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Aberrant methylation of *p16^{INK4a}* and deletion of *p15^{INK4b}* are frequent events in human esophageal cancer in Linxian, China

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p16^{INK4a}* and *p15^{INK4b}* genes, which encode two functionally related CDK inhibitors, recently emerged as candidate tumor suppressor genes since they were both localized to 9p21, which frequently undergoes hemizygous and homozygous deletion in a variety of tumor types. To determine the mode of inactivation of these two genes in human esophageal squamous cell carcinoma (ESCC), we performed multiple molecular analyses in 60 ESCC specimens from Linxian, China using DNA methylation assay, LOH analysis, deletion screening and SSCP-sequencing. We observed that *p16^{INK4a}* inactivation was predominantly associated with aberrant methylation in the CpG island of its promoter region, whereas *p15^{INK4b}* frequently had homozygous deletions. Compared with aberrant methylation, which occurred in 17 of 34 cases, homozygous deletion of *p16^{INK4a}* and LOH at its nearby D9S942 microsatellite marker were observed at a much lower frequency (17%). Intragenic mutation in *p16^{INK4a}* gene was rare. In contrast, homozygous deletion in *p15^{INK4b}* and LOH at the nearby D9S171 marker were observed at frequencies of 35 and 47%, respectively, and the two events were significantly associated with each other. On the other hand, aberrant methylation of *p15^{INK4b}* was relatively infrequent (6/34) and occurred concomitantly with *p16^{INK4a}* methylation. Among the 60 cases, only four contained a continuous homozygous deletion spanning both *p15^{INK4b}* and *p16^{INK4a}*. Six cases were exclusively deleted at *p16^{INK4a}* and 17 exclusively deleted at *p15^{INK4b}*. LOH at D9S942 and D9S171 was also found to be mutually exclusive. Our results suggest that the alteration mode at 9p21 was not uniform, and the two genes were inactivated by distinct mechanisms. Altogether, 68% of the samples harbor at least one type of alteration in *p16^{INK4a}* gene and 50% of the samples were altered in *p15^{INK4b}* gene, indicating that they are the frequent inactivating targets during ESCC development.

Introduction

The putative tumor suppressor genes *p16(*INK4a/MTS1/CDKN2) and *p15*(INK4b/MTS2) encode two important cyclin dependent kinase (CDK) inhibitors which negatively regulate G_1–S transition of the proliferating cells by contributing to the maintenance of pRb in an active (hypophosphorylated) state (1,2). p16^{INK4a} binds to and inhibits CDK4/6 activity during G_1 stage (3). p15^{INK4b} binds to the cyclin D-dependent kinase and prevents p27 association. p27 then binds to, and inactivates, cyclin E-CDK2 complex, thereby blocking the cell cycle at the G_1–S boundary (4). The absence of the inhibitory effect of p16^{INK4a} or p15^{INK4b} on the cyclin/CDK enzymes predisposes the cells to a risk of uncontrolled cell growth.

Inactivation of *p16^{INK4a}* and *p15^{INK4b}* genes has been observed in many types of human cancers including esophageal squamous cell carcinoma (ESCC). Multiple mechanisms seem to be involved in causing gene inactivation (5–18). Some studies showed that germline mutations in *p16^{INK4a}* gene might be related to familial melanoma (6), but in general the mutation is relatively infrequent in sporadic melanoma and most other cancers (7). Allelic loss, observed as loss of heterozygosity (LOH) at 9p21 is common in various cancer types and is often an early event (8,9). Homozygous deletions of *p16^{INK4a}* and *p15^{INK4b}* also occur at significant frequencies and sometimes correlate with a high risk of metastasis (10,11). Hemizygous and homozygous deletion at 9p21 are widely considered to be one of the primary mechanisms of *p16^{INK4a}*/*p15^{INK4b}* inactivation and may play an important role in the invasiveness of many cancers during their late stage. It was hypothesized that, due to the existence of multiple tumor suppressor genes including *p16^{INK4a}*, *p15^{INK4b}* and *p19^{ARF}* (which is translated from an alternatively spliced product of *p16^{INK4a}* gene using a different open reading frame) (12) in this region, deletion of a large piece of genomic material would be more effective over point mutation to eliminate two or more critical genes (13). Recently, aberrant methylation of the CpG island at the promoter regions of *p16^{INK4a}* and *p15^{INK4b}* genes was reported in many cancers (14) and was associated with loss of transcription (15,16). Interestingly, it was observed that for most epithelial-derived tumors, inactivation via promoter methylation occurs exclusively for *p16^{INK4a}*, whereas in most hematopoietic malignancies and gliomas, this mechanism seems to involve the *p15^{INK4b}* gene only (17,18). This suggests that promoter hypermethylation could be involved in the selective inactivation of targets at 9p21 region in different types of tumor.

In human ESCC, the functional significance of *p16^{INK4a}* alteration is still unclear. Although *p16^{INK4a}* gene has been shown to be deleted, mutated or hypermethylated in a number of ESCC cell lines, molecular analysis from primary tumors has not consistently indicated the alterations of this gene *in vivo (*19–24). For example, frequencies of homozygous deletion in cell lines range from 33 to 92% (19), whereas in primary tumors, only 0–16% have been observed (20,21). This apparent discrepancy could be due to (i) *p16^{INK4a}* deletion as a result of *in vitro* immortalization or selection during culturing; (ii) loss of *p16^{INK4a}* during very late stages of ESCC progression or (iii) technical difficulties in detecting homozygous deletion from primary tumor DNA due to contamination of DNA from normal tissue. The frequency of *p16^{INK4a}* mutations in primary tumors also vary among reports, from 0 to 50%, and differed

Abbreviations: CDK, cyclin-dependent kinase; ESCC, esophageal squamous cell carcinoma; LOH, loss of heterozygosity; SSCP, single strand conformation polymorphism; MI, microsatellite instability.

Table I. Oligonucleotides for determining $p16^{INK4a}$ and $p15^{INK4b}$ aberrations

Primer set	Targets	Product size (bp)	Primer sequence
Set 1	D9S171	159–177	5′-AGCTAAGTGAACCTCATCTCTGTCT-3′ 5′-ACCCTAGCACTGATGGTATAGTCT-3′
Set 2	$p15^{INK4b}$ promoter-exon 1 region containing CpG island	137	5′-CGCACCCTGCGGCCAGA-3′ 5′-AGTGGCCGAGCGGCCGG-3′
Set 3	$p15^{INK4b}$ exon 2	172	5′-ACCGGTGCATGATGCT-3′ 5′-TCAGTCCCCCGTGGCTGT-3′
Set 4	D9S942	100–130	5′-GCAAGATTCCAAACAGTA-3′ 5′-CTCATCCTGCGGAAACCATT-3′
Set 5	$p16^{INK4a}$ promoter-exon 1 region containing CpG island	122	5′-AGCCTTCGGCTGACTGGCTGG-3′ 5′-CTGGATCGGCCTCCGACCGTA-3′
Set 6	$p16^{INK4a}$ exon 1	208	5′-GAGAGGGGGAGAGCAGGC-3′ 5′-GCTGCAGACCCTCTACCCAC-3′
Set 7	$p16^{INK4a}$ exon 2	386	5′-CCTGGCTCTGACCATTCTGT-3′ 5′-GCTTTGGAAGCTCTCAGGGT-3′
Set C	β-actin fragment (internal control)	187	5′-CTGTGGCATCCACGAAACTA-3′ 5′-AGGAAAGACACCCACCTTGA-3′

significantly between different ethnic groups if not due to experimental variations (19 and references therein). Recent study also showed *de novo* promoter hypermethylation of $p16^{INK4a}$ in a group of Japanese ESCC patients (21). However, due to the lack of comparability of works from different groups, our knowledge of the mode of $p16^{INK4a}$ inactivation in ESCC is still sketchy. Our recent results from immunohistochemical analysis indicated that $p16^{INK4a}$ expression was present in only three out of 22 ESCC cases (22), suggesting that the $p16^{INK4a}$ gene is frequently inactivated in primary ESCC. The molecular basis for this $p16^{INK4a}$ immunohistochemical negativity needs to be established. Furthermore, it has been suggested that $p15^{INK4b}$ may also be an important target for inactivation in certain type of tumors (17). Recently, Tanaka *et al.* showed that this gene was co-deleted with $p16^{INK4a}$ in 53% of the ESCC cell lines they analysed (20). However, the status of $p15^{INK4b}$ gene in primary ESCC has not been reported.

To better understand the role of both $p16^{INK4a}$ and $p15^{INK4b}$ gene alterations in ESCC, we analysed 60 ESCC samples from a high incidence area in Henan, China. Multiple molecular analyses were performed in parallel to examine several potential types of aberrations affecting the $p16^{INK4a}$ and $p15^{INK4b}$ genes in the tumor samples. Matched pairs of normal and tumor DNA samples were screened for homozygous deletion using multiplex-PCR, and for frequency of LOH at 9p21 using two microsatellite markers D9S942 and D9S171. Among these samples, 34 were further analysed by PCR-methylation assay to evaluate aberrant methylation at the gene promoter regions and by SSCP-sequencing to examine $p16^{INK4a}$ mutation.

Materials and methods

Tumor samples and DNA preparation

Sixty surgically-resected esophageal specimens containing both tumor and nearby normal tissues were collected between 1991 and 1993 from patients with primary ESCC in Linxian (now Linzhou City), Henan Province, China, and were fixed in 80% ethanol. Approximately 20 mg of normal tissue from each sample was used directly for DNA extraction. All tumor tissues were embedded in paraffin, cut into 5 μm serial sections and deparaffinized before use. H&E staining and histopathological examination were performed on representative sections from each sample block to determine the tumor region. Tumor tissues were dissected from the neighboring four successive sections. Generally, contamination of the normal cells was ~20–25% mainly due to the fibroblast and inflammatory cells surrounding the cancer nests which were difficult to remove. Genomic DNA was extracted by proteinase K digestion and phenol–chloroform extraction.

PCR amplification

Primer sequences for all PCR amplifications in this study are shown in Table I. Sets 1 (GDB:188218) and 4 (GDB:370738) were obtained from the GDB database through internet, and were used for LOH assay. Sets 2 and 5 (21) were used in the PCR-methylation assay. Set 3 was used for $p15^{INK4b}$ gene deletion analysis. Sets 6 and 7 were used in deletion assay and single strand conformation polymorphism (SSCP)-sequencing of $p16^{INK4a}$ gene. All primers not given the source were designed using a primer picking software developed by Whitehead Institute/MIT Center for Genome Research. For PCR reaction, genomic DNA was amplified in a total volume of 25 μl containing 20 pmol of each primer, 250 μmol of dNTP, 1.4–2.0 mM $MgCl_2$, standard buffer and 0.5 U Taq polymerase (all from Gibco BRL). Mg^{2+} concentration, PCR cycles and annealing temperature (T_m) were optimized for each assay in pilot experiments.

PCR-based gene deletion and LOH analysis

Homozygous deletion at the $p16^{INK4a}$ and $p15^{INK4b}$ loci was analysed by comparative multiplex PCR using the D9S171 product or β-actin fragment as internal standards, and a closely sized PCR fragment of the gene as the primary target. The D9S171 microsatellite marker was originally selected to access LOH at 9p21. It was also used as an internal control in addition to β-actin because it was never found to be homozygously deleted and is relatively close to the target genes on the same chromosome. This provides a similar exposure rate of both the target and the control to the Taq polymerase, and can also identify LOH at D9S171 simultaneously. However, $p15^{INK4b}$ deletion was only analysed with the β-actin internal control, because the $p15^{INK4b}$ exon 2 has a size overlap with the D9S171 product.

To ensure linear amplification, PCR cycle number for tumor and normal DNA reactions were experimentally determined based on the product intensity versus cycle number gradient curve using a modified protocol originally described by Gonzales-Zulueta *et al.* (16). For PCR amplification, about 200 ng normal and 60 ng tumor genomic DNA were used. Forward primers of both target gene and control were radiolabeled at the 5′ end with [γ-^{33}P]dATP. Typically, the following PCR conditions were used: 5 min denaturation at 95°C before adding the Taq polymerase; 2 min at 95°C, 2 min at T_m, 2 min at 72°C for initial cycle; then 27–29 cycles of 90 s at 95°C, 45 s at T_m, 45 s at 72°C; followed by 2 min at 72°C. The PCR products were resolved on 6 or 20% polyacrylamide gel depending on product size difference. After autoradiography for 4 h, the film was developed and the ratio of intensity of the target gene versus control was measured and calculated using a computer imaging system (Image-Pro Plus, Media Cybernetics). A homozygous deletion was scored if the normalized signal intensity (target/β-actin) in the tumor lane was <25% of that in the normal lane. The threshold was so chosen because of the possible 20–25% non-cancerous cell contamination in our tumor samples. LOH at D9S171 was determined simultaneously with $p16^{INK4a}$ deletion, whereas LOH at D9S942 was determined separately.

PCR-based methylation assay

Normal DNA (400 ng) and tumor DNA (80 ng) were digested for 4 h with 10 U methylation-sensitive (*Cfo*I, *Hpa*II, *Sma*I) or methylation-insensitive (*Msp*I) restriction enzymes. After 2 min incubation at 95°C to inactivate the enzymes, the DNAs were subject to a second round of digestion by another 10 U freshly added enzymes to improve specificity and completeness of

Table II. Inactivation of the *p16^{INK4a}* and *p15^{INK4b}* genes in ESCC[a]

Case	*p16^{INK4a}*	D9S942	*p15^{INK4b}*	D9S171	Patient	*p16^{INK4a}*	D9S942	*p15^{INK4b}*	D9S171
4	M	NI	+	NI	920972	M	NI	+	NI
6	–	NI	+	NI	920976	–	–	+	NI
704	–	–	–	NI	933334	M	–	–	+/–
705	+	+	–	NI	933495	–	–	–	NI
91755	+	+	–	NI	5[b]	+	NI	+	+
91779	+	+	–	NI	91702[b]	+	NI	–	NI
91787	M	NI	+	NI	91794[b]	+	+	–	+/–
91799	+	+	+	NI	91795[b]	–	NI	+	+
910609	M	NI	M	NI	91807[b]	–	+/–	+	NI
910666	M	+	–	NI	910468[b]	+	+	+	+
910669	+	+	+	NI	910473[b]	+	+	–	NI
910673	M	+	–	NI	910487[b]	+	+	+	+/–
910682	+	NI	+	NI	910497[b]	+	+/–	+	NI
910731	M	+	M	NI	910571[b]	+	NI	+	NI
910751	M	+	M	NI	910618[b]	–	+	+	+
910793	M	+	M	NI	910623[b]	+	+	–	+
910840	M	+	M	+/–	910634[b]	+	NI	–	+/–
910843	M	+	+	+/–	910649[b]	+	NI	+	NI
920920	M	+	+	NI	910657[b]	–	–	–	NI
920922	M|Mut	+	M	NI	910670[b]	–	–	–	NI
920925	M	+	+	NI	910715[b]	–	+	+	+
920928	+	+	–	+/–	910743[b]	+	+/–	+	NI
920934	+	+/–	+	NI	910782[b]	+	+	–	NI
920935	M	+/–	+	NI	910783[b]	+	+/–	+	NI
920937	+	+	+	NI	910804[b]	+	MI	–	+/–
920943	+	MI	–	NI	910809[b]	+	+/–	+	+
920947	+	+	+	+	920942[b]	+	+	–	NI
920950	M	+	–	+/–	920953[b]	+	+	+	NI
920951	+	+/–	+	+	920973[b]	+	+	+	NI
920957	+	+	+	NI	933131[b]	+	NI	+	+

[a]+, intact gene; –, homozygous deletion; +/–, heterozygous deletion (LOH); M, methylation; MI, microsatellite instability; Mut, mutation; NI, non-informative.
[b]Cases not analysed for methylation due to insufficient amount of DNA.

digestion (all of the enzymes are from Gibco BRL and digestion was performed under manufacturer's instructions). Since *p16^{INK4a}* and *p15^{INK4b}* promoter regions have common methylation-sensitive restriction sites (i.e. *Hpa*II), the same digested DNA samples were used to determine methylation status of both genes. One hundred nanograms of normal and 20 ng of tumor DNA were amplified by PCR using primer set 2 or 5 which flanks the restriction sites, and with β-actin as internal control. The PCR conditions were similar to those described in the deletion assay with cycle number fine-tuned to limit the amplification to a linear range. PCR products were electrophoresed on 3% agarose gels. Same amounts of undigested and *Msp*I-digested DNA were used as the controls in every sample examined. Experiments were repeated three times for all samples to ensure reproducibility Signal intensities normalized by the internal control were measured and calculated as described in the deletion analysis. Methylation at a specific restriction site was determined by the ratio of the normalized intensity (target/internal standard) of the samples pretreated with methylation-sensitive enzymes over that of the undigested samples. A ratio >0.5 was defined as aberrant methylation.

Single strand conformation polymorphism (SSCP) and sequencing analysis

PCR was carried out as described with 1 μCi of α-^{32}P-dATP. The labeled PCR products were denatured and electrophoresed on 6% polyacrylamide gel containing glycerol at 10–25 W for 7–10 h. Shifted bands from the tumor DNA reaction as compared with the adjacent normal sample, as well as the human placental control were eluted and amplified by a second PCR followed by agarose gel electrophoresis. The final PCR products were purified for DNA sequencing using the Wizard PCR Preps DNA Purification System from Promega (Madison, WI) according to manufacturer's instructions. DNA sequencing was performed using the same primer for PCR–SSCP. DNA was sequenced with the Thermal Sequenase Cycle Sequencing Kit from Amersham Life Science (Cleveland, OH) according to the manufacturer's instructions. In cycle sequencing, the thermal cycle consisted of 2 min at 94°C; 20 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C; 10 cycles of 1 min at 94°C and 1 min at 72°C; and 1 min at 72°C. The reaction mixture was loaded on a 6% polyacrylamide sequencing gel made from the Sequagel DNA Sequencing Solutions (National Diagnostics, Atlanta, GA).

Results

LOH at 9p21 loci in primary ESCC

Genomic DNA obtained from 60 matched pairs of primary tumors and nearby normal tissues were examined for incidence of LOH at 9p21 where both *p16^{INK4a}* and *p15^{INK4b}* reside. Altogether, 17 of the 51 informative cases were found to harbor LOH at least one of the two loci we analysed (Table II). However, frequency of LOH at individual locus was significantly different. Allelic loss at D9S942 (Figure 1a), which is located close to *p16^{INK4a}* in a region up to 35kb long between *p16^{INK4a}* and *p15^{INK4b}*, was evident in eight of the 47 informative cases. Whereas nine out of the 19 informative cases showed allelic imbalance at D9S171 (Figure 1b), which is ~2–3 Mb centromeric to the *p15^{INK4b}* gene. No case was found to contain LOH at both loci.

In all 60 pairs of tumor and normal DNA samples, retention of at least one allele of the D9S171 microsatellite marker was observed. However, there were six cases showing no PCR product for the similar sized D9S942 marker after several attempts. This implies a possible homozygous deletion at the D9S942 locus. Microsatellite instability (MI) at these two loci was also monitored in the same analysis. Only two cases were found to harbor MI in marker D9S942. Interestingly, in one case, variation of the number of nucleotide repeats was seen in both alleles (Figure 1a).

Homozygous deletion of p16^{INK4a} and p15^{INK4b} genes in primary ESCC

Comparative multiplex PCR showed that 10 out of the 60 primary ESCC cases had homozygous deletions in the *p16^{INK4a}*

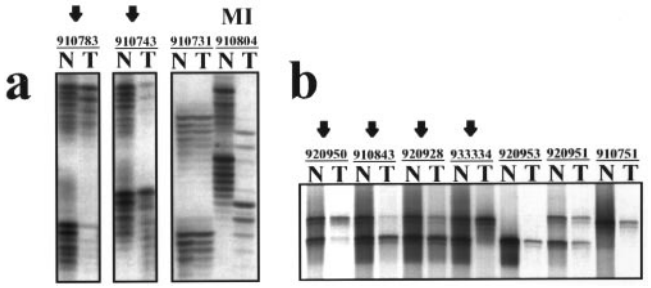

Figure 1. LOH and MI at 9p21 loci in primary ESCC. PCR products of matched pairs of normal (N) and tumor (T) were loaded next to each other for comparison. Hemizygous loss of each marker are indicated by an arrow. (**a**) Representatives of LOH and MI at microsatellite marker D9S942. Case 910731 showed retention of both alleles at this locus, whereas tumors from cases 910783 and 910743 showed allelic imbalance with loss of a lower or an upper allele, respectively. Case 910804 harbored a MI in tumor as evidenced by the down shift of both alleles caused by a deletion of two trinucleotide repeats in both alleles. (**b**) LOH at D9S171. Loss of either the upper or the lower allele was seen in tumors from the first four cases. Case 920951 retained both alleles at this locus, and cases 920953 and 910751 were examples of two types of non-informative cases.

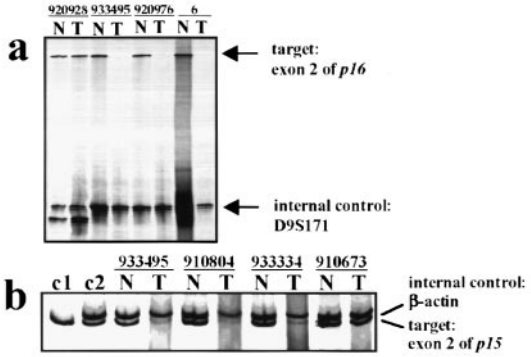

Figure 2. Homozygous deletion (HD) of $p16^{INK4a}$ and $p15^{INK4b}$ genes in primary ESCC detected by multiplex PCR. Deletion was defined by the target/control ratio in the tumor lane being <25% of that of the normal lane. (**a**) HD of $p16^{INK4a}$ was seen in the tumors from cases 933495, 920976 and 6. All of them showed the absence of the $p16^{INK4a}$ exon 2 product whereas retained the D9S171 band in the same PCR reaction. Case 920928 was a comparative case in which no HD was observed. (**b**) HD of the $p15^{INK4b}$ gene. c1, control DNA amplified with p15 amplifier only; c2, control DNA amplified with p15 and β-actin amplifiers. Except for 910673, all other cases contain HD as evidenced by no or very weak exon 2 product in the tumor samples. Case 933945 also contained codeletion at $p16^{INK4a}$ locus, as seen in (a).

locus. The deletion was evidenced by the loss of the exon 2 PCR product while retaining the D9S171 product in a multiplex PCR reaction (Figure 2a), and was confirmed by parallel PCR reactions targeting exon 1 with β-actin as control. A significantly higher frequency of homozygous deletion at the $p15^{INK4b}$ locus, however, was observed in the same sample collection, with 21 out of 60 devoid of exon 2 product (Figure 2b). Among these 21 cases, 17 contained deletion only at $p15^{INK4b}$ loci and four had dual deletions at both $p16^{INK4a}$ and $p15^{INK4b}$ loci. All of the four cases with dual deletions at both loci were among the six cases that failed to produce the D9S942 product during the LOH study, implying a large and contiguous deletion encompassing the $p16^{INK4a}$-$p15^{INK4b}$ region in these samples. For the remaining two cases with no D9S942 amplification, one was found to have a deletion at $p16^{INK4a}$, the other had a $p15^{INK4b}$ deletion. Altogether, 27 out of the 60 primary ESCC patients contain homozygous deletion at the 9p21 region, with $p15^{INK4b}$ being the most frequently targeted

Table III. Correlation of D9S942 or D9S171 LOH with homozygous deletion of $p16^{INK4a}$ or $p15^{INK4b}$ in ESCC

	$p16^{INK4a}$ deletion		$p15^{INK4b}$ deletion	
	Yes	No	Yes	No
D9S942				
LOH	1	7	0	8
No LOH	2	31[a]	14	19[b]
Deletion	5	1	5	1
D9S171				
LOH	0	9	6	3
No LOH	3	7[c]	1	9[d]
Deletion	0	0	0	0

Fisher's exact test. Data obtained from Table II.
[a]$P = 0.488$.
[b]$P = 0.023$.
[c]$P = 0.121$
[d]$P = 0.017$.

gene. The remaining 33 samples had no homozygous deletion in either of the genes (Table II).

Relationship between the $p16^{INK4a}$ and $p15^{INK4b}$ homozygous deletion and 9p21 LOH was studied (Table III). There was no significant association between LOH at D9S942 and homozygous deletion of the $p16^{INK4a}$ gene ($P = 0.488$). The incidence of D9S942 LOH, however, was significantly higher in samples with no $p15^{INK4b}$ deletion ($P = 0.023$). Nevertheless, LOH at D9S171 is strongly associated with homozygous deletion at $p15^{INK4b}$ ($P = 0.017$), but not with that at $p16^{INK4a}$.

Aberrant methylation of the CpG island in $p16^{INK4a}$ and $p15^{INK4b}$ promoter region

Among the 60 cases of ESCC we analysed, 34 had yielded enough genomic DNA for us to examine the methylation status of the $p16^{INK4a}$ and $p15^{INK4b}$ promoter regions depicted in Figure 3a. Aberrant methylation of $p16^{INK4a}$ in the tumor DNA was detected in 17 of the 34 cases (Figure 3b). Among them, 13 were found to be hypermethylated as manifested by their resistance to all three methylation-sensitive restriction enzymes while being sensitive to the methylation-insensitive enzyme *Msp*I. The remaining four cases contained partial methylation as shown by their sensitivity to less than three of the methylation-sensitive enzymes, a phenomena indicating that methylation in these cases did not cover the entire CpG island and, therefore, exposing some of the sites to enzymatic restriction. Among these four cases, two cases showed abnormal methylation pattern in which the targeted regions were resistant to *Msp*I restriction, but sensitive to one of the methylation-sensitive enzymes. This suggests the existence of an unconventional methylation pattern such as **^{m}C**CGG, rather than the standard C**^{m}C**GG pattern in these patients, which would confer their resistance to *Msp*I digestion. Out of the 17 methylated cases of ESCC, 14 cases did not have methylation in the normal DNA. In the remaining three cases, partial methylation, which covered a subset of the sites methylated in the tumors was seen in normal DNA (data not shown). There were 17 cases (50%) in which no PCR product was obtained from the genomic DNA after treatment with any one of the methylation-sensitive enzymes. Among them, four samples did not produce PCR products even when not treated with the enzymes, and they all corresponded to the $p16^{INK4a}$ homozygous deletion cases found in the deletion analysis. The other 13 cases could

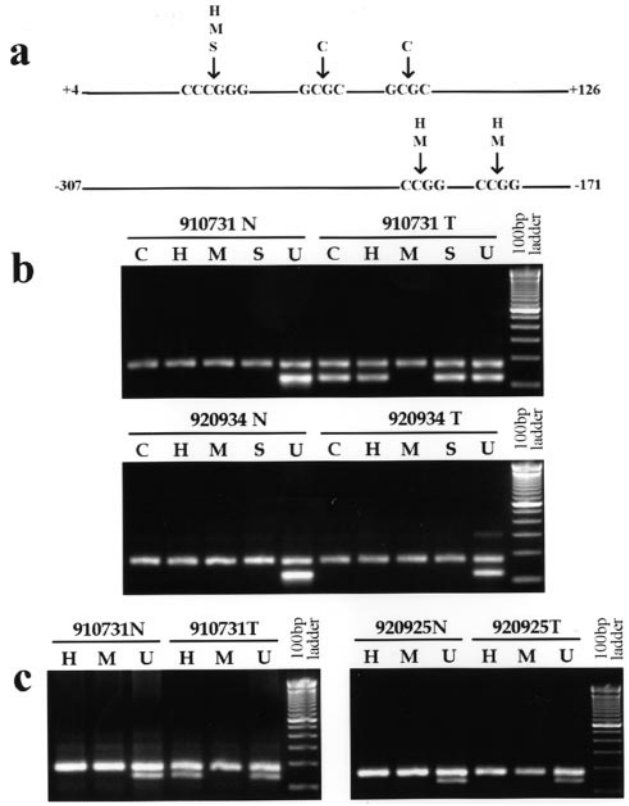

Figure 3. PCR-methylation assay of *p16*^{INK4a} and *p15*^{INK4b} genes in primary ESCC samples. Genomic DNAs were treated with methylation-sensitive (C, *Cfo*I; H, *Hpa*II; S, *Sma*I) or methylation-insensitive (M, *Msp*I) restriction enzymes, respectively, prior to PCR amplification. Aberrant methylation is defined with the criterion described in the Materials and methods. As controls, PCR using methylation-insensitive restriction enzyme (M) treated DNA yields no positive signal, and PCR of undigested DNA (U) always gives positive signal. Matched normal (N) and tumor (T) assays were loaded in adjacent panels for comparison. (**a**) Schematic representation of the promoter regions being analysed. The *p16*^{INK4a} target is a 122 bp fragment 4 bp downstream from the start codon with three sensitive sites (21). The *p15*^{INK4b} target is a 137 bp fragment 171 bp upstream from the start codon with two sensitive sites. The choice of this target region is based on previous description by Herman *et al.* (17), promoter sequence is available at Genbank with accession no. S75756. (**b**) Upper, hypermethylation of *p16*^{INK4a} in tumor of case 910731; lower, no methylation in tumor of case 920934. The upper band in the gel is the β-actin internal standard. (**c**) Left, hypermethylation of *p15*^{INK4b} in tumor of case 910731; right, no methylation in tumor of case 910925.

undergo PCR normally when no restriction enzymes were applied and, therefore, were believed to contain no methylation at *p16*^{INK4a} promoter region.

A much lower frequency of aberrant methylation in the *p15*^{INK4b} promotor region was observed by analysing the same digested DNA samples used in *p16*^{INK4a} methylation assay with amplifiers specific for *p15*^{INK4b} promoter region. This region contains 2 *Hpa*II/*Msp*I sites and is 171 bp upstream exon 1. Only six samples out of 34 harbored aberrant methylation (Figure 3c), all of them also contained aberrant methylation in the *p16*^{INK4a} promoter. Among the 28 cases where no PCR product was obtained from the *Hpa*II or *Msp*I treated genomic DNA, 11 actually correspond to the cases previously detected with a homozygous deletion at *p15*^{INK4b} locus. The remaining 17 samples were determined as having no aberrant methylation of *p15*^{INK4b} promotor.

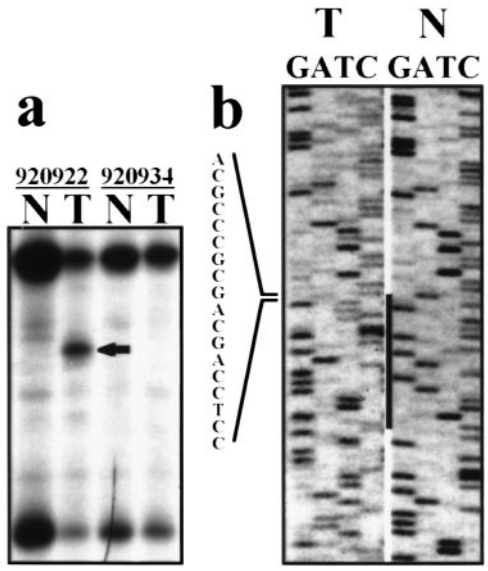

Figure 4. Detection of a rare microdeletion in exon 1 of the *p16*^{INK4a} gene in primary ESCC. (**a**) PCR–SSCP analysis of tumor DNA and normal control showed a mobility shift in tumor DNA indicated by an arrow from case 920922. No shifted band was observed from case 920934. (**b**) Sequencing of the recovered shifted band from case 920922 revealed a microdeletion of 18 bp starting at codon 20 (nucleotide 59) compared with the normal DNA.

Intragenic mutation of p16^{INK4a} *gene in ESCC*

PCR products of exons 1 and 2 of *p16*^{INK4a} gene from the same 34 pairs of primary ESCC and normal tissues were analysed by SSCP. Except for four cases, which showed homozygous deletion at *p16*^{INK4a} locus, all others had specific PCR products. Only one mobility shift was detected in exon 1 of the 31 tumor samples, whereas all of the other normal and tumor DNAs showed normal SSCP pattern (Figure 4a). Upon sequencing, the exon 1 shifted band was determined to contain a microdeletion of 18 nucleotides starting at codon 20 (Figure 4b). This in frame microdeletion will result in a truncated form of *p16*^{INK4a} protein. Sequencing analysis of all the other cases showed normal *p16*^{INK4a} sequence.

Discussion

In this study, a detailed analysis of the inactivation patterns of two related genes, *p16*^{INK4a} and *p15*^{INK4b}, was conducted in primary human ESCC. As summarized in Figure 5, preferential aberrant methylation of the *p16*^{INK4a} promoter and homozygous deletion of *p15*^{INK4b} gene were found to be frequent events in primary ESCC, along with some other less frequent aberrations.

Previously, we have observed frequent absence of p16^{INK4a} immuno-reactivity in ESCC samples (22). In the present study, among the 28 cases in which we were able to analyse different types of *p16*^{INK4a} aberration status, 19 harbored at least one type of molecular alteration within the gene or in its vicinity that potentially inactivates the gene (Table IV). In those samples that had been previously examined for p16^{INK4a} immunohistochemistry (22), their p16^{INK4a} staining patterns generally agree with the *p16*^{INK4a} gene status. These results suggest that *p16*^{INK4a} is a frequent inactivating target comparable with p53 in ESCC (25). However, unlike p53, mutation in *p16*^{INK4a} gene is rare in our cases. Although we could not exclude the possibility that some mutations may remain undetected by our SSCP method, our observation is consistent

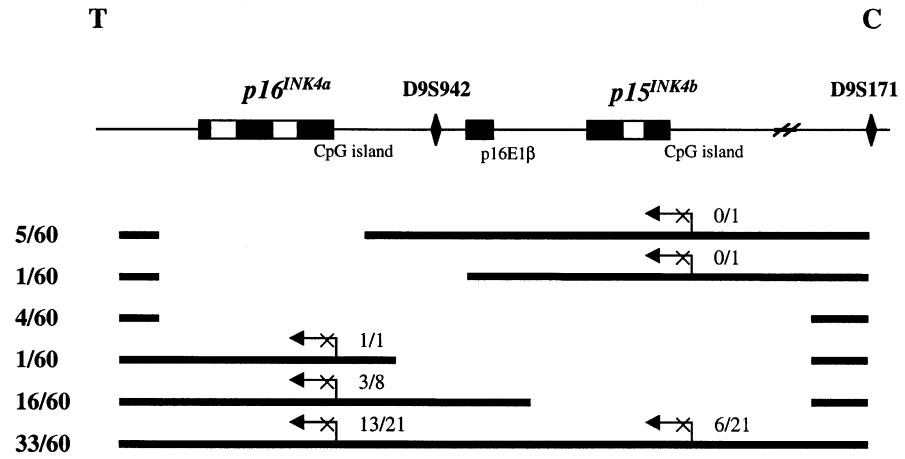

Figure 5. Schematic representation of the predominant mechanisms inactivating *p16*^{INK4a} and *p15*^{INK4b} in ESCC. The first lane shows the location of the genes and polymorphic loci we analysed on chromosome 9p21. The telomeric end is on the left side and the centromeric end is on the right side. ESCC samples are classified into six classes according to their homozygous deletion (HD) pattern as shown in the lower part. The numbers left of the bars indicate the incidence of HD of each class and the deleted region is depicted as the breakage at the corresponding position. Aberrant methylation is represented as an arrow intersecting with a cross. The numbers next to it indicate the methylation incidence among samples analysed for methylation status in the corresponding class. The map is not drawn to scale. Result of methylation status is only available in a subset of samples in each class. It can be seen that aberrant methylation occurred more frequently in *p16*^{INK4a} gene, whereas homozygous deletion was more frequent in *p15*^{INK4b} gene. Furthermore, deletions in *p16*^{INK4a} and *p15*^{INK4b} were not correlated, but methylation at *p15*^{INK4b} was always accompanied by a concomitant methylation at *p16*^{INK4a}.

with the recent report that *p16*^{INK4a} mutation is in general infrequent in ESCC (19). Among several inactivation modes, aberrant methylation, which can result in transcriptional silencing of the targeted gene, was the most frequent alteration found in *p16*^{INK4a} locus. Our frequency of aberrant methylation (50%) is significantly higher than that (19%) reported by Maesawa *et al.* (21) for a group of Japanese ESCC patients, but is consistent with the observation by Merlo *et al.* (15) in several other epithelium-derived cancers. To ensure data reliability, efforts were made to avoid incomplete digestion and false amplification using strict quality control procedures including double digestion, linear stage PCR and independent repeats. All results were based on semi-quantitative measurements. In most cases we studied (14/17), aberrant methylation of *p16*^{INK4a} was only observed in the tumor, but not in the normal tissues, which implied a transcription-silencing state of the gene only in tumor cells. However, in three cases, a less severe aberrant methylation was also detected in the 'normal' samples dissected from regions close to the tumors. Whether this is due to aberrant methylation in the *p16*^{INK4a} promoter region of the preneoplastic cells needs further investigation. It has been reported for colon cancer that promoter methylation can be a very early event which precedes many genetic abnormalities such as *p53* and *Ras* mutations (26). Recently, Wong *et al.* (27) also observed aberrant methylation in early lesions of esophageal adenocarcinoma. It is possible that in ESCC, promoter methylation also plays an important role in *p16*^{INK4a} inactivation during the early carcinogenic process.

The moderate frequency (17%) of homozygous deletion of the *p16*^{INK4a} gene in comparison with the frequent aberrant methylation, suggested that deletion is not a major cause of *p16*^{INK4a} inactivation in ESCC. Previous studies showed that *p16*^{INK4a} homozygous deletions were predominantly observed in ESCC cell line and metastatic lesions (20,21), which imply deletion as a late event involved in the acquisition of invasiveness. We observed that LOH at D9S942, an indication of allelic imbalance of the nearby *p16*^{INK4a} gene, had the same frequency to that of the *p16*^{INK4a} homozygous deletion, although there was no correlation between them. Glendening *et al.* (28) have suggested that deletion of a single copy of *p16*^{INK4a} could potentially drive cancer development. This is probably because p16^{INK4a} functions as a CDK inhibitor by direct protein–protein binding. Even a 50% reduction of p16^{INK4a} expression could weaken its ability to suppress the CDK activity, thereby triggering a proliferation cascade. Hemizygous and homozygous deletions may represent mechanisms for *p16*^{INK4a} inactivation in addition to aberrant methylation in ESCC. In particular, since homozygous deletion is far more frequent during metastasis, it might be related to the acquisition of metastatic potential during late stage ESCC development, as suggested by Maesawa *et al.* (21).

For *p15*^{INK4b}, frequency of the aberrant promoter methylation was relatively moderate in contrast to the 50% frequency affecting *p16*^{INK4a}. Furthermore, all six cases with methylated *p15*^{INK4b} also contained a concomitantly methylated *p16*^{INK4a}; no *p15*^{INK4b} methylation was observed in samples where

Table IV. Summary of inactivation of the *p16*^{INK4a} and *p15*^{INK4b} genes by different mechanisms in ESCC

	p16^{INK4a}	*p15*^{INK4b}
Aberrant methylation	17/34 (50%)	6/34 (18%)
Homozygous deletion	10/60 (17%)	21/60 (35%)
LOH at pertinent marker [a]	8/47 (17%)	9/19 (47%)
MI at pertinent marker	2/60 (3.4%)	0/60 (0 %)
Mutation	1/34 (3%)	ND[b]
Overall alteration rate	19/28 (68%)[c]	17/34 (50%)[d]

[a]D9S942 is considered as pertinent to *p16*^{INK4a} because it has a close LOH rate and deletion frequency to *p16*^{INK4a} deletion, and because of their physical closeness on 9p21. D9S171 is considered as pertinent to *p15*^{INK4b} because its LOH is strongly associated with *p15*^{INK4b} deletion and both events have a close frequency.
[b]ND, not determined.
[c]Statistics was made over those samples subjected to all five types of alteration analysis and yielded conclusive results. Non-informative cases were not taken into the frequency calculation.
[d]Alterations only include homozygous deletion and aberrant methylation since many samples lack LOH information due to low informative ratio.

p16^{INK4a} was not methylated or deleted (Figure 5). This suggests that *p16*^{INK4a} is the primary target of aberrant methylation on 9p, and *p15*^{INK4b} is also methylated in some cases during this process. On the other hand, homozygous deletion was more frequently involved in *p15*^{INK4b} inactivation. Observed in 21 out of the 60 ESCC samples, our frequency is higher than many previous reports (13,29,30). The frequent *p15*^{INK4b} inactivation in this study may be related to the special patient population, which was from Linxian, China, a region with high incidence of ESCC. Except for four cases, all the *p15*^{INK4b} homozygous deletions were observed without a concomitant *p16*^{INK4a} deletion, which contrasts previous findings in many cell line studies (20). In studies of ESCC cell lines, Tanaka *et al*. (20) reported highly frequent deletion (50–60%) of both *p16*^{INK4a} and *p15*^{INK4b}, but no exclusive homozygous deletion of *p15*^{INK4b} was observed. It is possible that cellular immortalization during culturing imposes a selective pressure upon the accumulation of genetic defects. Quick and effective removal of the cell cycle regulatory genes like *p16*^{INK4a} and *p15*^{INK4b} can provide immediate growth advantage and may quickly accumulate during culturing process. However, in primary ESCC, previous reports showed that *p16*^{INK4a} deletion in primary ESCC ranges from 0 to 16% (20,21), which are close to our observation and are significantly lower than the frequency in cell line.

In our study, different frequencies of LOH were observed at D9S942 and D9S171, and no cases harbored LOH at both loci, suggesting a non-uniform alteration mode on 9p21. The LOH frequency at the 9p21 region centromeric to *p15*^{INK4b} (D9S171) is 47%, much higher than that of D9S942 in the region between *p16*^{INK4a} and *p15*^{INK4b} (17%), and has a statistically significant association with deletion at *p15*^{INK4b} ($P = 0.017$). LOH on chromosome 9p region has been suggested as an early alteration in cancers such as head and neck SCC and esophageal adenocarcinoma (8,9,31). It is possible that the non-uniformity of LOH at 9p21 may also have originated during the early stage of ESCC development in our samples. The *p15*^{INK4b} gene may be lost through an early, followed by a late, deletion of one copy each of the gene. Since the D9S171 locus where frequent LOH was observed is ~2 Mb upstream *p15*^{INK4b}, we cannot exclude the possibility of the existence of additional critical gene(s) in the vicinity of *p15*^{INK4b}, such as *p19*^{ARF} (12,32), being co-targeted. The frequent *p15*^{INK4b} alteration (Table IV) suggests that it is an important factor involved in ESCC development. The importance of *p15*^{INK4b} inactivation in the neoplastic transformation may be directly linked to its role in controlling proliferation in normal cells. p15^{INK4b} can displace p27 from the cyclin D-CDK4 complex, thereby promoting p27 binding and inactivation of cyclin E-CDK2 (5). This non-constitutive CDK/CDI/pRb pathway can be induced by TGF-β and plays an essential role of growth inhibition in normal cells (33). An early loss of *p15*^{INK4b} may cause the cell to be insensitive to the growth inhibition signal.

The observation that different mechanisms are involved in the inactivation of closely located genes like *p16*^{INK4a} and *p15*^{INK4b} is not unusual. Herman *et al.* (17) has reported the preferential methylation of *p15*^{INK4b} gene along with selective deletion of *p16*^{INK4a} gene in leukemia and glioma. Gene specific selective inactivation of *p16*^{INK4a} and *p15*^{INK4b} by different mechanisms might be due to differences of the local chromosomal structure of these two loci.

In summary, consistent with the previous finding that 9p21 genetic alteration is closely related to multiple types of tumor development, we showed that both *p16*^{INK4a} and *p15*^{INK4b} genes were subject to frequent inactivation in human primary ESCC. Our results imply that (i) inactivation of *p16*^{INK4a} and *p15*^{INK4b} involves different mechanisms, with *p16*^{INK4a} predominantly affected by aberrant methylation and *p15*^{INK4b} by deletion; (ii) the non-uniform alteration pattern at 9p21 in our ESCC samples may originate during early ESCC stage with the preferential LOH at the *p15*^{INK4b}-D9S171 loci where other critical genes may also exist and be targeted. Loss of function of *p16*^{INK4a} or *p15*^{INK4b} genes could have profound consequences, such as releasing the tight control of CDK/CDI/pRb pathway over cell cycle progression. To fully understand the role of *p16*^{INK4a} and *p15*^{INK4b} inactivation in ESCC development, it is important to analyse these inactivating events in precancerous lesions and their relationship with other gene alterations.

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