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Clinical significance and the correlation of expression between *Let-7* and *K-ras* in non-small cell lung cancer

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Abstract. To detect the expression levels of the *lethal-7* (*let-7*) and *K-ras* genes in non-small cell lung cancer (NSCLC) and investigate their significance, the expression of *let-7* and *K-ras* was determined in cancerous tissues and pericancerous normal tissues of 31 NSCLC patients using reverse transcription-polymerase chain reaction. Our results using 31 NSCLC patient samples showed that 67.74% had a low *let-7* expression (P<0.05), 64.52% had a high *K-ras* expression (P<0.05), and a significant correlation was observed between *let-7* and *K-ras* gene expression (r=-0.6336) in tumor tissues. Patients with a low *let-7* expression had a significantly shorter survival than those with a moderate *let-7* expression (P<0.05). Patients with a high *K-ras* expression also had a significantly shorter survival than those with a moderate *K-ras* expression (P<0.05). In conclusion, a low expression of *let-7* and a high expression of *K-ras* are correlated with the pathogenesis and prognosis of NSCLC.

Introduction

Small RNAs (microRNAs, miRNAs) are recently discovered non-coding RNA molecules comprising approximately 18-25 nucleotides. The biological function of miRNAs remains to be clarified. miRNAs are believed to play an important role in the post-transcriptional regulation of mRNA (1). miRNAs have been shown to be closely associated with the pathogenesis and differentiation of non-small cell lung cancer (NSCLC) (9). In the human genome, approximately 50% of all miRNAs are located on chromosomes at sites associated with tumors, and their location suggests that miRNAs play an important role in the pathogenesis of tumors (2). *Lethal-7* (*let-7*) miRNAs are a family of miRNAs whose expression has been reported to be decreased in patients with lung cancer (3). *Ras* is a major oncogene, and the overexpression of ras proteins suppresses apoptosis, promoting the pathogenesis and development of tumors. In this study, the expression of the *let-7* and *K-ras* genes in NSCLC was examined, and the expression levels of these genes in NSCLC patients were determined by performing reverse transcription-polymerase chain reaction (RT-PCR).

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Materials and methods

Materials. A total of 31 patients with NSCLC [22 males and 9 females; mean age, 61.3 (5.1) years; range, 45-68] who underwent radical resection at our hospital between January 2007 and June 2007 were enrolled in the study. Fresh lung cancer and normal lung tissues were harvested away from the tumor (pericancerous tissues which served as the control) from specimens excised from the 31 patients within 30 min. The specimens were stored in Cryule vials and were immediately frozen by placing them in liquid nitrogen. Histopathological examinations performed after the operation using sections prepared from paraffin-embedded slices confirmed that all of the specimens were lung cancers, including 12 cases of squamous cell carcinoma and 19 cases of adenocarcinoma. Following the operation, the cancer specimens were staged according to the TNM staging system: 9 cases were in stage IIa, 7 in stage IIb, 14 in stage IIIa and 1 was in stage IIIb.

Reagents. TRIzol was purchased from Life Technologies, USA. RNase inhibitor, MMLV reverse transcriptase and Taq polymerase were from Promega. Oligo $(dT)_{18}$ was from Shanghai Sangon, China, and 10,000X SYBR-Green was purchased from Molecular Probes, USA. For the primer design, software Primer 5.0 and Rotor-gene 6.0 were used, which were provided by Shanghai Sangon and Corbett Research, respectively.

Real-time quantitative PCR. The TRIzol method was used to extract total RNA. Subsequently, the ultraviolet absorption spectrum was examined and denaturing RNA agarose gel electrophoresis was performed to determine the purity and integrity of the RNA. RT-PCR was performed to synthesize cDNA. Real-time Q-PCR was performed using the DK-8D Electro-Thermostatic Water Cabinet (Shanghai Sibas Biotechnology Development Co., Ltd., China), FeroTec Gradient PCR thermal cycler (Ferotec, Germany),

Table I. Primers used for real-time Q-PCR.

Gene name	Bidirectional primer sequences
β-actin	F: 5'CCTGTACGCCAACACAGTGC3' R: 5'ATACTCCTGCTTGCTGATCC3'
K-ras	F: 5'TTGCCTCCCTACCTTCCACA3' R: 5'GTTCAAAGCATCAGCCACCAC3'
Let-7	F: 5'TCTTATGAATGGCCCAAA3' R: 5'CAGTTATCTCCCTTGATGTAA3'

F, forward; R, reverse.

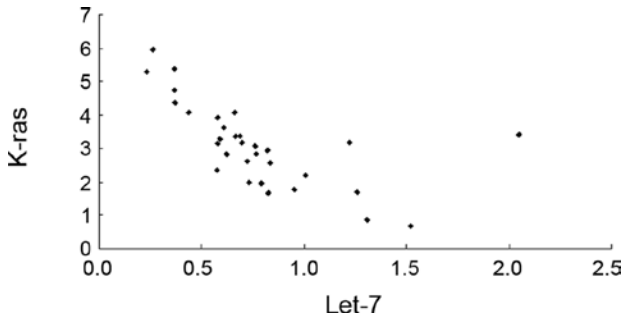

Figure 1. Correlation between *let-7* and *K-ras*.

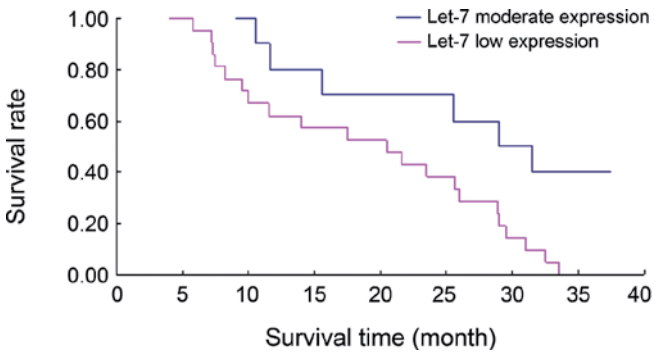

Figure 2. Survival curves of the groups with a low and moderate *let-7* expression.

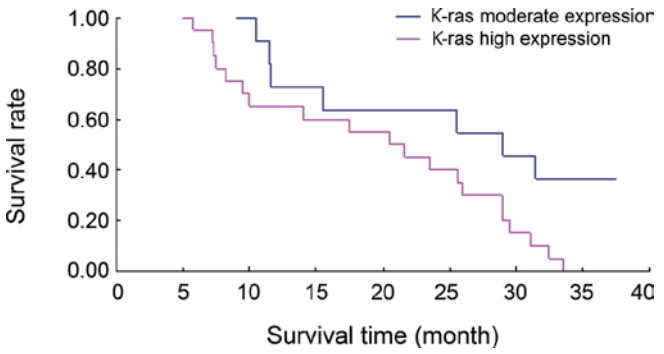

Figure 3. Survival curves of the groups with a moderate and high *K-ras* expression.

DYY-8 electrophoresis system (Shanghai Qite Analytical Instruments Co., Ltd., China), DY-32 mini-electrophoresis chamber (Xinghua Analytical Instrument Factory, China) and Rotor-Gene 3000 real-time PCR amplifier (Corbett Research, Australia). Primer sequences (Table I) were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA).

Calculation of relative expression. Using real-time Q-PCR the *let-7*, *K-ras* and *β-actin* genes were amplified from each sample. The corrected value of expression was obtained by dividing the value of *let-7* and *K-ras* expression by the value of β-actin (internal control). The value of relative expression of these genes was obtained by dividing the corrected values of *let-7* and *K-ras* expression in lung tumors by those of their expression in normal lung tissues.

Statistical analysis. SPSS13.0 was used for statistical analysis. Data were expressed as the mean (standard deviation, SD). The t-test was performed using the corrected values of the expression levels of *let-7* and *K-ras* in the lung cancer and normal lung tissues. Pearson's correlation analysis was performed for the corrected values of the expression levels of *let-7* and *K-ras* in the lung cancers. P<0.05 was considered to indicate a statistically significant difference.

The relative expression was considered to be low when its value was <0.5, as high expression when its value was >2, and as moderate when its value was between 0.5 and 2. On the basis of the values of the relative expression of *let-7* and *K-ras*, the 31 patients were assigned into low-, median- and high-expression groups. Follow-up studies were conducted for 1 to 37 months to determine the survival status of each group. Kaplan-Meier survival curves were plotted using these results and a log-rank test was carried out to determine differential survival between the two groups.

Results

Expression of let-7 and K-ras in lung cancer. Let-7 expression was low in 21 cases (67.74%) and moderate in 10 cases. However, a high expression of *let-7* was not detected in any of the lung cancer specimens. *K-ras* expression was high in 20 cases (64.52%) and moderate in 11 cases. However, a low expression of *K-ras* was not detected in any of the lung cancer specimens.

Corrected values of let-7 and K-ras in cancer and pericancerous tissues. The corrected values of *let-7* and *K-ras* expression in the 31 specimens were expressed as the mean (SD) (Table II). The results indicate that the corrected values of *let-7* or *K-ras* were significantly different between the cancer and normal tissues ($|t|_{let\ 7}$=6.658 or $t_{K\text{-}ras}$=6.617, P<0.05, respectively).

Correlation analysis. Fig. 1 shows the importance of the corrected values of *let-7* and *K-ras* in the cancer tissues. The results show that the expression of *let-7* and *K-ras* in tumor tissues was closely correlated (r=-0.6336, P<0.05).

Survival curves associated with let-7 and K-ras expression. Figs. 2 and 3 show Kaplan Meier survival curves associated

Table II. Correction of *let-7* and *K-ras* values in the cancer and pericancerous tissues.

Let-7		*K-ras*	
Cancer tissue	Pericancerous tissues	Cancer tissue	Pericancerous tissues
0.772 (0.383)	2.235 (1.162)	3.117 (1.253)	1.430 (0.671)

Values are the mean (SD). n=31.

with *let-7* and *K-ras* expression in the 31 lung cancer cases. The results from the log-rank differential survival test indicated statistically significant differences in the survival rates between the groups with a low and moderate *let-7* expression (χ^2=6.1577) and between those with a moderate and high *K-ras* expression (χ^2=5.0152); P<0.05.

Discussion

miRNAs are ubiquitous in eukaryotic genomes. These small RNA molecules bind to specific target mRNAs through base pairing and inhibit translation or negatively regulate gene expression by the degradation of target mRNA. This method of gene expression regulation plays an essential role in development, cell differentiation and apoptosis (4). *Let-7* is a member of the miRNA family. It was first found in nematodes and serves as a sequential control factor for cell fate determination (3).

Let-7 expression was found to be decreased in human lung cancer, and a low expression was correlated with the postoperative survival time of patients (3,5,6). Takamizawa *et al* (3) studied 143 NSCLC patients undergoing radical resection, and a COX proportional hazard model analysis was performed to determine the factors that may affect prognosis, such as age, gender, histological type, smoking history, TNM stage and the *let-7* expression level. Results of these authors showed that the *let-7* expression level was an independent postoperative prognostic factor for NSCLC.

Our results revealed that *let-7* expression was significantly lower than normal in 67.74% of the 31 lung cancer patients. Follow-up studies of these patients showed that patients in the low-expression group had a significantly lower survival rate than those in the median-expression group. The results show that *let-7* expression is low in lung cancer and that patients with a low *let-7* expression have a short survival time.

Ras is an important human oncogene. *H-ras*, K-ras and *N-ras*, three closely related members of the ras family, are the most common oncogenes in human cancer. *K-ras* is associated with lung cancer. Slebos *et al* (7) found a marked decrease in the survival rate of patients with a *K-ras* mutation. Nemunaitis *et al* (8) studied *K-ras* mutations and the expression of ras and c-erbB-2 proteins, and found that *K-ras* is a significant prognostic factor for lung adenocarcinoma. In our study, *K-ras* expression was high in 64.52% of the lung cancer patients, and the postoperative survival rate of these patients was significantly lower than that of the patients in the median-expression group. These results show that *K-ras* plays an important role in the pathogenesis of lung cancer.

Eder and Scherr (9) found that the *let-7* expression declined as the *K-ras* expression increased in NSCLC, suggesting their significance. Johnson *et al* (10) found that the 3'-UTR of *ras* mRNA contains a number of complementary binding sites for *let-7* and inferred that *let-7* may regulate the expression of *ras*. These authors reported that the target of *let-7* is the *K-ras* oncogene and that a decrease in *let-7* expression resulted in an increase in *ras* expression or the promotion of tumor growth. Tam (11) reported that a decrease in *let-7* expression caused an approximately 70% increase in the level of the ras protein expression in transfected HeLa cells.

In the present study, Pearson's correlation analysis revealed a negative correlation between the corrected values of *let-7* and *K-ras* in the NSCLC tissues (r=-0.6336). This result suggests that during the pathogenesis of NSCLC, a decrease in the level of *let-7* expression may lead to an enhanced expression of *K-ras*. Furthermore, our results showed that a polygene was involved in the pathogenesis and progression of NSCLC and that these genes act in synergy with each other, thus promoting the pathogenesis and progression of lung cancer and worsening patient prognosis.

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