Promoter Hypermethylation and Quantitative Expression Analysis of *CDKN2A* (*$p14^{ARF}$* and *$p16^{INK4a}$*) Gene in Esophageal Squamous Cell Carcinoma

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Abstract. *Background: Abnormal hypermethylation of the CDKN2A ($p14^{ARF}$ and $p16^{INK4a}$) gene can lead to repression of gene expression and contribute to carcinogenesis and tumor progression. Materials and Methods: In esophageal squamous cell carcinoma (ESCC), the promoter methylation of the $p14^{ARF}$ and $p16^{INK4a}$ gene was investigated in 38 cases by methylation-specific PCR and the expression of each gene in 18 cases was quantified by real-time quantitative reverse transcription-PCR. Results: Aberrant methylation of $p14^{ARF}$ and of $p16^{INK4a}$ was detected in 23 (61%) and 29 (76%) cases, respectively. No relationship was found between clinicopathological variables and $p14^{ARF}$ or $p16^{INK4a}$ promoter methylation. A statistically significant association between $p14^{ARF}$ methylation status and mRNA expression was found (p=0.0441). Regarding $p14^{ARF}$, a low expression group showed a significantly higher proportion of cases with deep invasion of tumor, lymph node metastasis, and a high TNM stage of disease (p=0.0474, 0.0474, and 0.0441, respectively), and a significantly poor prognosis (p=0.0402). Regarding $p16^{INK4a}$, no relationship was found among the methylation status, mRNA expression and clinicopathological variables, including survival. Conclusion: Our results suggest that methylation is the predominant mechanism of inactivation of the $p14^{ARF}$ gene in ESCC. The decrease in $p14^{ARF}$ gene expression associated with invasive and metastatic phenotypes may be significant as an indicator of the malignant potential of human ESCC.*

Esophageal squamous cell carcinoma (ESCC) is one of the most common types of cancer in the world, with extremely poor prognosis due to late presentation, rapid progression and poor efficacy of treatment. The 5-year survival rate for patients who underwent three-field lymph node dissection has been reported as 40-50% (1, 2). An understanding of the molecular mechanisms in carcinogenesis and tumor progression will not only provide biomarkers for early detection, but also enable us to improve treatment modalities. The genesis of human cancers, including that of esophageal cancer, is known to be a multistep process involving cumulative genetic alterations that include activation of oncogenes and/or inactivation of tumor-suppressor genes. Although frequent allelic deletions and other genetic abnormalities have been detected in ESCC (3), the precise molecular mechanisms of development and/or progression of ESCC remain unknown.

The *CDKN2A* (*$p14^{ARF}$* and *$p16^{INK4a}$*) locus is located on chromosome 9p21 and has the unique distinction of encoding two cell cycle regulatory genes, *$p14^{ARF}$* and *$p16^{INK4a}$* (4). Briefly, alternative splicing of the first exon and a common downstream exon permits one gene to encode two different products which function *via* two distinct pathways to inhibit cell cycle progression. *$p14^{ARF}$* interacts with the oncogenic protein MDM2, inducing stabilization of *p53* and enhancing *p53*-related functions (5). Thus, it has been suggested that concomitant *$p14^{ARF}$* and *p53* gene inactivation in the same tumor must be a rare event (6). In contrast, the tumor-suppressive activity of *$p16^{INK4a}$* is ascribed to its ability to bind both cdk4 and cdk6. This in turn inhibits the catalytic activity of the cdk4/6-cyclin D complex, blocks retinoblastoma phosphorylation, and

Abbreviations: ESCC, esophageal squamous cell carcinoma; MSP, methylation-specific PCR; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Key Words: Esophageal squamous cell carcinoma, *$p14^{ARF}$*, *$p16^{INK4a}$*, methylation, methylation-specific PCR, real-time quantitative RT-PCR, prognosis.

Table I. *PCR primer sequences and amplification conditions used in this study.*

Target gene[a]	Primer	Sequence	Product size (bp)	Annealing temperature (˚C)	Reference no.
MSP					
p14-M	Forward	5'-GTGTTAAAGGGCGGCGTAGC-3'	122	60	(21)
	Reverse	5'-AAAACCCTCACTCGCGACGA-3'			
p14-U	Forward	5'-TTTTTGGTGTTAAAGGGTGGTGTAGT-3'	132	60	(21)
	Reverse	5'-CACAAAAACCCTCACTCACAACAA-3'			
p16-M	Forward	5'-TTATTAGAGGGTGGGGCGGATCGC-3'	234	65	(22)
	Reverse	5'-CCACCTAAATCGACCTCCGACCG-3'			
p16-U	Forward	5'-TTATTAGAGGGTGGGGTGGATTGT-3'	234	65	(22)
	Reverse	5'-CCACCTAAATCAACCTCCAACCA-3'			

[a]M, methylated-specific primers; U, un-methylated-specific primers.

ultimately prevents cell-cycle progression (4). Mice with the *CDKN2A* locus deleted are prone to spontaneous tumor formation and have increased susceptibility to oncogenic effects of Ras, Myc, and an activated epidermal growth factor receptor (7-9), indicating the importance of this locus in tumor suppression *in vivo*.

Many tumor-suppressor genes show evidence of methylation silencing, providing a new potential pathway for tumor-suppressor gene inactivation (10). $p14^{ARF}$ and $p16^{INK4a}$ methylation have been reported in many types of cancer such as stomach, colon, lung, and uterine (11-13). Inactivation of the *CDKN2A* gene, which includes mutations, homozygous deletion and promoter methylation in ESCCs, has been reported at various frequencies (14-17). However, the association between $p14^{ARF}$ and $p16^{INK4a}$ promoter hypermethylation and the loss of its accurate expression in ESCC samples remains unclear. In addition, the biological significance of $p14^{ARF}$ and $p16^{INK4a}$ methylation and expression, as an indicator of the biomarker of carcinogenesis and/or the malignant potential of ESCC, has not been elucidated.

In the present study, we performed methylation-specific PCR (MSP) for $p14^{ARF}$ and $p16^{INK4a}$ genes and compared the results with their expression as determined by real-time quantitative reverse transcription-PCR (RT-PCR). The potential clinical implications of hypermethylation and expression of $p14^{ARF}$ and $p16^{INK4a}$ genes in ESCC were investigated. The purpose of this study was to clarify the significance of the *CDKN2A* gene in carcinogenesis and tumor progression of ESCC.

Materials and Methods

Specimens and extraction of genomic DNA. Thirty-eight consecutive Japanese patients with ESCC, who were surgically treated without radiation or chemotherapy prior to surgery in our institute from March 1994 to December 2001 and whose genomic DNA from resected specimen was available, were entered into this study.

Immediately after resection, the specimens were placed in liquid nitrogen and used for analyses of genomic DNA and total RNA. The remaining tissues were routinely processed for histopathological analyses. All the tumors were diagnosed by histopathological specialists at our hospital. In addition, to define the use of alcohol and cigarettes, a questionnaire was given to all patients, as described elsewhere (18-20).

Frozen tissues were broken up in liquid nitrogen and lysed in digestion buffer (10 mM Tris-Cl; pH 8.0, 0.1 M EDTA; pH 8.0, 0.5% SDS, 20 g/ml pancreatic RNase). After treatment with proteinase K and extraction with phenol, DNA was precipitated with ethanol, then dissolved in 1xTE (10 mM Tris-Cl, pH 7.5, 1 mM EDTA).

Bisulfite modification and MSP. The CpGenome DNA Modification Kit (Intergen Company, Purchase, NY, USA) was used for bisulfite treatment according to the manufacturer's protocol. One microgram of DNA was denatured by NaOH and modified by sodium bisulfite. Modified DNA was stored at –70˚C until used. The primer sequences of $p14^{ARF}$ and $p16^{INK4a}$ genes for both the methylated and unmethylated forms, product size and annealing temperatures are summarized in Table I. Methylated-specific and unmethylated-specific primers were taken from the literature (21, 22). The primer sequences of $p14^{ARF}$ spanned six CpGs within the 5' region of the gene. The 5' position of the sense unmethylated and methylated primers corresponds to bp 195 and 201 of GeneBank sequence number L41934. Both antisense primers originate from bp 303 of this sequence. The location of the 5' nucleotide of the $p16^{INK4a}$ sense primer is +167 in relation to the major transcriptional start site defined in the literature (23) and GeneBank sequence number X94154.

MSP was carried out essentially as described elsewhere (22), based on the principle that treating DNA with bisulfite would result in the conversion of unmethylated cytosine residues into uracil, and subsequent PCR using primers designed for either methylated or modified unmethylated DNA was performed. The PCR mixture contained 10xPCR buffer II (Applied Biosystems, Foster City, CA, USA), 2.7 mM $MgCl_2$, 0.18 mM of each dNTP, 1.0 μM of each primer, 100 ng modified DNA templates and 1 unit of AmpliTaq Gold (Applied Biosystems) in a total volume of 20 ml. PCR was carried out in a Perkin-Elmer GeneAmp PCR System 9700 (Applied Biosystems) using an unmethylated-specific primer pair or

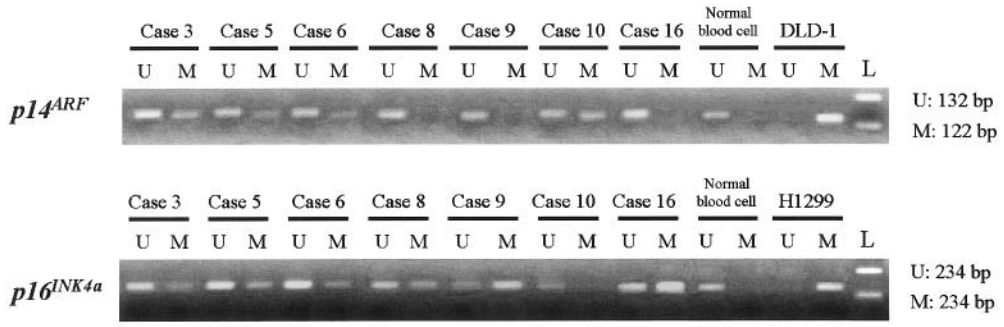

Figure 1. *Representative examples of MSP analysis of the $p14^{ARF}$ and $p16^{INK4a}$ promoter in ESCCs. At the left of each panel, the gene studied. Lane U, amplified product with primers recognizing an unmethylated sequence; lane M, amplified product with primers recognizing a methylated sequence; L, 100-base marker. The PCR product sizes of both genes are summarized in Table I. Normal peripheral blood mononuclear cells were used as a negative control for methylation. In each case, modified DNA from DLD-1 and H1299 cells was used as a positive control for the methylated allele of $p14^{ARF}$ and $p16^{INK4a}$, respectively.*

a methylated-specific primer pair after the first denaturation at 95˚C for 10 min. Each cycle consisted of denaturation at 95˚C for 30 s, annealing at each temperature listed in Table I for 30 s and extension at 72˚C for 30 s. After the last cycle of amplification, the extension was continued for an additional 10 min at 72˚C.

DNA from DLD-1 (colorectal cancer cell line) and H1299 (lung cancer cell line) was used as positive control for $p14^{ARF}$ and $p16^{INK4a}$ methylated alleles, respectively. DNA from normal peripheral blood mononuclear cells was used as a negative control for methylated alleles of both genes.

PCR products were analyzed on 2% agarose gels containing ethidium bromide. Hypermethylation of each gene was defined as occurring when methylated molecules were detected using a methyl-detected primer pair (Figure 1). Unmethylation of each gene was defined as occurring when only unmethylated molecules were detected using MSP analysis. Results were confirmed by repeating bisulfite treatment and MSP.

Total RNA extraction and first-strand cDNA synthesis. Tumor tissues from 18 ESCC patients were available for extraction of total RNA. Total RNA was extracted using an ISOGEN RNA extraction kit (Nippon Gene, Inc., Tokyo, Japan) and synthesis of cDNA was carried out with 4.0 μg of total RNA using a First-strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Inc., NJ, USA) according to the manufacturer's protocol. The final volume of the first-strand cDNA was 50 μl, 5 μl of which was used for each PCR test of $p14^{ARF}$, $p16^{INK4a}$ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression analysis.

Real-time quantitative RT-PCR. Real-time RT-PCR is a sensitive, quantitative and highly reliable method for RNA quantitation. The theoretical basis of the methods employed here has been described elsewhere (24). $p14^{ARF}$ and $p16^{INK4a}$ gene expression was measured using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Primers and Taqman Probe of human $p14^{ARF}$ and $p16^{INK4a}$ were purchased from Applied Biosystems (catalogue no. 4319444 and 4319445). Reaction mixtures of 50 μl contained: 1xTarget primers and Probe and 1xTaqMan PCR Master Mix (Applied Biosystems), which contained Ampli-Taq DNA polymerase, a reaction buffer, dNTP, dUTP and Amperase. As an internal reference, GAPDH mRNA of the same samples was checked using a TaqMan probe kit (Applied Biosystems). The sequences of the primers and hybridization probes for GAPDH were taken from the literature (25). Reaction mixtures of 50 μl contained: 300 nM of each primer, 200 nM specific TaqMan probe, and 1 xTaqMan PCR Master. The cycle parameters were 2 min at 50˚C, 10 min at 95˚C, and 40 cycles involving denaturation at 95˚C for 15 s and annealing/extension at 60˚C for 10 min. Experiments were run in duplicate and the average value of the threshold cycle (Ct) was calculated for $p14^{ARF}$, $p16^{INK4a}$ and GAPDH, respectively. The PCR reaction and the resulting relative increases in reporter fluorescent dye emission were analyzed by the 7700 sequence detector (Applied Biosystems). Analysis of the signal and construction of the standard curve was performed using Sequence Detector v1.7 software (Applied Biosystems).

Construction of plasmids containing a fragment of target gene. To obtain a standard curve corresponding to $p14^{ARF}$, $p16^{INK4a}$ and GAPDH mRNA in real-time quantitative RT-PCR, plasmids containing a fragment of cDNA were constructed. The PCR reaction was carried out to obtain the fragments using a Perkin-Elmer GeneAmp PCR System 9700 (Applied Biosystems). The first-strand cDNA of esophageal carcinoma samples was used as a template. The pair of primers for this PCR reaction was that used for the real-time quantitative RT-PCR. PCR products were checked by agarose gel electrophoresis, and the products of $p14^{ARF}$ and $p16^{INK4a}$ were subcloned into the PCRII Vector (Invitrogen, Groningen, The Netherlands). Ligation of the fragment into the plasmid was carried out using T-cloning methods (26). The products of GAPDH were ligated to a pT7Blue T-Vector (Novagen, Inc., Madison, WI, USA) with T4 DNA ligase (Takara, Tokyo, Japan). The cloned plasmid containing the PCR fragment was purified and the sequence of the ligated fragment was confirmed using an ABI Prism 310 fluorescence-based, semi-automated DNA sequencer (Applied Biosystems). The plasmids were quantified by spectrophotometry (absorbance at 260 nm) and the number of copies was calculated with the use of the molecular weight of the gene. Ten-fold serial dilutions of the plasmids were prepared to make the standard curve.

Data analysis. The association between the variables was tested by Fisher's exact test or Student's *t*-test. The survival rates of different

Table II. *The relationship between quantitative ratio by real-time quantitative RT-PCR and MSP in the 18 esophageal squamous cell carcinomas.*

	$p14^{ARF}$ methylation		*P*-value[a]	$p16^{INK4a}$ methylation		*P*-value[a]
	+ (n=14)	– (n=4)		+ (n=15)	– (n=3)	
Low expression	12	1		12	2	
High expression	2	3	0.0441	3	1	1.0000

[a]Fisher's exact test.

groups were compared by the log-rank test. A multivariate survival analysis was calculated according to backward stepwise survival analysis with Cox's proportional hazards model. A level of $p<0.05$ was taken to indicate significance.

Results

Frequency of methylation of the $p14^{ARF}$ and $p16^{INK4a}$ genes. Using MSP, among 38 ESCC samples, aberrant methylation of $p14^{ARF}$ and $p16^{INK4a}$ was detected in 23 (61%) and 29 (76%) cases, respectively. The presence of methylated allele was indicated by the presence of a positive PCR product with the methylated-specific primers.

Methylation status and expression of the $p14^{ARF}$ and $p16^{INK4a}$ mRNA. The relationships between methylation status and mRNA expression of the $p14^{ARF}$ and $p16^{INK4a}$ were examined (Table II). To determine the $p14^{ARF}$ and $p16^{INK4a}$ expression pattern in ESCCs, the mRNA levels of each gene were analyzed by real-time quantitative RT-PCR in 18 available cases of all 38 cases. The $p14^{ARF}$ and $p16^{INK4a}$ mRNA expression in ESCCs was expressed as the ratio of each gene to the GAPDH product. The relative expression levels of $p14^{ARF}$ and $p16^{INK4a}$ were obtained by normalizing the copy number of 0.4 mg mRNA by dividing it by that of GAPDH 0.4 mg mRNA in each tumor sample, respectively. Low and high groups were determined by the threshold level of $p14^{ARF}$ and $p16^{INK4a}$ mRNA (0.093, 0.041; mean + SE), respectively. There was a statistically significant association detectable between $p14^{ARF}$ methylation status and mRNA expression. In contrast, no relationship was found between the $p16^{INK4a}$ methylation status and mRNA expression.

Methylation of the $p14^{ARF}$ and $p16^{INK4a}$ gene and clinicopathological characteristics. After completion of MSP analysis in all specimens, clinicopathological data were examined for relationships with the molecular analysis (Table III). Clinicopathological variables such as the age, sex, size of tumor, location, histology depth of invasion, lymph node metastasis, vascular invasion, and TNM stage were not associated with the $p14^{ARF}$ or $p16^{INK4a}$ methylation status. In addition, the associations of methylation with cigarette smoking and alcohol drinking, which are considered to be important risk factors of ESCC in the Japanese (27) were examined. All patients were distributed into three groups on the basis of their drinking index and pack-years (19). Exposure to alcohol and cigarettes was not significantly associated with $p14^{ARF}$ or $p16^{INK4a}$ promoter methylation. However, there was a tendency toward $p16^{INK4a}$ promoter methylation in the high-risk group. Neither methylation status at $p14^{ARF}$ nor $p16^{INK4a}$ was associated with survival (data not shown).

Expression of $p14^{ARF}$ and $p16^{INK4a}$ mRNA and clinicopathological characteristics. The clinicopathological factors analyzed are shown in Table IV in relation to the $p14^{ARF}$ and $p16^{INK4a}$ mRNA expression status. Regarding the $p14^{ARF}$, low expression was significantly associated with higher depth of invasion and lymph node metastasis. Furthermore, the TNM stage of disease in the low $p14^{ARF}$ mRNA expression group was significantly higher than that in the high expression group. In contrast, other pathological variables were not significantly associated with the $p14^{ARF}$ expression status. No significant association was found between $p16^{INK4a}$ mRNA expression and any clinicopathological factor.

The cumulative survival rate of the low and high expression groups of $p14^{ARF}$ and $p16^{INK4a}$ were also compared as shown in Figure 2. The low $p14^{ARF}$ mRNA expression group showed a significantly poorer prognosis than the high expression group. A multivariate survival analysis was used to select a set of independently significant prognostic factors, such as $p14^{ARF}$ mRNA expression, age, sex, size of tumor, location, histology, depth of invasion, lymph node metastasis, vascular invasion and TNM stage in the 18 cases. Adjusting for age, size of tumor and depth of invasion, which were selected in the model by the backward stepwise survival analysis, the low $p14^{ARF}$ mRNA expression group did not have a significantly shorter survival time (hazards ratio, 1.514; 95% confidence interal, 0.143-16.020, $p=0.7305$) than the high expression group. Expression of $p16^{INK4a}$ mRNA did not correlate with survival.

Discussion

To date, two mechanisms have been postulated as primary causes of inactivation of the potential tumor-suppressor genes on 9p21: homozygous deletion and promoter hypermethylation (14-16). The frequency of $p14^{ARF}$ and $p16^{INK4a}$ promoter methylation in each tumor type is different (11-13). In ESCC, the frequencies of

Table III. *Clinicopathological features of methylation of $p14^{ARF}$ and $p16^{INK4a}$ in the 38 esophageal squamous cell carcinomas of this study.*

Variables	$p14^{ARF}$ methylation			$p16^{INK4a}$ methylation		
	+ (n=23)	– (n=15)	*P*-value[a]	+ (n=29)	– (n=9)	*P*-value[a]
Age (years)[b]	61.7±8.1	64.2±9.0	0.3703	62.4±9.3	63.4±4.8	0.7538
Gender						
Male	19	14		26	7	
Female	4	1	0.6295	3	2	0.5741
Size of tumor (cm)[b]	4.1±2.6	4.1±2.5	0.9447	4.2±2.7	3.8±1.9	0.6550
Location						
Upper	2	3		5	0	
Middle	11	7		13	5	
Lower	10	5	0.5979	11	4	0.5917
Histology[c]						
Well	4	5		5	4	
Moderate	13	8		18	3	
Poor	6	2	0.4526	6	2	0.2605
Depth of invasion						
Within the wall	11	11		17	5	
Invaded into adventitia/ neighbouring structures	12	4	0.1816	12	4	1.0000
Lymph node metastasis						
Negative	10	11		16	5	
Positive	13	4	0.1000	13	4	1.0000
Vascular invasion						
Negative	7	9		13	3	
Positive	16	6	0.0978	16	6	0.7060
TNM stage						
0-I	7	9		12	4	
II-IV	16	6	0.0987	17	5	1.0000
Drinking index[d]/pack-years[e]						
High risk group	6	5		10	1	
Middle risk group	11	8		15	4	
Low risk group	6	2	0.7502	4	4	0.1294

[a]Fisher's exact test or Student's *t*-test; [b]Mean ±SD; [c]Well, well-differentiated SCC; Moderate, moderately differentiated SCC; Poor, poorly differentiated SCC; [d]Number of drinks per week x number of years of drinking; [e](Number of cigarettes per day/20) x number of years of smoking.

hypermethylation are as high as 15-52% for $p14^{ARF}$ and 19-82% for $p16^{INK4a}$, respectively (16, 28, 29). In the present study, the frequency of $p14^{ARF}$ and $p16^{INK4a}$ promoter methylation was 61% and 76% respectively. In previous reports, the frequency of hypermethylation has been analyzed by various methods. Although MSP is a rapid and qualitative method for analysis of the presence of methylation in a given region of DNA, careful selection of primers is very important because it is possible to obtain false-positives with both methylated and unmethylated primer pairs, making it difficult to interpret the results. Incomplete bisulfite modification of genomic DNA will also give false-positives for methylated cytosine (13).

Cigarette smoking and alcohol drinking are considered to be important risk factors of ESCC in the Japanese (27). However, the precise mechanism by which smoking and alcohol act to induce alterations of normally functional genes remains unknown. In ESCCs, frequent p53 protein accumulations have been found (30), and cigarette smoking and alcohol drinking are associated with p53 protein accumulations (19, 20). However, the association between the hypermethylation of $p14^{ARF}$ and $p16^{INK4a}$ and these risk factors in ESCC is not well documented. Tobacco carcinogen exposure is associated with methylation of the $p16^{INK4a}$ gene in lung cancer (31). Methylated $p16^{INK4a}$ is present in bronchial epithelia before clinical evidence of lung cancer in chronic smokers (32). We did not detect any association between the hypermethylation of $p14^{ARF}$ and these risk factors in ESCCs. However, the hypermethylation of $p16^{INK4a}$ tended to be present in the high-risk group, thus

Table IV. *The relationship between the $p14^{ARF}$ and $p16^{INK4a}$ mRNA expression and clinicopathological features in the 18 esophageal squamous cell carcinomas of this study.*

Variables	$p14^{ARF}$			$p16^{INK4a}$		
	Low expression (n=13)	High expression (n=5)	*P*-value[a]	Low expression (n=14)	High expression (n=4)	*P*-value[a]
Age (years)[b]	62.2±19.3	56.0±15.9	0.2244	59.8±20.9	62.8±8.7	0.5941
Gender						
Male	12	3		12	3	
Female	1	2	0.1716	2	1	
Size of tumor (cm)[b]	4.3±2.8	3.8±1.6	0.7372	3.9±2.7	4.9±1.3	0.5074
Location						
Upper	1	2		2	1	
Middle	5	3		6	2	
Lower	7	0	0.0654	6	1	1.0000
Histology[c]						
Well	2	1		3	0	
Moderate	6	2		6	2	
Poor	5	2	1.0000	5	2	1.0000
Depth of invasion						
Within the wall	3	4		5	2	
Invaded into adventitia/ neighbouring structures	10	1	0.0474[d]	9	2	1.0000
Lymph node metastasis						
Negative	3	4		5	2	
Positive	10	1	0.0474[d]	9	2	1.0000
Vascular invasion						
Negative	1	2		3	0	
Positive	12	3	0.1716	11	4	1.0000
TNM stage						
0-I	1	3		3	1	
II-IV	12	2	0.0441[d]	11	3	1.0000

[a]Fisher's exact test or Student's *t*-test; [b]Mean±SD; [c]Well, well-differentiated SCC; Moderate, moderately differentiated SCC; Poor, poorly differentiated SCC; [d]statistically significant.

suggesting the potential of using methylated DNA as a tumor marker in ESCC screening, monitoring of chemoprevention and the treatment of cancer.

In recent years, several investigations have demonstrated that methylation of CpG islands in promoter regions of several genes, including known tumor-suppressor genes, results in a subsequent failure to express their functional proteins. Consequently, DNA methylation represents an early and fundamental step in the pathway by which normal tissue undergoes neoplastic transformation (33). In a previous study of brain tumors, loss of $p14^{ARF}$ protein expression was found to be associated with the gene status, *i.e.* homozygous deletion or promoter hypermethylation (34). In ESCC, previous studies have found a concordance between $p14^{ARF}$ and $p16^{INK4a}$ promoter methylation status and the expression of these genes using comparative RT-PCR; these results, however, do not provide complete quantitative evidence of reduced $p14^{ARF}$ and $p16^{INK4a}$ expression (16).

In the present study, we performed expression analysis with real-time quantitative RT-PCR, which makes it possible to quantify the amount of mRNA based on determining the PCR cycle during which the reaction enters the exponential phase (24). To our knowledge, this is the first report to focus on the association between hypermethylation of $p14^{ARF}$ and $p16^{INK4a}$ and the accurate mRNA expression of these genes in ESCC based on real-time quantitative RT-PCR. Using this method, we found a statistically highly significant association between $p14^{ARF}$ methylation and low levels of $p14^{ARF}$ mRNA. Thus, epigenetic regulation (promoter hypermethylation) is likely to play a role in the regulation of $p14^{ARF}$ expression in ESCC. As regards $p16^{INK4a}$, the results of the present study suggest that there are other mechanisms of $p16^{INK4a}$ silencing such as deletion (including loss of heterozygosity (LOH) and homozygous deletion) and mutation. In our plans for future research, it will be important to determine the deletional and mutational status of $p16^{INK4a}$ gene in

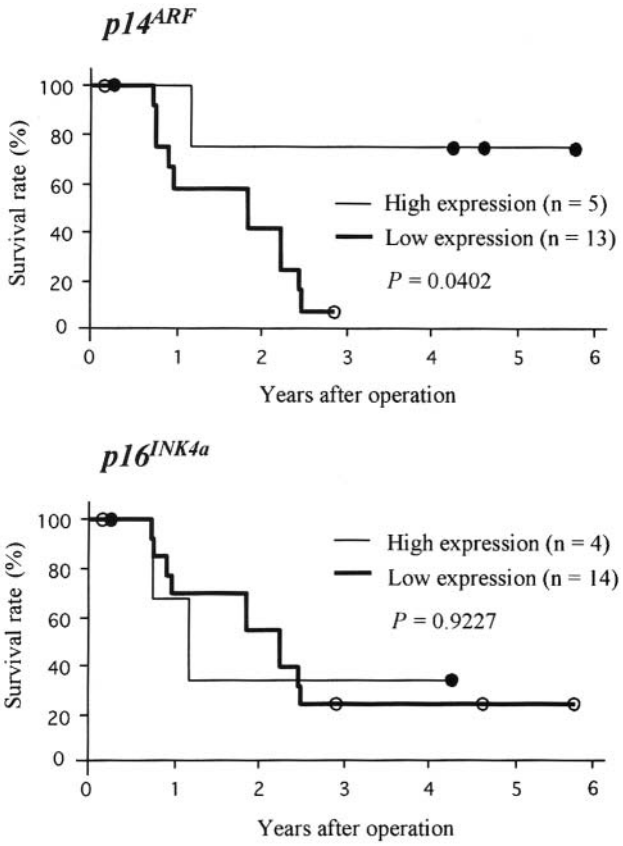

Figure 2. *Comparison of the Kaplan-Meier survival curves in the high expression group versus the low expression group of $p14^{ARF}$ and $p16^{INK4a}$. The survival rate for patients in the low expression group was significantly lower than that for patients in the high expression group with regard to $p14^{ARF}$ mRNA expression.*

addition to the methylation status and to examine the significance of the association between these aberrations and mRNA expression found in ESCCs.

Inactivation of multiple tumor-suppressor genes by aberrant hypermethylation is a fundamental process involved in the progression of many malignant tumors, including gastrointestinal cancer (11, 35). The incidence of aberrations (point mutation, homozygous deletion, and methylation) of the *CDKN2A* gene are a predominant mechanism of inactivation of the *CDKN2A* gene and may be associated with a metastatic and invasive phenotype of ESCC (36). In this study, we quantified the expression levels of $p14^{ARF}$ mRNA which were found to have a significant positive relationship with the invasiveness of ESCCs, such as depth of invasion and lymph node metastasis. In addition, high-stage tumors were found more frequently in the low $p14^{ARF}$ mRNA expression group. These observations suggest that disruption of the $p14^{ARF}$ gene produced by methylation might confer a growth advantage and play an important role in the acquisition of metastatic potential.

Although the inactivating mechanisms of $p16^{INK4a}$ have been widely examined, the alterations selectively affecting $p14^{ARF}$ have been poorly analyzed. In previous studies, $p14^{ARF}$ methylation combined with methylation of other genes and *p53* mutation has been found to correlate with poor prognostic information in acute lymphoblastic leukemia, astrocytoma, breast carcinoma, colon carcinoma and bladder carcinoma (37-39). A low mRNA expression of $p14^{ARF}$ combined with mRNA expression of other *CDKN2A* pathway genes was found to be associated with aggressive clinical behavior and unfavorable prognosis in gastrointestinal stromal tumor (40). Loss of $p14^{ARF}$ protein expression is associated with poor outcome independently in squamous cell carcinoma of the anterior tongue (41). However, $p14^{ARF}$ methylation and $p14^{ARF}$ expression independently failed to provide statistically prognostic information in ESCC. In this study, although a multivariate analysis demonstrated that $p14^{ARF}$ mRNA expression is not an independent prognosticator in patients with ESCC, our data indicate that a decrease in $p14^{ARF}$ mRNA expression due to $p14^{ARF}$ methylation may be associated with the progression of ESCC. $p16^{INK4a}$ inactivation is associated with a more aggressive phenotype and poorer prognosis in a wide range of neoplasms, including non-small cell lung cancer, pancreatic carcinoma and ESCC (42-44). We found no statistically significant association between $p16^{INK4a}$ mRNA expression and clinicopathological variables, including survival. In a previous study, loss of $p16^{INK4a}$, *Rb*, $p21^{CIP/WAF-1}$ or *Bax* and overexpression of *cyclin D1* were found to be associated with individually shorter overall survival in ESCC (45). Our results may be relevant to the expression of these Rb-pathway and apoptosis-controlling genes.

In conclusion, our results demonstrate that methylation of the $p14^{ARF}$ gene is associated with reduced expression in ESCCs, suggesting that methylation is the predominant mechanism of inactivation of the $p14^{ARF}$ gene. The decrease in $p14^{ARF}$ gene expression associated with invasive and metastatic phenotypes may be significant as an indicator of malignant potential of the human ESCC.

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