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-7miRNA 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-4, 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 triiodothyronine, penicillin (100 IU/ml), and streptomycin (100 µg/ml) at 37°C with 5% CO2. The tumor specimens were homogenized in guinidine 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’TACTATA-CAACCTACTACCTCAATTGCC and 5’S TTAGCTTCCGAGATCA-SSC and 1% SDS at 37.5°C.

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Note: J. Takamizawa and H. Konishi contributed equally to the present study. H. Konishi is currently at the Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD.

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3753
CAGTCGGGAGAAGAGTGTC); and 5S–S (sense; 5′-TACGGCATACTACCCCTGAA) and 5S–AS (antisense; 5′-CAGTCGGAGAAGAAGTGTAC); and 5S–S (sense; 5′-TACGGCCATAC/H11032


let-7 genes, standard curves were made using serially diluted pBluescriptISK (−) 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 RNA 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.

Colonies Formation Assay. The let-7 expression construct and a control plasmid were constructed by the cloning of annealed oligonucleotides of let-7a (sense, 5′-GATCCCTGGAGTATGAGTTGTATAGTTTT and antisense, 5′-AGCTAAAAACTATAAACCTACTACCTCACGGG), let-7f (sense, 5′-GATCCCTGGAGTATGAGTTGTATAGTTTT and antisense, 5′-AGCTAAAAACTATAAACCTACTACCTCACGGG), or control (sense, 5′-GATCCCTGGAGTATGAGTTGTATAGTTTT and antisense, 5′-AGCTAAAAACTATAAACCTACTACCTCACGGG), into pH1-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. 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 HPLID, 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.

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 \( (P = 0.004 \text{ by the } \chi^2 \text{ test}) \), no other significant associations were found between the clusters and various clinicopathological features including age, sex, histology, primary tumor status \( (\text{pT}) \), 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 \( (P = 0.0003 \text{ by the log-rank test; Fig. 2B}) \). 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 \( (P = 0.03 \text{ by the log-rank test; Fig. 2, C and D}) \).

Univariate Cox regression analysis was then performed for the entire cohort and showed that, in addition to disease stage \( (P < 0.001; \text{Table 1}) \), 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 \( (\text{Table 1}) \). 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 2.97 (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 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.

![Fig. 3.](image)

**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

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

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazard ratio (95% CI&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>Unfavorable/favorable</th>
<th>P</th>
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<td>Univariate analysis</td>
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<tr>
<td>Age (yr)</td>
<td>1.70 (0.97–2.99)</td>
<td>≥62/62</td>
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<td>Sex</td>
<td>1.34 (0.75–2.38)</td>
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<td>Histology</td>
<td>1.30 (0.67–2.52)</td>
<td>Squamous/non-squamous</td>
<td>0.443</td>
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<tr>
<td>Smoking history</td>
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<td>Smoker/non-smoker</td>
<td>0.233</td>
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<td>Disease stage</td>
<td>3.89 (2.14–7.08)</td>
<td>II/III</td>
<td>&lt;0.001</td>
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<td>let-7</td>
<td>2.78 (1.56–4.89)</td>
<td>Cluster 1/cluster 2</td>
<td>&lt;0.001</td>
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<tr>
<td>Multivariate analysis</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Age (yr)</td>
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<td>≥62/62</td>
<td>0.076</td>
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<tr>
<td>Sex</td>
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<td>0.741</td>
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<tr>
<td>Histology</td>
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<td>Non-squamous/squamous</td>
<td>0.942</td>
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<tr>
<td>Smoking history</td>
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<td>Non-smoker/smoker</td>
<td>0.899</td>
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<tr>
<td>Disease stage</td>
<td>3.49 (1.89–6.42)</td>
<td>II–III</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>let-7</td>
<td>2.17 (1.21–3.89)</td>
<td>Cluster 1/cluster 2</td>
<td>0.009</td>
</tr>
</tbody>
</table>

<sup>a</sup> 95% CI, 95% confidence interval.
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 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 consequent 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.

References
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