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Advances in Brief

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-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% CO_2. 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 triiode thyronine, penicillin (100 IU/ml), and streptomycin (100 μg/ml) at 37°C with 5% CO_2. 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′-TTAGCTTCCGAGATCAGACGA) were generated by T4 polynucleotide kinase (New England Biolabs, Beverly, MA) mediated end-labeling of DNA oligonucleotides with [γ-^{32}P]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-1*S (sense; 5′-CCTGGATGTTCTCTTCACTG) and *let-7a-1*AS (antisense; 5′-GCCTGGATGCAGACTTTTCT); *let-7a-2*S (sense; 5′-TTCCAGCCATTGTGACTGCA) and *let-7a-2*AS (antisense; 5′-CTCACCATGTTGTTTAGTGC); *let-7a-3*S (sense; 5′-ACCAAGACCGACTGCCCTTT) and *let-7a-3*AS (antisense; 5′-CTCTGTCCACCGCAGATATT); *let-7f-1*S (sense; 5′-TGTACTTTCCATTCCAGAAG) and *let-7f-1*AS (antisense; 5′-TAATGCAGCAAGTCTACTCC); *let-7f-2*S (sense; 5′-TGAAGATGGACACTGGTGCT) and *let-7f-2*AS (antisense; 5′-

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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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