchical clustering was applied using the complete linkage method to investigate whether there was evidence for natural groupings of tumor samples based on correlations between gene-expression profiles.

Statistical Analysis. The Kaplan-Meier method was used to estimate survival as a function of time, and survival differences were analyzed by the log-rank test. Cox regression analysis of factors potentially related to survival was performed to identify which independent factors might jointly have a significant influence on survival.

Colony Formation Assay. The *let-7* expression construct and a control plasmid were constructed by the cloning of annealed oligonucleotides of *let-7a* (sense, 5'-GATCCCCTGAGGTAGTAGTTGTATAGTTTTT and antisense, 5'-AGCTAAAAACTATACAACCTACTACCTCAGGG), *let-7f* (sense, 5'-GATCCCCTGAGGTAGTAGATTGTATAGTTTTT and antisense, 5'-AGCTAAAACATACTACTACCTCAGGG), or control (sense, 5'-GATCCCCTTTTTTTGGAAA and antisense, 5'-AGCTTTTCCAAAAAAAAGGG) into pHI-RNAPuro, in which expression of a gene is under the control of the RNA polymerase III H1-RNA gene promoter prepared by PCR amplification of human genomic DNA. The *let-7a* and *-7f* expression constructs were

transfected into A549 lung adenocarcinoma cell line using the FuGENE 6 reagent (Roche, Inc. Basel, Switzerland) according to the manufacturer's instructions. Cells were selected by the addition of puromycin (2 µg/ml) 3 days after the transfection and cultured at 37°C for 2 weeks. After 2 weeks of puromycin selection, the plates were stained with Giemsa and scored for the number of resistant colonies.

Results

Reduced Expression of *let-7* in Human Lung Cancers in Both *in Vitro* and *in Vivo*. Northern blot analysis was first performed to analyze *let-7* expression in 20 human lung cancer cell lines as well as in two immortalized human normal lung epithelial cell lines (Fig. 1A). The mature form of *let-7* miRNA was readily detectable in both immortalized lung epithelial cell lines at a level comparable with that in normal lung tissues. In marked contrast, a significant reduction (>80%) in the expression levels of *let-7* was observed in 60% (12 of 20) of lung cancer cell lines. Expression levels of *let-7* in primary human lung cancer tissues taken directly from surgically treated patients, in which sufficient RNA were available, were further analyzed by Northern blot analysis. Consequently, 44% (7

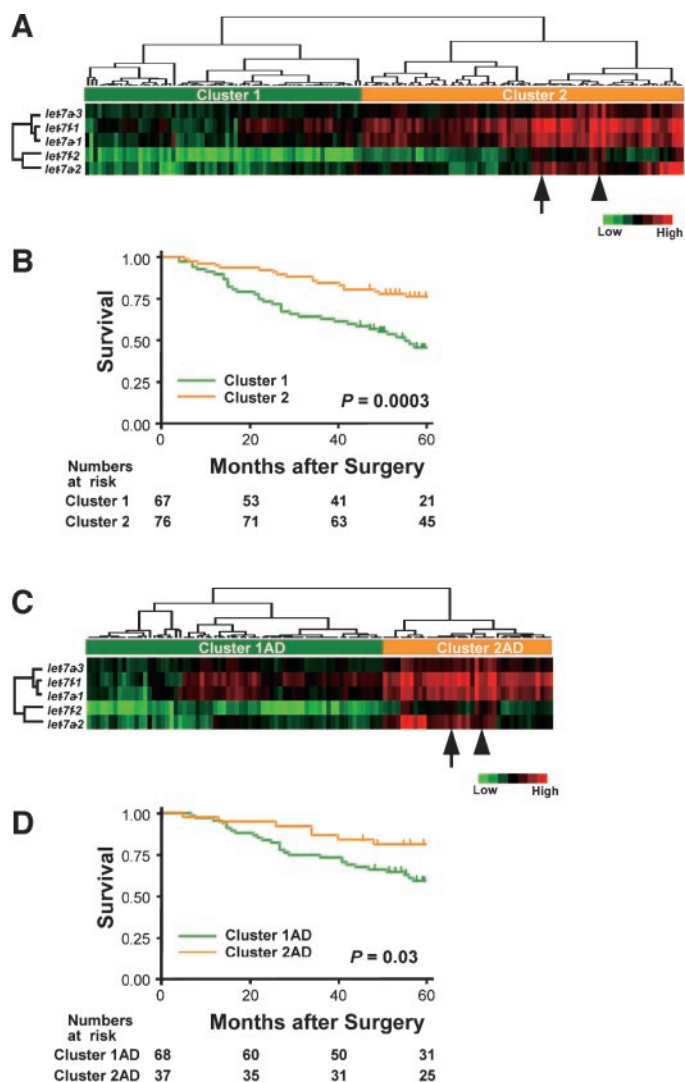


Fig. 2. Hierarchical clustering and Kaplan-Meier survival curves based on expression of *let-7* microRNA (miRNA) isoforms. A, results of unsupervised hierarchical clustering of the entire cohort of 143 nonsmall cell lung carcinoma (NSCLC) cases. B, Kaplan-Meier survival curves for NSCLC patients who were classified into clusters 1 and 2. The difference in postoperative survival between clusters 1 and 2 was highly significant ($P = 0.0003$ by log-rank test). C, results of unsupervised hierarchical clustering of the 105 adenocarcinoma cases. D, Kaplan-Meier survival curves for adenocarcinoma cases belonging to either cluster 1AD or 2AD. The difference in postoperative survival between clusters 1AD and 2AD was also statistically significant ($P = 0.03$ by log-rank test). Arrows and arrow heads, mixture of RNAs of 38 and 120 normal human peripheral lung tissues, respectively.

of 16) of the cases examined were found to exhibit >80% reduction in *let-7* expression when compared with that in the corresponding normal lung tissues (Fig. 1B). A more frequent occurrence of reduced *let-7* expression in cell lines *in vitro* may be related to the inevitable contamination of normal stromal/inflammatory cells in tumor tissues *in vivo* or, alternatively, this may reflect *in vitro* selection of cells with reduced *let-7* in the process of the establishment of cell lines. These findings thus clearly showed the frequent occurrence of a significant reduction in *let-7* miRNA expression in lung cancers.

Prognostic Impact of Reduced *let-7* Expression in Surgically Treated Lung Cancer Patients. We next wished to investigate whether reduced *let-7* expression has any relation to clinicopathological characteristics of lung cancers in an isoform-specific manner. To this end, 143 lung cancer cases, which had undergone potential curative resection of NSCLCs, were examined by real-time reverse transcription-PCR analysis using *let-7* isoforms-specific oligonucleotide primers. Expression levels of *let-7* pri-miRNAs were consequently shown to vary significantly among lung cancer cases, although they tended to be coordinately regulated. The most abundant species were *let-7a-1* and *let-7f-1*, which are known to be clustered within a few hundred bases in the human genome (6) and could be amplified together by reverse transcription-PCR (data not shown). We used unsupervised hierarchical clustering to classify the 143 resected human NSCLC cases in an unbiased manner without using any information on the identity of the samples. This procedure resulted in the classification of NSCLC cases into two major classes based on similarities in *let-7* expression (Fig. 2A). Except for a significant association between cluster 1 with low *let-7* expression and higher disease stages ($P = 0.004$ by the χ^2 test), no other significant associations were found between the clusters and various clinicopathological features including age, sex, histology, primary tumor status (pT), and differentiation grade. Of special interest was a striking difference in the postoperative survival of patients between the two clusters. The Kaplan-Meier survival curves demonstrated that patients belonging to cluster 1 were at a significantly greater risk of an earlier death than those classified as cluster 2 ($P = 0.0003$ by the log-rank test; Fig. 2B). A separate study analyzed the prognostic significance of *let-7* in adenocarcinomas, which constitute the major proportion of lung cancers in Japan as well as in other countries such as the United States. We found that adenocarcinoma cases can also be divided into two major clusters, again showing that patients in cluster 1AD with low *let-7* expression had significantly shorter survival than those in cluster 2AD with high *let-7* expression ($P = 0.03$ by the log-rank test; Fig. 2, C and D).

Univariate Cox regression analysis was then performed for the entire cohort and showed that, in addition to disease stage ($P < 0.001$; Table 1), classification into cluster 1 with characteristically low *let-7* expression is a significant predictive factor for poor prognosis ($P < 0.001$). Cox proportional hazards modeling was then conducted to identify which independent factors would jointly have a significant influence on survival (Table 1). The inter-relationship of possible prognostic factors and survival was analyzed, using age, sex, histological type, smoking history, disease stage, and the *let-7*-defined cluster as variables, resulting in the identification of *let-7*-defined cluster as a significant, independent prognostic factor in surgically treated NSCLC patients after potentially curative resection ($P = 0.009$) in addition to disease stage ($P < 0.001$). The hazard ratio of earlier death was 2.17 (95% confidence interval, 1.21–3.89) for clusters 1 versus 2 and 3.49 (95% confidence interval, 1.89–6.42) for pathological stage II/III versus pathological stage I. Taken

Table 1 Cox regression analysis of various prognostic factors for postoperative survival of lung cancer patients

Variables	Hazard ratio (95% CI) ^a	Unfavorable/favorable	P
Univariate analysis			
Age (yr)	1.70 (0.97–2.99)	≥62/<62	0.063
Sex	1.34 (0.75–2.38)	Male/female	0.323
Histology	1.30 (0.67–2.52)	Squamous/non-squamous	0.443
Smoking history	1.42 (0.80–2.51)	Smoker/non-smoker	0.233
Disease stage	3.89 (2.14–7.08)	II–III/I	<0.001
<i>let-7</i>	2.78 (1.56–4.89)	Cluster 1/cluster 2	<0.001
Multivariate analysis			
Age (yr)	1.68 (0.95–2.97)	≥62/<62	0.076
Sex	1.18 (0.44–3.13)	Male/female	0.741
Histology	1.03 (0.49–2.16)	Non-squamous/squamous	0.942
Smoking history	1.07 (0.41–2.82)	Non-smoker/smoker	0.889
Disease stage	3.49 (1.89–6.42)	II–III/I	<0.001
<i>let-7</i>	2.17 (1.21–3.89)	Cluster 1/cluster 2	0.009

^a 95% CI, 95% confidence interval.

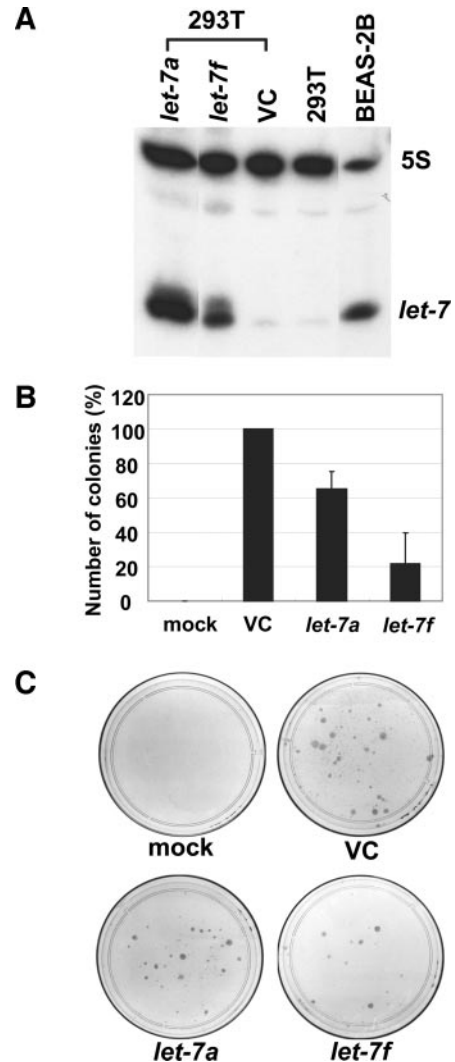


Fig. 3. Introduction of *let-7* into A549 lung adenocarcinoma cell line. A, results of Northern blot analysis confirming expression of *let-7a* and *let-7f* isoforms. B, graphic presentation of a representative colony formation assay by the introduction of exogenous *let-7*. Similar results were obtained in five independent assays done in triplicate. C, representative dishes showing reduced colony formation by overexpression of exogenously introduced *let-7*.

together, expression levels of *let-7* seemed to have a significant impact on the postoperative survival of NSCLC patients.

Growth Suppression of Lung Cancer Cells by Overexpression of Exogenous *let-7*. The identification of a reduced expression of *let-7* in lung cancers, in association with a shortened survival, prompted us to explore the possible biological significance of *let-7* in lung cancer development. As an initial step, we introduced *let-7* into a lung cancer cell line by using expression constructs, which were designed to synthesize mature miRNAs of two predominant *let-7* isoforms, *let-7a* and *let-7f*, under the control of the RNA polymerase-III H1-RNA gene promoter. We confirmed that these expression constructs could work as expected using 293T cells (Fig. 3A). Overexpression of *let-7f* in A549 lung adenocarcinoma cell line resulted in a 78.6% reduction in the number of colonies, whereas the introduction of *let-7a* also showed similar but a more modest growth-inhibitory effect (Figs. 3, B and C). Similar results were obtained in five independent experiments, which were done in triplicate using three independent preparations of plasmid DNAs.

Discussion

It has become apparent that genomic information for transcribing miRNAs is indeed implemented in the human genome (6, 9), but extremely little information is available regarding their physiological

and pathological roles. This is the first demonstration that expression levels of *let-7* miRNA, which to date is one of the best-studied miRNAs, are altered in human lung cancers. Furthermore, we have shown that reduced *let-7* expression is significantly associated with shortened postoperative survival and that overexpression of *let-7* results in the inhibition of lung cancer cell growth. Altogether, these findings suggest that reduced expression of *let-7* may play a role in the pathogenesis of lung cancers.

Very little information is available at the moment with regard to the potential pathological roles of miRNAs. Two proteins (Gemin 3 and Gemin 4), which are components of the protein complex related to spinal muscular atrophy, are also known to be components of a ribonucleoprotein complex containing miRNAs (microRNP; Ref. 9), whereas the *Drosophila* homologue of fragile X mental retardation protein has been shown to be a component of RNA-induced silencing complex/microRNPs (20, 21). This circumstantial evidence suggests the possibility of the involvement of miRNA machineries in these diseases. As for links between cancer and miRNA, Calin *et al.* (22) reported frequent down-regulation of *miR15* and *miR16* in chronic lymphocytic leukemia, whereas Michael *et al.* (23) recently reported reduced expression of *miR-143* and *miR-145* in human colon cancers. In contrast to these studies, which did not address the question of whether reduced expression of miRNAs has any influence on clinicopathological features, this study clearly shows that reduced *let-7* expression is indeed significantly associated with the shortened survival of patients. Because no changes in *let-7* expression were reported in colon cancers (23), it is possible that miRNAs may be distinctly involved in the pathogenesis of these two most common cancers of adults and possibly in other types of human cancers.

It has been shown that the *let-7* gene regulates developmental timing in *C. elegans* and that mutant worms lacking *let-7* fail to properly execute a larval-to-adult switch in hypodermal cell development (13). Although *lin-41* is known to be post-transcriptionally repressed by *let-7* (24), it is not inconceivable that other genes may also be targeted by *let-7*, because of the requirement of imprecise base-pairing for miRNA-mediated translational repression (1). Indeed, *hbl-1/lin-57* was recently reported to be targeted by *let-7* (14, 15), whereas a few additional genes have also been predicted to be a potential target for *let-7* (24, 25). Interestingly, such potential targets include *LIM kinase 2* (25), which belongs to a gene family having a role in the regulation of cell shape and motility as well as possibly in metastasis. One could speculate that the change in miRNA expression as is seen in this study might be an efficient strategy for cancer cells to simultaneously alter the expression profile of a series of genes. Alterations in miRNA expression may accordingly confer cancer cells with selective growth advantage, allowing them to form a distant metastasis and resulting in the consequential death of the patient. This scheme may be consistent with the present finding of the significant prognostic impact of *let-7* expression. One might argue that reduced expression of *let-7* in lung cancers may merely reflect its oncofetal regulation, because fetal lung exhibited considerably lower *let-7* expression than adult lung (data not shown). However, growth-inhibitory effects of overexpressed *let-7* in A549 adenocarcinoma cell line argue against this possibility. Taken together, these findings suggest the potential involvement of reduction in *let-7* expression in the pathogenesis of this fatal disease, although the results obtained with overexpression of mature miRNA need to be interpreted cautiously and await further experimental clarification.

In this study, we observed that various *let-7* pri-miRNA isoforms were coordinately regulated, *let-7a-1* and *let-7f-1* being the most predominant. In this connection, it should be noted that some of the *let-7* pri-miRNAs give rise to identical mature miRNA isoforms, and the others may also have presumably very similar, if not identical,

spectra of the target genes (6). It is uncertain at the moment how the expression levels of various *let-7* isoforms are coordinated, and this remains an intriguing question awaiting further investigation.

In conclusion, we have shown for the first time that *let-7* expression is frequently reduced in lung cancers and that alterations in the miRNA expression may have a prognostic impact on the survival of surgically treated lung cancer patients. These findings warrant additional studies to investigate whether *let-7* alterations are also involved in other types of human cancers and how altered miRNA expression would manifest the biological and biochemical consequences in the development of human cancers. Accordingly, future identification of the downstream targets for *let-7* may provide clues to develop a novel therapeutic means. It is envisaged that such future studies may ultimately provide a foundation for a new paradigm of the involvement of noncoding small RNA species, miRNA, in human oncogenesis.

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