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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 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's clinicopathological characteristics. The p stage II–IV tumors had significantly lower $p16^{INK4a}$ to $p14^{ARF}$ ratios than 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, frequently deleted in many 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 1α for $p16^{INK4a}$ and exon 1β 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 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 can occur with more tumor-specific 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,[6, 18)] while no point mutations in the specific exon 1β of $p14^{ARF}$ have been reported. Specific deletion of exon 1β has been described in melanoma cell lines[19)] 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).

Table I. 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 III 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.

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 Biotech, 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 μl 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′-TGT GGC 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 a 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′-CGG AGT CAA CGG ATT TGG TCG TAT-3′; and (antisense), 5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′, for 22 cycles of 94°C for 60 s, 58°C for 60 s, and 72°C for 60 s. To rule out the possibility of false positives, competitive RT-PCR was performed at least twice in independent experiments.

Evaluation of $p16^{INK4a}$ and $p14^{ARF}$ expression The intensities of $p16^{INK4a}$ and $p14^{ARF}$ bands were quantified using NIH Image. The relationship between the ratio of the intensity of $p16^{INK4a}$ to $p14^{ARF}$ band and clinicopathological characteristics was examined.

Statistical analyses Associations between categorical variables were analyzed by Mann-Whitney's *U* test,

Fisher's exact probability test and simple linear regression (Stat View 4.0). All tests were two-sided and the criterion of significance was $P<0.05$.

RESULTS

Competitive RT-PCR We investigated the expression of p16INK4a and p14ARF using competitive RT-PCR in 60 tumors and matching normal lung tissues from the same patients. Using specific sense primers in exon 1α and exon 1β 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 p16INK4a and p14ARF. As shown in Fig. 1, the expression levels of p16INK4a in the normal lung tissues (n=60) were similar, and the intensities of the p16INK4a and p14ARF bands were nearly equal or p16INK4a expression slightly exceeded that of p14ARF. In most tumor samples, the intensities of the p16INK4a and p14ARF bands were different from those in the corresponding normal lung tissues. In 38 cases the intensity of the p16INK4a band was similar to or slightly weaker than that of p14ARF (represented by patient 1 in Fig. 1). In 6 cases the intensity of the p16INK4a band was weaker than that of p14ARF, but the band of p16INK4a was clearly visible (represented by patient 3). In 15 cases the intensity of p16INK4a band was much weaker than that of p14ARF and the band of p16INK4a was barely visible (represented by patients 4 and 5). In only one tumor was the intensity of p16INK4a band much stronger than that of p14ARF, and the band of p14ARF 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 p16INK4a and p14ARF 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 p16INK4a to p14ARF band and clinicopathological characteristics in NSCLCs We next analyzed the relationship between the ratio of the intensity of p16INK4a to p14ARF band and clinicopathological characteristics. The p stage II–IV tumors had significantly lower p16INK4a to p14ARF ratios than the p stage I tumors (Fig. 2).

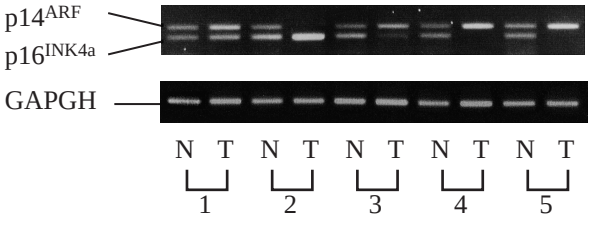

Fig. 1. Competitive RT-PCR of representative cases of tumor specimens (T) and matching normal lung tissues (N). A common primer and two specific primers were used to amplify the transcripts of *p16INK4a* and *p14ARF* genes in a single reaction. p16INK4a and p14ARF transcripts showed approximately equal intensity in normal lung tissues, whereas the intensities varied considerably among the tumors.

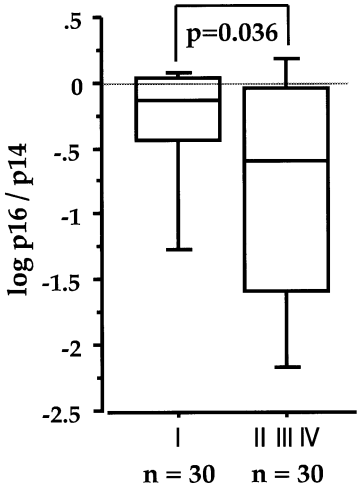

Fig. 2. Ratio of the intensity of the p16INK4a to the p14ARF band according to p stage of NSCLCs. The boxes represent 75% confidence interval; the long horizontal line, the median; the short horizontal bars, 90% confidence interval. Statistical analysis by Mann-Whitney's U test.

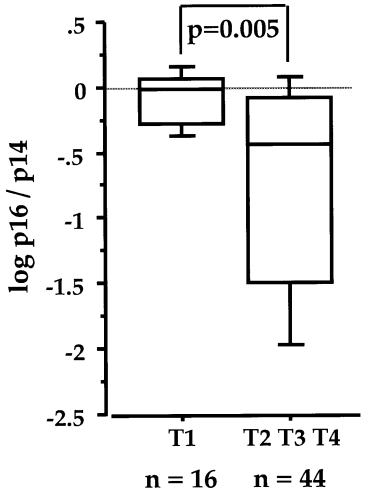

Fig. 3. Ratio of intensity of the p16INK4a to the p14ARF band according to tumor status of NSCLCs. Symbols are the same as in the legend to Fig. 2.

The T2–4 tumors had significantly lower $p16^{INK4a}$ to $p14^{ARF}$ ratios than the T1 tumors (Fig. 3). The N1–3 tumors had significantly lower $p16^{INK4a}$ to $p14^{ARF}$ ratios than the N0 tumors (Fig. 4). We also examined the relationship between clinicopathological characteristics and $p16^{INK4a}$ or $p14^{ARF}$ separately using each of the bands in the competitive RT-PCR assay. Tendencies toward lower intensity of the $p16^{INK4a}$ band were observed in the p stage

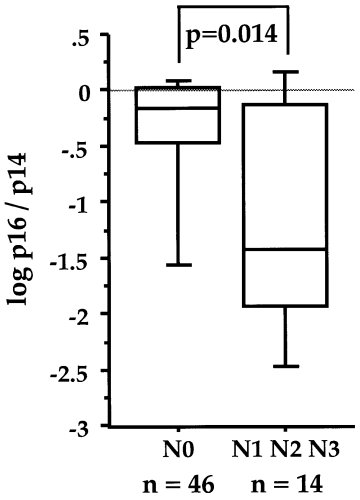

Fig. 4. Ratio of intensity of the $p16^{INK4a}$ to the $p14^{ARF}$ band according to nodal status of NSCLCs. Symbols are the same as in the legend to Fig. 2.

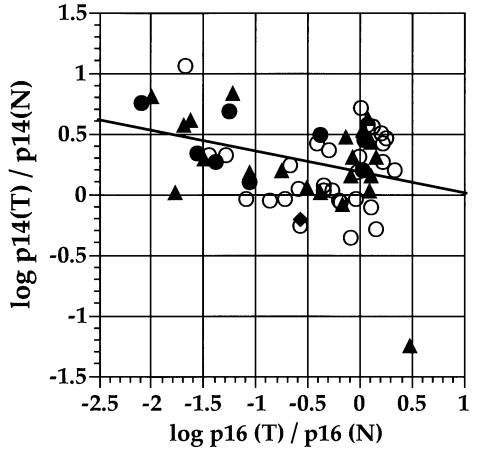

Fig. 5. Relationship between the intensity ratios of the $p16^{INK4a}$ and $p14^{ARF}$ bands with respect to those in corresponding normal lung tissue, in individual NSCLC. The line in the figure indicates the simple linear regression line ($r=-0.336$, $P=0.0087$). Pathological stage is indicated as follows: ○, stage I; ●, stage II; ▲, stage III; ◆, stage IV.

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 $p16^{INK4a}$ 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 $p14^{ARF}$ band (data not shown).

Then we classified these tumors into two groups according to the ratio of the intensity of $p16^{INK4a}$ to $p14^{ARF}$; 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 ($P=0.033$). 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 ($P=0.287$). 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 $p16^{INK4a}$ to $p14^{ARF}$ tends to decrease during the progression of NSCLC.

Expression levels of $p16^{INK4a}$ and $p14^{ARF}$ 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 $p16^{INK4a}$ band was plotted against that of $p14^{ARF}$, and its relation to the p stage of the tumor was analyzed (Fig. 5). The tumor to normal tissue ratio of the intensity of $p16^{INK4a}$ band varied from -2 to 0.5. All tumors but one had a log $p14^{ARF}(T)/p14^{ARF}(N)$ value of above -0.5. Stage I tumors tended to segregate to the quadrant with a log $p14^{ARF}(T)/p14^{ARF}(N)$ above -0.5 and a log $p16^{INK4a}(T)/p16^{INK4a}(N)$ above -1. There were more stage II–IV tumors than stage I tumors with a log $p16^{INK4a}(T)/p16^{INK4a}(N)$ value below -1 ($P=0.039$). Stage II–IV tumors were distributed fairly evenly. These results indicate that lower expression of $p16^{INK4a}$ correlated to a higher grade of malignancy. It should also be pointed out that tumors with decreased expression of $p16^{INK4a}$ have significantly higher expression of $p14^{ARF}$ (Fig. 5).

DISCUSSION

We measured the intensity of the bands of expressed $p16^{INK4a}$ and $p14^{ARF}$ 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 $p16^{INK4a}$ to $p14^{ARF}$ band was a good indicator of increased grade of malignancy in NSCLC. The intensity ratio of $p16^{INK4a}$ to

p14^{ARF} bands was 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 p16^{INK4a} expression is a significant event in the progression of melanomas[23] and NSCLCs.[24] When each of the bands for p14^{ARF} and p16^{INK4a} was analyzed separately, it was also clear that the intensity of the p16^{INK4a} 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 p16^{INK4a} to p14^{ARF} band was used instead of the intensity of the p16^{INK4a} band alone. Thus, we used the ratio of the intensity of the p16^{INK4a} to p14^{ARF} 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 p14^{ARF} does not necessarily accompany decreased p16^{INK4a} expression. Thus, under the conditions used in our experiment, the intensities of p16^{INK4a} and p14^{ARF} 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 p16^{INK4a}, the intensity of the p14^{ARF} band alone was not significantly correlated with any of the clinical characteristics (data not shown). However, when we plotted the intensity of the p14^{ARF} band (expressed as the ratio to the corresponding normal tissue) against that of p16^{INK4a}, there was an interesting correlation. Compared with tumors having a normal intensity of the p16^{INK4a} band, tumors with a low intensity of the p16^{INK4a} band had an elevated intensity of the p14^{ARF} band (Fig. 5). It is an interesting possibility that p14^{ARF} is more highly expressed in cells with decreased p16^{INK4a} expression to compensate for the impaired control of the cell cycle by bringing the p14^{ARF}/p53 pathway into action. Because the tumors with the lowered p16^{INK4a} and elevated p14^{ARF} band intensities were mostly advanced (Fig. 5), this compensation may not have been an effective one in these tumors. For p14^{ARF} to effectively suppress tumor growth, intact p53 function is required.[1, 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.

Aberrant methylation of the p14^{ARF} promoter independent of that of the p16^{INK4a} promoter has been demonstrated in some cancer cell lines and human cancers.[21, 22] The results shown in Fig. 5 may also indicate that the control of the expression of p14^{ARF} is independent of that of p16^{INK4a}, and that simultaneous loss of expression of both p16^{INK4a} and p14^{ARF} at the mRNA level is a rare event. However, expression of p14^{ARF} protein needs to be analyzed because disparity of protein and mRNA expressions of p14^{ARF} has been reported.[25] In addition, loss of both p16^{INK4a} and p14^{ARF} at the protein level was demonstrated in NSCLC.[26] It was also reported that some of these tumors did not exhibit any methylation of exon 1α or mutations in exon 1α and exon 2, suggesting that there may be a common mechanism for the loss of p16^{INK4a} and p14^{ARF} expression. Since most tumors with loss of p16^{INK4a} maintained p14^{ARF} at the mRNA level (Fig. 5), analysis of the function of wild-type p14^{ARF} in cancer progression should also be the focus of further study.

Our results showed that the intensities of the p16^{INK4a} and p14^{ARF} bands in the competitive RT-PCR assay were approximately equal or the intensity of the p16^{INK4a} band slightly exceeded that of p14^{ARF} in the normal lung tissue. This may indicate that expression of both p16^{INK4a} and p14^{ARF} is necessary to maintain the quiescent state of normal lung tissue. The loss of p16^{INK4a} and maintenance of p14^{ARF} in many tumors may suggest a significant role of p16^{INK4a}, but not of p14^{ARF}, in the oncogenesis of NSCLC.

Esteller *et al.*[22] reported that loss of p14^{ARF} expression in the presence of p16^{INK4a} expression was observed in 14% of colorectal tumors. However, only one of 60 (1.7%) showed reduced intensity of the p14^{ARF} band and increased intensity of the p16^{INK4a} band in our study (Fig. 1 patient 2 and the tumor shown in the lower right corner of Fig. 5). This patient had a stage III tumor and it is possible that a rare loss of p14^{ARF} expression may have contributed to tumor progression even with high p16^{INK4a} expression. Collection of more cases and functional studies of the expressed protein will be necessary to further elucidate the role of p14^{ARF} in NSCLC.

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