Ratio of Expression of p16\(^{INK4a}\) to p14\(^{ARF}\) Correlates with the Progression of Non-small Cell Lung Cancer

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The CDKN2 gene is located on the short arm of chromosome 9p and encodes two unrelated proteins, p16\(^{INK4a}\) and p14\(^{ARF}\), through the use of independent first exons and shared exons 2 and 3. p16\(^{INK4a}\) is a cyclin-dependent kinase inhibitor, whereas p14\(^{ARF}\) regulates the cell cycle through a p53 and MDM2-dependent pathway. We have examined the expression of p16\(^{INK4a}\) and p14\(^{ARF}\) using competitive RT-PCR in 60 non-small cell lung cancers (NSCLCs) and matching normal lung tissues. The intensities of bands for p16\(^{INK4a}\) and p14\(^{ARF}\) were nearly equal or the intensity of the p16\(^{INK4a}\) band slightly exceeded that of p14\(^{ARF}\) in the normal lung tissues (n=60). In 38 tumors the intensity of the p16\(^{INK4a}\) band was similar to or slightly weaker than that of p14\(^{ARF}\). In 6 tumors the intensity of the p16\(^{INK4a}\) band was very strong, while the band of p14\(^{ARF}\) was barely visible. In only one tumor was the p14\(^{ARF}\) band slightly exceeded that of p16\(^{INK4a}\) in the normal lung tissues.

The intensities of bands for p16\(^{INK4a}\) and p14\(^{ARF}\) were nearly equal or the intensity of the p16\(^{INK4a}\) band was similar to or slightly weaker than that of p14\(^{ARF}\). In 15 tumors the intensity of the p14\(^{ARF}\) band was very strong and the p16\(^{INK4a}\) band was barely visible. In only one tumor was the intensity of the p16\(^{INK4a}\) band weaker than that of p14\(^{ARF}\). In 15 tumors the intensity of the p14\(^{ARF}\) band was very strong and the p16\(^{INK4a}\) band was barely visible. In only one tumor was the intensity of the p16\(^{INK4a}\) band very strong, while the band of p14\(^{ARF}\) was barely visible. The ratio of the intensity of p16\(^{INK4a}\) to p14\(^{ARF}\) had an interesting correlation with the tumor characteristics. The p stage I tumors (P=0.036). The T2–4 tumors had significantly lower p16\(^{INK4a}\) to p14\(^{ARF}\) ratios than the T1 tumors (P=0.005). The N1–3 tumors had significantly lower p16\(^{INK4a}\) to p14\(^{ARF}\) ratios than the N0 tumors (P=0.014). Our results suggest that the ratio of expression of p16\(^{INK4a}\) to p14\(^{ARF}\) tends to decrease during the progression of NSCLC.

Key words: CDKN2 — p16\(^{INK4a}\) — p14\(^{ARF}\) — Non-small cell lung cancer

Many different pathways and proteins are involved in the regulation of the cell cycle. The CDKN2 gene on the short arm of chromosome 9p, is frequently deleted in human cancer cell lines, encodes two different cell cycle regulatory proteins, p16\(^{INK4a}\) and ARF (murine p19\(^{ARF}\), human p14\(^{ARF}\)).\(^1\) p16\(^{INK4a}\) and p14\(^{ARF}\) have different first exons (exon 1\(\alpha\) for p16\(^{INK4a}\) and exon 1\(\beta\) for p14\(^{ARF}\)) and share common exons, exon 2 and exon 3. They use different reading frames to translate two distinct proteins.\(^2,\)\(^3\)

This genetic arrangement is unusual in mammalian cells.\(^4,\)\(^5\) p16\(^{INK4a}\) and p14\(^{ARF}\) play an important role in independent cell cycle regulatory pathways, the retinoblastoma pathway and the p53 pathway, respectively.

p16\(^{INK4a}\) is a tumor suppressor, which specifically inhibits its cyclin-dependent kinases, CDK4 and CDK6.\(^6\) Several observations indicate an important role of p16\(^{INK4a}\) in the development of many tumors. Mutations are frequently observed in melanomas, including familial types,\(^7,\)\(^8\) and head and neck squamous cell carcinomas.\(^9\) While homozygous deletions are a common event in some types of human cancers and cancer cell lines,\(^6\) such alterations are infrequent in the majority of solid tumors. However, hypermethylation of p16\(^{INK4a}\) promoter may occur with more tumorspecific patterns than found for homozygous deletions.\(^10\)–\(^13\)

The other protein, p14\(^{ARF}\), also functions as a growth suppressor. p14\(^{ARF}\) overexpression induces G1/S and G2/M cell cycle arrest by a mechanism distinct from that of the CDK inhibition.\(^14\) Recent studies have suggested that p14\(^{ARF}\) interacts in vivo with MDM2 and neutralizes MDM2-mediated inhibition of p53.\(^1,\)\(^15\)–\(^17\) Mutations in shared exons 2 and 3 have been demonstrated in some tumors.\(^5,\)\(^18\) while no point mutations in the specific exon 1\(\beta\) of p14\(^{ARF}\) have been reported. Specific deletion of exon 1\(\beta\) has been described in melanoma cell lines\(^9\) and genomic alterations of p14\(^{ARF}\) are found in some T-cell acute lymphocytic leukemias.\(^20\) However, the deletion of p14\(^{ARF}\) also targets p16\(^{INK4a}\) in the majority of cases. Recent studies demonstrated that hypermethylation of the human p14\(^{ARF}\) promoter is independent of that of p16\(^{INK4a}\) promoter in some cancer cell lines and human cancers.\(^21,\)\(^22\)

Thus, hypermethylation of p14\(^{ARF}\) promoter may be associated with p14\(^{ARF}\) inactivation in some human tumors.

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Abbreviations: INK4a, inhibitor of CDK4a; ARF, alternative reading frame; MDM2, multiple double minute 2; NSCLC, non-small cell lung cancer; RT-PCR, reverse transcription PCR; CDK, cyclin-dependent kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
In the present study, we examined the intensities of p16\(^{INK4a}\) and p14\(^{ARF}\) bands by competitive RT-PCR in 60 NSCLCs and matching normal lung tissues to investigate the relationship between the expression of p16\(^{INK4a}\) and p14\(^{ARF}\) and the progression of NSCLC. Our results indicated that the ratio of the intensity of p16\(^{INK4a}\) to p14\(^{ARF}\) band was related to the progression of NSCLC.

### MATERIALS AND METHODS

#### Tumor specimens and clinicopathological data

Sixty paired samples of primary NSCLC and matching normal lung tissue from the same patients were obtained at the Nagoya City University Hospital from January 1998 to June 1999. All patients had undergone potentially curative resections with lobectomy or pneumonectomy. None had received chemotherapy or radiotherapy prior to their surgery. Written consent was obtained from all the patients to make the resected material available for our research. The 60 patients included 40 men and 20 women, and the median age at surgery was 65 years (range, 43–88 years). The pathological stage of each patient was evaluated according to the UICC criteria (1997): 30 cases were at p stage I, 8 were at p stage II, 21 were at p stage III, and 1 was at p stage IV. The histological type of all tumors was determined according to the standard criteria of the WHO (1999): 39 cases were adenocarcinoma, 15 were squamous cell carcinoma, 3 were large cell carcinoma, and 3 were adenosquamous carcinoma. The patient’s smoking history (number of cigarettes per day and duration of smoking) was obtained from preoperative personal interviews (Table I).

#### RNA extraction

Total RNA was extracted from freshly frozen tissue samples using ISOGEN (Nippon Gene, Toyama) according to the manufacturer’s recommendations.

#### Competitive RT-PCR

To compare relative levels of p16\(^{INK4a}\) and p14\(^{ARF}\) transcripts we performed competitive RT-PCR. Competitive RT-PCR was carried out using Ready-To-Go RT-PCR Beads (Amersham Pharmacia Bio-tech, Foster City, CA) according to the Two-Step Protocol recommended by the manufacturer. Two hundred nanograms of total RNA was subjected to reverse transcription using oligo(dT) primer in a 50 \(\mu\)l reaction volume for 30 min at 42°C. The following primers were then added for PCR, in exon 1α (sense 1) 5'GGA GGC CGA TCC AGG TCA-3', exon 1β (sense 2) 5'GGT GCC CCT CGT GCT GAT-3' and exon 2 (antisense) 5'ACC ACC AGC GTG TCC AGG AA-3'. PCR conditions were as follows: 95°C for 5 min, 32 cycles of 94°C for 60 s, 58°C for 60 s, and 72°C for 90 s, followed by incubation at 72°C for 5 min. PCR conditions were determined from the cycle curve and RNA concentration curve. Ten microliters of each product was run on 2% agarose gel, and the bands were visualized by 10 mg/ml ethidium bromide staining for 15 min. As an internal control, a fragment of human GAPDH was amplified from parallel samples by PCR using the following primers: (sense) 5’-AGC ATG TGG TCG TAT-3'; (antisense) 5’-AGC CTT CTC CAT GGT GGT GAA GAC-3'.

#### Table 1. Correlation of p16\(^{INK4a}\) and p14\(^{ARF}\) Expression in NSCLCs with Clinicopathological Characteristics

| Parameters                          | log p16/p14 ≤−0.6 | log p16/p14 <−0.6 | \(P\) value |
|-------------------------------------|-------------------|-------------------|-------------|
| Age at surgery                      |                   |                   |             |
| ≤60                                 | 10                | 8                 | 0.381       |
| >60                                 | 29                | 13                |             |
| Sex                                 |                   |                   |             |
| Male                                | 23                | 17                | 0.150       |
| Female                              | 16                | 4                 |             |
| Pathological stage                  |                   |                   |             |
| I                                   | 25                | 5                 | 0.006       |
| II II IV                            | 14                | 16                |             |
| Tumor factor                        |                   |                   |             |
| T1                                  | 15                | 1                 | 0.005       |
| T2 T3 T4                            | 24                | 20                |             |
| Nodal factor                        |                   |                   |             |
| N0                                  | 35                | 11                | 0.003       |
| N1 N2 N3                            | 4                 | 10                |             |
| Metastasis factor                   |                   |                   |             |
| M0                                  | 38                | 21                | >0.999      |
| M1                                  | 1                 | 0                 |             |
| Histology                           |                   |                   |             |
| Adenocarcinoma                      | 28                | 11                | 0.033       |
| Squamous cell carcinoma             | 6                 | 9                 |             |
| Differentiation                     |                   |                   |             |
| Adenocarcinoma                      |                   |                   |             |
| Well+Moderately                     | 25                | 6                 | 0.028       |
| Poorly                              | 3                 | 5                 |             |
| Squamous cell carcinoma             |                   |                   |             |
| Well+Moderately                     | 5                 | 4                 | 0.287       |
| Poorly                              | 1                 | 5                 |             |
| Smoking history                     |                   |                   |             |
| ≤25 pack-year                       | 20                | 4                 | 0.008       |
| >25 pack-year                       | 12                | 15                |             |

\(P\) value: Fisher’s exact probability test.
RESULTS

Competitive RT-PCR We investigated the expression of p16\textsuperscript{INK4a} and p14\textsuperscript{ARF} using competitive RT-PCR in 60 tumors and matching normal lung tissues from the same patients. Using specific sense primers in exon 1\textalpha{} and exon 1\textbeta{} and a common reverse primer in exon 2, both transcripts were amplified simultaneously in a single reaction. This allowed us to compare directly the amounts of transcripts of p16\textsuperscript{INK4a} and p14\textsuperscript{ARF}. As shown in Fig. 1, the expression levels of p16\textsuperscript{INK4a} in the normal lung tissues (n=60) were similar, and the intensities of the p16\textsuperscript{INK4a} and p14\textsuperscript{ARF} bands were nearly equal or p16\textsuperscript{INK4a} expression slightly exceeded that of p14\textsuperscript{ARF}. In most tumor samples, the intensities of the p16\textsuperscript{INK4a} and p14\textsuperscript{ARF} bands were different from those in the corresponding normal lung tissues. In 38 cases the intensity of the p16\textsuperscript{INK4a} band was similar to or slightly weaker than that of p14\textsuperscript{ARF} (represented by patient 1 in Fig. 1). In 6 cases the intensity of the p16\textsuperscript{INK4a} band was weaker than that of p14\textsuperscript{ARF}, but the band of p16\textsuperscript{INK4a} was clearly visible (represented by patient 3). In 15 cases the intensity of p16\textsuperscript{INK4a} band was much weaker than that of p14\textsuperscript{ARF} and the band of p16\textsuperscript{INK4a} was barely visible (represented by patients 4 and 5). In only one tumor was the intensity of p16\textsuperscript{INK4a} band much stronger than that of p14\textsuperscript{ARF}, and the band of p14\textsuperscript{ARF} was barely visible (patient 2). In cases such as patients 4, 5, or 2 in Fig. 1, it is possible that the band of one product may have obscured the other product because of the competition in the PCR. We also performed the RT-PCR for p16\textsuperscript{INK4a} and p14\textsuperscript{ARF} separately for each of these patients. The intensity of the bands in the competitive RT-PCR correlated reasonably well with that of RT-PCR performed separately (data not shown). Thus, the faint band in the competitive RT-PCR actually represents a small amount of transcript.

Ratio of the intensity of p16\textsuperscript{INK4a} to p14\textsuperscript{ARF} band and clinicopathological characteristics in NSCLCs We next analyzed the relationship between the ratio of the intensity of p16\textsuperscript{INK4a} to p14\textsuperscript{ARF} band and clinicopathological characteristics. The p stage II–IV tumors had significantly lower p16\textsuperscript{INK4a} to p14\textsuperscript{ARF} ratios than the p stage I tumors (Fig. 2).

Fig. 1. Competitive RT-PCR of representative cases of tumor specimens (T) and matching normal lung tissues (N). A common primer and two specific primers were used to amplify the transcripts of p16\textsuperscript{INK4a} and p14\textsuperscript{ARF} genes in a single reaction. p16\textsuperscript{INK4a} and p14\textsuperscript{ARF} transcripts showed approximately equal intensity in normal lung tissues, whereas the intensities varied considerably among the tumors.

Fig. 2. Ratio of the intensity of the p16\textsuperscript{INK4a} to the p14\textsuperscript{ARF} band according to p stage of NSCLCs. The boxes represent 75% confidence interval; the long horizontal line, the median; the short horizontal bars, 90% confidence interval. Statistical analysis by Mann-Whitney’s U test.

Fig. 3. Ratio of intensity of the p16\textsuperscript{INK4a} to the p14\textsuperscript{ARF} band according to tumor status of NSCLCs. Symbols are the same as in the legend to Fig. 2.
T2–4 tumors had significantly lower p16INK4a to p14ARF ratios than the T1 tumors (Fig. 3). The N1–3 tumors had significantly lower p16INK4a to p14ARF ratios than the N0 tumors (Fig. 4). We also examined the relationship between clinicopathological characteristics and p16INK4a or p14ARF separately using each of the bands in the competitive RT-PCR assay. Tendencies toward lower intensity of the p16INK4a band were observed in the p stage II–IV tumors as compared with the p stage I tumors (P=0.059) and in the T2–4 tumors as compared with the T1 tumors (P=0.056). The N1–3 tumors had a significantly lower intensity of the p16INK4a band than the N0 tumors (P=0.037). There was no difference in the p stage, in the T status, or in the N status with regard to the intensity of the p14ARF band (data not shown).

Then we classified these tumors into two groups according to the ratio of the intensity of p16INK4a to p14ARF: group A (n=39/60: 65%), log p16/p14≥−0.6 and group B (n=21/60: 35%), log p16/p14<−0.6 (Table I). No difference was observed in terms of age and sex between the two groups. Group B tumors had a significantly higher p stage (P=0.006), p T factor (P=0.005), and p N factor (P=0.003) than group A tumors, but there was no difference in M factor between the two groups. There was more squamous cell carcinoma than adenocarcinoma in group B as compared with group A (P=0.03). Adenocarcinoma in group B was more poorly differentiated than that in group A (P=0.028), although the difference was not significant in squamous cell carcinoma (P=0.28). In addition, group B patients had a history of heavier smoking than group A patients (P=0.008). These results suggest that the ratio of the expression of p16INK4a to p14ARF tends to decrease during the progression of NSCLC.

Expression levels of p16INK4a and p14ARF in lung cancer with reference to those in matching normal lung tissue and its relationship with p stage Tumor to normal tissue ratio of the intensity of the p16INK4a band was plotted against that of p14ARF, and its relation to the p stage of the tumor was analyzed (Fig. 5). The tumor to normal tissue ratio of the intensity of p16INK4a band varied from −2 to 0.5. All tumors but one had a log p14ARF(T)/p14ARF(N) value of above −0.5. Stage I tumors tended to segregate to the quadrant with a log p14ARF(T)/p14ARF(N) value of −0.5 and a log p16INK4a(T)/p16INK4a(N) value below −1 (P=0.039). Stage II–IV tumors were distributed fairly evenly. These results indicate that lower expression of p16INK4a correlated to a higher grade of malignancy. It should also be pointed out that tumors with decreased expression of p16INK4a have significantly higher expression of p14ARF (Fig. 5).

DISCUSSION

We measured the intensity of the bands of expressed p16INK4a and p14ARF in 60 NSCLC samples and corresponding normal lung tissues. We took advantage of the shared exons and performed a competitive PCR so that the expression levels of these genes were directly comparable (Fig. 1). The lower ratio of the intensity of p16INK4a to p14ARF band was a good indicator of increased grade of malignancy in NSCLC. The intensity ratio of p16INK4a to
p14ARF bands were significantly lower in tumors with advanced stage (Fig. 2), with higher local invasion (Fig. 3) and with more extensive nodal involvement (Fig. 4).

Loss of p16INK4a expression is a significant event in the progression of melanomas23) and NSCLCs.24) Each of the bands for p14ARF and p16INK4a was analyzed separately, it was also clear that the intensity of the p16INK4a band was significantly correlated with clinical stage, T and N factors. However, the level of significance was better when the ratio of the intensity of the p16INK4a to p14ARF band was used instead of the intensity of the p16INK4a band alone. Thus, we used the ratio of the intensity of the p16INK4a to p14ARF band for further analysis. Under our experimental conditions, the intensities of the bands in the competitive PCR assay correlated to those of the individual PCRs (data not shown), indicating that the competition for the common primer is not so limiting as to totally obscure the bands for the less expressed gene. It is also clear from Fig. 5 that higher expression of p14ARF does not necessarily accompany decreased p16INK4a expression. Thus, under the conditions used in our experiment, the intensities of p16INK4a and p14ARF bands of the competitive PCR roughly correspond to the absolute amounts of the transcript. The use of simultaneous PCR is a convenient way to compare the intensities of the bands for the two genes under the same amplification conditions.

In contrast to p16INK4a, the intensity of the p14ARF band alone was not significantly correlated with any of the clinical characteristics (data not shown). However, when we plotted the intensity of the p14ARF band (expressed as the ratio to the corresponding normal tissue) against that of p16INK4a, there was an interesting correlation. Compared with tumors having a normal intensity of the p16INK4a band, tumors with a low intensity of the p16INK4a band had an elevated intensity of the p14ARF band (Fig. 5). It is an interesting possibility that p14ARF is more highly expressed in cells with decreased p16INK4a expression to compensate for the impaired control of the cell cycle by bringing the lowered p16INK4a and elevated p14ARF band intensities were mostly advanced (Fig. 5), this compensation may not have been an effective one in these tumors. For p14ARF to effectively suppress tumor growth, intact p53 function is required.15, 17) We also studied the p53 expression using immunohistochemistry. Tumors with increased p53 immunostaining did not segregate on the plot shown in Fig. 5 (data not shown). These results need to be confirmed by means of p53 sequence studies.

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